Veterinary Microbiology 232 (2019) 156–161

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Inhibition of calmodulin increases intracellular survival of *Salmonella* in chicken macrophage cells

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ARTICLE INFO

Keywords:
Calmodulin
Kinome
Nitric oxide
Salmonella
Macrophage cell
Chicken

ABSTRACT

Calcium (Ca^{2+}) is a pivotal intracellular second messenger and calmodulin (CaM) acts as a multifunctional Ca^{2+}-binding protein that regulates downstream Ca^{2+} dependent signaling. Together they play an important role in regulating various cellular functions, including gene expression, maturation of phagolysosome, apoptosis, and immune response. Intracellular Ca^{2+} has been shown to play a critical role in Toll-like receptor-mediated immune response to microbial agonists in the HD11 chicken macrophage cell line. The role of that the Ca^{2+}/CaM pathway plays in the intracellular survival of *Salmonella* in chicken macrophages has not been reported. In this study, kinome peptide array analysis indicated that the Ca^{2+}/CaM pathway was significantly activated when chicken macrophage HD11 cells were infected with *S.* Enteritidis or *S.* Heidelberg. Further study demonstrated that treating cells with a pharmaceutical CaM inhibitor W-7, which disrupts the formation of Ca^{2+}/CaM, significantly inhibited macrophages to produce nitric oxide and weaken the control of intracellular *Salmonella* replication. These results strongly indicate that CaM plays an important role in the innate immune response of chicken macrophages and that the Ca^{2+}/CaM mediated signaling pathway is critically involved in the host cell response to *Salmonella* infection.

1. Introduction

Salmonella enterica serovar Enteritidis and Heidelberg are the most prevalent poultry *Salmonella* strains associated with human salmonellosis (Hoffmann et al., 2012). In contrast to humans, chickens infected with non-host specific *Salmonella* serovars largely display no symptoms (Barrow and Freitas Neto, 2011). In chickens, *Salmonella* that cross the intestinal barrier are taken up by polymorphonuclear heterophils and macrophages. The phagocytized *Salmonella* inside the heterophils are effectively killed (Genovese et al., 2013). However, *Salmonella* can survive inside the chicken macrophages, despite chicken macrophage's ability to produce an array of bactericidal substances, including reactive radical oxygen species (ROS), nitric oxide (NO), lysozyme, and proteolytic enzymes when exposed to *Salmonella* (Barrow and Freitas Neto, 2011; Braukmann et al., 2015). It is well established that *Salmonella* rely on the pathogenicity islands-coded Type III Secretion System (T3SS) that produces nearly 40 different virulence effectors to enable invasion, survival, and replication within the *Salmonella*-containing vacuole (SCV) inside phagocytes (Haraga et al., 2008; Ibarra and Steele-Mortimer, 2009; Malik-Kale et al., 2011). However, robust macrophage functionality has also been implicated to increase the resistance to systemic spread (Wigley et al., 2006) and intestinal colonization (Sun et al., 2008) by *Salmonella*. Macrophages are the important mediator in the interaction between the host and *Salmonella* and play a key role determining the outcome of the infection.

Calcium is a pivotal intracellular second messenger, regulating many cellular functions in all eukaryotes by forming a complex with calmodulin (CaM), a 17-kDa acidic protein with four Ca^{2+} high-affinity binding motifs (Soderling, 1999). Bound Ca^{2+}/CaM activates Ca^{2+}/CaM-dependent Ser-Thr kinases (CaMKs), including the CaM-dependent protein kinase I (CaMKI) subfamily, CaMKII subfamily, CaMKIV, and the CaMKK subfamily (Soderling, 1999). Through these CaMKs, CaM is directly or indirectly involved in activation of transcription factors CREB (Dash et al., 1991), NF-κB (Jang et al., 2001; Bae et al., 2003), and AP-1 (Ho et al., 1996); phagosome-lysosome fusion process (Malik et al., 2001; Stockinger et al., 2006); immune function (Herrmann et al., 2005; Zhang et al., 2011; Racioppi et al., 2012); inhibition of apoptosis through activation of Akt/PkB (Yano et al., 1998); and regulation of mitogen-activated protein (MAP) kinase pathway (Enslen et al.,1996). Additionally, calcium-bonded CaM interacts directly with and activates

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https://doi.org/10.1016/j.vetmic.2019.02.013
Received 10 December 2018; Received in revised form 1 February 2019; Accepted 5 February 2019
0378-1135/ Published by Elsevier B.V.

nitric oxide synthase (NOS) (Piazza et al., 2015), thus playing a critical role in killing intracellular *Salmonella* Typhimurium in murine macrophage RAW 264.7 (Smallwood et al., 2006). In chickens, Ca^{2+}/CaM signaling has also been shown to regulate the Toll-like receptor mediated immune response in chicken macrophage HD-11 (He at al., 2006, 2008; Arsenault et al., 2013).

Although CaM is involved in immune response and phagosome-lysosome fusion, its role in *Salmonella* infection in chicken macrophages has not been reported. In the present study, we used a CaM inhibitor, W-7, to evaluate the role of CaM in chicken macrophage immune response to *Salmonella* infection and intracellular survival of *Salmonella*.

2. Materials and methods

2.1. Reagents

Cell culture medium and reagents were obtained from Sigma (St. Louis, MO, USA). The CaM antagonist W-7 was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Ultra-pure lipopolysaccharide (LPS) from *Salmonella* Minnesota R595 was obtained from InvivoGen (San Diego, CA, USA). All other products used in this study were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

2.2. Cell line

The MC29 virus-transformed chicken macrophage cell line HD11 (Beug et al., 1979) was maintained in a complete Dulbecco's Modified Eagles Medium (DMEM) containing 10% chicken serum, antibiotics (100 U penicillin/ml and 100 µg streptomycin/ml), and 1.5 mM L-glutamine at 39 °C, 5% CO_2, and 95% humidity. Aliquots of cell suspension (2×10^6 cells/ml) was seeded into each well at 500 µl/well for 24-well plate (BD) and allowed to grow to about 85% confluence (~36 h) before being used for infection.

2.3. Bacterium

Salmonella Enteritidis and *S.* Heidelberg used in the present study were initially field isolates from poultry farms and were serotyped by the National Veterinary Services Laboratory (Ames, IA, USA). These isolates were selected to resist carbenicillin-novobiocin (C–N) and have been used in our previous studies (He et al., 2012). *Salmonella* stocks were stored in 75% Tryptic Soy Broth (TSB) +25% sterile glycerol at −80 °C until used. The aliquots of the stocks were cultured overnight at 39 °C in BD's TSB, the overnight cultures were diluted at 1:10 into fresh TSB and cultured at 39 °C for 4 h to reach exponential growth phase, and the bacteria were collected using a centrifuge, washed, and suspended in PBS at a final concentration of ~1×10^9 (cfu, colony-forming unit)/ml, determined by colony counts on BD's Difco's xylose-lysine tergitol 4 (XLT4) agar plates containing C–N. HKSE was prepared by incubating the bacterial suspension in a 75 °C water bath for 15 min and verified by overnight culture.

2.4. Cell infection with Salmonella

Culture medium was removed from the HD11 cells and infected with 500 µl of *Salmonella* suspensions (~5×10^8 cfu/ml in plain DMEM) were added to each well with multiplicity of infection (MOI) at about 50:1 and three replicate wells for each serovar and incubated for 1 h at 39 °C in a 5% CO_2 humidified incubator. At 1 h post infection (hpi), the infection medium was removed, and the cells were washed once with plain DMEM, treated with 100 µg/ml of gentamicin sulfate for 30 min to kill extracellular bacteria and then replaced with fresh complete DMEM containing 25 µg/ml of gentamicin sulfate. Treatment of inhibitor W-7 (at 0, 25, 50, 100 µM for NO experiment and 50 µM for *Salmonella* intracellular survival experiment) started at 1 hpi and continued for the duration of the experiment (20 hpi).

Intracellular viable *Salmonella* were determined at 1.5 and 20 hpi as described previously (He et al., 2012). Briefly, infected cells were washed twice with PBS and lysed for 10 min in 1% Triton X-100 (in PBS). Serial 1:10 dilutions of the lysates were plated onto XLT4 agar plates containing C and N and incubated at 39 °C for 24 h. Colonies were counted to determine the cfu of intracellular viable bacteria.

2.5. Nitrite assay

Nitrite, a stable metabolite of NO, produced by activated macrophages was measured by the Greiss assay (Green et al., 1982). Cells in 24-well plates were either infected with *Salmonella* and treated with or without inhibitor W-7 as described above or stimulated with 1 µg/ml of LPS in the presence of W-7 at various concentrations for 20 h at 39 °C in a 5% CO_2 humidified incubator. After 20 hpi with *Salmonella* infection or LPS stimulation, aliquots of 100 µl culture supernatant from each well were transferred to the wells of a new flat-bottom 96-well plate and mixed with 50 µl of 1% sulfanilamide and 50 µl of 0.1% naphthylenediamine (both were prepared in 2.5% phosphoric acid solution) sequentially. After 10 min incubation at room temperature, the nitrite concentration was determined by measuring optical density (OD_{550}) of each well using a microplate reader. Sodium nitrite was used as a standard to determine nitrite concentrations in the cell-free medium.

2.6. Intracellular Ca^{2+} assay

Intracellular Ca^{2+} level was measured by fluorescence using a membrane-permeable calcium indicator, Fura-2-acetoxymethyl ester (Fura-2AM, Molecular Probes Life Technologies, Eugene, OR, USA). Briefly, chicken macrophage HD11 cells at 2×10^6/mL were treated with *Salmonella* as described above for indicated periods (hpi) in 6 well tissue culture treated plates, harvested, and resuspended at 2×10^6 cells/mL in modified Krebs-Ringer-HEPES (KRH) buffer (pH 7.4) containing 0.1% BSA, 118 mM NaCl, 4.6 mM KCl, 24.9 mM $NaHCO_3$, 1.0 mM KH_2PO_4, 11.1 mM glucose, 1.1 mM $MgSO_4$, 5.0 mM HEPES, and 1.0 mM $CaCl_2$ in a 96-well plate (Greiner bio-one Cellstar). The cells were incubated with 2 µM Fura-2AM at 37 °C in 5% CO_2 for 30 min and then the cells were centrifuged at 300 × *g* for 8 min, replaced with fresh KRH buffer, and incubated for an additional 20 min. Fluorescence was measured at 339 nm excitation/505 nm emission using a SpecraMax $M2^e$ plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.7. Peptide array protein phosphorylation analysis

Peptide arrays were made by JPT Peptide Technologies (Berlin, Germany) which contain 771 unique chicken kinase substrate target peptide sequences, derived from phosphorylation sites of 572 proteins were printed in replicate 9 times (Arsenault et al., 2014). Infected cells, in two replicates for each time points, were collected at 1.5, 3, and 7 hpi and stored at − 80 °C. The frozen cells were lysed using 100 ul of lysis buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 µg/ml leupeptin, 1 g/ml aprotinin and 1 mM Phenylmethylsulphonyl fluoride. Lysate was incubated on ice for 10 min then spun in a microcentrifuge at 14,000 g for 10 min at 4 °C. The final protein concentration was measured (Pierce Modified Lowry Protein Assay Kit) and adjusted to 1.5 mg/ml. The peptide array assay was carried out as described (Arsenault et al., 2012). Data were analyzed by the PIIKA2 peptide array analysis software (Trost et al., 2013).

2.8. Data analysis

Three independent experiments were conducted to determine the effect of W-7 on NO production and *Salmonella* intracellular survival. Within each experiment, three replicates were measured for each

treatment. Data were analyzed by One Way ANOVA followed by multiple comparisons (Tukey test) using SigmaStat® software (Jandel Scientific, San Rafael, CA). The value of $p < 0.05$ is considered to be significant.

3. Results and discussion

3.1. Salmonella infection induced significant phosphorylation changes in many members of the Ca^{2+}/CaM signaling pathway in chicken macrophage cells

Protein kinases, along with phosphatases, control protein phosphorylation and regulate signaling pathways and cellular processes involved in nearly every aspect of cell life (Johnson, 2009). This reversible phosphorylation modification can rapidly regulate and fine-tune the protein function and activity in response to environmental signals. *Salmonella* infection has been shown to cause phosphorylation changes in a large number of proteins in mammalian (Rogers et al., 2011; Imami et al., 2013) and chicken (He et al., 2018) macrophages. Most of the changes are induced by cellular defense mechanisms in response to infection; however, *Salmonella* are also known to manipulate the host kinase network to benefit their intracellular survival (Rogers et al., 2011). In the present study, we focused on the effect of *Salmonella* infection on the macrophage Ca^{2+}/CaM signaling pathway using poultry-specific immunometabolism kinome peptide arrays analysis (Arsenault et al., 2014). The results showed that infection with both *Salmonella* Enteritidis and Heidelberg caused significant changes of phosphorylation in many members of the KEGG (http://www.genome.jp/kegg/pathway.html) calcium signaling pathway (Table 1). These proteins with significant phosphorylation change, depicted in the diagram of the calcium signaling pathway (Fig. 1), include growth factor receptors (EGFR and PDGFR), G protein–coupled receptor (HRH1 and GRM1), Na^+/Ca^{2+} exchanger channel protein (SLC8A1), protein kinases PKA, phospholipase C (PLC) and Ca^{2+}/CaM downstream targets, such as Ca^{2+}/CaM-dependent protein kinase (CAMK), protein kinases (PKC and PHK), nitric oxide synthase (iNOS), and ATPase (ATP2B1). CaM acts as a central component of the Ca^{2+}/CaM signaling pathway that transduces extracellular signals to cellular functions through either regulating intracellular Ca^{2+} or as Ca^{2+}/CaM complex directly binding to downstream targets. Mammalian CaM is known to subject to phosphorylation-dependent modulation by multiple kinases (Benaim and Villalobo, 2002). Similarly, our study also showed that chicken macrophage CaM had significantly increased phosphorylation at the conserved serine residue (S82) during the *Salmonella* infection (Fig. 2). The data suggest a significant role of the Ca^{2+}/CaM signaling pathway in the cellular response of chicken macrophages to *Salmonella* infection. Because of the prominent role of CaM in the Ca^{2+}/CaM signaling pathway, its function in chicken macrophage innate immune response, represented by NO production, and in controlling intracellular *Salmonella* replication were further evaluated using its selective inhibitor W-7.

3.2. Calmodulin antagonist W-7 reduced NO response of HD11 cells to microbial stimulation

Nitric oxide is an important innate immune response of chicken macrophage cells to microbial stimulation. It is well established that chicken macrophages are activated to produce NO when exposed to pathogens (Lillehoj and Li, 2004; Okamura et al., 2005; Smith et al., 2005; He et al., 2012) and pathogen associated molecules (He et al., 2006, 2008). Although inducible NOS (iNOS) is less insensitive to Ca^{2+} than other forms of NOS (endothelial eNOS and neuronal nNOS) which are Ca^{2+}/CaM-dependent, iNOS activity requires Ca^{2+} and binding of functional CaM (Spratt et al., 2007). In mouse macrophage cell line RAW 264.7, CaM was found to form a stable complex that is required for rapid activation of iNOS in response to *S.* Typhimurium infection (Smallwood et al., 2006). In the present study, a CaM antagonist W-7 that binds and prevents Ca^{2+}/CaM from interacting with its normal target proteins (Osawa et al., 1998) was used to evaluate the role of CaM in chicken macrophage NO response. Inhibition of CaM with W-7 resulted in a significant dose-dependent reduction in NO production in response to both LPS stimulation and infection by *S.* Heidelberg (Fig. 3), indicating functional CaM is critical for iNOS activity in chicken macrophage cells. Given that *S.* Enteritidis can effectively suppress the NO production in infected HD11 cells (He et al., 2012), the heat-killed *S.* Enteritidis (HKSE) was used instead in this experiment. The result showed that the inhibitor W-7 similarly inhibited the NO production induced by HKSE (Fig. 3). In a previous study (He et al., 2006), depletion of intracellular Ca^{2+} by a selective Ca^{2+} chelator BAPTA-AM was shown to effectively inhibit the LPS induced NO production. Together, these results demonstrated the critical role of Ca^{2+}/CaM in NO immune response to microbial stimulation in chicken macrophage cells.

Table 1
Members of the KEGG calcium signaling pathway displaying significant change in phosphorylation ($p \leq 0.05$) in HD11 cells infected with *Salmonella enterica* serotypes.

S. Enteritidis			*S.* Heidelberg		
1.5 hpi (9.46E-12)	3 hpi (1.51E-09)	7 hpi (1.21E-09)	1.5 hpi (3.84E-09)	3 hpi (3.05E-09)	7 hpi (6.15E-08)*
–	–	ATP2B1	–	ATP2B1	–
CaM	CaM	CaM	CaM	CaM	CaM
CAMK2A	CAMK2A	CAMK2A	CAMK2A	CAMK2A	CAMK2A
–	CAMK2D	CAMK2D	CAMK2D	–	CAMK2D
CAMK2G	CAMK2G	CAMK2G	CAMK2G	CAMK2G	CAMK2G
CHRNA7	CHRNA7	–	–	–	–
EGFR	EGFR	EGFR	EGFR	EGFR	EGFR
–	GRM1	–	–	GRM1	–
HRH1	HRH1	HRH1	HRH1	–	HRH1
MYLK	MYLK	MYLK	MYLK	MYLK	–
–	NOS2	NOS2	NOS2	NOS2	NOS2
PDGFRA	PDGFRA	PDGFRA	PDGFRA	PDGFRA	PDGFRA
PDGFRB	PDGFRB	PDGFRB	–	PDGFRB	PDGFRB
PHKA1	PHKA1	PHKA1	PHKA1	PHKA1	PHKA1
–	PHKA2	–	–	PHKA2	–
PHKB	PHKB	PHKB	–	PHKB	PHKB
–	PHKG1	PHKG1	PHKG1	PHKG1	PHKG1
PLCG1	PLCG1	PLCG1	PLCG1	PLCG1	PLCG1
PLCG2	–	PLCG2	PLCG2	PLCG2	PLCG2
PRKACA	PRKACA	PRKACA	PRKACA	PRKACA	PRKACA
PRKCA	PRKCA	PRKCA	PRKCA	PRKCA	PRKCA
–	PTK2B	PTK2B	PTK2B	PTK2B	PTK2B
SLC8A1	SLC8A1	SLC8A1	SLC8A1	–	SLC8A1

Abbreviation: ATP2B1 (ATPase plasma membrane Ca2+ transporting 1), CaM (calmodulin), CAMK2A (calcium/calmodulin dependent protein kinase II alpha), CAMK2D (calcium/calmodulin dependent protein kinase II delta), CAMK2G (calcium/calmodulin-dependent protein kinase type II gamma), CHRNA7 (cholinergic receptor nicotinic alpha 7 subunit), EGFR (epidermal growth factor receptor), GRM1 (glutamate metabotropic receptor 1), HRH1 (histamine receptor H1), MYLK (myosin light chain kinase), NOS2 (nitric oxide synthase 2), PDGFRA (platelet derived growth factor receptor alpha), PDGFRB (platelet derived growth factor receptor beta), PHKA1 (phosphorylase kinase regulatory subunit alpha 1), PHKA2 (phosphorylase kinase regulatory subunit alpha 2), PHKB (phosphorylase kinase regulatory subunit beta), PHKG1 (phosphorylase kinase catalytic subunit gamma 1), PLCG1 (phospholipase C gamma 1), PLCG2 (phospholipase C gamma 2), PRKACA (protein kinase cAMP-activated catalytic subunit alpha), PRKCA (protein kinase C alpha), PTK2B (protein tyrosine kinase 2 beta), SLC8A1 (solute carrier family 8 member A1).

* *P*-value (FDR)for the KEGG calcium signaling pathway.

3.3. Calmodulin antagonist w-7 increased intracellular survival rate of Salmonella

Macrophages are professional phagocytes that destroy phagocytized bacterial pathogens by exposing them to hydrolytic enzymes produced

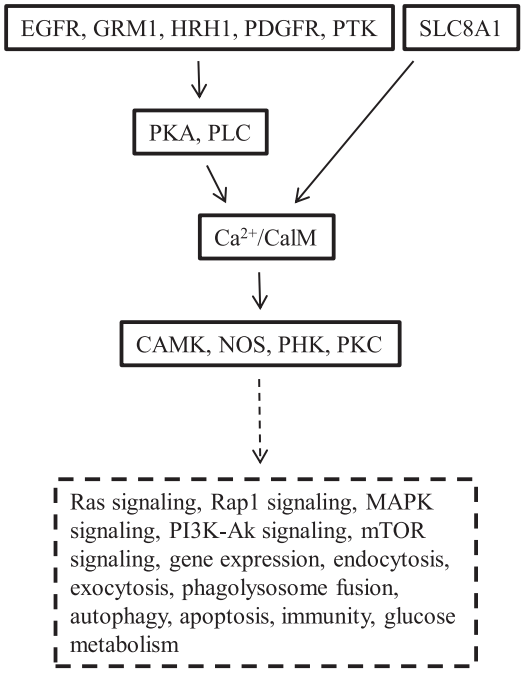

Fig. 1. Schematic depiction of the central role of CaM in the Ca^{2+}/CaM signaling pathway, showing members (solid lined boxes) of the pathway with significantly altered phosphorylation identified by the peptide array analysis. Abbreviation: CaM (calmodulin), CAMK (calcium/calmodulin dependent protein kinase), EGFR (epidermal growth factor receptor), GRM1 (glutamate metabotropic receptor 1), HRH1 (histamine receptor H1), NOS2 (nitric oxide synthase 2), PDGFR (platelet derived growth factor receptor), PHK (phosphorylase kinase), PLC (phospholipase C), PKA (cAMP-dependent protein kinase), PKC (protein kinase C), PTK2 (protein tyrosine kinase 2), SLC8A1 (aka, NCX1, sodium/calcium exchanger 1).

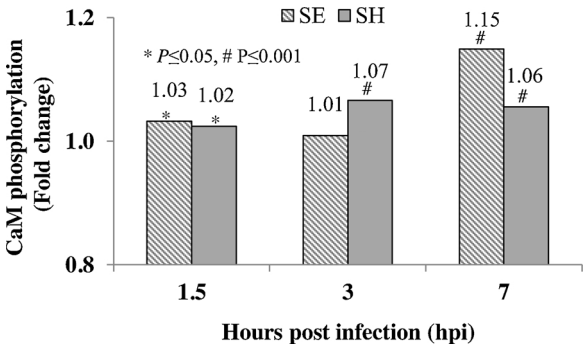

Fig. 2. Phosphorylation changes at the conserved serine residue (S82) of calmodulin (CaM) induced by *Salmonella* infection in chicken macrophage HD11 cells. Cells were infected with *S.* Enteritidis (SE) and *S.* Heidelberg (SH) and samples were harvested at 1.5, 3, and 7 hpi. Infected cells were collected and stored at − 80 °C. The frozen cells were lysed and the phosphorylation changes of proteins in lysed samples were analyzed by the peptide arrays containing unique chicken kinase substrate target peptide sequences. *P*-values and fold changes of the CaM phosphorylation were calculated from comparison of infected calls to uninfected cells at respective time point.

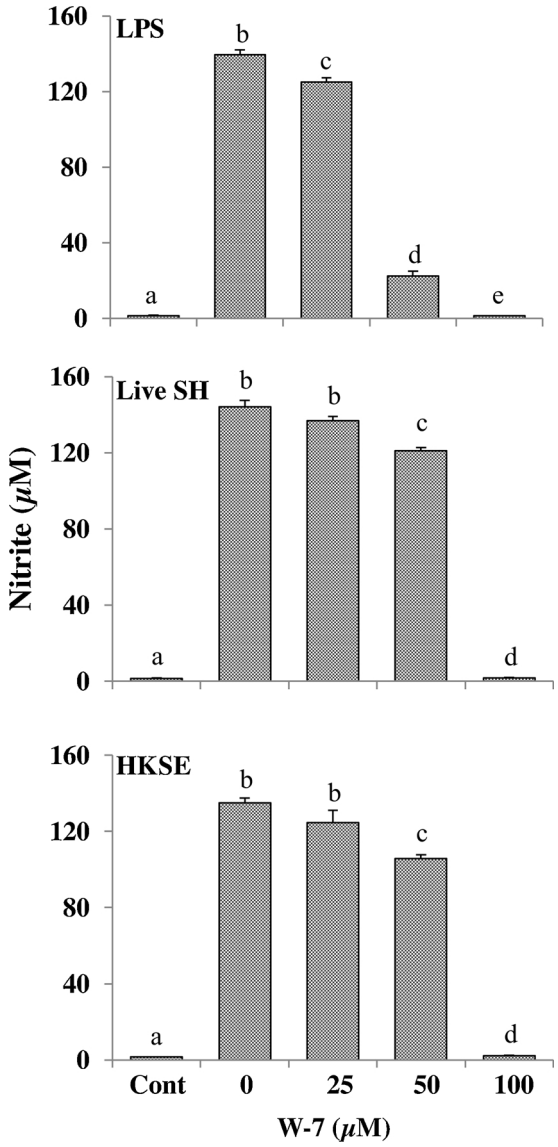

Fig. 3. Effect of the selective CaM inhibitor W-7 on nitric oxide (NO) production in chicken macrophage cell HD11 induced by lipopolysaccharide (LPS), live *S.* Heidelberg (SH), and heat-killed *S.* Enteritidis (HKSE). Cells in 24-well plates were either infected with *Salmonella* or stimulated with LPS (1 μg/ml) in the absence or presence of W-7 at various concentrations with (0, 25, 50, and 100 μM) as indicated for 20 h at 39 °C in a 5% CO_2 humidified incubator. Negative control cells (Cont) were not treated. The nitrite concentrations in the cell culture supernatants were determined by measuring optical density (OD_{550}) using a microplate reader. Different letters indicate that the difference between the treatments is statistically significant ($p \leq 0.05$).

by lysosomes (Poirier and Av-Gay, 2015). It has been well established that phagosome-lysosome fusion is CaM-dependent (Colombo et al., 1997; Malik et al., 2001; Yates et al., 2005). Inhibition of CaM prevents phagosome-lysosome fusion, resulting in enhanced survival of intracellular *Mycobacterium tuberculosis* in human macrophages (Malik et al., 2001). To evade the destruction by the host cells, *Salmonella* have developed a highly complex type III secretion system (T3SS) that produces virulence effectors to delay phagolysosomal fusion, thereby enabling some bacteria to avoid exposure to lysosomal contents and facilitating their survival and replication within *Salmonella*-containing vacuoles (SCV) (Haraga et al., 2008). Although chicken macrophages seem able to express bactericidal activity and to a certain degree to limit replication of intracellular *Salmonella*, many different strains of *Salmonella* can survive inside chicken macrophage cells (He et al., 2012). Because of the role of CaM in lysosome fusion process (Malik et al., 2001; Stockinger et al., 2006), we hypothesized that a functional CaM is required for controlling the intracellular *Salmonella* replication in chicken macrophages. To test the hypothesis, we used the CaM antagonist W-7 to treat the *Salmonella*-infected chicken macrophage HD11 cells and evaluated its effect on the survival of intracellular *Salmonella*. Indeed, treating infected macrophage cells with W-7 at 50 μM resulted in significantly increased intracellular survival of both *Salmonella*

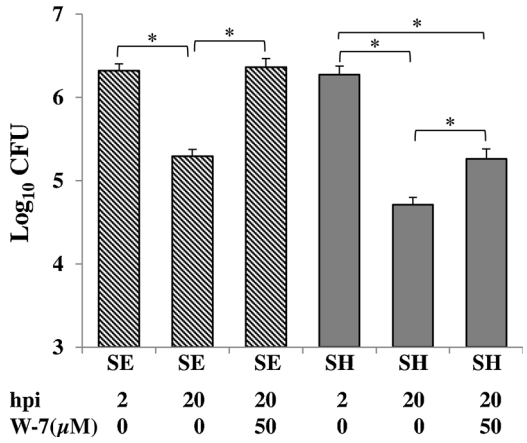

Fig. 4. Effect of the selective CaM inhibitor W-7 on intracellular survival of *S.* Enteritidis (SE) and *S.* Heidelberg (SH) at 20 h post infection (hpi). Cells were infected with *Salmonella* for 1 h at 39 °C in a 5% CO_2 humidified incubator. At 1 h post infection (hpi), extracellular bacteria were killed with 100 μg/ml of gentamicin sulfate for 30 min and then incubated in the fresh complete DMEM containing 25 μg/ml of gentamicin sulfate for the duration of the experiment (20 hpi). At 1.5 and 20 hpi, infected cells were lysed in 1% Triton X-100 (in PBS) for 10 min. Intracellular viable *Salmonella* were determined by plating serial 1:10 dilutions of the lysates on XLT4 agar plates containing carbonicillin-novobiocin at 39 °C for 24 h. Colonies were counted to determine the colony-forming unit (cfu) of intracellular viable bacteria. Symbol (*) indicates that the difference between the treatments is statistically significant *($p \leq 0.05$)*.

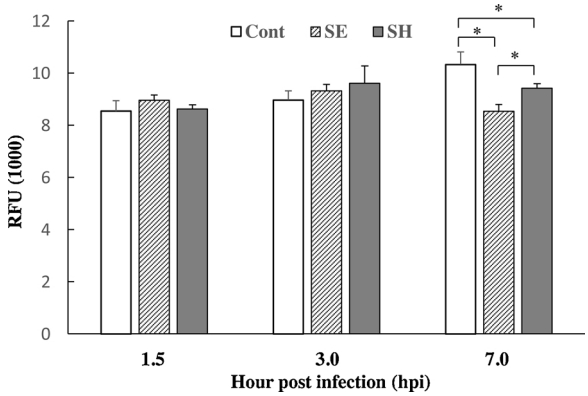

Fig. 5. Effect of *Salmonella* infection on intracellular Ca^{2+} level. Intracellular Ca^{2+} level was measured by fluorescence using a membrane-permeable calcium indicator, Fura-2-acetoxymethyl ester (Fura-2AM). Cells at 2×10^6/mL were infected with *Salmonella* for indicated periods (hpi) in 6 well plates, harvested, and resuspended at 2×10^6 cells/mL in modified Krebs-Ringer-HEPES (KRH) buffer (pH 7.4). The cells were incubated with 2 μM Fura-2AM at 39 °C in 5% CO_2 for 30 min, washed once with fresh KRH buffer, and incubated in KRH buffer for an additional 20 min. Fluorescence intensity of cells was measured at 339 nm excitation/505 nm emission as relative fluorescence unit (RFU) using a SpecraMax M2^e plate reader. Symbol (*) indicates that the difference between the treatments is statistically significant ($p \leq 0.05$). Cont, negative control; SE, *S.* Enteritidis; and SH, *S.* Heidelberg.

strains (Fig. 4). This observation suggests that disruption of the CaM function may interrupt the phagosome-lysosome fusion process; thereby diminishing the lysosome mediated bactericidal activity and improving the survival of intracellular *Salmonella*. Since NO is bactericidal and has been implicated in controlling intracellular *Salmonella* (Smallwood et al., 2006), inhibition of iNOS activity by the CaM antagonist W-7 may also contribute to diminished ability of chicken macrophage to control the replication of intracellular *Salmonella*.

3.4. Effect of Salmonella infection on intracellular Ca^{2+}

Interaction of bacteria with host cells has been shown to induce oscillation of intracellular Ca^{2+}. Several bacterial toxins can induce release of the intracellular Ca^{2+} from storage or form pores on the cell membrane to increase the influx of Ca^{2+} (Tran Van Nhieu et al., 2004). Bacterial structural molecules (e.g. LPS and flagellin) are also known to induce Ca^{2+} influx, which in turn leads to activation of NF-κB and genes of inflammatory immune response (Liu et al., 2016; Chen et al., 2007). For some bacterial pathogens, e.g. *Shigella* (Tran Van Nhieu et al., 2013), *Salmonella* (Pace et al., 1993), enteropathogenic *E. coli* (Brown et al., 2008), *Campylobacter* (Hu et al., 2005), and *Listeria* (Dramsi and Cossart, 2003), studies have shown that transient increase of intracellular Ca^{2+} is necessary for the cytoskeleton reorganization to facilitate bacterial internalization and intracellular trafficking. However, the dependence of *Salmonella* invasion on an increase of intracellular Ca^{2+} has been challenged in a later study in which mutant *S.* Typhimurium demonstrated higher levels of bacterial internalization and induction of inflammatory cytokine IL-8 without increase of intracellular Ca^{2+} (Figueiredo et al., 2009). In the present study, intracellular Ca^{2+} was not affected during early infection with both *Salmonella* strains and significant reduction of intracellular Ca^{2+} was observed at 7 hpi (Fig. 5). The Ca^{2+} homeostasis in the host cells is tightly controlled and influx of Ca^{2+} has been shown to promote inflammatory immune response (Gewirtz et al., 2000; Ye et al., 2012). In chicken macrophage cell HD11, depletion of intracellular Ca^{2+} has been reported to inhibit NO response to microbial agonist stimulation (He at al., 2006, 2008). Therefore, the reduction of intracellular Ca^{2+} may dampen the antibacterial inflammatory response and create an environment to be advantageous for *Salmonella* survival once inside the host macrophages. Because of the important role of Ca^{2+} in bacterial infection, there is a significant interest in using Ca^{2+} release inhibitors and antagonists as possible treatments for a variety of infectious diseases, or as a co-therapy with current antimicrobial measures (Clark et al., 2013). Our data shown that disruption of Ca^{2+}/CaM leads to diminished control of *Salmonella* replication inside the chicken macrophage HD11 cells, indicating that certain downstream elements of Ca^{2+}/CaM signaling must have played a critical role in controlling the replication of *Salmonella*.

In conclusion, *Salmonella* infection induced significant phosphorylation changes in many members of the Ca^{2+}/CaM signaling pathway, suggesting significant involvement of the pathway in chicken macrophage response to the infection. Inhibition of CaM, a central player of the Ca^{2+}/CaM signaling pathway, resulted in diminished NO production in response to *Salmonella* infection and weakened control of intracellular replication of *Salmonella*. Our data strongly support that CaM and likely many of CaM-dependent proteins are critically involved in controlling the fate of intracellular *Salmonella* in chicken macrophages. Identifying the key controlling element may provide useful information for designing novel intervention strategies that target the Ca^{2+}/CaM signaling pathway to reduce or eliminate colonization and systemic carriage of *Salmonella* in poultry.

Acknowledgements

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