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***Salmonella enterica* serovar Typhimurium-induced internalization and IL-8 expression in HeLa cells does not have a direct relationship with intracellular Ca²⁺ levels**

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Abstract

The invasion-associated type III secretion system (T3SS-1) of *S. Typhimurium* is required to initiate and sustain an acute inflammatory response in the intestine. We investigated the relationship of *S. Typhimurium* T3SS-1-induced IL-8 expression and invasion with intracellular Ca²⁺ mobilization in HeLa cells. Compared to the *sipAsopABDE2* mutant, strains carrying a mutation in *sipA*, or mutations in *sopABDE2* induced higher levels of IL-8 and greater bacterial internalization despite the fact that these mutants elicited similarly low intracellular concentrations of Ca²⁺. Likewise, complemented *sipAsopABDE2* mutant with *sopE2* did not affect intracellular Ca²⁺ concentrations or IL-8 expression, but significantly increased bacterial internalization. Treating HeLa cells with the calcium chelator BAPTA-AM or with D-BAPTA-AM, a derivative with greatly reduced Ca²⁺ chelating activity, yielded strong evidence that BAPTA-AM does not affect invasion and inhibits IL-8 secretion by a calcium-dependent mechanism. These findings suggest that, although wild-type *S. Typhimurium*-induced IL-8 expression and bacterial internalization in HeLa cells coincides with increased cytosolic Ca²⁺, the differing levels of IL-8 and invasion induced by strains carrying different effector proteins are unrelated to levels of intracellular Ca²⁺.

Keywords

Salmonella enterica serovar Typhimurium; sipA; calcium; IL-8; T3SS-1

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1. Introduction

The *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) effector proteins SipA, SopA, SopB, SopD and SopE2, translocated through the *Salmonella* Pathogenicity Island-1 (SPI-1) encoded type III secretion system (T3SS-1), play important roles in generating an inflammatory response in the bovine ligated ileal loop model. A *S. Typhimurium* strain carrying mutations in *sipA*, *sopA*, *sopB*, *sopD* and *sopE2* causes 60% less inflammatory responsiveness and decreased fluid accumulation relative to wild type infected loops. On the other hand, mutations in the *slrP*, *avrA*, *sspH1* or *sptP* genes that encode other T3SS-1 effector proteins did not reduce fluid accumulation or the degree of inflammation in bovine ligated ileal loops. The degree of attenuation observed in a *sipAsopABDE2* mutant was similar to that of a *S. Typhimurium sipB* mutant [1], a strain deficient for translocating T3SS-1 effector proteins into host cells. These findings suggest that SipA, SopA, SopB, SopD and SopE2 are the major effector proteins required for *S. Typhimurium*-induced enteritis in calves.

S. Typhimurium T3SS-1-translocated effector proteins, SipA, SopA, SopB, SopD and SopE2, induce cytoskeletal rearrangements leading to bacterial internalization [2]. SopA associates with a host ubiquitin E3 ligase inducing bacterial escape into the cytosol of epithelial cells [3]. SopE2, is a guanine nucleotide exchange factor [4], and SopB, is an inositol phosphate phosphatase [5]. Phosphatidylinositol phosphates (generated by SopB) and activated Rho-family GTPases (produced by SopE2) act in concert to activate WASP/Scar proteins, which in turn recruit the Arp2/3 complex to initiate the formation of new branches on actin filaments [6]. SopD acts cooperatively with SopB to promote membrane fission and macropinosome formation during the invasion process [7]. SipA, which is delivered into host cells as quickly as 10 sec after exposure [8], localizes in the host cell plasma membrane [9] where it acts as an actin-binding protein [10].

Once in contact with host cells, *S. Typhimurium* was found to alter cytosolic Ca^{2+} concentrations within 2 to 4 minutes after infection [11-14]. Ca^{2+} is an important intracellular messenger involved in many cellular functions, including vesicular trafficking [15], cytoskeletal rearrangements [16] and gene expression [17]. Therefore, changes in intracellular Ca^{2+} level, depending on the amplitude of transients, may influence different cell signaling pathways [18].

Other bacterial pathogens have been found to alter Ca^{2+} homeostasis in host cells. Although intracellular Ca^{2+} has been implicated in mediating cytoskeletal rearrangements, the dependence of bacterial invasion on changes in Ca^{2+} fluxes is controversial. Invasion of *Shigella flexneri* in epithelial cells induces an increase in intracellular Ca^{2+} that is dependent on a functional T3SS, but cytoskeletal rearrangements are also observed in cells with no detectable Ca^{2+} response [19]. *Listeria monocytogenes* and *Campylobacter jejuni* induce intracellular Ca^{2+} changes in host cells [20,21]. While chelation of intracellular Ca^{2+} interferes with *C. jejuni* internalization [20], it has no effect on *L. monocytogenes* invasion [21]. Previous studies suggest that *S. Typhimurium* invasion is dependent on the induction of cytosolic Ca^{2+} increases [12], and that chelation of Ca^{2+} inhibits bacterial internalization [22]. However, a non-invasive *S. Typhimurium* $\Delta hila$ mutant induces cytosolic Ca^{2+} changes [11], indicating that host cell internalization is not a prerequisite for inducing Ca^{2+} mobilization.

Because Ca^{2+} is central to many cell signaling events, including the expression of IL-8 and the induction of cytoskeletal changes, and the *S. Typhimurium* effector proteins SipA, SopA, SopB, SopD and SopE2 induce inflammatory responses and bacterial invasion, we focused our studies on the role(s) of intracellular Ca^{2+} in *S. Typhimurium*-induced invasion and the induction of IL-8 expression.

2. Materials and Methods

2.1. Cell line and bacteria strains

To correlate cytosolic calcium changes with IL-8 expression, bacterial internalization, and effector proteins, we used HeLa S3 cells, in which, as opposed to T84 cells, different profiles of responses to infection of *S. Typhimurium* wild type and mutant can be detected [23], therefore, questions related to specific effector proteins can be addressed. HeLa S3 cells (human cervical epithelial cells – ATCC) were grown in Kaighn's modification of Ham's F12 medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum. Before performing assays, HeLa cells were grown to a concentration of 0.2×10^5 cells/cm² in 24 well polystyrene plates (Corning, Acton, MA), or in 0.1 mg of collagen type I-pretreated Coverglass Chambers (Nalge Nunc International, Rochester, NY) for 48 hr. Strains of bacteria used in this study are listed in Table 1. *S. Typhimurium* strains were grown in Luria-Bertani (LB) medium overnight with nalidixic acid 50 mg/liter, then sub-cultured in LB for 4 hr [1].

2.2. Gene expression

Expression levels of IL-8 in *S. Typhimurium* infected cells were measured by quantitative real time-PCR (qRT-PCR). HeLa cells were infected with *Salmonella* strains (Table 1) at multiplicity of infection (MOI) 200 [24,25] for 1 hr. After infection, total RNA was extracted and reverse transcription was performed using the Cells to cDNA kit (Applied Biosystem, Foster City, CA). The qRT-PCR was performed in a Smart Cycler II (Cepheid, Sunnyvale, CA) by mixing the cDNA with OmniMix beads (Cepheid, Sunnyvale, CA), SYBG Green I 1× (Invitrogen Corporation, Carlsbad, CA) and 500 nM of each primer set (IL-8 forward primer: AAGGAACCATCTCACTGTGTGTAAAC, IL-8 reverse primer: ATCAGGAAGG CTGCCAAGAG). The cDNA concentration of each sample was normalized to an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GAPDH forward primer: GAAGGTGAAGGTCGGAGTC, GAPDH reverse primer: GAAGATGGTGATGGGATTTC). The normalized levels of gene expression in infected cells were calculated relative to uninfected cells as previously described [26]. The effect of an intracellular Ca²⁺ chelator on gene expression was performed by treating HeLa cells with 50 μM BAPTA-AM (Calbiochem, San Diego, CA), or D-BAPTA-AM (provided by Dr. Rousseau, CEA/Saclay, France) in HBSS media for 45 min [11] prior to *Salmonella* infection. Gene expression analysis of BAPTA-AM treated cells was performed as described above.

Expression levels of *sipA* in bacteria culture were measured by qRT-PCR as described above, using *sipA* specific primers (SipA forward primer: AAGATTGCTGCGGGTTAACG, SipA reverse primer: TGGCTGCCAGAAACAA AGAA). *S. Typhimurium* strains were grown overnight and sub-cultured for 4 hr. *S. Typhimurium* RNA was extracted using the SV total RNA isolation kit (Promega Corporation, Madison, WI). The cDNA concentration of each sample was normalized to an internal control, *rrsB* 16S ribosomal RNA (RrsB forward primer: GGCAGGCCTAACACATGCA, RrsB reverse primer: CTTGCGACGTTATGCGG TATT). The normalized levels of gene expression in *sipA**sopABDE2* mutant complemented with *sipA* and *sopABDE2* mutant were calculated relative to *S. Typhimurium* wild type [26].

2.3. Bacterial protein analysis

Bacteria were grown in LB medium overnight with appropriate antibiotics [1] then sub-cultured in LB for 4 hr. The bacteria cultures were centrifuged, and the bacteria extracts were obtained by lysing cells with 20 mM MOPS, pH 7.0, 0.5% Triton X-100, and protease inhibitor mixture. The homogenates were sonicated twice for 15 sec, centrifuged for 30 min at $16,000 \times g$, and the supernatant removed for further assays. The protein concentration of bacteria cell extracts was determined using a commercial Bradford assay (Sigma, St. Louis, MO). An equal amount

of protein obtained from bacteria cell extract was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to immobilon transfer membrane (Millipore Corporation, Bedford, MA), and subsequently reacted with mouse anti-*S. Typhimurium* SipA antibody (Center for Innovations in Medicine, Biodesign Institute, Arizona State University, Tempe, Arizona), and anti-mouse peroxidase secondary antibody (Sigma, St. Louis, MO).

2.4. Invasion assay

HeLa cells were infected with *Salmonella* strains (Table 1) at MOI 200 [24,25] for 1 hr. After infection, plates were centrifuged for 5 min at 1000 rpm and incubated at 37°C for 1 hr. Cells were washed three times with 500 µl/well of phosphate buffer saline (PBS) and treated with 100 µg/ml of gentamicin (Invitrogen Corporation, Carlsbad, CA) for 1 hr at 37°C. After antibiotic treatment, cells were washed again with PBS and incubated with 1% Triton X-100 for 5 min at 37°C. The number of internalized bacteria was determined by plating ten-fold serial dilutions of the cell lysates in nalidixic acid containing LB plates. To analyze the effect of BAPTA-AM on bacteria internalization, cells were treated with 50 µM BAPTA-AM (Calbiochem, San Diego, CA), or D-BAPTA-AM in HBSS media for 45 min [11] followed by *Salmonella* infection, and an invasion assay was performed as described above.

2.5. Intracellular Ca²⁺ measurements

The intracellular Ca²⁺ levels were assessed by confocal microscopy as described previously [18,27,28]. HeLa cells grown on Coverglass Chamber slides (Nalge Nunc International, Rochester, NY) were loaded with 3 µM of Fluo-4 (Invitrogen Corporation, Carlsbad, CA) for 1 hr at 37°C and washed with serum- and phenol red-free medium. Fluo-4 loaded epithelial cells were then examined with Meridian Ultima confocal microscope using 488 nm wavelength for excitation and 530 nm for emission. Basal intracellular Ca²⁺ level was obtained during the first four scans, then the cells were infected with *S. Typhimurium* strains and Ca²⁺ was monitored for 5 min. We examined cells using differential interference contrast and confocal microscopy immediately after infection, and we were able to readily detect bacterial contact with the cell surface (data not shown). The changes in intracellular Ca²⁺ after infection at any time (t) were normalized to basal Ca²⁺ level at time (0). Cells treated with 10 µM thapsigargin (Sigma, St. Louis, MO) were used as a positive Ca²⁺ control and PBS as a negative control. To verify that the magnitude of intracellular Ca²⁺ responses is biologically meaningful, absolute concentrations of cytosolic Ca²⁺ were assessed using the Ca²⁺ calibration kit #2 (Invitrogen Corporation, Carlsbad, CA). To chelate intracellular Ca²⁺, HeLa cells were treated with 50 µM BAPTA-AM (Calbiochem, San Diego, CA), or D-BAPTA-AM in HBSS media for 45 min [11] followed by *Salmonella* infection.

2.6. Statistical analysis

To analyze the effects of *S. Typhimurium* effector proteins on intracellular Ca²⁺, induction of gene expression, and invasion in HeLa cells, geometric means and standard deviations were determined and statistical significance was calculated using Student's t-test or one-way ANOVA followed by Tukey's multiple comparison test at $P < 0.05$.

3. Results and discussion

3.1. Role of T3SS-1 effector proteins, especially SipA and SopE2, in *S. Typhimurium* induced cellular responses in HeLa cells

Infesting HeLa cells with a series of defined *S. Typhimurium* mutants defective in secreting individual or combinations of T3SS-1 effector proteins demonstrated that the *S. Typhimurium* effector proteins SipA, SopA, SopB, SopD and SopE2 act in concert to trigger IL-8 expression

in HeLa cells, as previously demonstrated [23]. As shown in Fig. 1A, strains carrying *sipA* (*sopABDE2* mutant and *sipAsopABDE2* mutant complemented with plasmid encoding *sipA*), or strains lacking *sipA*, but carrying the genes *sopA*, *sopB*, *sopD* and *sopE2* induced higher levels of IL-8 expression than strains lacking the genes encoding all five effector proteins (*sipAsopABDE2* mutant). Previously, we have shown, that induction of IL-8 expression detected by qRT-PCR leads to secretion of IL-8 [23]. Most observations on *S. Typhimurium* invasion of HeLa cells paralleled those on triggering IL-8 expression. The *sipAsopABDE2* mutant complemented with a plasmid encoding *sipA*, the *sopABDE2* mutant, and the *sipA* mutant invaded HeLa cells at significantly higher levels than the *sipAsopABDE2* mutant (Fig. 1B). However, the *sipAsopABDE2* mutant complemented with a plasmid encoding *sopE2* did not induce IL-8 expression, but it partially complemented the invasion defect of the *sipAsopABDE2* mutant (Fig. 1B).

3.2. *S. Typhimurium*-induced cellular response is accompanied by but is not proportional to changes in intracellular Ca^{2+}

Because bacteria-host cell interactions induce Ca^{2+} responses in the early stages of infection [11-14,19-21], and intracellular Ca^{2+} is involved in the activation of many cell signaling pathways [15-17], we investigated whether the level of Ca^{2+} is proportional to the level of invasion and gene expression in *S. Typhimurium* infected HeLa cells. We found that the *S. Typhimurium* wild type significantly increased the intracellular Ca^{2+} concentration (from resting $[\text{Ca}^{2+}] \sim 70\text{nM}$ to $\sim 519\text{nM}$) at 30 sec after infection (Fig. 2A and 2B), which has been previously shown [11-14]. Changes in the Ca^{2+} concentration were also observed when cells were infected with other *S. Typhimurium* strains (Fig. 2C). However, *S. Typhimurium* wild type increased intracellular Ca^{2+} concentrations in HeLa cells at significantly higher levels than the *sipAsopABDE2* mutant. Complementation of *sipAsopABDE2* mutant with the cloned *sipA* gene increased intracellular Ca^{2+} concentrations at levels similar to that induced by wild type (Fig. 2C). The *sipAsopABDE2* mutant complemented with *sopA* induced intermediate levels of Ca^{2+} . On the other hand, the *sipAsopABDE2* mutant complemented with *sopD*, the *sipA* mutant, and the *sopABDE2* mutant induced lower levels of Ca^{2+} as compared to *sipAsopABDE2* mutant (Fig. 2C). The reduced Ca^{2+} response induced by *sipA* mutant, *sopABDE2* mutant and *sopD* complemented strains were unexpected and to further understand it, experiments in parallel with SPI-1 mutant using multiple mutants will need to be performed. The current study, however, was focused on finding whether levels of Ca^{2+} are responsible for the different levels of IL-8 and invasion induced by several *S. Typhimurium* strains.

Compared to the *sipAsopABDE2* mutant, strains carrying a mutation in *sipA*, or mutations in *sopABDE2* induced higher levels of IL-8 and greater bacterial internalization despite the fact that these mutants elicited similarly low intracellular concentrations of Ca^{2+} . Likewise, the *sipAsopABDE2* mutant complemented with *sopE2* induced higher levels of bacterial internalization while the intracellular concentrations of Ca^{2+} remained low (Table 2). These findings suggest that the level of Ca^{2+} induced was not proportional to the levels of IL-8 expression and invasion induced by each *S. Typhimurium* strain.

In summary, infection of HeLa cells with *S. Typhimurium* was generally accompanied by a significant increase in the intracellular Ca^{2+} concentration when compared to uninfected cells, which averaged approximately 70nM; however, the cellular events (invasion and IL-8 expression) induced by different *S. Typhimurium* strains are more likely due to the presence of effector proteins (SipA or SopE2 or combination of SopA, SopB, SopD and SopE2) rather than the levels of Ca^{2+} (Table 2).

3.3. Analysis of SipA expression

We performed additional experiments to investigate the discrepancies regarding alterations in Ca^{2+} generated by strains carrying the *sipA* gene cloned on a low copy number plasmid (*sipAsopABDE2* mutant complemented with *sipA*) and a strain carrying a chromosomal copy of *sipA* (*sopABDE2* mutant). qRT-PCR indicated that *sipA* mRNA levels were similar in the *sopABDE2* mutant and the *S. Typhimurium* wild type. In contrast, the *sipAsopABDE2* mutant complemented with the cloned *sipA* gene expressed *sipA* at 177.5-fold higher levels than the wild type (Fig. 3A). Western blot analysis of the bacteria whole cell lysate confirmed a higher level of SipA in *sipAsopABDE2* mutant complemented with *sipA* than in *sopABDE2* mutant and in wild type (Fig. 3B). The higher level of Ca^{2+} found in cells infected with *sipAsopABDE2* mutant complemented with *sipA* may be due to the artificially increased expression of *sipA* by cloning the gene on a plasmid. Extracellular addition of SipA (up to 40 μg), however, failed to mobilize Ca^{2+} (data not shown). Interestingly, it has been found that SipA is delivered into host cells through T3SS-1 beginning at 10-90 sec after bacteria-cell contact [8], which may explain the SipA-induced increases in cytosolic Ca^{2+} levels at 30 sec after infection. Immediately after cell contact, the *sipAsopABDE2* mutant complemented with *sipA* may secrete higher amounts of SipA into the host cell, thereby triggering higher levels of cytosolic Ca^{2+} responses.

While high levels of SipA (expressed from *sipAsopABDE2* mutant complemented with *sipA*) induced Ca^{2+} responses at wild type levels, IL-8 expression and IL-8 secretion [23] were observed when *sipA* was present either on the chromosome (low levels of SipA) or on a plasmid (high levels of SipA). As mentioned previously, the Ca^{2+} response occurs immediately after bacteria-cell contact [11-14,19-21], whereas IL-8 expression was detected at 1 hr post infection. Thus, even low concentrations of internalized SipA or other *S. Typhimurium* proteins (SopA, SopB, SopD and SopE2) are able to interact with host cell signaling molecules and induce IL-8 expression at relatively low levels of cytosolic Ca^{2+} .

3.4. Effect of BAPTA-AM on *S. Typhimurium* pathogenesis

Previous studies indicated that chelating intracellular Ca^{2+} with BAPTA-AM inhibits *S. Typhimurium* invasion and IL-8 expression, suggesting that those cellular responses are mediated through changes in Ca^{2+} homeostasis [11,22]. We tested the effect of BAPTA-AM on *S. Typhimurium* invasion and IL-8 gene expression. Treating HeLa cells with 50 μM of BAPTA-AM had no significant effect on *S. Typhimurium* internalization (Fig. 4A), which was consistent with results from a previous study demonstrating that 10 μM of BAPTA-AM had no effect on *S. Typhimurium* or *L. monocytogenes* invasion in Hep2 cells [21]. It should be mentioned that the use of BAPTA-AM to study bacteria internalization has yielded conflicting results, as others have found that 200 μM of BAPTA-AM reduces *S. Typhimurium* invasion by 87% in HeLa cells [22], and also affects *C. jejuni* internalization in human intestinal cells [20].

It has been previously demonstrated that *S. Typhimurium* stimulates Ca^{2+} mobilization in epithelial cells [11-14], which was required to activate IL-8 production, yet increases in Ca^{2+} levels and IL-8 secretion were absent in *S. Typhimurium* infected T84 cells previously treated with BAPTA-AM [11]. In agreement with these studies [11-14], we found that *S. Typhimurium* alters Ca^{2+} levels as early as 30 sec after infection, and that the use of BAPTA-AM inhibited cytosolic Ca^{2+} transients and *S. Typhimurium* and thapsigargin-induced IL-8 expression in HeLa cells (Fig. 4B). Several other studies have demonstrated, however, that BAPTA-AM can interfere with cell signaling events independently of changes in the Ca^{2+} concentration [29,30]. In different cell types, 10-50 μM of BAPTA-AM acts on actin and microtubule disassembly and affects mitochondrial function independent of its Ca^{2+} chelating activity [30]. Additionally, BAPTA-AM has a dose-dependent-neurotoxic effect on primary

neuronal cells, reduces global protein synthesis and induces an endoplasmic reticulum (ER)-specific stress response by influencing the ER Ca^{2+} homeostasis [29]. Therefore, the use of BAPTA-AM to elucidate the role of Ca^{2+} on cell signaling pathways may lead to controversial results not only due to dose dependent effects, but also due to non-specific effects on metabolic pathways.

To test whether down-regulation of IL-8 in BAPTA-AM-treated cells is due to reduced levels of Ca^{2+} or BAPTA-AM-side effects, we treated HeLa cells with BAPTA-AM or D-BAPTA-AM, a BAPTA-AM derivative lacking one acetic acid group with reduced Ca^{2+} chelating activity [30]. As shown previously [30], BAPTA-AM but not D-BAPTA-AM inhibited thapsigargin-induced Ca^{2+} transients (data not shown). Treating HeLa cells with either D-BAPTA or BAPTA-AM reduced *S. Typhimurium*-induced IL-8 expression (Fig. 4C), indicating that down-regulation of IL-8 in BAPTA-AM treated cells is due to effects of this reagent unrelated to its ability to chelate Ca^{2+} .

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Figure 1.

(A) qRT-PCR of IL-8 gene in uninfected (negative control), thapsigargin treated (positive control), and *S. Typhimurium*-infected HeLa cells. (B) Invasion of *S. Typhimurium* strains in HeLa cells. Values represent mean $\pm$ standard deviation of at least three experiments. Asterisk

indicates significant difference from *S. Typhimurium sipAsopABDE2* mutant at $P < 0.05$. WT: *S. Typhimurium* wild type.

Before infection

After infection

Figure 2.

Evaluation of intracellular Ca^{2+} in HeLa cells by confocal microscopy. (A) An example of *S. Typhimurium* wild type-induced intracellular Ca^{2+} transients. The dotted vertical line indicates the time of addition of *S. Typhimurium* wild type. (B) Changes in cytosolic Ca^{2+} before *S. Typhimurium* wild type infection (left) and after infection (right). (C) Fluorescence intensity of Fluo-4 in *S. Typhimurium* infected HeLa cells. The changes in intracellular Ca^{2+} after infection at any time (t) were normalized to basal Ca^{2+} level at time (0). Values represent mean normalized fluorescence intensity $\pm$ SEM induced following addition of each treatment. For each experiment, data were recorded from 10 to 15 cells per well and 4 wells per treatment were analyzed. Two to four replicate experiments per treatment on separate days were performed. Comparable results were obtained in replicate experiments. Different letters indicate significant difference at $P < 0.05$. WT: *S. Typhimurium* wild type.

Figure 3.

Comparison of SipA expression in the *sopABDE2* mutant and the *sipAsopABDE2* mutant complemented with *sipA*. (A) qRT-PCR of *sipA* in cultures of *S. Typhimurium* WT, *sopABDE2* mutant and *sipAsopABDE2* mutant complemented with *sipA*. Values represent mean $\pm$ standard deviation of at least three experiments. Asterisk indicates significant difference in *sipA* level compared to other strains at $P < 0.05$. (B) Western blot analysis using anti-SipA antibody of proteins present in crude lysates of *S. Typhimurium* strains. WT: *S. Typhimurium* wild type; lane 1: *S. Typhimurium* wild type; lane 2: *S. Typhimurium sipAsopABDE2* mutant; lane 3: *S. Typhimurium sopABDE2* mutant; lane 4: *S. Typhimurium sipAsopABDE2* mutant complemented with *sipA*; and lane 5: molecular marker.

Figure 4.

Analysis of *S. Typhimurium* pathogenesis in BAPTA-AM treated HeLa cells. (A) Invasion with or without BAPTA-AM treatment. (B) *S. Typhimurium*-induced IL-8 gene expression. (C) *S. Typhimurium*-induced IL-8 expression with or without BAPTA-AM, or D-BAPTA-AM treatment. Values represent mean $\pm$ standard deviation of at least three experiments. WT: *S. Typhimurium* wild type.

Table 1*S. Typhimurium* strains and plasmid used in this study.

Strains or plasmids	Genotypes	References
<i>S. Typhimurium</i> strains		
IR715	ATCC 14028 wild type, <i>nal^r</i>	
ZA10	ATCC 14028 <i>nal^rΔsipA</i>	[1]
ZA21	ATCC 14028 <i>nal^rΔsipAΔsopAsopBDE2</i>	[1]
ZA20	ATCC 14028 <i>nal^rΔsopAsopBDE2</i>	[1]
Plasmids		
pWSK29	Cloning vector	[2]
pMR15	pWSK29 carrying the <i>sopD</i> gene	[2]
pMR17	pWSK29 carrying the <i>sopE2</i> gene	[2]
pMR26	pWSK29 carrying the <i>sopB</i> gene	[2]
pMR28	pWSK29 carrying the <i>sopA</i> gene	[2]
pMR29	pWSK29 carrying the <i>sipA</i> gene	[2]

Table 2Activation of Ca²⁺ response, IL-8 expression and invasion in HeLa cells by different *S. Typhimurium* strains

<i>S. Typhimurium</i> strains	Ca ²⁺ response	IL-8 expression	Invasion
IR715	+	+	+
<i>sipA</i> mutant	-	+	+
<i>sopABDE2</i> mutant	+/-	+	+
<i>sipAsopABDE2</i> mutant complemented with <i>sipA</i>	+	+	+
<i>sipAsopABDE2</i> mutant complemented with <i>sopA</i>	+/-	+/-	+/-
<i>sipAsopABDE2</i> mutant complemented with <i>sopB</i>	+/-	+/-	+/-
<i>sipAsopABDE2</i> mutant complemented with <i>sopD</i>	-	+/-	+/-
<i>sipAsopABDE2</i> mutant complemented with <i>sopE2</i>	+/-	+/-	+

(+): cellular responses induced at significant higher level than *sipAsopABDE2* mutant.

(-): cellular responses induced at significant lower level than *sipAsopABDE2* mutant.

(+/-): cellular responses induced at the same level of *sipAsopABDE2* mutant.