Anaerobiosis-induced virulence of *Salmonella enterica* subsp. *enterica* serovar Typhimurium: role of phospholipase Cγ signalling cascade

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*Salmonella enterica* subsp. *enterica* serovar Typhimurium (S. Typhimurium) can initiate entry into non-phagocytic epithelial cells by triggering certain signal transduction pathways, thereby allowing the pathogen to invade and establish a niche within host cells. Anaerobiosis has been shown to be an important inducer of the invasion process of S. Typhimurium. However, the effect of anaerobiosis on modulation of cell signalling cascades by S. Typhimurium is not known. In the present study, the phospholipase Cγ signalling cascade was investigated in mice enterocytes, following interaction with S. Typhimurium grown under aerobic and anaerobic growth conditions. Significant increases in enterocyte intracellular calcium and inositol 1,4,5-triphosphate levels were observed on interaction with S. Typhimurium grown anaerobically compared with S. Typhimurium grown aerobically. An increased membrane/cytosolic ratio of protein kinase C was also seen with anaerobic S. Typhimurium in enterocytes compared with aerobic S. Typhimurium. These data suggest that anaerobically grown organisms are more efficient in initiating cell-signalling events than are aerobically grown bacteria. These enhanced cell signals may contribute to the increased virulence of S. Typhimurium grown anaerobically.

**INTRODUCTION**

*Salmonella enterica* subsp. *enterica* serovar Typhimurium (S. Typhimurium) is a broad-host-range serotype that causes disease in humans, livestock, domestic fowl, rodents and birds (Rabsch et al., 2002). The initial site of infection by S. Typhimurium is the distal ileum, where these organisms are exposed to a relatively anaerobic environment (pO2 of 5–40 mmHg). S. Typhimurium grown under anaerobic growth conditions have been found to be more virulent and invasive (Finlay et al., 1989; Lee & Falkow, 1990). Anaerobically grown S. Typhimurium have been shown to cause effective cytoskeletal rearrangements and morphological changes in infected HEp-2 cells (Galan, 1996). A number of *Salmonella*-secreted proteins, termed Sips (*Salmonella* invasive proteins) and Sops (*Salmonella* outer proteins), have been characterized and have been shown to participate in the invasion of epithelial cells (Hermant et al., 1995; Wood et al., 1996). A 'hyperinvasive' (hil) locus has also been identified in the genome of S. Typhimurium, which is involved in the anaerobic induction of invasion (Lee et al., 1994).

Bacterial pathogens are known to exploit host-cell machinery to their advantage by activating signal transduction events within host cells. It has been shown that these signalling events play an important role in mediating invasion of non-phagocytic cells by bacteria (Falkow et al., 1992). Bliska et al. (1993) reported that S. Typhimurium could initiate uptake into non-phagocytic cells by activating cell signalling events in these cells. A specific type-III secretion system consisting of Inv–Spa complex located on a pathogenicity island had been proposed to be involved in signalling, uptake and invasion of S. Typhimurium (Kaniga et al., 1995; Galan, 1999). Membrane ruffling, cytoskeletal rearrangements and intracellular calcium fluxes have been observed in mammalian cells following activation of epidermal growth factor receptor (Pace et al., 1993), which also serves as a receptor for *Salmonella typhi*. Interaction of S. Typhimurium with HEp-2 cells was also observed to be accompanied by a marked increase in [Ca2+]i (Gimochio et al., 1992) and was found to be necessary for internalization of bacteria (Brumell et al., 1999), suggesting that these cell signalling events are important determinants in bacterial pathogenicity. Exposure of S. Typhimurium to anaerobic conditions has been observed to enhance its virulence (Singh et al., 2000) and invasiveness.
METHODS

Bacterial strain and growth conditions. S. Typhimurium (virulent strain 140284) (Singh et al., 2000) used in the present study was obtained from the National Escherichia coli and Salmonella Center, Central Research Institute, Kasauli, India. The bacterial strain was checked for purity by biochemical methods, serotyping and by inoculation on xylose lysine deoxycholate and MacConkey agar plates. Bacterial strains were stored as lyophilized ampoules and fresh cultures were started from the stock every month. S. Typhimurium was grown under aerobic and anaerobic conditions in brain heart infusion (BHI) broth and BHI agar. Anaerobic conditions were maintained in a gas jar with a mixture of 85 % nitrogen, 10 % hydrogen and 5 % carbon dioxide. The bacterial culture was incubated under static conditions at 37 °C. Bacteria were harvested in stationary phase (18 h) and stored at −80 °C. The remainder of the bacterial culture was designated as a control. Subsequently, enterocytes were treated with 1 ml LiCl (10 mM) for 30 min and incubated with 0.5 μM H²-myosin-inositol for 30 min at 37 °C.

The entocyte suspension was centrifuged at 2000 g for 10 min to wash off excess labelled inositol and enterocytes were resuspended in 1 ml Tris/HCl (pH 7.4). The suspension was treated immediately with 2 vols ice-cold 20 % perchloric acid and kept on ice for 20 min. Proteins were removed by centrifugation at 2000 g for 20 min at 4 °C. Siliconized glassware was used in further steps to minimize losses of inositol phosphates. The pH of the supernatant was brought to 7.5 with ice-cold 5 M KOH on ice and the supernatant was centrifuged at 2000 g for 20 min at 4 °C to remove KClO₃ precipitate.

S. Typhimurium or An. S. Typhimurium at different times (0, 5, 10, 20 and 30 min). Enterocytes were centrifuged and resuspended in HEPES buffer and placed on ice. Fluorescence measurements (excitation wavelength, 340 nm; emission wavelength, 510 nm) were made in a Kontron spectrophotometer (model SFM25). The basal fluorescence measurement of test cutvete was designated as F. The intracellular free calcium was calculated from the formula: $[Ca^{2+}]_i = \frac{1}{c_0} \times \left( F - F_{bas} \right)$, where $c_0$ is the $K_M$ for Fura-2 AM, $F_{bas}$ and $F$ were measured by permeabilizing the cells with 10 μM 1,2- bis(3-(o-(9-anthryl)amino)propyl)-dipyrromethene dibromide (ABQ) and measuring fluorescence at zero $Ca^{2+}$ and at saturating $Ca^{2+}$ concentrations, respectively.

Measurement of inositol 1,4,5-triphosphate (IP₃) levels. IP₃ turnover in enterocytes was measured using H²-labelled myo-inositol (Sigma). Enterocytes (1 × 10⁶ cells ml⁻¹ in 20 mM Tris/HCl, pH 7.4) were incubated for different time periods (0, 1, 5, 10, 20, 30 and 45 min) with Ae. S. Typhimurium or An. S. Typhimurium under aerobic and anaerobic conditions. Measurement of protein kinase C (PKC) levels. PKC translocation and its activity in cytosolic and membrane fractions of enterocytes were determined as described by Howcroft et al. (1988). Briefly, enterocytes (1 × 10⁶ cells ml⁻¹) in serum-free RPMI-1640 (pH 7.2) were used in enterocytes isolated from mice by the method of Laux et al. (1986). Briefly, the intestines were flushed with cold PBS, pH 7.4, and incubated with PBS containing DTT (pH 7.4) at 37 °C for 15 min, after tying the loose ends of the intestine. This was followed by incubation of intestines with PBS containing 1.5 mM DTT (pH 7.4). The solution inside the intestines was collected and centrifuged at 3000 r.p.m. for 10 min and the pellet was collected and suspended in Tris/mannitol buffer (2 mM Tris/HCl, 50 mM mannitol, pH 7.2). The viability of the cells was checked by staining with Trypan blue (0.2 %) and the purity of cells was determined under phase-contrast microscope. Enterocytes having more than 90 % viability were chosen for experimental purposes and were divided into the following groups. Group I was a control group, and signal transduction assays were assayed in enterocytes without interaction with S. Typhimurium. Group II included enterocytes incubated with Ae. S. Typhimurium, and group III included enterocytes incubated with An. S. Typhimurium. Measurement of intracellular calcium ($[Ca^{2+}]_i$) levels. In enterocytes, was determined using Fura-2 AM (2 μmol per 10⁷ cells) (Sigma) (Chang et al., 1986). Briefly, isolated enterocytes in HEPES buffer (pH 7.2) were loaded with Fura-2 AM (dissolved in DMEM) and incubated with Ae. S. Typhimurium or An. S. Typhimurium for different time intervals (0, 5, 10, 20 and 30 min). Enterocytes were centrifuged and resuspended in HEPES buffer and placed on ice. Fluorescence measurements (excitation wavelength, 340 nm; emission wavelength, 510 nm) were made in a Kontron spectrophotometer (model SFM25). The basal fluorescence measurement of test cutvete was designated as F. The intracellular free calcium was calculated from the formula: $[Ca^{2+}]_i = \frac{1}{c_0} \times \left( F - F_{bas} \right)$, where $c_0$ is the $K_M$ for Fura-2 AM, $F_{bas}$ and $F$ were measured by permeabilizing the cells with 10 μM 1,2- bis(3-(o-(9-anthryl)amino)propyl)-dipyrromethene dibromide (ABQ) and measuring fluorescence at zero $Ca^{2+}$ and at saturating $Ca^{2+}$ concentrations, respectively.

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scintillation fluid. Radioactivity incorporated was determined as outlined above. PKC activity was calculated by subtracting the amount of $^{32}$P incorporation into histone in the presence of EGTA and the absence of added phosphatidyl serine, diolein and Ca$^{2+}$ from the amount of $^{32}$P incorporated in the presence of phosphatidyl serine, diolein and Ca$^{2+}$.

**Statistical analysis.** Results were expressed as means±SD and were compared by one-way analysis of variance (ANOVA) in multiple groups and by Student’s unpaired *t*-test between two groups. *P*, 0·05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Enterocyte [Ca$^{2+}$]$_i$**

Changes in [Ca$^{2+}$]$_i$ in enterocytes upon interaction with An. S. Typhimurium and Ae. S. Typhimurium are shown in Fig. 1. A significant increase (*P* < 0·01) in [Ca$^{2+}$]$_i$, was observed in enterocytes stimulated with An. S. Typhimurium compared with Ae. S. Typhimurium. Moreover, the maximal increase in [Ca$^{2+}$]$_i$, was seen at 10 min with An. S. Typhimurium compared with 20 min with Ae. S. Typhimurium.

**IP$_3$ levels**

A significant and time-dependent increase in IP$_3$ was observed in enterocytes treated with either An. S. Typhimurium or Ae. S. Typhimurium compared with untreated enterocytes (*P* < 0·001). However, the increase in IP$_3$ was significantly greater with An. S. Typhimurium compared with Ae. S. Typhimurium (*P* < 0·01) (Fig. 2).

**PKC activity**

PKC activity was measured in cytosolic and membrane fractions of enterocytes following incubation with Ae. S. Typhimurium and An. S. Typhimurium and was expressed as a ratio of membrane/cytosolic activity (Fig. 3). A significant increase in the membrane/cytosolic ratio of PKC activity was observed in enterocytes treated with An. S. Typhimurium compared with those treated with Ae. S. Typhimurium (*P* < 0·05).

**Conclusions**

In the present study, we investigated host-cell signalling events mediated by PLC-γ in enterocytes upon interaction with An. S. Typhimurium. We observed that An. S. Typhimurium caused a significant increase in [Ca$^{2+}$]$_i$ in enterocytes, compared with Ae. S. Typhimurium. This observed increase could result from either increased Ca$^{2+}$ influx or...
increased mobilization of Ca\(^{2+}\) from intracellular stores. A significant increase in [Ca\(^{2+}\)], caused by anaerobic bacteria was seen even in the absence of calcium from extracellular medium (M. Khullar, unpublished), indicating that An. S. Typhimurium triggered increased mobilization of [Ca\(^{2+}\)], from intracellular stores. Moreover, S. Typhimurium has been shown to induce an inositol phosphate flux in infected epithelial cells (Galan & Curtiss, 1989). Thus, the increased mobilization of intracellular Ca\(^{2+}\) in our study might be due to the increased production of IP\(_3\) by PLC-\(\gamma\), which, in turn, could initiate the mobilization of Ca\(^{2+}\) from intracellular stores. Changes in [Ca\(^{2+}\)], are an important cell signal leading to various cellular activities. Increases in [Ca\(^{2+}\)], have been reported to play an important role in bacterial internalization by the formation of membrane ruffles so as to allow bacterial entry (Pace et al., 1993; Brumell et al., 1999). The induction of membrane ruffles is critical for entry of S. Typhimurium, since mutants unable to induce Ca\(^{2+}\) flux are severely impaired in their ability to enter cultured HEp-2 cells (Pace et al., 1993). We observed that enterocytes incubated with An. S. Typhimurium showed significantly higher IP\(_3\) levels compared with those incubated with aerobic bacteria. This enhanced IP\(_3\) response suggests specific and avid interaction between these bacteria and the enterocytes, which could lead to initiation of signal cascading via IP\(_3\) and [Ca\(^{2+}\)]. This interaction could be via specific ligand binding to a hitherto unknown receptor on enterocytes. Hence, the increased [Ca\(^{2+}\)], in enterocytes observed in the present study may contribute to enhanced virulence and penetration of host cells by anaerobic bacteria.

Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) by PLC-\(\gamma\) is accompanied by production of diacylglycerol (DAG) along with IP\(_3\), and second messengers like DAG, Ca\(^{2+}\) and IP\(_3\) are known to be potent activators of PKC (Galan, 1996). The activation of PKC results in the translocation of PKC activity from cytosol to membrane, thereby causing the phosphorylation of membrane-bound proteins (Gupta et al., 1999). We observed that interaction of An. S. Typhimurium with enterocytes resulted in increased translocation of PKC activity from cytosol to membrane compared with aerobic bacteria. PKC activation is also essential for diarrheagenic organisms, as it results in phosphorylation of specific membrane proteins responsible for the efflux of ions, electrolytes and water from intestinal epithelial cells, leading to the manifestation of diarrhoea (Ruschkowski et al., 1992; Ganguly & Kaur, 1996). Since S. Typhimurium does not cause diarrhoea in mice, its seems that activation of PKC in mice enterocytes may help in phosphorylation of membrane proteins involved in adhesion and entry of bacteria into these cells. The relative increase in PKC activation following interaction of anaerobic bacteria with enterocytes observed in our study suggests it to be an important cell signalling event aiding entry of the organism into the host cell by specific phosphorylation of specific membrane proteins.

In conclusion, we observed that anaerobic S. Typhimurium could initiate potent host-cell signal responses. These enhanced cell signalling responses may lead in turn to increased bacterial virulence and invasion of host cells.

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M. Khullar and others


