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Production of β-defensin-2 by human colonic epithelial cells induced by *Salmonella enteritidis* flagella filament structural protein

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Abstract We recently showed that FliC of *Salmonella enteritidis* increased human β-defensin-2 (hBD-2) expression, and now describe the signaling responsible pathway. FliC increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$) in Caco-2 cells. The $[Ca^{2+}]_{in}$ increase induced by FliC was prevented by U73122 and heparin, but not by chelating extracellular Ca^{2+} or pertussis toxin. The FliC-induced increase in hBD-2 promoter activity via nuclear factor κB (NF-κB) was also inhibited by chelation of intracellular Ca^{2+} or by U73122. We conclude that FliC increased $[Ca^{2+}]_{in}$ via inositol 1,4,5-trisphosphate, which was followed by up-regulating hBD-2 mRNA expression via an NF-κB-dependent pathway.

Key words: Phospholipase C; Inositol 1,4,5-trisphosphate; Intracellular Ca^{2+} concentration; Nuclear factor κB; Human β-defensin-2

1. Introduction

Salmonella species are Gram-negative organisms that cause gastroenteritis and enteric fever in humans. These symptoms appeared to result from the mucosal inflammatory response induced by this pathogen.

We recently reported that FliC of *Salmonella enteritidis* increased human β-defensin-2 (hBD-2) mRNA levels and time course correlated with nuclear factor κB (NF-κB) activation in a human colon carcinoma (Caco-2) cell line [1]. Bacterial flagella filament structural proteins have also been implicated in the production of cytokines [2,3]. Purified phase 1 *Salmonella* flagellin and FliC synthesized in *Escherichia coli* each enhanced release of tumor necrosis factor α and interleukin (IL)-1 from a human promonocytic cell line [2,3]. *Salmonella typhimurium* flagella impaired antigen uptake and presentation by human macrophages [4], and induced a cytokine production cascade in these cells [5]. Key factors in activating cytokine production were the increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$) and subsequent NF-κB activation.

Intracellular Ca^{2+} mobilization is a widely used signaling mechanism and there are many reports of *Salmonella*-induced increases in $[Ca^{2+}]_{in}$ in model epithelium. Pace et al. demonstrated that blocking *S. typhimurium* invasion with cytochalasin D, an inhibitor of actin polymerization, did not prevent an increase in $[Ca^{2+}]_{in}$ [6] and that the invasion-defective *S. typhimurium* mutant HilΔ, which lacks the type III secretion apparatus, was also able to increase $[Ca^{2+}]_{in}$ leading to NF-κB activation and IL-8 secretion [7]. We postulated that the NF-κB-dependent stimulation of hBD-2 mRNA expression induced by FliC of *S. enteritidis* might result from increases in $[Ca^{2+}]_{in}$.

As reported here, we found that FliC of *S. enteritidis* did, indeed, stimulate $[Ca^{2+}]_{in}$, apparently via inositol 1,4,5-trisphosphate (IP_3) leading to increase hBD-2 mRNA expression.

2. Materials and methods

2.1. FliC from S. enteritidis cells

FliC of *S. enteritidis* was prepared as previously described with the following modification [1,8]. *S. enteritidis* was grown on tryptic soy agar broth for 16 h at 37°C, and was pelleted by centrifugation ($5000 \times g$, 30 min, 4°C) and suspended in phosphate-buffered saline (PBS), which was adjusted to pH 2 with 1 M HCl and maintained at that pH with constant stirring for 30 min at room temperature. After centrifugation at $100\,000 \times g$ for 1 h at 4°C, the pH of the supernatant containing detached monomeric flagellin was adjusted to 7.2 with 1 M NaOH and $(NH_4)_2SO_4$ was added to 65% saturation. After incubation overnight at 4°C, the solution was centrifuged ($15\,000 \times g$, 15 min, 4°C). The precipitate was dissolved in distilled water, dialyzed against distilled water, heated at 65°C for 15 min and centrifuged at $100\,000 \times g$ for 1 h at 4°C. To the supernatant solid $(NH_4)_2SO_4$ was added to a concentration of 0.7 M. After storage overnight at 4°C, polymerized FliC was collected by centrifugation at $100\,000 \times g$ for 1 h at 4°C and dissolved in PBS.

2.2. Preparation of anti-FliC IgG

Anti-FliC IgG was prepared as previously described. Briefly, a peptide, NH_2-QFTFDDKTKNESAKL (amino acids 339–353) of *S. enteritidis* FliC with an added carboxy-terminal Cys, was synthesized and coupled to keyhole limpet hemocyanin (KLH). Rabbits were immunized with peptide-coupled KLH (200 μg) or purified FliC pro-

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Abbreviations: FliC, flagella filament structural protein; hBD-2, human β-defensin-2; $[Ca^{2+}]_{in}$, intracellular Ca^{2+} concentration; IL, interleukin; NF-κB, nuclear factor κB; IP_3, inositol 1,4,5-trisphosphate; PBS, phosphate-buffered saline; KLH, keyhole limpet hemocyanin; DMEM, Dulbecco's modified Eagle's medium; Fura-2/AM, 1-(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2(2ino-5′-methyl-phenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxy methyl ester; FCS, fetal calf serum; PTX, pertussis toxin; PLC, phospholipase C; BAPTA-AM, 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester)

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tein (40 μg) in complete Freund adjuvant (1:1 v/v) subcutaneously on day 0, day 21, and at weekly intervals thereafter until the desired serum titer against the peptide was reached. Rabbits were bled 7 weeks after the first injection.

2.3. Caco-2 cells in culture

Caco-2 cells, a colonic epithelial cell line, were purchased from the American Type Culture Collection (MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 100 U/ml gentamicin. The media and supplements were purchased from Gibco BRL (MD, USA). Cells were grown in 75-cm^2 flasks in an atmosphere of 5% CO_2 at 37°C with medium changed twice weekly.

2.4. Intracellular Ca^{2+} measurements

The $[Ca^{2+}]_{in}$ was assessed by microfluorometry, using the fluorescent dye Fura-2/AM (1-(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2(2ino-5′-methyl-phenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxy methyl ester) at excitation wavelengths of 340 nm and 380 nm and at an emission wavelength of 510 nm with an ARGUS-50/CA system (Hamamatsu Photonics, Japan). Cells were grown on round coverslips (12 mm) for 2 days, after which they were incubated with 2 μM Fura-2/AM (Molecular Probes, OR, USA) for 30 min and washed twice with PBS, before coverslips were inserted into the chamber for measuring $[Ca^{2+}]_{in}$ at 37°C.

2.5. Heparin loading into Caco-2 cells

Heparin (molecular weight 2000 ~ 5000) was loaded into the Caco-2 cells using Influx™ pinocytic cell loading reagent (Molecular Probes, OR, USA). Briefly, Caco-2 cells growing on coverslip were incubated in hypertonic loading medium with 2 μM Fura-2/AM with or without heparin (5 mg/ml) at 37°C for 10 min, and were incubated for 2 min in

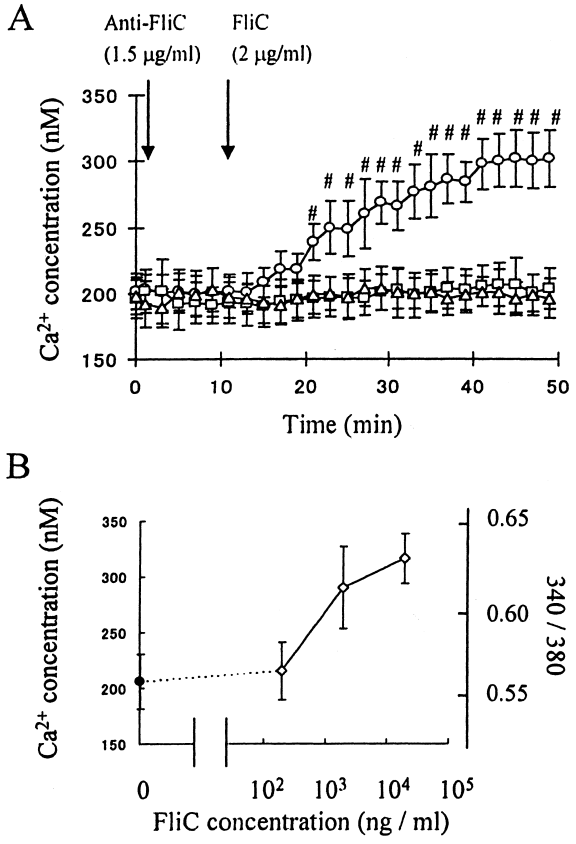

Fig. 1. Effects of FliC on $[Ca^{2+}]_{in}$ of Caco-2 cells. A: Time courses of $[Ca^{2+}]_{in}$ in Caco-2 cells after addition of FliC and/or anti-FliC at the indicated time. (square) No addition; (circle) FliC; (triangle) FliC plus anti-FliC. # Significant difference, $P < 0.05$ vs. (square). B: Effect of FliC concentration on $[Ca^{2+}]_{in}$ of Caco-2 cells. Data are means ± S.D. ($n = 10$).

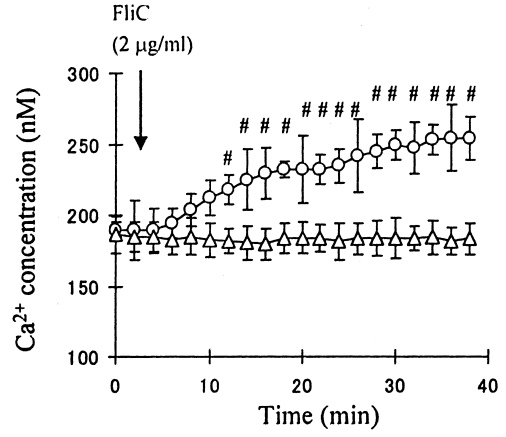

Fig. 2. Effect of Ca^{2+}-free medium on FliC-induced increase in $[Ca^{2+}]_{in}$. $CaCl_2$ was omitted from the medium and 1 mM EGTA was added. (circle) FliC was added to cells at the indicated time. (triangle) No addition of FliC. Data are means ± S.D. ($n = 10$). # Significant difference, $P < 0.05$ vs. (triangle).

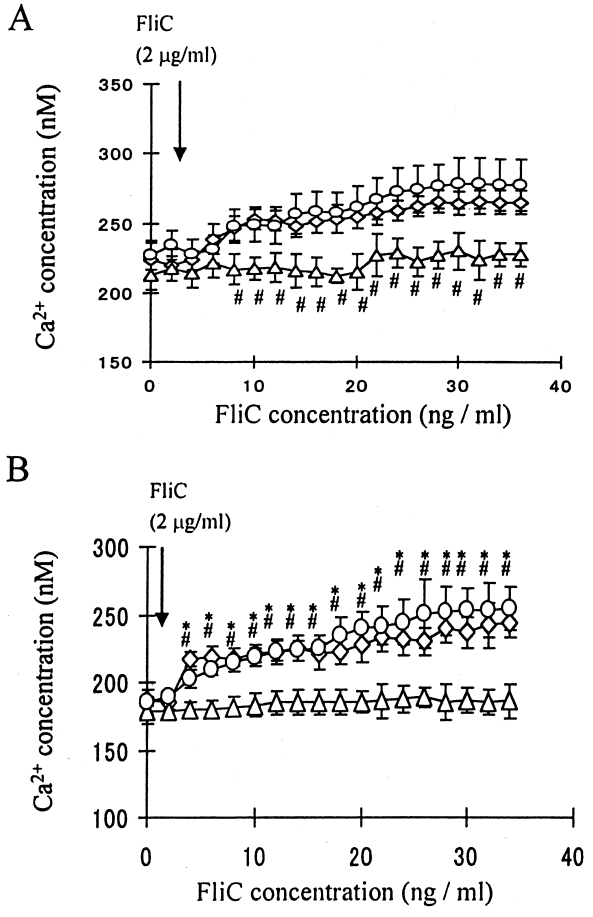

Fig. 3. A: Effects of ryanodine and U73122 on $[Ca^{2+}]_{in}$ of Caco-2 cells treated with FliC. (circle) FliC was added to cells at the indicated time (positive control). (rhomb) Ryanodine (10 μM) or (triangle) U73122 (1 μM) was added to cells 30 min before. Data are expressed as means ± S.D. ($n = 10$). # Significant difference, $P < 0.05$ vs. positive control. B: Effect of PTX on $[Ca^{2+}]_{in}$ response to FliC. (circle) FliC was added to cells at the indicated time. * Significant difference, $P < 0.05$ vs. (triangle). (triangle) No addition of FliC. (rhomb) Cells were incubated with PTX (200 ng/ml) for 24 h before the addition of FliC at the indicated time. Data are means ± S.D. ($n = 10$). # Significant difference, $P < 0.05$ vs. (triangle).

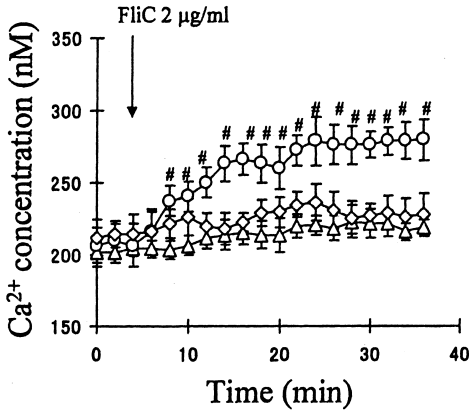

Fig. 4. Effect of heparin on $[Ca^{2+}]_{in}$ response to FliC. (circle) FliC was added to cells at the indicated time. (triangle) No additions. (rhomb) Heparin was delivered into cells by pinocytosis (see Section 2) before addition of FliC. Data are means ± S.D. ($n = 10$). # Significant difference, $P < 0.05$ vs. (rhomb).

the hypotonic lysis medium with 2 μM Fura-2/AM with or without heparin (5 mg/ml), before washing twice with PBS.

2.6. Luciferase assay

To assess hBD-2 promoter activity, Caco-2 cells were distributed in 24-well plates (1×10^5 cells in 1 ml of DMEM per well) and incubated for 24 h at 37°C. The hBD-2 promoter or 5′-deletion constructs of the hBD-2 promoter pGL3-2110, each sample 2.5 μg, linked to a luciferase reporter gene [9] were incubated with 0.5 μg of (internal control) a *Renilla* luciferase expression vector (pRL-TK) and 10 μl of 3.2 mM dendritic poly-(L-lysine) (KG6) [10] in 250 μl of fetal calf serum (FCS)-free DMEM for 15 min at room temperature before the mixture containing DNA–peptide complexes was added to Caco-2 cells and incubated for 3 h at 37°C. 1 ml of DMEM was added 24 h after culture medium was replaced with 1 ml of fresh FCS-free DMEM. Samples (300 μl) of the preparation to be assayed were then added. After incubation for 6 h at 37°C, cells were washed with 1 ml of PBS and lysed in 100 μl of lysis buffer (Tokyo Ink Co., Japan). After 15 min at room temperature, the lysate was centrifuged ($18\,000 \times g$, 5 min, 4°C). Luciferase activity of the supernatant, measured using a luminometer (Berthold), was normalized to activity of the control *Renilla* luciferase activity.

2.7. Immunocytochemical staining

Caco-2 cells were grown on coverslips (18×18 mm) for 2 days. After treatment of the cells with FliC, BAPTA-AM (1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester)), or U73122, they were washed three times with PBS and fixed with 3.5% formaldehyde in PBS for 20 min. After washing with PBS, the cells were treated with 0.01% (v/v) Triton X-100 for 30 s, and then they were incubated with 4% milk casein for 1 h. After washing, the cells were incubated with 200 times diluted rabbit antibody against p65 (Santa Cruz, CA, USA) for 1 h at room temperature. The cells were washed again and incubated for 1 h with 500 times diluted Alexa Fluor® 594 conjugated goat anti-rabbit IgG (Molecular Probes, OR, USA).

3. Results

3.1. FliC increased intracellular free Ca^{2+}

$[Ca^{2+}]_{in}$ in Caco-2 cells increased at a relatively constant

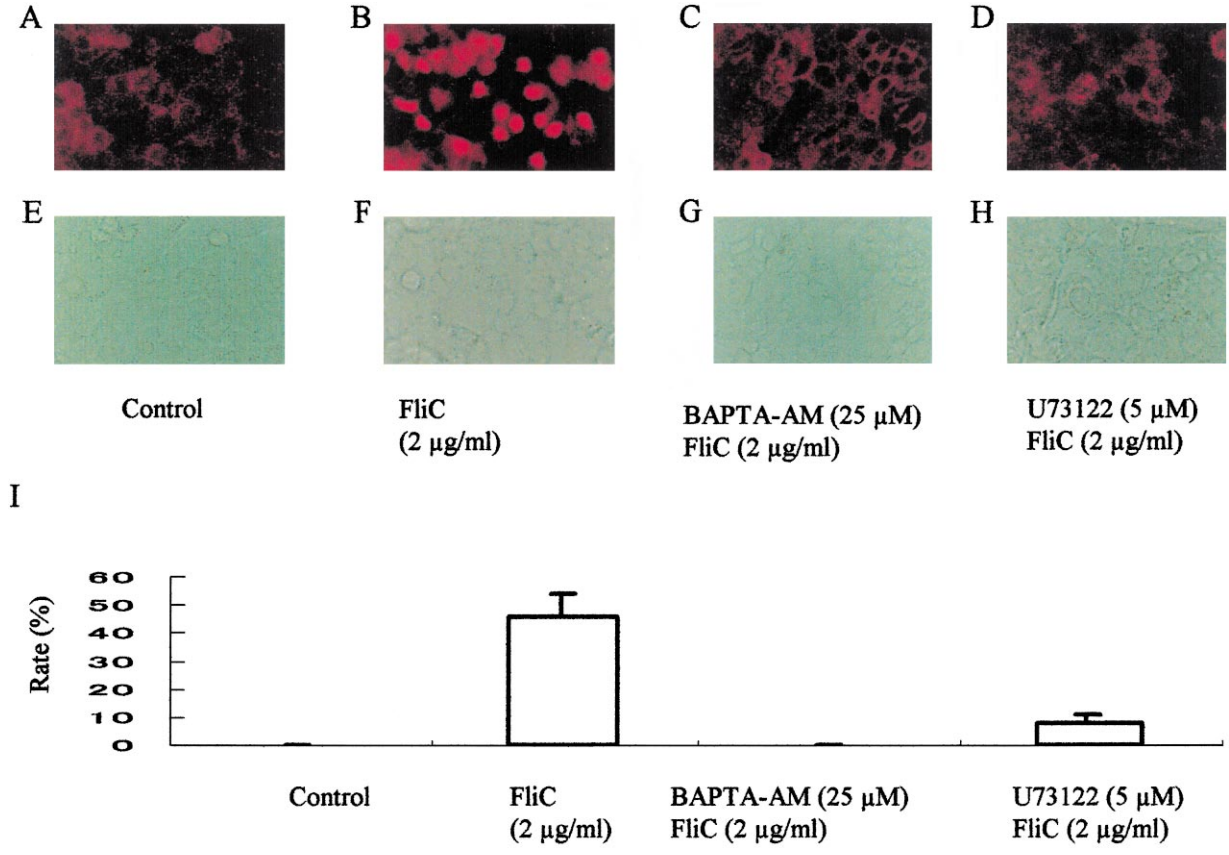

Fig. 5. Effect of BAPTA-AM or U73122 on NF-κB distribution. Cells were incubated for 30 min with the indicated concentration of BAPTA-AM or U73122 before addition of FliC (2 μg/ml). The cells were fixed 3 h later. A–D: Fluorescence photomicrograph. E–H: Photomicrograph on each of the same area as in A–D. I: Rate of the cells that p65 translocated to nucleus.

rate for 15 min after the addition of FliC (2 μg/ml) and was slowly decreased after. This effect was inhibited by prior addition of ant-FliC antibody (1.5 μg/ml) (Fig. 1A), confirming that FliC increased $[Ca^{2+}]_{in}$ in Caco-2 cells. The magnitude of the $[Ca^{2+}]_{in}$ increase at 15 min was dependent on FliC concentration (Fig. 1B).

3.2. Signaling pathway for FliC-induced intracellular Ca^{2+} mobilization

The FliC effect on $[Ca^{2+}]_{in}$ persisted in Ca^{2+}-free medium containing 1 mM EGTA (Fig. 2), suggesting that the increase in $[Ca^{2+}]_{in}$ was due to release from intracellular Ca^{2+} stores.

Two well-characterized pathways evoked release from internal Ca^{2+} stores, ryanodine receptor- and IP_3 receptor-mediated pathways. Ryanodine can deplete the ryanodine-sensitive Ca^{2+} stores in cells [11], however, 10 μM ryanodine failed to inhibit the $[Ca^{2+}]_{in}$ increase induced by FliC (Fig. 3A). To assess involvement of the IP_3 receptor-mediated pathway in FliC-induced release from internal Ca^{2+} stores, the cells were incubated with pertussis toxin (PTX; 200 ng/ml) for 24 h before experiments (Fig. 3B). PTX treatment had no $[Ca^{2+}]_{in}$ response to FliC, suggesting that the FliC-evoked increase in $[Ca^{2+}]_{in}$ was not mediated by a PTX-sensitive G protein. U73122, a phospholipase C (PLC) inhibitor that has been reported to inhibit PLC-mediated effects in several cell types [12,13], completely prevented the FliC-mediated $[Ca^{2+}]_{in}$ increase (Fig. 3A). Heparin, a known inhibitor against IP_3 receptors [14,15], was delivered into the Caco-2 cells using pinocytosis (see Section 2) and completely inhibited the effect on $[Ca^{2+}]_{in}$ (Fig. 4).

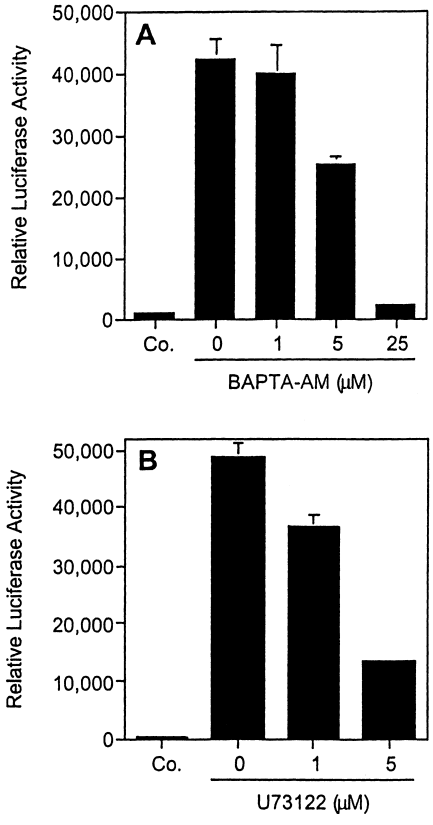

Fig. 6. Effect of BAPTA-AM or U73122 on hBD-2 production. Cells were incubated for 30 min with the indicated concentration of BAPTA-AM or U73122 before addition of FliC (2 μg/ml). Luciferase activity was determined 3 h later. A: BAPTA-AM. B: U73122. Data are means ± S.D. of values from three separate experiments with assays in duplicate.

3.3. hBD-2 promoter activity via NF-κB

FliC induced the translocation of NF-κB to nucleus (Fig. 5). On the other hand, treatment with BAPTA-AM and U73122 prevented the translocation of NF-κB to nucleus. These results indicated that the translocation of NF-κB to nucleus induced by FliC was PLC- and $[Ca^{2+}]_{in}$-dependent.

To assess FliC-induced hBD-2 promoter activity via NF-κB was measured. Activation of the hBD-2 promoter via NF-κB was inhibited by $[Ca^{2+}]_{in}$ chelation with BAPTA-AM or U73122 in a dose-dependent manner (Fig. 6), consistent with the conclusion that $[Ca^{2+}]_{in}$ increases were essential for hBD-2 production.

4. Discussion

Purified FliC from *S. enteritidis* increased $[Ca^{2+}]_{in}$ in Caco-2 cells via a mechanism that apparently involves PLC-dependent stimulation of IP_3 production and release of Ca^{2+} from ryanodine-insensitive intracellular Ca^{2+} stores. The pathways that induce Ca^{2+} release from intracellular Ca^{2+} stores are well-characterized in many cells. The signals are often initiated via heptahelical G protein-coupled receptors, but can also be initiated by cell-surface molecules that lack an intracellular domain (for example, via glycophosphatidyl inositide-anchored Fcγ receptors on human neutrophils), presumably by clustering signaling molecules [16]. Recently, it was reported that the Toll-like receptor 5 was a receptor for *S. typhimurium* flagellin [17]. Although Toll-like receptor 5 may be also a receptor for FliC of *S. enteritidis*, recent knowledge about Toll-like receptor 5 cannot explain the pathways of $[Ca^{2+}]_{in}$ increases, clearly [18–22]. But, it seems reasonable that a receptor-mediated mechanism of these types could mediate FliC of *S. enteritidis*-induced $[Ca^{2+}]_{in}$ mobilization. Receptor-mediated events have been reported in other systems. In airway epithelial cells, *Pseudomonas aeruginosa* activated NF-κB via interaction with the cellular pilin receptor [22]. FliC-induced $[Ca^{2+}]_{in}$ mobilization was PTX-insensitive (Fig. 3B). The existence of PTX-insensitive G protein–PLC–IP_3 signaling in a P2Y purine receptor signaling pathway was correlated with a PTX-insensitive G protein of the Gq/11 family [23,24]. It seems likely that a protein of the Gq/11 family is involved in FliC-mediated $[Ca^{2+}]_{in}$ mobilization but this remains to be established.

The increase with $[Ca^{2+}]_{in}$ initiated by *S. enteritidis* FliC occurred rather slowly. The $[Ca^{2+}]_{in}$ increases induced by *S. typhimurium* [7,25] and by activation of Toll-like receptor RP-105 in B cells [26] were similarly slow. In muscle cell, however, increase in $[Ca^{2+}]_{in}$ coupled to PLC-dependent stimulation of IP_3 production and release of Ca^{2+} from intracellular stores are very rapid [27,28]. On the other hand, the recently reported increases in $[Ca^{2+}]_{in}$ coupled to PLC activity to stimulate IP_3 production and release of Ca^{2+} from intracellular Ca^{2+} stores in primary neurons were slow [29]. The reasons for the very different kinetics of seemingly similar mechanisms of $[Ca^{2+}]_{in}$ increases remain to be determined.

Gerwirts et al. indicated that *S. typhimurium* infections increased $[Ca^{2+}]_{in}$ during 3 h, when extracellular Ca^{2+} was chelated by EGTA (named 'initial' increase). When extracellular

Ca^{2+} was chelated, however, the $[Ca^{2+}]_{in}$ increases were smaller 3–8 h following *Salmonella* infections [7]. Because, FliC-mediated $[Ca^{2+}]_{in}$ increases were not inhibited by chelating extracellular Ca^{2+} and did not return to baseline after about 30 min in this study. These changes in $[Ca^{2+}]_{in}$ are consistent with the 'initial' phase (during the first 3 h after *Salmonella* infection as described by Gerwirts et al.). Moreover, we demonstrated that FliC mediated $[Ca^{2+}]_{in}$ increases, which are consistent with the 'initial' phase of $[Ca^{2+}]_{in}$ increases upregulated by hBD-2 mRNA expression via NF-κB activation. Thus, the 'initial' increases in $[Ca^{2+}]_{in}$ are a sufficient stimulus to induce NF-κB activation.

References

[1] Ogushi, K., Wada, A., Niidome, T., Mori, N., Oishi, K., Nagatake, T., Takahashi, A., Hojo, H., Nakahara, Y., Hatakeyama, T., Aoyagi, H., Kurazono, H., Moss, J. and Hirayama, T. (2001) J. Biol. Chem. 276, 30521–30526.

[2] Ciacci-Woolwine, F., Blomfield, I.C., Richardson, S.H. and Mizel, S.B. (1998) Infect. Immun. 66, 1127–1134.

[3] Ciacci-Woolwine, F., McDermott, P.F. and Mizel, S.B. (1999) Infect. Immun. 67, 5176–5185.

[4] Wyant, T.L., Tanner, M.K. and Sztein, M.B. (1999) Infect. Immun. 67, 1338–1346.

[5] Wyant, T.L., Tanner, M.K. and Sztein, M.B. (1999) Infect. Immun. 67, 3619–3624.

[6] Pace, J., Hayman, M.J. and Galan, J.E. (1993) Cell 72, 505–514.

[7] Gewirtz, A.T., Rao, A.S., Simon Jr., P.O., Merlin, D., Carnes, D., Madara, J.L. and Neish, A.S. (2000) J. Clin. Invest. 105, 79–92.

[8] Ibrahim, G.F., Fleet, G.H., Lyons, M.J. and Walker, R.A. (1985) J. Clin. Microbiol. 22, 1040–1044.

[9] Wada, A., Ogushi, K., Kimura, T., Hojo, H., Mori, N., Suzuki, S., Kumatori, A., May, Sie, Nakahara, Y., Nakamura, M., Moss, J. and Hirayama, T. (2001) Cell Microbiol. 3, 115–123.

[10] Ohsaki, M., Urakawa, M., Niidome, T. and Aoyagi, H. (1999) Pept. Sci. 1998, 453–456.

[11] Pozzan, T., Rizzuto, R., Volpe, P. and Meldolesi, J. (1994) Physiol. Rev. 74, 595–636.

[12] Bleasdale, J.E. and Swanson, M.L. (1993) Biochim. Biophys. Acta 1181, 240–248.

[13] Hildebrandt, J.P., Plant, T.D. and Meves, H. (1997) Br. J. Pharmacol. 120, 841–850.

[14] Ehrlich, B.E. and Watras, J. (1998) Nature 336, 583–586.

[15] Mayrleitner, M., Chadwick, C.C., Timerman, A.P., Fleischer, S. and Schindler, H. (1991) Cell Calcium 12, 505–514.

[16] Strohmeier, G.R., Brunkhorst, B.A., Seetoo, K.F., Meshulam, T., Bernardo, J. and Simons, E.R. (1995) J. Leukoc. Biol. 58, 415–422.

[17] Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M. and Aderem, A. (2001) Nature 410, 1099–1103.

[18] Medzhitov, R. and Janeway Jr., C. (2000) N. Engl. J. Med. 343, 338–344.

[19] Akira, S. (2000) Biochem. Soc. Trans. 28, 551–556.

[20] O'Neill, L. (2000) Biochem. Soc. Trans. 28, 557–563.

[21] Muzio, M., Polentarutti, N., Bosisio, D., Manoj Kumar, P.P. and Mantovani, A. (2000) Biochem. Soc. Trans. 28, 563–566.

[22] DiMango, E., Ratner, A.J., Bryan, R., Tabibi, S. and Prince, A. (1998) J. Clin. Invest. 101, 2598–2605.

[23] Dubyak, G.R. and el-Moatassim, C. (1993) Am. J. Physiol. 265, C577–C606.

[24] Harden, T.K., Boyer, J.L. and Nicholas, R.A. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 541–579.

[25] Ruschkowski, S., Rosenshine, I. and Finlay, B.B. (1992) FEMS Microbiol. Lett. 74, 121–126.

[26] Chan, V.W., Mecklenbrauker, I., Su, I., Texido, G., Leitges, M., Carsetti, R., Lowell, C.A., Rajewsky, K., Miyake, K. and Tarakhovsky, A. (1998) J. Exp. Med. 188, 93–101.

[27] Yu, P., Chen, Q., Xiao, Z., Harnett, K., Biancani, P. and Behar, J. (1998) Am. J. Physiol. 275, G203–G211.

[28] van Helden, D.F., Imtiaz, M.S., Nurgaliyeva, K., von der Weid, P. and Dosen, P.J. (2000) J. Physiol. 524, 245–265.

[29] Yang, F., He, X., Feng, L., Mizuno, K., Liu, X.W., Russell, J., Xiong, W.C. and Lu, B. (2001) Nat. Neurosci. 4, 19–28.