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Reorganization of cytoskeletal proteins by *Escherichia coli* heat-stable enterotoxin (STa)-mediated signaling cascade

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ABSTRACT

Background: IP_3-mediated calcium mobilization from intracellular stores activates and translocates PKC-α from cytosol to membrane fraction in response to STa in COLO-205 cell line. The present study was undertaken to determine the involvement of cytoskeleton proteins in translocation of PKC-α to membrane from cytosol in the *Escherichia coli* STa-mediated signaling cascade in a human colonic carcinoma cell line COLO-205.

Methods: Western blots and consequent densitometric analysis were used to assess time-dependent redistribution of cytoskeletal proteins. This redistribution was further confirmed by using confocal microscopy. Pharmacological reagents were applied to colonic carcinoma cells to disrupt the microfilaments (cytochalasin D) and microtubules (nocodazole).

Results: STa treatment in COLO-205 cells showed dynamic redistribution and an increase in actin content in the Triton-insoluble fraction, which corresponds to an increase in polymerization within 1 min. Moreover, pharmacological disruption of actin-based cytoskeleton greatly disturbed PKC-α translocation to the membrane.

Conclusions: These results suggested that the organization of actin cytoskeleton is rapidly rearranged following *E. coli* STa treatment and the integrity of the actin cytoskeleton played a crucial role in PKC-α movement in colonic cells. Depolymerization of tubulin had no effect on the ability of the kinase to be translocated to the membrane.

General significance: In the present study, we have shown for the first time that in colonic carcinoma cells, STa-mediated rapid changes of actin cytoskeleton arrangement might be involved in the translocation of PKC-α to membrane.

1. Introduction

Heat-stable enterotoxin (STa), a major cause of watery diarrhea in human, is secreted by enterotoxigenic *Escherichia coli* (ETEC) [1]. Two hundred eighty million episodes of diarrhea due to ETEC are estimated annually in children less than 5 years old in developing countries [2]. STa binds with cell surface receptor guanylyl cyclase C (GC-C) and leads to the rise of intracellular cGMP level. cGMP stimulates the cGMP-dependent protein kinases that regulate the activation of CFTR channels and Cl^- secretion [3,4]. But the specific signaling pathway behind *E. coli* STa induced rise in cGMP activity and its secretory response is still not clear [5]. Previous reports from our laboratory [6–8] and others [9,10] suggested that besides cGMP, other signaling molecules have been anticipated in the STa-mediated intestinal secretion. It has been reported earlier by our laboratory that STa causes an inositol-triphosphate (IP_3)-mediated release of Ca^{2+} from intracellular store [6,7]. It is also reported that by perturbing the Ca^{2+} homeostasis, toxin may also affect the cytoskeletal architecture and alter the barrier function of epithelial cells, because of the critical role of Ca^{2+} in the regulation of cytoskeleton dynamics induced by bacteria [11–13]. However, the basis of this dynamic regulation of cytoskeleton remains to be defined [11]. Furthermore, it has also been reported by us that STa induces an increase in $[Ca^{2+}]_i$ that leads to the activation and translocation of the protein kinase C-α (PKC-α) from cytosol to membrane, which might be required for the stimulation of a membrane-bound guanylyl cyclase [8]. However, the molecular mechanism behind the translocation of PKC-α is still not clear [14]. The present study has been aimed to investigate the reorganization of cytoskeletal proteins and their role in translocation of PKC-α to membrane in the *E. coli* STa-mediated signaling mechanism in a human colonic carcinoma cell line COLO-205. Our results clearly demonstrate that actin network is significantly rearranged in STa-treated COLO-205 colonic carcinoma cell line and this rearrangement leads to the translocation of PKC-α.

Abbreviations: cGMP, cyclic GMP; STa, *E. coli* heat-stable enterotoxin; PKC-α, protein kinase C-α; PMA, phorbol 12-myristate-13-acetate; BSA, bovine serum albumin; Ca^{2+}, calcium

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2. Materials and methods

2.1. Materials

The chemicals used in the present study were obtained from the following sources: RPMI-1640 medium, fetal bovine serum (FBS), trypsin-EDTA, Hank's balanced salt solution, sodium bicarbonate, glucose and sodium pyruvate from Gibco BRL, USA; Dimethyl sulfoxide (DMSO), Phenylmethylsulphonylfluoride (PMSF), Dithiothreitol (DTT), Leupeptin, Aprotinin, *E. coli* heat-stable enterotoxin (STa), phorbol 12-myristate-13-acetate (PMA), penicillin, streptomycin sulfate, cytochalasin D, Nocodazole, taxol, PIPES, TRITC (tetramethylrhodamine B isothiocyanate)-conjugated phalloidin , and isoform-specific antibody against PKC-α from Sigma, USA; Nonidet P-40 from Pierce; anti-actin antibody from Santa Cruz Biotechnology; anti-tubulin antibody from Cell Signaling and secondary antibodies from Jackson ImmunoResearch. All other reagents were of analytical grade, and deionized water was used throughout the study.

2.2. Cell culture and preparation of viable cells and cell treatment

COLO-205 cells, which were procured from National Centre for Cell Sciences (NCCS), Pune, India, were routinely cultured in tissue culture flasks and grown up to monolayer in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 2.4 g/l sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate in humidified 5% CO_2 atmosphere at 37 °C.Confluent monolayer were passaged using trypsin-EDTA. Viability of the cells was routinely checked by trypan blue exclusion. After reaching 70% confluence, cells were washed with Hank's balanced salt solution (HBSS) and cultured in serum-free RPMI 1640 medium for 24 h and then used for experimental purpose. For the dynamics study of the cytoskeleton, proteins COLO-205 cells were treated with or without 5nM STa in serum-free medium for different time periods. COLO-205 cells were exposed to different drugs at the following concentrations: 1 mM cytochalasin D, 1 mM nocodazole in serum free medium for 1 h at 37 °C.

2.3. Preparation of microtubules

Microtubules were prepared from COLO-205 cells (2×10^6 cells/ml) according to Solomon et al. (1986) [15] with some modification. The cells were treated with 1 mM taxol for 2 h before extraction of microtubules, which in turn stabilizes microtubules without promoting polymerization. The culture medium was then aspirated and replaced with 0.1 M PIPES buffer(PIPES buffer containing 2 M glycerol, 5 mM $MgCl_2$, 2 mM EGTA, 0.04 TIU/ml aprotinin, 2 mM phenylmethylsulfonylfluoride, and 1 mM benzamidine, pH 6.9) containing 1 mM taxol. Cells were scraped from the substratum with a cell scraper and centrifuged at 1000×*g* for 5 min at room temperature. The cell pellet was then resuspended with PIPES buffer (0.1 M PIPES buffer containing 2 M glycerol, 5 mM $MgCl_2$, 2 mM EGTA, 0.04 TIU/ml aprotinin, 2 mM phenylmethylsulfonylfluoride, and 1 mM benzamidine, pH 6.9) containing 1% Nonidet P-40 and 1 mM taxol and incubated for 15 min at 37 ª C. After the incubation, the suspension was centrifuged at 1000×*g* for 5 min at 37 °C. The pellet consisting of the microtubules was then solubilized in 125 mM Tris buffer, pH 6.8, containing 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol and heated to 100 °C for 5 min. After centrifugation at 10,000×*g* for 5 min, the supernatant was stored at −20 ª C for subsequent Western blot analysis. For total cell extracts, same number of cells was taken and washed twice with HBSS buffer and solubilized as described above.

2.4. Preparation of microfilaments

Microfilament-enriched preparations were extracted from COLO-205 cells (2×10^6 cells/ml) as described by Phillips et al. (1980) [16,17]. Culture medium was aspirated and changed to HBSS buffer. Cells were scraped from the tissue culture flasks with a cell scraper. Cells were centrifuged at 100×*g* for 5 min at room temperature. The pellet was dissolved in 500 μl of Triton solution (1% Triton X-100, 10 mM EGTA, and 0.1 M Tris–HCl, pH 7.4) and kept on ice for 10 min. The preparation was then centrifuged at 8000×*g* for 5 min at room temperature. Triton-soluble G-actin fraction was contained in the supernatant. The pellet, which corresponds to the Triton-insoluble fraction, was solubilized in 2% SDS-2% 2-mercaptoethanol (v/v) by boiling at 100 °C for 10 min.

2.5. Preparation of cytosolic and membrane-bound PKC-α

Cytosolic and membrane-bound PKC-α were prepared according to Datta Gupta et al. (2005) [8] with little modification. Confluent monolayers of COLO-205 cells (2×106 cells/ml) were treated in the presence and absence of STa or with disruptors for 1 h before stimulation of STa for 1 min. The reactions were terminated using the ice-cold Hank's balanced salt solution (HBSS). Cells were washed twice in 20 mM Tris–HCl (pH 7.5), suspended in the same buffer containing PMSF, and leupeptin and then homogenized with a motor driven Teflon homogenizer (REMI Udyog, India). The homogenate was centrifuged for 60 min at 30,000 rpm at 4 °C. The Supernatant was separated from pellet and used for measuring the cytosolic PKC-α [18]. The pellet was then resuspended in 20 mM Tris–HCl (PH 7.5) containing PMSF and leupeptin, homogenized briefly in the presence of 1% Triton X-100 to solubilize the membrane-bound proteins and recentrifuged as before (for 60 min at 30,000 rpm at 4 °C). The resulting supernatant served as the source of membrane-bound PKC-α [19].

2.6. Isolation of cytosolic and membrane fraction

Membrane and cytosolic fractions were obtained by using the method of Cote et. al. (1996) [20]. Briefly, the confluent monolayer of COLO-205 cells (2×10^6 cells/ml) were treated with STa (5nM) and incubated at 37 °C for 0–120 s, cells were washed twice with HBSS buffer and then with 10 mM ice-cold Tris–HCl buffer (containing 0.5 mM EDTA, 1 mM $MgCl_2$, 1 μg/ml leupeptin, 0.21 μg/ml aprotinin1mM PMSF, and10 μM benzimidine, pH 8.0). The cells were then scraped and homogenized in the same buffer. Cell extracts were centrifuged at 800×*g* for 5 min, and the supernatant was then centrifuged at 15,000×*g* for 30 min. After centrifugation, the resulting supernatant was used as cytosolic fraction. The pellet was then resuspended in 50 mM Tris–HCl buffer (containing 2 mM EDTA, 5 mM $MgCl_2$, and 250 mM sucrose) to obtain the membrane fractions.

2.7. Immunoprecipitation

Immunoprecipitation was performed according to Ron et al. (1999) [21] with little modification. Each membrane fraction (prepared as described above) was pre-cleaned by incubation with 50 μl of protein A/G plus agarose (Santa Cruz Biotechnology) for 2 h at 4 °C. The samples were centrifuged at 200×*g* for 1 min, to collect the supernatant, and protein content was measured. Immunoprecipitation was performed overnight at 4 °C with 5 μg of actin antibody per 500 μg protein diluted with an equal volume of immunoprecipitation buffer (2% Triton X-100, 300 mM NaCl, 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 0.4 mM sodium vanadate, 1 mM PMSF, leupeptin and aprotinin). Fifty microliters of protein A/G plus agarose was then added, and the samples were incubated at 4 °C for 2 h. Finally, the samples were centrifuged at 14,000×*g* for 10 min, the supernatant was discarded, and the pellet was resuspended in SDS protein gel loading solution. It was then subjected to immunoblotting by using anti-PKC-alpha antibody. It was also subjected to immunoblotting by using anti-PKC-βII antibody to exclude unspecific binding to actin.

2.8. Immunoblotting and densitometric analysis

Western blot analysis was done to detect the actin, tubulin, and PKC-α by using anti-actin, anti-tubulin, and anti-PKC-α antibodies.