Available online at www.sciencedirect.com

SCIENCE DIRECT

Toxicology and Applied Pharmacology 206 (2005) 9–16

Toxicology and Applied Pharmacology

www.elsevier.com/locate/ytaap

Involvement of protein kinase C in the mechanism of action of *Escherichia coli* heat-stable enterotoxin (STa) in a human colonic carcinoma cell line, COLO-205

Dyuti Datta Gupta, Subhrajit Saha, Manoj K. Chakrabarti*

Division of Pathophysiology, National Institute of Cholera and Enteric Diseases, P-33, C. I. T. Road, Scheme-XM, Beliaghata, Kolkata-700 010, India

Received 30 June 2004; accepted 1 November 2004
Available online 23 December 2004

Abstract

The present study was undertaken to determine the involvement of calcium-protein kinase C pathway in the mechanism of action of *Escherichia coli* heat stable enterotoxin (STa) apart from STa-induced activation of guanylate cyclase in human colonic carcinoma cell line COLO-205, which was used as a model cultured cell line to study the mechanism of action of *E. coli* STa. In response to *E. coli* STa, protein kinase C (PKC) activity was increased in a time-dependent manner with its physical translocation from cytosol to membrane. Inhibition of the PKC activity in membrane fraction and inhibition of its physical translocation in response to IP_3-mediated calcium release inhibitor dantrolene suggested the involvement of intracellular store depletion in the regulation of PKC activity. Among different PKC isoforms, predominant involvement of calcium-dependent protein kinase C (PKCα) was specified using isotype-specific pseudosubstrate, which showed pronounce enzyme activity. Inhibition of enzyme activity by PKCα-specific inhibitor Gö6976 and immunoblott study employing isotype-specific antibody further demonstrated the involvement of calcium-dependent isoform of PKC in the mechanism of action of *E. coli* STa. Moreover, inhibition of guanylate cyclase activity by PKCα-specific inhibitor Gö6976 suggested the involvement of PKCα in the regulation of guanylate cyclase activity.

Keywords: Calcium; Protein kinase C; *Escherichia coli* heat-stable enterotoxin; COLO-205 cell line; Guanylate cyclase

Introduction

The heat stable enterotoxin (STa) of *Escherichia coli* is a major causative agent of secretory diarrhea. STa is an infant mouse active class of stable toxin consisting of 18 to 19 amino acid residues (Aimoto et al., 1982; Takao et al., 1983). Binding of *E. coli* STa with its receptor increased the intracellular cGMP level which regulates the chloride secretion through the activation of a chloride channel known as cysteic fibrosis transmembrane conductance regulator (CFTR) (Giannella and Mann, 2003; Tien et al., 1994). Although it was reported that STa stimulation of GC-C is greater in the human small intestine than in the colon (Guarino et al., 1987; Kuhn et al., 1994) and in general, fewer STa receptors are present in human colon (Cohen et al., 1988; Krause et al., 1994; Sears and Kaper, 1996), the short circuit current in response to STa is greater in colon. The reason behind this is still unknown. Therefore, to study the mode of action of STa, colonic cells may help to understand the in-depth mechanism. Moreover, it is still not clear whether cGMP alone accounts for full secretory response to STa (Beubler et al., 1992). In addition to this, involvement of specific signaling pathway for STa-mediated activation of cGMP is still not clear (Sears and Kaper, 1996). It was reported that in rat intestinal epithelial cells, *E. coli* STa not only increased the intestinal cGMP level (Crane and Shanks, 1996; Field et al., 1978) it also involves intracellular calcium and protein kinase C (PKC) activation in its mechanism of action (Ganguly et al., 2001; Ghosh Chaudhury and Ganguly, 1995; Knoop et al., 1991).

* Corresponding author. Fax: +91 3323505066.
E-mail address: mkc_niced@yahoo.co.in (M.K. Chakrabarti).

0041-008X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2004.11.001

However, correlation of these signalling mediators with intestinal secretion is still not clear. On the other hand, in T-84 human colonic carcinoma cell line, it was found that although STa bound and accumulate cGMP (Guarino et al., 1987; Visweswariah et al., 1994), it did not rise the intracellular calcium level (Huott et al., 1988). But we reported earlier that in another hitherto unreported human colonic carcinoma cell line COLO-205, *E. coli* STa not only bound and accumulated cGMP but also involved IP_3-mediated calcium mobilization from intracellular calcium store (Bhattacharya and Chakrabarti, 1998; Bhattacharya et al., 1997). In the present study, we have shown that in COLO-205 cell line, IP_3-mediated calcium mobilization activates and translocates protein kinase C (PKC) from cytosol to membrane fraction of human colonic carcinoma cell line, COLO-205 in response to *E. coli* STa. This activated PKC may be involved in the rise in intracellular cGMP level by the activation of guanylate cyclase.

Materials and methods

Materials

RPMI-1640 medium, Foetal Bovine Serum (FBS), Hank's Balanced Salt Solution (HBSS) and Sodium pyruvate were obtained from Gibco BRL, USA. Dimethyl sulfoxide (DMSO), Phenylmethylsulphonylfluoride (PMSF), Phosphatidylserine (PdSer), Diolein, Histone IIIS, Dithiothreitol (DTT), Leupeptin, *Escherichia coli* heat stable enterotoxin (STa), Dantrolene and isoform-specific monoclonal antibody against PKCα were obtained from Sigma, USA. Secondary antibody was obtained from Bangalore Genei, India. Gö6976 was procured from Calbiochem, UK, and [γ-^{32}P] ATP was purchased from Board of Radiation and Isotope Technology, Hyderabad. [^{3}H] cGMP radioimmunoassay kit was purchased from Amersham Biosciences (UK). All other reagents were of analytical grade and deionized double distilled water was used throughout the study.

Methods

Cell culture and preparation of viable cells. COLO-205 cells which were procured from National Centre for Cell Sciences, Pune, were routinely cultured in tissue culture flasks and grown up to monolayers in RPMI-1640 medium supplemented with 10% Foetal Bovine Serum (FBS), in a humidified 5% CO_2 atmosphere at 37 °C. Confluent monolayers were subcultured at 3–4 day intervals. The viability of the cells were routinely checked by trypan blue exclusion.

Preparation of cytosolic and membrane-bound protein kinase C (PKC). Confluent monolayers of COLO-205 cells (2×10^6 cells/ml) were treated with or without *E. coli* STa (5 nM) and incubated at 37 °C for a given time interval. 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 and then homogenized with a motor-driven Teflon/glass homogenizer (REMI Udyog, India). The homogenate was centrifuged for 60 min at 100,000 × *g*. The supernatant was separated from the pellet and used for measuring the cytosolic PKC (Ganguly and Ghosh Chaudhury, 1996; Kraft and Anderson, 1983.) The pellet was then resuspended in 20 mM Tris–HCl (pH 7.5), homogenized briefly in the presence of 1% Triton-X-100 to solubilize the membrane-bound proteins and recentrifuged as before. The resulting supernatant served as the source of membrane-bound PKC (Ganguly et al., 2001; Ghosh Chaudhuri et al., 1993; Rush et al., 1992). The efficiency of the separation of cytosolic and membrane fractions was determined by measuring the activities of alkaline phosphatase, a membrane enzyme marker (Bernard et al., 1992), and lactate dehydrogenase, a cytosolic marker (Giovanazzi et al., 1997).The enzyme preparations were stored in aliquots at −70 °C until use.

Assay of protein kinase C. Protein kinase C activity was assayed using the standard method (Ganguly et al., 2001; Ghosh Chaudhuri et al., 1993). The standard assay mixture contained 10 μg histone IIIS, 5 μg Phosphatidylserine, 2.5 μg diolein, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.5), 5 mM DTT, 2 μg leupeptin, 10 μM ATP and 1.0 μCi [γ-^{32}P] ATP (specific activity 5000 Ci/mmol) to a final volume of 100 μl. The reaction was started by the addition of cytosolic and membrane fractions (5 μg protein) followed by incubation at 30 °C for 10 min. The reaction was terminated by transferring 60 μl of the reaction mixture to a 3 mm whatman filter paper (2×2 cm^2) as described by Racker (1991). The phosphocellulose filter paper was then dried in air, placed in a plastic container (4×6 in.) and washed with 100 ml of 10% trichloroacetic acid (TCA) plus 10 mM Na_2HPO_4. The progress of the washing was monitored using a Geiger counter. Each filter paper was removed by a forcep, dried in an oven, and then placed in a vial containing 3 ml of liquid scintillation cocktail (ready solvent HP, Beckman, USA.). Radioactivity was determined using a liquid scintillation counter (Beckman, LS1801). All assays were done in duplicate and repeated at least thrice. Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Detection of PKC isoform in the cytosolic and membrane fractions by immunoblotting. *E. coli* STa treated and untreated cytosolic and membrane fractions were prepared from COLO-205 cell line as described earlier in Materials and methods. Protein concentration of each sample was 50 μg per 25 μl of sample buffer (Laemmli, 1970). Samples were heated for 5 min, subjected to 10% SDS-PAGE for separation and then transferred to nitrocellulose membranes