ELSEVIER

FEMS Microbiology Letters 160 (1998) 125–129

FEMS MICROBIOLOGY LETTERS

Rise of cytosolic Ca^{2+} and activation of membrane-bound guanylyl cyclase activity in rat enterocytes by heat-stable enterotoxin of *Vibrio cholerae* non-01

Alok Ghosh Chaudhuri [a], Jayanta Bhattacharya [a], G. Balakrish Nair [a], Tae Takeda [b], Manoj K. Chakrabarti [a,*]

[a] *National Institute of Cholera and Enteric Diseases, P-33, C.I.T Road, Scheme-XM, Beliaghata, Calcutta-700 010, India*

[b] *National Children's Medical Research Institute, Tokyo, Japan*

Received 9 September 1997; revised 9 October 1997; accepted 12 October 1997

Abstract

The cytosolic calcium level ($[Ca^{2+}]_i$) and the membrane-bound guanylyl cyclase activity in the isolated rat intestinal epithelial cells were investigated. Heat-stable enterotoxin of *Vibrio cholerae* non-01 (NAG-ST) was found to increase both the $[Ca^{2+}]_i$ and the enzyme activity. These changes occur similarly until 5 min of incubation with NAG-ST, indicating that these changes might be involved in NAG-ST induced signal transduction in rat enterocytes. © 1998 Published by Elsevier Science B.V.

Keywords: *Vibrio cholerae* non-01; Heat-stable enterotoxin; Intracellular calcium ion concentration; Guanylyl cyclase; Epithelial cell

1. Introduction

Vibrio cholerae strains of the non-01 serovar, which are referred to as nonagglutinable (NAG) vibrios, produce severe gastroenteritis in humans [1] and the clinical features are sometimes indistinguishable from cholera. The virulence of the NAG vibrios depends on their ability to colonize the intestines and to elaborate a heat-stable enterotoxin (NAG-ST) [2]. NAG-ST consists of 17 amino acid residues and structurally resembles the heat-stable enterotoxins of *Escherichia coli* and *Yersinia enterocolitica* [3]. Heat-stable enterotoxin of *E. coli* [4] and *Y. enterocolitica* [3] triggers intestinal secretion by stimulating the membrane-bound guanylyl cyclase and thereby elevating the intracellular levels of cyclic guanosine 3′5′ monophosphate (cyclic GMP). *E. coli* heat-stable enterotoxin (STa) has been found to elevate the intracellular levels of calcium ($[Ca^{2+}]_i$) [5] and the Ca^{2+}-dependent protein kinase C appears to regulate the activation of guanylyl cyclase [6]. In the present study, we have demonstrated that similar to *E. coli* STa, NAG-ST increases the $[Ca^{2+}]_i$ besides the activation of membrane-bound guanylyl cyclase in isolated rat enterocytes.

* Corresponding author. Tel.: +91 (33) 350-4478; Fax: +91 (33) 350-5066; E-mail: icmrnicd@ren.nic.in

PII S0378-1097(98)00021-4

2. Materials and methods

2.1. Materials

Phenylmethylsulfonylfluoride (PMSF), leupeptin, cyclic GMP, fura-2/acetoxymethyl ester (AM), bovine serum albumin (BSA), GTP, creatine phosphate, creatine phosphokinase, calcium chloride ($CaCl_2$), dimethylsulfoxide (DMSO) and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma Chemical Company (USA). A [^{3}H]-cyclic GMP radioimmunoassay kit was obtained from Amersham International, UK NAG-ST enterotoxin was prepared as described in [3]. All other chemicals were analytical grade reagents and deionized double distilled water was used throughout the study.

2.2. Suckling mouse assay

The biological activity of the NAG-ST was assayed in the 2–3 days old suckling mouse model [3]. The minimum quantity of the enterotoxin (8 ng) which gave in the suckling mouse assay a fluid accumulation ratio (intestinal weight/remaining body weight) of 0.090 after 3 h was taken as 1 mouse unit.

2.3. Preparation of intestinal epithelial cells

Rat intestinal epithelial cells were prepared from the jejunum as described earlier [7]. The cells were finally suspended in balanced salt solution (BSS) containing 135 mM NaCl, 4.5 mM KCl, 5.6 mM glucose, 0.5 mM $MgCl_2$, 10 mM HEPES and 1 mM $CaCl_2$, pH 7.4, plus 20 μg/ml leupeptin. Cell numbers and viability were determined with a hemocytometer and by trypan blue exclusion respectively.

2.4. Measurement of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ was measured using fura-2 fluorescence according to Grynkiewicz et al. [8]. Briefly, cells (4×10^6/ml) suspended in BSS (pH 7.4) with 0.1% BSA were loaded with 10 μM of fura-2/AM in DMSO and incubated at 37°C under constant shaking in the dark. After 40 min the cells were washed, resuspended in BSS without any BSA and transferred to a thermostated, magnetically stirred quartz cuvette of Hitachi spectrofluorometer (model 3010). Fluorescence (F) was measured at 37°C with excitation at 340 nm (slit 5 nm) and emission at 495 nm (slit 5 nm). Maximum fluorescence (F_{max}) was measured in the presence of 0.1% Triton X-100, which reflected the efficiency of fura-2 loading. Subsequent addition of 20 μM $MnCl_2$ caused quenching of intracellular Ca^{2+}-fura-2 fluorescence (F_{min}). The $[Ca^{2+}]_i$ was then obtained using the formula: $[Ca^{2+}]_i = 224(F-F_{min})/(F_{max}-F)$, where 224 was the association constant of Ca^{2+} with fura-2 at physiological pH. Autofluorescence was measured in cells sham loaded with DMSO and subtracted from the values obtained in experimental samples.

2.5. Preparation and assay of guanylyl cyclase

Enterocytes (4×10^6/ml) suspended in BSS (pH 7.4) containing 1 mM IBMX were treated with NAG-ST and incubated at 37°C for a given period of time. After stopping the reaction with ice-cold BSS (pH 7.4) without IBMX, the cells were resuspended in 50 mM Tris buffer (pH 7.6) containing 4 mM EDTA and homogenized. The homogenate thus obtained was immediately used as a source of guanylyl cyclase.

The guanylyl cyclase activity was measured by the method of Gazzano et al. [9] with slight modifications. The assay mixture contained 50 mM Tris-4 mM EDTA (pH 7.6), 4 mM $MgCl_2$, 1 mM GTP, 7.5 mM creatine phosphate, 20 μg (200 units/mg) creatine phosphokinase and 1 mM IBMX to a final volume of 120 μl. The reaction was then initiated by the addition of enzyme preparation (50–100 μg protein) and carried out at 37°C for 10 min. The reaction was terminated by the addition of 30 μl ice-cold 50 mM Tris-4 mM EDTA buffer (pH 7.6) rapidly followed by boiling at 95°C for 3 min. The mixture was then centrifuged at $12\,000\times g$ for 10 min. The supernatant was collected to the volume of 100 μl and the amount of cyclic GMP produced was measured using a [^{3}H]-cyclic GMP radioimmunoassay kit.

2.6. Protein determination

The protein concentrations were estimated by the method of Bradford [10] using bovine serum albumin (BSA) as standard.

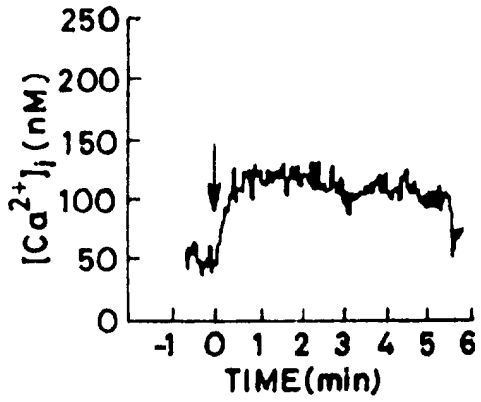

Fig. 1. Time course of NAG-ST action on the $[Ca^{2+}]_i$ in rat enterocytes. Arrow indicates the time of addition of toxin (16 ng). The result shown here was the tracing from a representative experiment and similar results were obtained in two other experiments.

3. Results

3.1. Time course of STa action on $[Ca^{2+}]_i$

As shown in Fig. 1, treatment of enterocytes with NAG-ST (16 ng) provoked a rapid increase of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ increased as early as 30 s and became maximal within 1 min of NAG-ST incubation followed by a sustained phase up to 5 min. There was a 2-fold rise of $[Ca^{2+}]_i$ as compared to basal value (50 ± 4 nM vs. 116 ± 7 nM, $P < 0.01$).

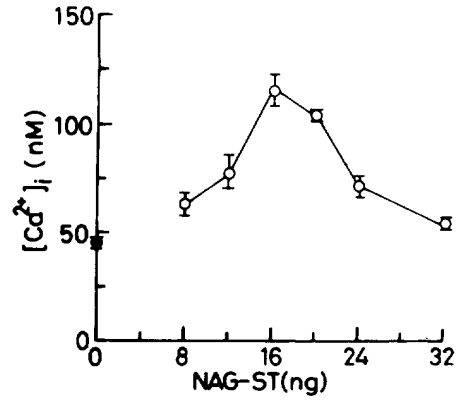

Fig. 2. Effect of different concentrations of NAG-ST on the $[Ca^{2+}]_i$ in rat enterocytes. Data represent the mean ± S.E.M of three determinations.

3.2. Effect of different concentrations of NAG-ST on $[Ca^{2+}]_i$

Dose related effects of NAG-ST on $[Ca^{2+}]_i$ are shown in Fig. 2. The $[Ca^{2+}]_i$ increased significantly within 1 min from basal concentrations of 46 ± 4 nM to 64 ± 4 nM, 80 ± 6 nM and 116 ± 5 nM with 8, 12 and 16 ng of NAG-ST respectively, indicating optimal level of $[Ca^{2+}]_i$ was achieved with 16 ng of NAG-ST, which was used in the subsequent study. However, with increasing concentrations (20, 24 and 32 ng) the $[Ca^{2+}]_i$ underwent a gradual decrement probably due to a toxic effect of the enterotoxin on the cell.

3.3. Effect of NAG-ST on guanylyl cyclase activity

The effect of NAG-ST on guanylyl cyclase activity is shown in Fig. 3. The enzyme activity increased as early as 30 s after NAG-ST treatment and maximum stimulation was observed after 2 min of NAG-ST incubation. There was a 5-fold rise of enzyme activity in the enterotoxin treated cells as compared to that in control cells (1.46 ± 0.3 vs. 6.93 ± 0.5 pmole cyclic GMP produced/mg protein, $P < 0.001$). Thereafter, the enzyme activity decreased slightly and remained greater than the basal value even up to 5 min.

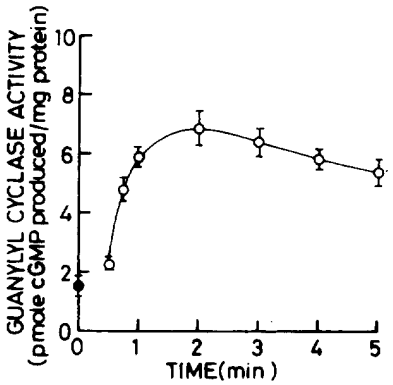

Fig. 3. Time course of NAG-ST action on the guanylyl cyclase activity in rat enterocytes. Cells were treated with 16 ng of NAG-ST for the indicated times. The enzyme activity was assayed as described in Section 2. Data represent the mean ± S.E.M ($n = 3$). ●, control; ○, NAG-ST treated.

4. Discussion

V. cholerae non-01 strains are recognized as the causative agents of gastroenteritis [1]. Proposed virulence factors of non-01 vibrios include toxins similar to cholera toxin [11], ElTor hemolysin [12], Kanagawa hemolysin [13], Shiga like toxin [14], various cell-associated hemagglutinins [15] and NAG-ST. NAG-ST closely resembles the heat-stable enterotoxin (STa) of *E. coli* with respect to their amino acid sequences especially at the carboxyl-terminal region [16]. *E. coli* STa has been demonstrated to stimulate the membrane bound guanylyl cyclase activity in rat enterocytes [4] and cultured human colonic carcinoma T84 cells [17]. Increased levels of cyclic GMP within the cells are hypothesized to lead to enhanced chloride ion secretion from the intestinal cells. The rise of cytosolic Ca^{2+} by *E. coli* STa is evident in rat enterocytes [5] and has been found to be involved in initiating the pathophysiological events leading to fluid accumulation in the suckling mouse model [18,19].

In the present study, the suckling mouse positive NAG-ST has also been demonstrated to raise the intracellular level of Ca^{2+} in rat enterocytes (Fig. 1) and the effects are dose-dependent (Fig. 2). Moreover, there is an enhanced activity of membrane bound guanylyl cyclase (Fig. 3) and this observation is conforming to what was found in T84 cells [20]. In NAG-ST treated enterocytes the time course of $[Ca^{2+}]_i$ rise and the activation of membrane bound guanylyl cyclase occur in the same time frame, suggesting $[Ca^{2+}]_i$ might be consonant with the signal transduction mechanism associated with intracellular cyclic GMP accumulation resulting in fluid loss and subsequent diarrhea.

Acknowledgments

The authors wish to thank Dr. S.K. Bhattacharya, Director, NICED, for encouragement throughout the studies.

References

[1] Bagchi, K., Echeverria, P., Arthur, J.D., Sethabutr, O., Serichantalergs, O. and Hoge, C.W. (1993) Epidemic of diarrhoea caused by *Vibrio cholerae* non-01 that produced heat-stable toxin among Khmers in a camp in Thailand. J. Clin. Microbiol. 31, 1315–1317.

[2] Ogawa, A., Katop, J.I., Watanabe, H., Nair, G.B. and Takeda, T. (1990) Cloning and nucleotide sequence of a heat-stable enterotoxin gene from *Vibrio cholerae* non-01 isolated from a patient with traveller's diarrhoea. Infect. Immun. 58, 3325–3329.

[3] Yoshimura, S., Takao, T., Shimonishi, Y., Hara, S., Arita, M., Takeda, T., Imaishi, H., Honda, T. and Miwatani, T. (1986) A heat-stable enterotoxin of *Vibrio cholerae* non-01: Chemical synthesis, and biological and physicochemical properties. Biopolymers 25, S69–S83.

[4] Field, M., Graf, L.H., Laird, W.J. and Smith, P.L. (1978) Heat-stable enterotoxin of *Escherichia coli*: in vitro effects on guanylate cyclase activity, cyclic GMP concentration and ion transport in small intestine. Proc. Natl. Acad. Sci. USA 75, 2800–2804.

[5] Ghosh Chaudhuri, A. and Ganguly, U. (1995) Evidence for stimulation of the inositol triphosphate-Ca^{2+} signalling system in rat enterocytes by heat stable enterotoxin of *Escherichia coli*. Biochim. Biophys. Acta 1267, 131–133.

[6] Crane, J.K., Wehner, M.S., Bolen, E.J., Sando, J.J., Linden, J., Guerrant, R.L. and Sears, C.L. (1992) Regulation of intestinal guanylate cyclase by the heat-stable enterotoxin of *Escherichia coli* (STa) and protein kinase C. Infect. Immun. 60, 5004–5012.

[7] Ghosh Chaudhuri, A., Sen, P.C. and Ganguly, U. (1993) Evidence for protein kinase C stimulation in rat enterocytes pretreated with heat-stable enterotoxin of *Escherichia coli*. FEMS Microbiol. Lett. 110, 185–190.

[8] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

[9] Gazzano, H., Wu, H.I. and Waldman, S.A. (1991) Activation of particulate guanylate cyclase by *Escherichia coli* heat-stable enterotoxin is regulated by adenine nucleotides. Infect. Immun. 59, 1552–1557.

[10] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.

[11] Yamamoto, K., Takeda, Y., Miwatani, T. and Craig, J.P. (1983) Evidence that a non-01 *Vibrio cholerae* produces enterotoxin that is similar but not identical to cholera enterotoxin. Infect. Immun. 41, 896–901.

[12] Yamamoto, K., Ichinose, Y., Naaksone, N., Taanbe, M., Nagahama, N., Sakurai, J. and Iwanaga, M. (1986) Identity of hemolysins produced by *Vibrio cholerae* non-01 and *Vibrio cholerae* 01, biotype ElTor. Infect. Immun. 51, 927–931.

[13] Yoh, M., Honda, T. and Miwatani, T. (1985) Production by non-01 *Vibrio cholerae* of hemolysin related to thermostable direct hemolysin of *Vibrio parahaemolyticus*. FEMS Microbiol. Lett. 29, 197–200.

[14] O'Brien, A.D., Chen, M.E., Holmes, R.K., Kaper, J.B. and Levine, M.M. (1984) Environmental and human isolates of

Vibrio cholerae and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae 1* (Shiga-like) cytotoxin. Lancet i, 77–78.

[15] Yamamoto, T. and Yokota, T. (1988) *Vibrio cholerae* non-01: production of cell-associated hemagglutinins and in vitro to mucous coat and epithelial surfaces of the villi and lymphoid follicles of human small intestines treated with formalin. J. Clin. Microbiol. 26, 2018–2024.

[16] Ogawa, A. and Takeda, T. (1993) The gene encoding the heat-stable enterotoxin of *Vibrio cholera* is flanked by 123-base pair direct repeats. Microbiol. Immunol. 37, 607–616.

[17] Visweswariah, S.S., Ramachandran, V., Ramamohan, S., Das, G. and Ramachandran, J. (1994) Characterization and partial purification of the human receptor for the heat-stable enterotoxin. Eur. J. Biochem. 219, 727–736.

[18] Thomas, D.D. and Knoop, F.C. (1982) The effect of calcium and prostaglandin inhibitors on the intestinal fluid response to heat-stable enterotoxin of *Escherichia coli*. J. Infect. Dis. 145, 141–147.

[19] Greenberg, R.N., Murad, F. and Guerrent, R.C. (1982) Lanthum chloride inhibition of the secretory response to *Escherichia coli* heat-stable enterotoxin. Infect. Immun. 35, 483–488.

[20] Visweswariah, S.S., Shanti, G. and Balganesh, T.S. (1992) Interaction of heat-stable enterotoxins with human colonic (T84) cells: modulation of the activation of guanylyl cyclase. Microb. Pathog. 12, 209–218.