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Stimulation of phosphoinositides breakdown by the heat stable *E. coli* enterotoxin in rat intestinal epithelial cells

Napur Banik and Uma Ganguly

National Institute of Cholera and Enteric Diseases, P-33 CIT Road, Scheme XM, Beliaghata, PO Box 177, Calcutta 700010, India

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Rat intestinal epithelial cells were labelled with [^{32}P]P_i and extracted, and the phospholipids were analysed by thin-layer chromatography. ^{32}P-incorporation in phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-phosphate (PIP_2) were measured in control and heat stable enterotoxin (ST)-treated cells. ST was found to induce rapid degradation of PIP and PIP_2. The degradation of inositol lipids was accompanied by an increase of water soluble inositol phosphate (IP_1, IP_2, IP_3) compounds. There was a two-fold increase of radioactivity in IP_2 and IP_3 but no significant change was observed in IP_1. Phospholipase C activity was increased tenfold with substrate PIP_2 in ST-pretreated cells. The present study indicates that ST triggers another second messenger system by increasing the PIP_2 hydrolysis with the enzyme phospholipase C.

Heat stable enterotoxin; Inositol lipid; Inositol phosphate; (Epithelial cell)

1. INTRODUCTION

The phosphoinositide (PI) and polyphosphoinositides (PIP and PIP_2) have been implicated in the signal transduction of external stimuli such as hormones, neurotransmitters and various agonists [1]. It was proposed that inositol triphosphate (IP_3) thus produced acts as a second messenger for intracellular Ca^{2+} mobilisation which in turn modulates many cellular events including regulation of electrolyte transport [2].

We reported [3] that a heat stable eneterotoxin of *E. coli* stimulated the PI-specific phospholipase C activity of the rat intestine. This observation was correlated with increased uptake of Ca^{2+} by ST-treated rat brush border membrane [4] and increased activity of calmodulin in the ST-treated intestinal microvillar core [5]. These findings suggested that ST may induce some kind of signal via the PI cycle to promote Ca^{2+} translocation across the plasma membrane of stimulated cells which may in turn induce intestinal secretion.

The experiments reported in this communication demonstrated the formation of the inositol phosphates from their respective inositol phospholipids upon the activation of rat intestinal epithelial cells with ST.

Correspondence address: U. Ganguly, National Institute of Cholera and Enteric Diseases, P-33 CIT Road, Scheme XM, Beliaghata, PO Box 177, Calcutta 700010, India

2. MATERIALS AND METHODS

L-α-phosphatidylinositol, L-α-phosphatidylinositol 4-monophosphate, L-α-phosphatidylinositol 4,5-bisphosphate, *myo*-inositol and inositol phosphate compounds, triethanolamine, phenylmethylsulfonyl fluoride (PMSF) and ditheothreitol (DTT) were obtained from Sigma and Dowex 1×8 (chloride form 100-200 mesh) from BioRad. [^{32}P]P_i was purchased from Bhaba Atomic Research Centre, Trombay, Bombay.

The ST was prepared from *E. coli* strain 90 as described earlier [4]. The minimal amount of ST given in suckling mouse, produced a fluid accumulation (FA) ratio of 0.085 after 3 h and was defined as 1 mouse unit (MU).

2.1. *Preparation of epithelial cells*

Rat intestinal epithelial cells were prepared from jejunum [6]. After removing the debris with 0.9% NaCl plus 1 mM DTT, jejunum was filled with buffer containing 1.5 mM KCl, 96 mM NaCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 27 mM Na_2-citrate,

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pH 7.3, plus 1 mM DTT and 0.23 mM PMSF and incubated for 15 min at 37°C. Jejunum was emptied and filled again with EDTA buffer containing 0.9% NaCl, 9.0 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 1.5 mM Na_3 EDTA, pH 7.6, plus 0.5 mM DTT and 0.23 mM PMSF. Fluid was collected and cells were spun down in a Sorvall RS5 fixed angle rotor for 5 min at 1500 rpm. The pellet was then resuspended in buffer containing 250 mM sucrose, 10 mM triethanolamine HCl, pH 7.6, plus 0.23 mM PMSF and centrifuged at 2000 rpm for 5 min. The pellet suspended in PBS buffer represents mucosal epithelial cells.

2.2. *Labelling of cells with $H_3{}^{32}PO_4$*

Epithelial cells were labelled with [^{32}P]P_i according to the method of Sugimoto et al. [7]. The cells were incubated in a medium containing 116 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM Hepes, 500 mg/ml glucose, pH 7.2, and carrier free [^{32}P]P_i (50 μci/ml cell suspension) for 60 min at 37°C. Subsequently, the excess of extracellular [^{32}P]P_i was removed by thoroughly washing with ice-cold buffer and resuspended in the same buffer. Thereafter, the cells were incubated with ST (2 MU) and the samples withdrawn after 0,30,60,90 and 120 s of incubation. Incubation was terminated by the addition of ice-cold chloroform/methanol/HCl.

2.3. *Extraction [8] and analysis of phosphoinositides [9]*

To 1 ml of aqueous cell suspension, 3.75 ml of chloroform/methanol/1.2 N HCl (2:1:1.6 ml, v/v) was added and allowed to stand (at room temperature in ice cold conditions) for 20–30 min. This was followed by 1.25 ml of chloroform. During extraction, 0.05% 2,6-di-*tert*-butyl-*p*-cresol was used as an antioxidant. Phase separation was carried out by centrifugation. The chloroform layer was withdrawn and dried in vacuo and lipids were dissolved in chloroform/methanol (2:1, v/v).

Inositol lipids were separated by one-dimensional TLC on Silica gel 60 H plates impregnated with 1% potassium oxalate (E. Merck, Darmstadt, FRG) using the solvent system chloroform/methanol/28% NH_4OH (65:35:5, v/v). The labelled phospholipids were visualised by iodine vapour and spots were identified when matched against authentic standards. The corresponding area of each phospholipid was scraped off and radioactivity counted by a liquid scintillation counter (Beckman LS 1801).

2.4. *Identification of inositol compounds [10]*

Water-soluble inositol compounds present in the polar phase of cell extracts were separated on a small column containing 1 ml of Dowex AG1×8 in formate form (200–400 mesh, BioRad). The column was washed with distilled water (7 ml) to remove *myo*-inositol then the phosphate esters were eluted by stepwise addition of (i) 0.025 M ammonium formate (glycerophosphoinositol), (ii) 0.1 M ammonium formate (Ins cyclic phosphate), (iii) 0.2 M ammonium phosphate (IP), 0.5 M ammonium formate (IP_2), and 1 M ammonium formate (IP_3).

A volume of 2 ml of the elute was diluted to 10 ml with water and lyophilized to remove both the water and ammonium formate. The dried extract was suspended in 0.1 ml water and the sample was chromatographed on Whatman no. 1 paper using the solvent system *n*-propanol/conc. NH_3/H_2O (5:4:1, v/v). The spots were identified with ammonium molybdate spray [11]. The identity of each spot was confirmed by comparison with authentic standard. The radioactivity was measured from a spot corresponding to IP_1, IP_2 and IP_3. The recovery of radioactivity from the applied sample was only 60%. PI-specific phospholipase C (PLC) activity was measured [3] in ST-pretreated cells with substrates PIP and PIP_2. The specific ^{32}P-radioactivity of metabolic ATP was determined [12] and the mass of ATP was measured by the luciferin and luciferase method.

3. RESULTS

Fig.1 shows the time course for the incorporation of [^{32}P]P_i in rat intestinal epithelial cells for 90 min. The cells were taken out at desired intervals, washed thoroughly and their radioactivity measured. Under the control condition, the levels of the [^{32}P]P_i into the cells increased in a linear manner up to 60 min. Thus all of our experiments were done in 60 min incubation with [^{32}P]P_i.

The time course of ST action on PI and poly-PI turnover were shown in fig.2. The early response to ST was examined in cells which were prelabelled for 60 min and subsequently treated with ST for 2 min. ST was found to induce a degradation of PIP (fig.2B) and PIP_2 (fig.2C). At 2 min, about 40% of labelled PIP and PIP_2 had disappeared. The ^{32}P-radioactivity associated with PIP was reduced slowly starting from 30 s after addition of ST whereas the ^{32}P-radioactivity of PIP increased for 45 s and then started decreasing. The ^{32}P-radioactivity of PI (fig.2A) remained unchanged.

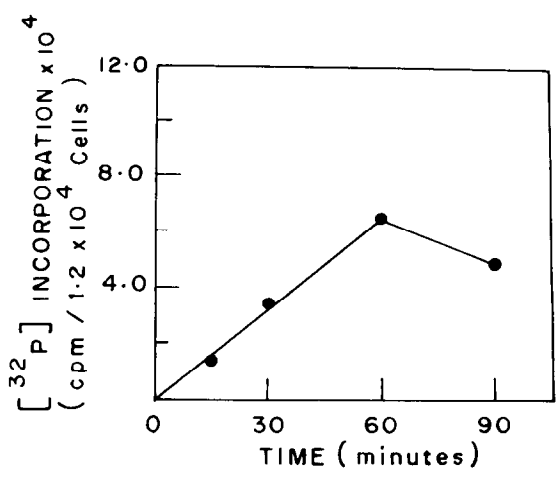

Fig.1. Time course of ^{32}P-incorporation in rat intestinal epithelial cells.

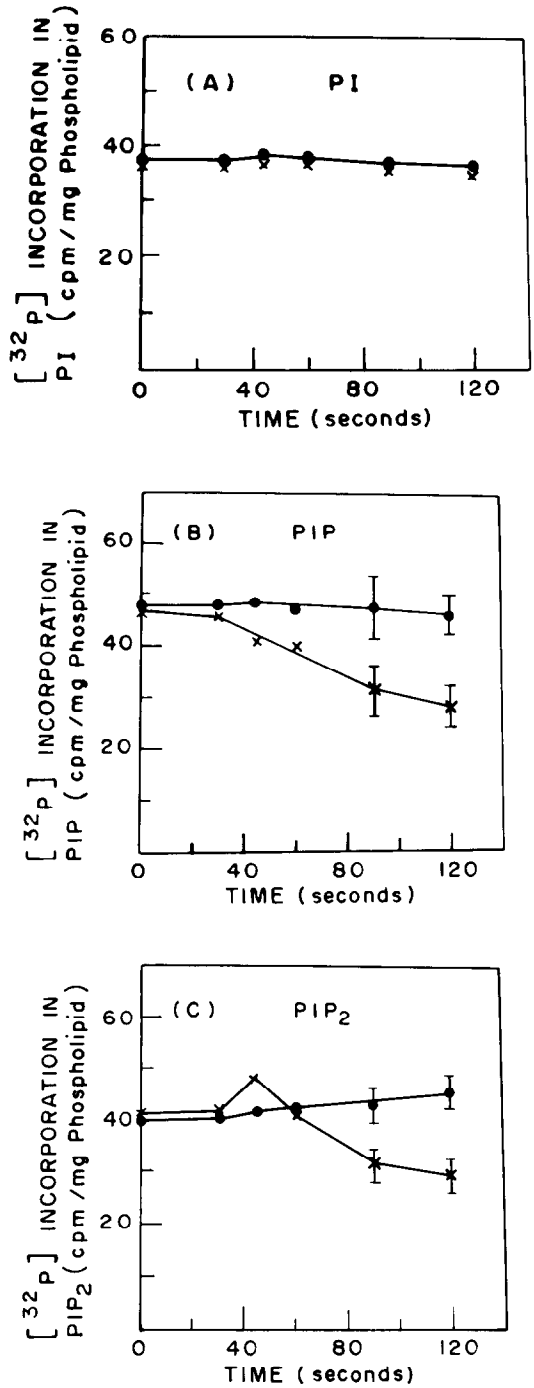

Fig.2. Time course of ST-induced degradation of inositol lipids. Epithelial cells prelabelled with [^{32}P]P_i were treated with ST for the time indicated. (●—●) Control; (×—×) PI(A), PIP (B) and PIP_2 (C). Each point is the mean of triplicate determination.

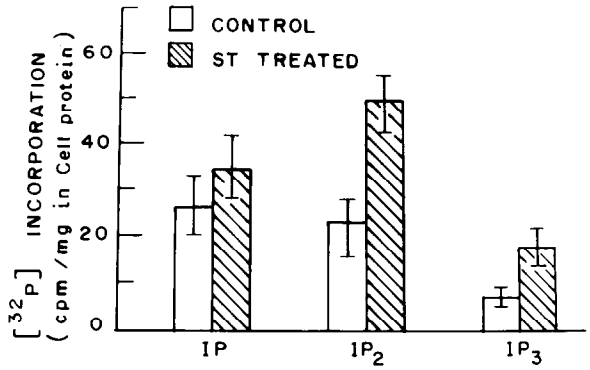

Fig.3. Effect of ST on the formation of inositol phosphate in rat intestinal epithelial cells. Cells were labelled with [^{32}P]P_i for 60 min and then treated with buffer or ST for further 2 min. IP_1, inositol phosphate; IP_2, inositol biphosphate; IP_3, inositol triphosphate.

The degradation of inositol lipids was accompanied by an increase of water-soluble inositol phosphates (fig.3). There was a 2-fold increase of radioactivity in IP_2 and IP_3 but no significant change was noticed for IP_1. The specific ^{32}P-radioactivity of metabolic ATP and PIP_2 was almost identical after labelling the epithelial cells for 60 min. The specific ^{32}P-radioactivity of ATP remained constant during the 2 min period of incubation with ST. Hence, it was assumed that the changes in radioactivity of inositol phosphates (fig.3) were due to the changes in the respective inositol lipids (fig.2).

The PI-specific PLC activity was measured in ST-pretreated cells and appeared to be stimulated 10-fold by substrate PIP_2 and 2-fold by substrate PIP at 2 MU ST (table 1).

Table 1

Effect of ST on the stimulation of rat intestinal phospholipase C

Substrate	Phospholipase C activity (mol P released/mg protein)	
	Control	ST treated
Phosphatidylinositol 4-monophosphate	0.386 ± 0.068	0.641 ± 0.12
Phosphatidylinositol 4,5-bisphosphate	0.168 ± 0.031	1.54 ± 0.17

Data represent the mean ± SE (N = 3) substrate (10 μM) and toxin (2 MU) were used

4. DISCUSSION

The mechanism by which ST stimulates intestinal secretion has been believed to be intimately and exclusively linked with activation of the enzyme guanylate cyclase [13]. Our previous [3,4] and the present study indicate that ST triggers another second messenger system by increasing the PIP_2 hydrolysis with the enzyme phospholipase C. With 2 MU ST, the phospholipase C activity increased 10-fold with substrate PIP_2 and 2-fold with substrate PIP. Since the hydrolysis of substrate PIP by the enzyme PLC was much lower than that of PIP_2, the conversion of PIP into PIP_2 involving kinase probably occurred during ST treatment. This may explain the early rise of ^{32}P-incorporation into PIP_2 (fig.2C). However, the formation of IP_3 (fig.3) was not great enough to explain the 10-fold increase of the PLC activity, thus reflecting the active breakdown cycle of $IP_3 \rightleftharpoons IP_2 \rightleftharpoons IP$ by the phosphatases.

It is not clear from the present experiments whether PI cycles have a role in the mediation of the effect of ST on intestinal secretion. It remains to be seen whether there is any correlation between PI turnover and increase of cellular cGMP.

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