Volume 250, number 2, 201–204 FEB 07198 July 1989

Diacylglycerol breakdown in plasma membrane of rat intestinal epithelial cells

Effect of *E. coli* heat-stable toxin

Nupur Dey Banik and Uma Ganguly

P-33 C.I.T. Road, Scheme XM, Beliaghata, Calcutta 700010, India

Received 27 March 1989

Rat intestinal epithelial cells were isolated and the activity of the enzyme diacylglycerol lipase (DG lipase, EC 3.1.1.3) was investigated. When cells were treated with *Escherichia coli* heat-stable toxin (ST) liberation of endogenous glycerol and fatty acids was observed. The enzyme responsible for this effect could be demonstrated to be a DG lipase by using specific substrates. It was found that the activity of DG lipase was increased 5–6-fold with the substrates diolein and 1,2-dioleyl-*rac*-glycerol and triolein being neutral lipid insensitive to DG lipase. ST had no direct effect on the DG lipase. The enzyme DG lipase was activated via a chain reaction due to the hydrolysis of phosphatidylinositol (PI) by the enzyme PI-specific phospholipase C stimulated by ST.

Diacylglycerol lipase; Toxin, heat-stable; (Epithelial cell)

1. INTRODUCTION

It is well established that inositol triphosphate (IP_3) and diacylglycerol (DG), two second messengers produced by hydrolysis of polyphosphoinositide (PIP_2), play important roles in the release of Ca^{2+} from intracellular stores and various cellular responses through the activation of protein kinase C [1,2]. A partially purified, *E. coli* heat-stable enterotoxin (ST) has been shown to increase $^{45}Ca^{2+}$ uptake by rat intestinal brush border membranes [3] and to stimulate the phosphatidylinositol (PI)-specific phospholipase C (PLC) activity of rat intestine [4]. In rat intestinal epithelial cells, the release of IP_3 in response to ST has recently been demonstrated [5]. To complete the picture of the metabolism of PI in ST-treated cells, it would be interesting to determine the fate of DG generated by PLC-induced hydrolysis of PIP_2. Here, we report that in ST-treated cells, DG lipase activity was increased and evaluate the role of DG as a source of arachidonic acid (AA).

Correspondence address: U. Ganguly, P-33 C.I.T. Road, Scheme XM, Beliaghata, Calcutta 700010, India

2. MATERIALS AND METHODS

2.1. *Materials*

Phosphatidic acid, diolein and 1,2-dioleoyl-*rac*-glycerol were obtained from Sigma. $^{32}P_i$ was purchased from Bhaba Atomic Research Centre (Trombay, Bombay). ST was prepared from *E. coli* strain 90 as in [3]. The minimal amount of ST giving in suckling mouse an FA ratio of 0.090 after 3 h was defined as one mouse unit (MU).

2.2. *Preparation of microsomes*

Four male rats weighing about 80–100 g were used for experiments. Animals were anesthetized and the abdomen opened. After washing intestinal debris with 0.9% NaCl, two 10-cm loops were made below the duodenum. Normal saline was introduced into one loop and 1 MU ST into the other. After 15 min, animals were killed and the loop was rinsed with ice-cold normal saline and everted. Mucosal scrapings from control and ST-treated loops of the four rats were homogenized separately with 9 vols (w/v) of 0.32 M sucrose and centrifuged at 800 × *g* for 10 min. The supernatant was centrifuged at 28000 × *g* using a Sorvall RC 5C apparatus. Microsomes were precipitated after further centrifugation of the supernatant

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

(100000 × g, 60 min). The final pellet was suspended in Hepes buffer (pH 7.4) and stored at −20°C [6]. The precipitate was used for studying the enzyme DG lipase.

2.3. *Diglycerol lipase assay*

Enzyme assays were according to Bell et al. [7]. The composition of the incubation mixture (total volume, 0.1 ml) was 5 mM Hepes buffer (pH 7.0), 100 μM NaCl, 5 mM $CaCl_2$, 2 mM reduced glutathione, 700 μM emulsified substrate diolein or 100 μM 1,2-dioleoyl-*rac*-glycerol and enzyme (70–80 μg protein). After 5 min incubation at 25°C, the reaction was terminated by addition of 1.5 ml chloroform/methanol/heptane (1.25:1.4:1) and 0.5 ml of 50 mM K_2CO_3 (pH 10) [8] and subjected to vigorous vortex-mixing. After separation of phases by slow centrifugation, a 0.5 ml aliquot of the upper phase was used for assaying glycerol [9]. Fatty acids were identified by acidification of the K_2CO_3 extract, extraction of fatty acids with hexane and comparison with authentic standards on TLC silica gel G plates with a solvent system comprising diethyl ether/hexane/acetic acid (70:30:1). Inhibition was studied with the use of *p*-bromophenacyl bromide, allowing the reaction to proceed for 30 min, followed by stopping the reaction as described above.

2.4. *Extraction and analysis of phosphatidic acid (PA)*

PA was extracted from $^{32}P_i$-labelled epithelial cells from rat intestinal jejunum as described [5]. PA was separated by one-dimensional TLC on silica gel 60H plates impregnated with 1% potassium oxalate using a solvent system composed of chloroform/methanol/28% NH_4OH (65:35:5, v/v). Labelled PA was visualized by means of iodine vaporization and spots were identified by referral to authentic standards. The corresponding area of PA was scraped off and the radioactivity determined by liquid scintillation counting (Beckman LS 1801).

2.5. *Extraction and identification of DG*

DG was extracted according to Bligh and Dyer [10] from epithelial cells and separated by TLC on silica gel G plates using a solvent system of hexane/diethyl ether/acetic acid (60:40:1, v/v). DG was visualized via the use of iodine vapor and identified by comparison with the standard diolein.

3. RESULTS

The fate of DG produced by PLC-induced hydrolysis of PIP_2 was examined by determination of endogenous glycerol in ST-treated cells; enhanced production of glycerol was observed as compared to controls (table 1), indicating that DG generated during hydrolysis of PIP_2 underwent degradation to yield glycerol and fatty acids.

To ascertain whether the increase in production of endogenous glycerol was due to degradation of DG or neutral lipid DG lipase activity in ST-treated cells was assayed using the specific substrates diolein, 1,2-dioleoyl-*rac*-glycerol and the neutral lipid, triolein. The activity of DG lipase

Table 1

Production of endogenous glycerol in ST-treated rat intestinal epithelial cells

Condition	Glycerol released (μmol/mg protein)
Control	0.323 ± 0.12
ST-treated	0.790 ± 0.48

Rat intestinal epithelial cells (6×10^4 cells/ml) were incubated with 2 MU of ST at 37°C for 15 min and the reaction then stopped by placing samples on ice until assay of glycerol. Data are means ± SE ($N = 4$)

increased 5–6-fold with the substrates diolein and 1,2-dioleoyl-*rac*-glycerol in ST-treated cells vs the control. Triolein, being a neutral lipid, was insensitive to the DG lipase (table 2), suggesting that during treatment with ST the activity of the enzyme was increased specifically. DG lipase showed 20% inhibition with 200 μM *p*-bromophenacyl bromide with which it is known to interact.

Furthermore, release of arachidonic acid (AA) due to metabolism of DG was demonstrated by TLC along with an authentic standard (fig.1). We observed that an appreciable amount of AA was released after 10 min of ST treatment.

The time course of production of PA and endogenous glycerol was evaluated in rat intestinal epithelial cells in response to ST vs controls. $^{32}P_i$-labelled epithelial cells pretreated with ST showed rapid incorporation of $^{32}P_i$ into PA which was maximal at 60 s (fig.2A) and then declined rapidly, reaching basal levels within 90 s. Over the same period, no change was detected in the endogenous

Table 2

Diacylglycerol lipase activity in rat intestine: effect of ST

Substrate	Concentration (μM)	Glycerol released (μmol/mg protein)	
		Control	ST-treated
Diolein	700	0.557 ± 0.15	3.17 ± 0.505
1,2-Dioleoyl-*rac*-glycerol	100	0.807 ± 0.069	4.32 ± 0.82
Triolein	700	0.27 ± 0.046	0.331 ± 0.038

Diacylglycerol lipase activity of rat intestinal epithelial cells was measured with different substrates as described in the text. Each value represents the mean of four different experiments ± SE

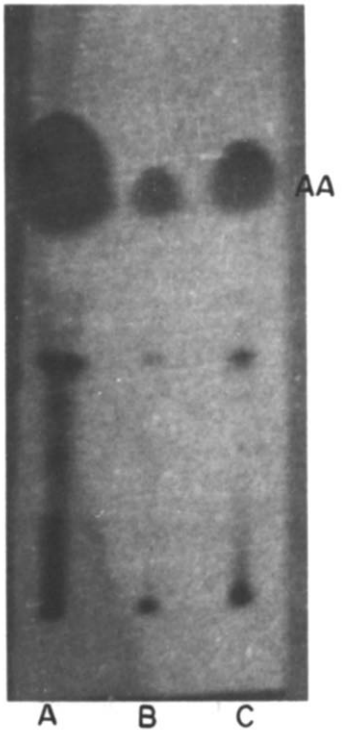

Fig.1. TLC of arachidonic acid extracted from epithelial cells 15 min after ST treatment. Solvent: diethyl ether/hexane/acetic acid (70:30:1). A, standard; B, control cells; C, ST-treated cells.

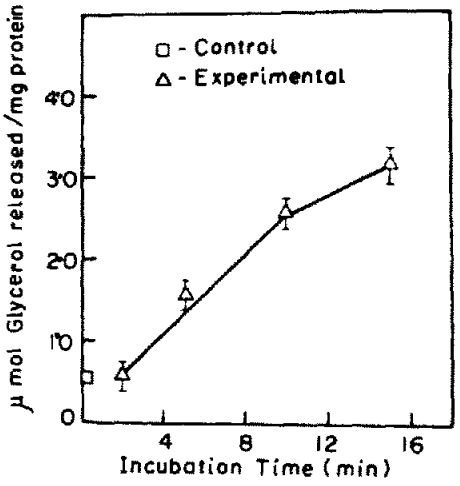

Fig.3. Effect of time-dependent ST treatment on diacylglycerol lipase activity in rat intestinal epithelial cells. Substrate was diolein, other conditions being the same as described in the text.

glycerol level (fig.2B), suggesting that stimulation of DG lipase activity was not an early event.

The degradation of PA was monitored by following the increase in DG lipase activity of epithelial cells (fig.3). Hydrolysis of DG was linear with time for at least 10 min under the assay conditions employed, the substrate used being diolein. DG was identified in both control and ST-treated cells on TLC plates (fig.4).

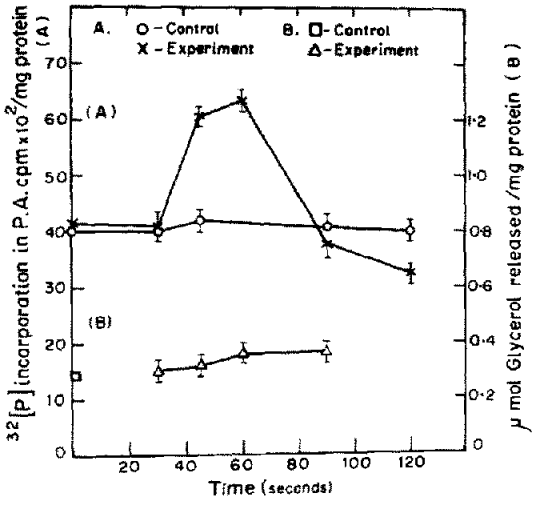

Fig.2. Time course of ST-induced production of phosphatidic acid (A) and endogenous glycerol (B) in rat intestinal epithelial cells. Each point is the mean of triplicate determinations.

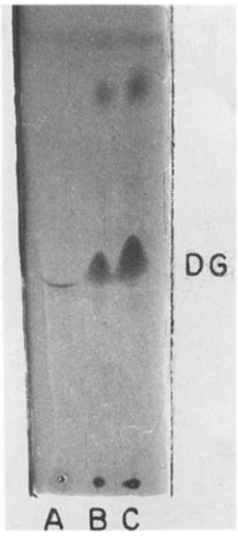

Fig.4. TLC of DG extracted from epithelial cells 2 min after ST treatment. Solvent: hexane/diethyl ether/acetic acid (60:4:11). A, standard; B, control; C, ST-treated.

4. DISCUSSION

The PI cycle has been proposed to control signal transduction through two messengers [11]: DG and IP_3. The former is an activator of protein kinase C and acts as substrate in release of AA through DG lipase.

Release of IP_3 in response to ST has recently been reported in rat intestinal epithelial cells [5]. However, the fate of DG in ST-treated cells was not described. The outcome for DG appears to be one of 3 possibilities: degradation by DG lipase, formation of PA by DG kinase and regeneration of phospholipid or accumulation. These processes may take place independently or in combination.

Here, we determined that combination of all three is possible in ST-treated cells. Transient accumulation of PA occurred within 60 s of ST stimulation. However, DG lipase activity increased only after 2 min of such stimulation. These observations suggested that competition for DG by DG kinase and DG lipase may occur and that DG lipase was only active when accumulation of DG had reached a certain threshold level. The activity of DG lipase rose significantly after 5 min treatment with ST, indicating that DG was generated during hydrolysis of PIP_2. Previously, it was reported [4] that ST stimulated a PLC specific for PI and that it did not affect phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Therefore, glycerol and AA were produced from DG generated by PI, not from PC or PE. However, it should be borne in mind that ST had no direct effect on the enzyme DG lipase. DG lipase was activated via a chain reaction due to PI hydrolysis by the enzyme PI-specific PLC. We have provided in this article further supporting evidence that the PIP_2 breakdown described previously [5] plays a role in eliciting the biological responses of ST-treated cells through the two second messengers, namely DG and IP_3.

REFERENCES

[1] Hokin, E.L. (1985) Annu. Rev. Biochem. 54, 205–235.
[2] Nishizuka, Y. (1984) Nature 308, 693–698.
[3] Ganguly, U. and Talukdar, S. (1985) FEMS Microbiol. Lett. 26, 255–257.
[4] Ganguly, U. and Talukdar, S. (1985) FEMS Microbiol. Lett. 30, 17–19.
[5] Banik, N. and Ganguly, U. (1988) FEBS Lett. 236, 489–492.
[6] Cabot, M.C., Weish, C.J., Zhang, Z. and Chabbott, H. (1987) Methods Enzymol. 141, 301–305.
[7] Bell, R.L., Kennerly, D.A., Stanford, N. and Majerus, W. (1979) Proc. Natl. Acad. Sci. USA 76, 3238–3241.
[8] Belfrag, P. and Vaughn, M. (1969) J. Lipid Res. 10, 341–345.
[9] Mosinger, B. and Vaughan, M. (1967) Biochim. Biophys. Acta 144, 556–560.
[10] Bligh, D. and Dyer, W. (1959) Can. J. Biochem. Physiol. 37, 30–39.
[11] Berridge, M.J. and Irvine, R.E. (1984) Nature 312, 315–321.