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DEPRESSION OF AMINO ACID TRANSPORT IN CULTURED RAT HEPATOCYTES BY PURIFIED ENTEROTOXIN FROM CLOSTRIDIUM PERFRINGENS

O. Giger and M. W. Pariza

Department of Food Microbiology and Toxicology
Food Research Institute, University of Wisconsin
Madison, WI 53706

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SUMMARY

Rat liver parenchymal cells (hepatocytes) were isolated by a collagenase perfusion technique and maintained as monolayers in serum-free medium in collagen-coated culture dishes. Glucagon, in combination with dexamethasone, induced α-aminoisobutyric acid transport in these cells. Addition of purified Clostridium perfringens enterotoxin to hepatocytes preinduced by glucagon and dexamethasone rapidly depressed (but did not abolish) α-aminoisobutyric acid transport. The toxin effect was dose dependent: 1000 or 300 ng/ml produced maximal depression whereas 100 or 40 ng/ml were without effect in 120 minutes. The effect was eliminated by pretreating the toxin with heat or specific antisera. The effect of enterotoxin on α-aminoisobutyric acid transport in two cultured rat hepatoma cell lines (H4-II-E-C3 and McA-RH 7777) was also investigated. Only the McA-RH 7777 cells were sensitive to the toxin suggesting that the enterotoxin may interact with specific membrane components of normal rat liver cells which are also present on some (but not all) cancerous rat liver cells.

Certain strains of Clostridium perfringens type A produce an enterotoxin which causes mild symptoms of food poisoning in humans. The enterotoxin has been purified and characterized as a heat labile protein with a molecular weight of approximately 35,000 and an isoelectric point of 4.3 (1). It possesses no detectable protease or phospholipase activities.

Little is known about the specific mechanism or site of action of this enterotoxin. McDonel (2) established that C. perfringens enterotoxin reverses net water, sodium, and chloride transport from absorption to secretion in the terminal rat ileum. This secretion of fluid and electrolytes is not mediated through elevated levels of cyclic AMP as appears to be the case with cholera and Escherichia coli enterotoxins nor is it dependent upon de novo protein

Abbreviations: AIB, α-aminoisobutyric acid, S77, Swim's S77 medium, H-35, H4-II-E-C3; 7777, McA-RH 7777.

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synthesis (3). On the basis of these results McDonel and Duncan (3) hypothesized that C. perfringens enterotoxin acts either via transport inhibition or by membrane damage causing subsequent disruption of cellular function.

Primary cultures of adult rat liver parenchymal cells (hepatocytes) have been used as a model for investigating amino acid transport in liver (4-11). Alpha-aminoisobutyric acid (AIB) is transported in these cultures by a process which is Na^+ and energy dependent (6,7) and is responsive to regulation by several different hormones alone and in combination (4-11).

In the present study, the effect of C. perfringens enterotoxin on AIB transport in cultured rat hepatocytes and two cultured rat hepatoma cell lines was investigated.

Methods

A. Enterotoxin. Enterotoxin was purified as described by Stark and Duncan (1). Lyophilized toxin was dissolved in Swim's S77 medium (S77) and added to cultures at a final concentration of 40-1000 ng/ml cell culture medium. This corresponds to approximately 0.1-2.5 erythemal units/ml cell culture medium.

B. Primary rat liver cell cultures. Hepatocytes were isolated from adult rats by a collagenase perfusion technique and maintained as monolayers in serum-free medium on 60 mm collagen-coated plates as described previously (4). Cells were initially inoculated into serum-free WO/BA-M2 supplemented with insulin (0.5 µg/ml) and gentamicin (Schering) (50 µg/ml) (4). Medium was changed to fresh WO/BA-M2 plus insulin and gentamicin 3-4 h after initial plating. At 30 h medium was changed to S77 containing 4 mM glutamine and gentamicin (50 µg/ml). Dexamethasone (1 µM) was added at 30 h (Table 1) or 36 h (Figure 1). Induction of AIB transport was started at 36 h by adding 0.2 µM glucagon (Lilly). Toxin additions were made 48 h after initial plating.

C. Hepatoma cell lines. The cell lines H4-II-E-C3 (H-35) derived from Reuber hepatoma H-35 (12) and McA-RH 7777 (7777), derived from Morris hepatoma 7777 (13), were grown as monolayers in 60 mm plastic dishes. The growth medium was S77 supplemented with 4 mM glutamine, 20% horse serum and 5% fetal calf serum. Eighteen hours before toxin addition the medium was changed to S77 $^+/_-$ 4 mM glutamine.

D. AIB transport determination. AIB uptake was measured during a 4 min exposure to 2-3 ml of Hank's-Hepes buffered salt solution containing 1 mM AIB and [^{14}C]-AIB (0.2 µCi/ml medium) as described previously (4-6). Such determinations approximate transport rates since uptake is linear for 8 minutes (10).

Results and Discussion

Figure 1 shows the effect of C. perfringens enterotoxin on the glucagon-induced AIB transport system in primary cultures of adult rat hepatocytes. As reported previously (4), pretreatment with dexamethasone alone caused no induc-

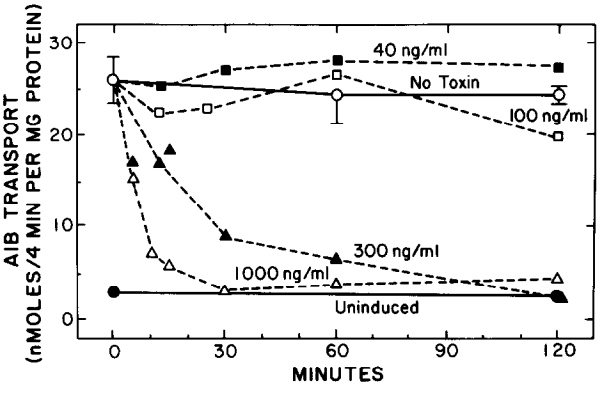

Figure 1. Effect of C. perfringens enterotoxin on the glucagon-induced AIB transport system in primary cultures of adult rat hepatocytes. Cells were pretreated for 12 h with 1 μM dexamethasone alone (●) or with 1 μM dexamethasone plus 0.2 μM glucagon (○). At 0 min, various amounts of enterotoxin were added to the medium as indicated. AIB transport was determined at intervals thereafter. Points are means of 2 or 3 plates with standard errors indicated where 3 plates were assayed.

tion of AIB transport. However, addition of glucagon resulted in approximately a ten-fold increase in AIB transport. Addition of C. perfringens enterotoxin at 1000 ng/ml caused decay of AIB transport to the uninduced basal level within 30 min. The inhibition was dose-dependent in that toxin at 300 ng/ml caused a slower decay rate while lower toxin doses (40 or 100 ng/ml) were without effect. The effect was abolished by heating the toxin (70 C for 30 min) or treating it with specific antiserum before addition to the cultures (Table 1).

The effect of enterotoxin on two cultured rat hepatoma cell lines is shown in Figure 2. These cell lines do not respond to glucagon but AIB transport is elevated in H-35 cells when glutamine is omitted from the medium for 18 h (panel A). A similar glutamine effect was observed by Kalckar et al. (14) when GIV cells (glutamine independent variant derived from the BHK Py cell line) were cultured in the absence of glutamine. No changes in AIB transport in H-35 cells were observed after enterotoxin addition (1000 ng/ml) regardless of whether or not transport had been previously elevated (glutamine removed for 18 h). Other similar experiments established that treatment with toxin at 10 μg/ml for 5.5 h did not affect AIB transport in H-35 cells (not shown).

Table 1. Inactivation of C. perfringens enterotoxin by heating or treating with specific antisera.

	AIB Transport* (nmole/4 min/mg protein)	
	2 h	6 h
Experiment A		
Uninduced	1.7	2.8
Induced	27.2	29.7
Enterotoxin (1000 ng/ml)	1.9	1.2
Heated Enterotoxin (1000 ng/ml)	26.4	31.1
Experiment B		
Uninduced	1.0	1.5
Induced	10.8	11.0
Enterotoxin (1000 ng/ml)	1.4	1.8
Enterotoxin (1000 ng/ml) pretreated with rabbit antienterotoxin	9.8	12.0

*AIB transport was induced in cultured hepatocytes by pretreatment with dexamethasone and glucagon as described in the text. AIB transport was determined 2 or 6 h after toxin addition. Values are means of two replicate plates.

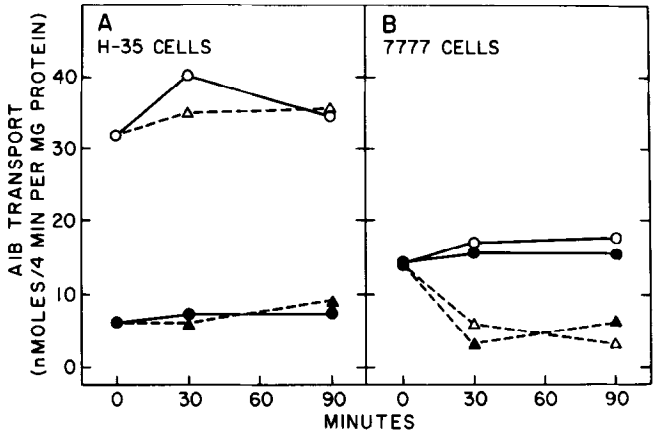

Figure 2. Effect of C. perfringens enterotoxin on AIB transport in H-35 hepatoma cells (A) and 7777 hepatoma cells (B). Cells were maintained in S77 (open symbols) or S77 containing 4 mM glutamine (closed symbols) for 18 h prior to measurement of AIB transport. At 0 min, enterotoxin (1000 ng/ml of medium) was added (Δ, ▲). Control plates without added toxin were also assayed (○, ●). Points are means of two replicate plates.

The presence or absence of glutamine had no effect on AIB transport in 7777 hepatoma cells (panel B). However, like normal hepatocytes, these cells were

sensitive to C. perfringens enterotoxin as evidenced by a rapid decrease in AIB transport following toxin addition.

At this time it is not clear whether the enterotoxin affects normal liver cells and the sensitive hepatoma cell line (7777) via the same mechanism. It is also uncertain why the H-35 cells are resistant to the toxin action. Possibly there exist specific toxin binding sites on the sensitive cells which have been lost from H-35 cells.

McDonel and Duncan (3) suggested that C. perfringens enterotoxin may act on intestine either via transport inhibition or by membrane damage causing subsequent disruption of cellular function. The rapidity of the response to the toxin by cultured hepatocytes and hepatoma 7777 cells (Fig. 1 and 2B) suggests that the site of action may be the membrane itself although neither protease nor phospholipase activities have been detected in purified toxin preparations. Futhermore, although AIB transport in glucagon-induced hepatocytes is decreased following toxin administration, it is not abolished. Therefore, the enterotoxin is probably not destroying components essential for membrane integrity since basal levels of AIB transport are still detectable in toxin-treated cells. The possibility of limited membrane damage still exists.

Increases in cAMP levels were not involved in the intestinal response to C. perfringens enterotoxin (3). By inference, cAMP is also probably not a primary target in the response of cultured hepatocytes to enterotoxin as elevating cAMP with hormones, phosphodiesterase inhibitors, or by adding dibutyryl cAMP increases or has no effect on AIB transport in these cells but never decreases this activity (4,5,11).

Other studies involving isolated rat liver mitochondria (3,15) indicated that enterotoxin may interfere with energy production possibly by altering membrane permeability. Further study is needed to determine if enterotoxin interferes with energy metabolism or energy availability for active transport in cultured hepatocytes and hepatoma 7777 cells.

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