Biochimica et Biophysica Acta, 595 (1980) 264—276

BBA 78595

MECHANISM OF ACTION OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN

EFFECTS ON MEMBRANE PERMEABILITY AND AMINO ACID TRANSPORT IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

OLARAE GIGER and MICHAEL W. PARIZA *

Department of Food Microbiology and Toxicology, Food Research Institute, University of Wisconsin-Madison, Madison, WI 53706 (U.S.A.)

(Received May 8th, 1979)

Key words: Enterotoxin action; Amino acid transport; Bacterial toxin; Membrane permeability; (C. perfringens, Rat hepatocyte)

Summary

Purified enterotoxin from the bacterium *Clostridium perfringens* rapidly decreased the hormonally induced uptake of α-aminoisobutyric acid in primary cultures of adult rat hepatocytes. At 5 min after toxin addition the decrease in α-aminoisobutyric acid uptake appeared not due to increased passive permeation (estimated with L-glucose) or to increased α-aminoisobutyric acid efflux. When short uptake assay times were employed a depression of α-aminoisobutyric acid influx was observed in toxin-treated hepatocytes. The depression of α-aminoisobutyric acid influx was correlated with a rapid increase in intracellular Na^+ (estimated using $^{22}Na^+$) apparently effected by membrane damage. In contrast, the uptake of cycloleucine in the presence of unlabeled α-aminoisobutyric acid (assay for Na^+-independent amino acid uptake) by hepatocytes treated with toxin for 5 min was decreased to only a small extent or not at all depending upon experimental design. At later times, *C. perfringens* enterotoxin increased the exodus of L-glucose, 3-*O*-methylglucose and α-aminoisobutyric acid from pre-loaded cells indicating that the toxin effects progressive membrane damage. When enterotoxin was removed by repeated washing after 5—20 min the decay of α-aminoisobutyric acid uptake ceased and appeared to undergo recovery towards the hormonally induced control level. The degree of recovery of α-aminoisobutyric acid uptake was inverse to the length of time of exposure to toxin. Adding at 10 min specific rabbit antiserum against *C.*

---

* To whom requests for reprints should be addressed.
Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

perfringens enterotoxin without medium change also reversed the effect of toxin on increased intracellular $^{22}Na^+$, and on the exodus (from preloaded cells) of α-aminoisobutyric acid, L-glucose, and 3-*O*-methylglucose.

Introduction

Certain strains of the bacterium *Clostridium perfringens* elaborate an enterotoxin which causes relatively mild symptoms of food poisoning in humans. The toxin has been purified and characterized as a heat-labile protein with a molecular weight of about 35 000 and an isoelectric point of 4.3 [1]. Studies employing mutants grown under different conditions [2], immunological techniques [3—5], and biological activity [5] have established that the enterotoxin is distinct from other toxic products of *C. perfringens*, including phospholipase C (α-toxin). Moreover, the purified toxin is free of detectable phospholipase and protease activity [6].

In previous reports it has been demonstrated that α-aminoisobutyric acid is transported in primary cultures of adult rat hepatocytes by a process which is dependent upon Na^+ and energy [7,8]. Glucagon increases the rate of α-aminoisobutyric acid uptake by decreasing the apparent K_m for transport, while dexamethasone potentiates the glucagon effect by increasing the apparent V for transport in glucagon-treated (but not control) cells [7—10]. Recently, we reported that *C. perfringens* enterotoxin produced a rapid and dose-dependent depression of glucagon-dexamethasone-induced α-aminoisobutyric acid uptake in cultured hepatocytes [11]. Pretreating the toxin with heat or specific antiserum abolished this effect. We report herein experiments which further elucidate the mechanism of action of *C. perfringens* enterotoxin on cultured rat hepatocytes.

Materials and Methods

Enterotoxin. Enterotoxin was purified from *C. perfringens* type A strain NCTC 8239 by the method of Stark and Duncan [1]. This procedure, which involves chromatography on Sephadex G-100, Cellex T anion exchange, and hydroxyapatite columns, resulted in a highly purified enterotoxin preparation which showed a single protein band after electrophoresis on disc polyacrylamide gels. Lyophilized toxin was dissolved in Swim's S-77 medium and added to cultures at a final concentration of 0.3 or 1 μg/ml cell culture medium. These concentrations were used based on our previous reports [11] that glucagon-dexamethasone-induced α-aminoisobutyric acid uptake was rapidly depressed by toxin at 1 μg/ml, less rapidly depressed by toxin at 0.3 μg/ml, and apparently unaffected by toxin at lower concentrations (0.1 μg/ml or 0.04 μg/ml).

Antiserum. Antiserum was prepared against purified enterotoxin in 2.0—2.5 kg female New Zealand white rabbits. The rabbits were injected intramuscularly with 0.25 mg of enterotoxin in either Freund complete adjuvant (first injection) or Freund incomplete adjuvant (subsequent injections). A total of four injections were made at 6-week intervals. Rabbits were bled every 2 weeks

from the ear vein or artery using a bleeding apparatus (Bellco) and a pump to apply suction. Blood was allowed to clot for 1 h at 37°C and then incubated at 7°C for several hours. Serum was removed, clarified by centrifugation, and stored frozen at −20°C.

Primary rat liver cell cultures. Hepatocytes were isolated from male Sprague-Dawley rats (200—300 g) by a collagenase perfusion technique and maintained as monolayers on 60-mm plastic tissue culture dishes coated with a thin layer of rat tail collagen as previously described [7,10]. Cells were initially cultured in modified serum-free Waymouth's MB 752/1 medium, previously designated WO/BA-M2 [7], containing insulin (0.5 μg/ml) and gentamicin (50 μg/ml). Medium was changed to fresh WO/BA-M2 plus insulin and gentamicin 3—4 h after initial plating. At 30 h the medium was changed to Swim's S-77 medium containing 4 mM glutamine and gentamicin (50 μg/ml) [10].

α-Aminoisobutyric acid uptake determinations. α-Aminoisobutyric acid transport was pre-induced in the cultured hepatocytes by the addition of 1 μM dexamethasone 30 h after initial plating and 0.2 μM glucagon at 45 h [10]. Experiments were started with the addition of enterotoxin 6 h after glucagon addition (52 h after initial plating) [11]. α-Aminoisobutyric acid uptake was measured by incubating the cells in 2—3 ml glucose-free Hanks' *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-buffered salt solution containing 1 mM α-aminoisobutyric acid and α-amino[^{14}C]isobutyric acid (0.2 μCi/ml medium) based on our previous reports [7,10]. Incubation was terminated by rinsing the cells several times with cold Hanks'/Hepes buffer and the cells were prepared for scintillation counting and protein determination as previously described [7].

Cycloleucine uptake determinations. Cells were pretreated with hormones as described above. Cycloleucine uptake was determined with a modification of the procedure of LeCam and Freychet [12] involving the incubation of hepatocytes in glucose-free Hanks'/Hepes buffer containing 1 mM cycloleucine and [^{14}C]cycloleucine (0.4 μCi/ml) with 20 mM α-aminoisobutyric acid. In some experiments cells were pretreated with 5 mM α-aminoisobutyric acid for 2 h prior to cycloleucine uptake determinations.

L-Glucose and 3-O-methylglucose studies. The effect of enterotoxin on the membrane permeability barrier was investigated 48 h after initial plating [7,8]. In experiments involving L-glucose, cells were incubated for 12 h in Swim's S-77 medium containing 1 mM L-glucose and L-[^{14}C]glucose (1 μCi/ml). The cells were then rinsed, and incubated in 3 ml Hanks'/Hepes buffer containing enterotoxin. The effect of *C. perfringens* enterotoxin on L-glucose exodus was determined at various times thereafter. In experiments involving 3-*O*-methylglucose (3-*O*-methyl-α-D-glucopyranoside), 48-h cultures were rinsed several times with glucose-free Hanks'/Hepes-buffered salt solution and reincubated at 37°C for 30 min with glucose-free Hanks'/Hepes buffer (3 ml/plate) containing 10 mM 3-*O*-methylglucose and 3-*O*-[^{14}C]methylglucose (0.4 μCi/ml medium). Enterotoxin was then added and the incubation continued for up to 45 min. At intervals after toxin addition, the capacity of phloretin to inhibit 3-*O*-methylglucose exodus was determined by rinsing the cultures several times with warm (37°C) glucose-free Hanks'/Hepes buffer containing 1 mM phloretin (to remove external 3-*O*-methylglucose) and reincubating the cells at 37°C for 5

min in 3 ml glucose-free Hanks'/Hepes buffer containing phloretin. Since the total cell water volume/plate was 3 μl or less [8], the incubation volume was large enough to prevent significant recapture of 3-*O*-methylglucose lost from cells. The cultures were harvested by rinsing several times with ice-cold glucose-free Hanks'/Hepes buffer containing phloretin.

Intracellular $^{22}Na^+$ determination. $^{22}Na^+$ was used as a marker in experiments examining the effect of enterotoxin on intracellular Na^+ levels [13]. The cultures were rinsed several times with Hanks'/Hepes buffer and reincubated with Hanks'/Hepes containing 8 mM glucose and 1.8 μCi/ml $^{22}NaCl$. Cells were incubated in this medium until there was no further increase in intracellular $^{22}Na^+$ (the system was at equilibrium in 2 min but incubations were usually carried out for 30 min). Enterotoxin was then added and the incubation continued for up to 45 min. At intervals after toxin addition, cultures were harvested by rinsing several times with ice-cold sodium-free Hanks'/Hepes buffer prepared by replacing NaCl and $NaHCO_3$ with choline chloride and choline bicarbonate [13]. Cells were harvested as above and the intracellular levels of $^{22}Na^+$ were determined in a γ scintillation counter. In other experiments, cultures at 48 h were rinsed with Earle's balanced salt solution [14] and reincubated for 3 h in Earle's balanced salt solution containing 6 mM ouabain and 1.75 μCi/ml $^{22}NaCl$. Enterotoxin (1 μg/ml) was added (without medium change) and the incubation continued. After 5 min, the cultures were rinsed with cold (4°C) Hanks'/Hepes buffer containing 6 mM ouabain. 3 ml of Hanks'/Hepes with 6 mM ouabain were added and the plates were reincubated at 4°C. At intervals the cultures were harvested and counted as described above. It was necessary to carry out incubations at 4°C to prevent the rapid exchange of Na^+ which was found to occur at 37°C.

Materials. Sources of most materials used in this study have been published elsewhere [7,9,10,12]. In addition unlabeled 3-*O*-methylglucose, cycloleucine, choline chloride, choline bicarbonate, and ouabain were purchased from Sigma Chemical Co. 3-*O*-[^{14}C]methyl-α-D-glucopyranoside, 1-aminocyclopentane-1-[*carboxyl*-^{14}C]carboxylic acid and $^{22}NaCl$ were obtained from New England Nuclear, and phloretin came from ICN Pharmaceuticals (K and K).

Results

The data of Fig. 1 show that *C. perfringens* enterotoxin at 1 μg/ml produces a rapid apparent inhibition of α-aminoisobutyric acid uptake in cultured rat hepatocytes previously treated with dexamethasone and glucagon to induce the rate of amino acid transport. This result is in agreement with our previous report [11] that the elevated level of α-aminoisobutyric acid uptake induced in cultured hepatocytes by glucagon in combination with dexamethasone was rapidly depressed by *C. perfringens* enterotoxin at 1 μg/ml (in addition, less rapid depression was effected by toxin at 0.3 μg/ml, whereas no effect was produced by toxin at lower concentrations, 0.1 μg/ml, or 0.04 μg/ml) [11]. α-Aminoisobutyric acid uptake is also depressed by toxin in cells not treated with hormones [15], and *C. perfringens* enterotoxin effects dose-dependent inhibition of the induction of elevated levels of α-aminoisobutyric acid uptake by the hormones glucagon in combination with dexamethasone [15], epineph-

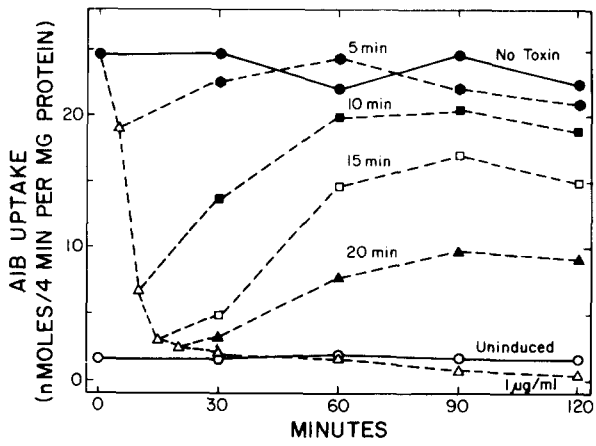


Fig. 1. Inhibition of α-aminoisobutyric acid (AIB) uptake by *C. perfringens* enterotoxin and its reversibility when toxin is removed. Cultured rat hepatocytes were pre-induced by treatment for 18 h with 1 μM dexamethasone and 6 h with 0.2 μM glucagon (except for uninduced controls). At '0' min the cells were treated with enterotoxin at 1 μg/ml. At 5, 10, 15 or 20 min toxin was removed by repeated washing and the cells were then incubated in toxin-free medium containing glucagon and dexamethasone. The uptake of 1 mM α-amino[^{14}C]isobutyric acid was determined in 4-min assays as described under Materials and Methods. Each value is the average of duplicate plates.

rine in combination with dexamethasone [15], and insulin alone (unpublished results). These findings indicate that the effects of *C. perfringens* enterotoxin on α-aminoisobutyric acid uptake in cultured hepatocytes are not limited to particular hormonally mediated processes but rather reflect a more general underlying phenomenon.

The data of Fig. 1 also show that if the toxin is removed after 5—20 min the apparent decrease in α-aminoisobutyric acid uptake ceases, and on additional incubation in toxin-free medium α-aminoisobutyric acid uptake appears to undergo some degree of recovery towards the fully induced level dependent upon the length of time of exposure to the toxin. Cycloheximide (10 μg/ml) or puromycin (23 μg/ml) [10] did not alter the enterotoxin-effected decay of preinduced α-aminoisobutyric acid uptake nor was recovery following toxin removal affected by puromycin (not shown).

To assess the possible role of increased α-aminoisobutyric acid efflux in this phenomenon, the experiment shown in Fig. 2 was conducted. α-Aminoisobutyric acid was lost from toxin-treated cultures more rapidly than from control cells, but the effect was not apparent until 10 min after toxin addition. When rabbit antiserum against *C. perfringens* enterotoxin was added without medium change to toxin-treated cultures at 10 min, the loss of α-aminoisobutyric acid was reduced at subsequent times.

The effect of *C. perfringens* enterotoxin on liver cell membrane permeability was investigated in the experiments shown in Figs. 3 and 4. In Fig. 3, the effect of enterotoxin on the exodus of L-glucose was examined. L-Glucose is not metabolized by liver cells and crosses the membrane by passive permeation [15, 16]. We have previously used L-glucose to assess membrane damage in liver cells just after isolation vs. cells in culture for 24 h [7]. The data show that at 5 min the amount of L-glucose in control and toxin-treated cultures was similar

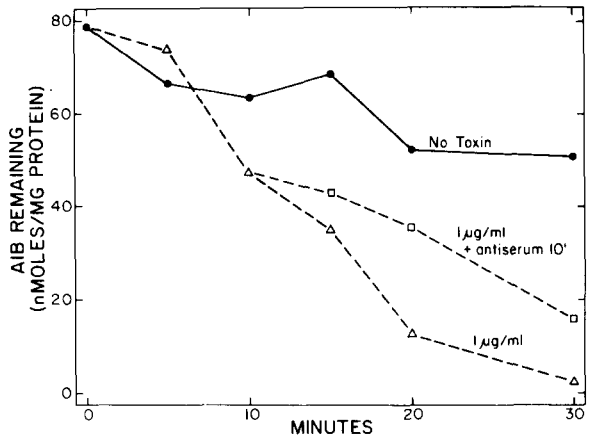


Fig. 2. The exodus of α-amino[^{14}C]isobutyric acid (AIB) from previously loaded hepatocytes. Cells fully induced by pretreatment for 12 h with 1 μM dexamethasone and 0.2 μM glucagon were incubated in Earle's balanced salt solution containing the hormones and 1 mM α-amino[^{14}C]isobutyric acid for 120 min. This medium was removed by aspiration, the cells rinsed with cold (4°C) Hanks'/Hepes, and then 3 ml of warm Hanks'/Hepes buffer containing 1 μg/ml *C. perfringens* enterotoxin was added. At 10 min specific rabbit antiserum against the enterotoxin was added to some plates without a medium change. At the indicated times duplicate plates were analyzed for the amount of α-amino[^{14}C]isobutyric acid remaining in the cells.

whereas by 10 min somewhat more L-glucose had been lost from toxin-treated than from control cells, indicating that passive permeation was more pronounced in liver cells treated with *C. perfringens* enterotoxin for 10 min. Adding specific antiserum to toxin-treated cells at 10 min without a medium

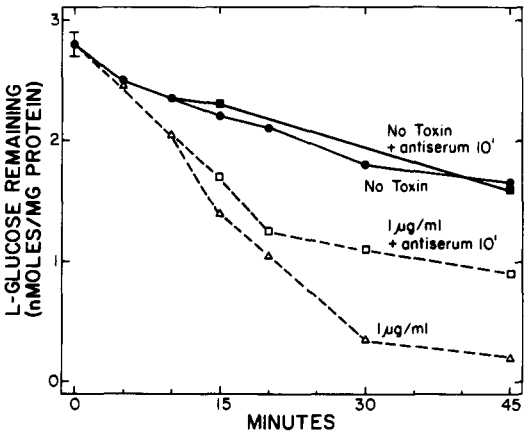


Fig. 3. Effect of *C. perfringens* enterotoxin on the passive migration of L-glucose from cultured rat hepatocytes. Cells were preincubated for 12 h in Swim's S-77 medium containing 1 mM L-glucose and L-[^{14}C]glucose (1 μCi/ml). The cells were then rinsed with warm Hanks'/Hepes buffer, and enterotoxin was added. The amount of L-glucose remaining in cells was determined at various times thereafter. Rabbit antiserum against *C. perfringens* enterotoxin was added to some toxin-treated or control cultures at 10 min without medium change. In cultures from the same liver cell preparation enterotoxin effected the following changes in the dexamethasone-glucagon-induced uptake of α-aminoisobutyric acid (nmol/4 min per mg protein): 0 min, 21.1; 5 min, 9.6; 10 min, 2.9; 15 min, 2.05; 20 min, 1.45.

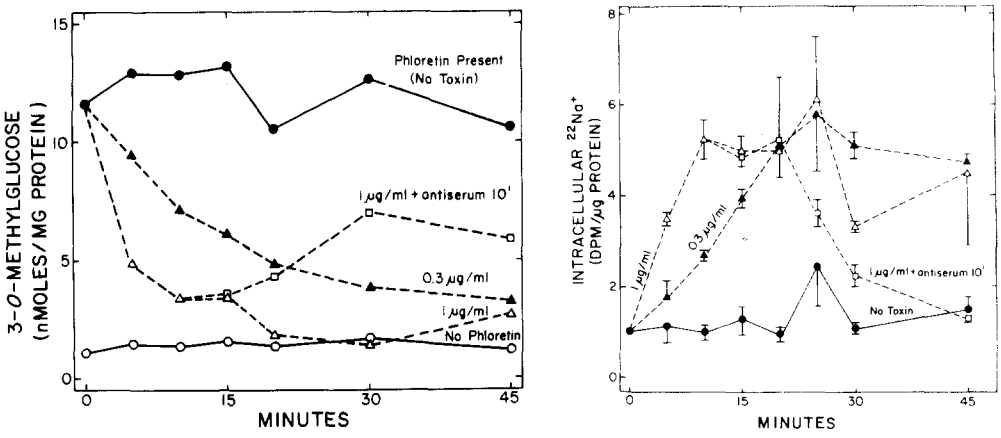


Fig. 4. Impairment of phloretin inhibition of 3-*O*-methylglucose exodus by *C. perfringens* enterotoxin. Cultured hepatocytes were pre-incubated for 30 min in glucose-free medium containing 10 mM 3-*O*-methylglucose (0.4 μCi 3-*O*-[^{14}C]methylglucose/ml). Toxin was added (1 μg/ml or 0.3 μg/ml) and at intervals thereafter the medium was removed, the cells rinsed and then incubated for 5 min in 3 ml of toxin and 3-*O*-methylglucose-free medium containing 1 mM phloretin. Phloretin present (no toxin), the control plates incubated with phloretin; no phloretin, control cells exposed to the 5 min incubation in phloretin-free medium. Note than when antiserum was added at 10 min to cells in medium containing toxin (1 μg/ml) and 3-*O*-methylglucose partial recovery was evident. Prior to the 5-min incubation periods the cells contained 19.8 nmol of 3-*O*-methylglucose/mg protein.

Fig. 5. Intracellular $^{22}Na^+$ concentration following treatment with *C. perfringens* enterotoxin. Cultured hepatocytes were pre-incubated for 30 min in Hanks'/Hepes containing 8 mM glucose and 1.8 μCi/ml ^{22}NaCl. Toxin was added (1 μg/ml or 0.3 μg/ml) and at intervals thereafter the medium was removed, the cells rinsed with sodium-free Hanks'/Hepes buffer and analyzed for the amount of $^{22}Na^+$ present in the cells. When antiserum was added 10 min after addition of 1 μg/ml toxin, reversal of the enterotoxin effect occurred. Values are the means of three replicate plates with S.E. indicated by the bars. The extracellular Na^+ concentration in Hanks'/Hepes buffer is 141 mM; assuming complete exchange between extra- and intracellular sodium, then based on dpm/μg protein and the water content of cultured hepatocytes (about 2.5 μl water/mg cell protein, Ref. 8 and unpublished results) the apparent intracellular Na^+ concentration in control (no toxin) cells was about 17 mM.

change decreased the rate of loss of L-glucose observed at later time points. In cells from this same liver preparation α-aminoisobutyric acid uptake fell from a value (nmol/4 min per mg protein) of 21.2 (range 18.2—24.1) to 9.6 at 5 min, and to 2.9 at 10 min (legend to Fig. 3). Increased passive permeation effected by enterotoxin in this time period seems alone insufficient to account for the dramatic drop in α-aminoisobutyric acid uptake since toxin-treated and control hepatocytes contained the same amounts of L-glucose at 5 min and only slightly dissimilar levels at 10 min.

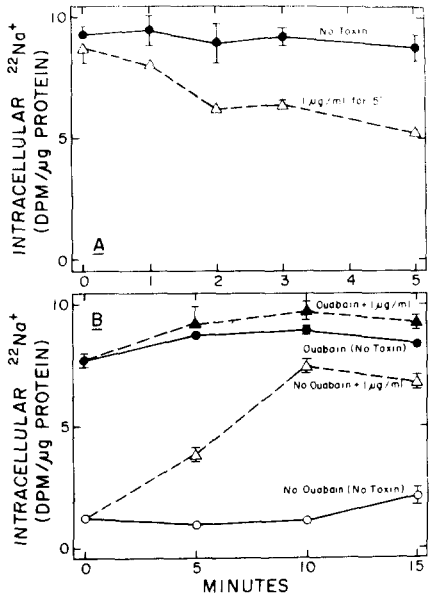
Fig. 4 shows the effect of *C. perfringens* enterotoxin on 3-*O*-methylglucose exodus from preloaded liver cells. The experiment is based on previous studies in which we employed this non-metabolizable hexose analog in combination with phloretin, a specific inhibitor of hexose transport, to determine intracellular water volume [7,8]. 3-*O*-Methylglucose is transported into animal cells via carrier-mediated processes which are not energy dependent except in kidney and intestine [16]. Phloretin inhibits the exodus of 3-*O*-methylglucose from preloaded cultures of intact hepatocytes presumably by directly interacting with hexose transport sites [7,8,16]. We postulated that if *C. perfringens*

enterotoxin damages the permeability barrier then phloretin should be less able to block 3-*O*-methylglucose exodus from toxin-treated cells than from control cells. The data of Fig. 4 show that following 5 min of exposure to toxin at 1 μg/ml the capacity of phloretin to inhibit 3-*O*-methylglucose exodus was impaired. The effect was dose dependent in that toxin at 0.3 μg/ml gave a smaller response (Fig. 4). In other experiments *C. perfringens* enterotoxin at 0.3 μg/ml gave similar reduced effects on α-aminoisobutyric acid uptake [11] and exodus (unpublished results). It should be noted that 5-min exposures to medium containing phloretin were required to unequivocally demonstrate the effect of toxin on this parameter. In preliminary experiments simply rinsing cells with buffer containing phloretin resulted in smaller and less convincing differences between control cells and cultures treated with toxin for 5—30 min, indicating that in this time period the toxin-treated hepatocytes had not become freely permeable. When specific antiserum against *C. perfringens* enterotoxin was added at 10 min a partial reversal of toxicity was observed (Fig. 4).

α-Aminoisobutyric acid uptake is dependent upon Na^+ cotransport [7,12,13, 18] and for this reason we investigated the effect of *C. perfringens* enterotoxin on intracellular Na^+ levels, using $^{22}Na^+$ as a marker [13]. As shown in Fig. 5, within 5 min of adding enterotoxin at 1 μg/ml the intracellular $^{22}Na^+$ level increased three fold, and by 10 min the level was about five times control. Toxin at 0.3 μg/ml produced a slower rise again demonstrating a dose-dependent effect. Adding antiserum against *C. perfringens* enterotoxin without medium change to cultures treated for 10 min with toxin (1 μg/ml) resulted in the return of intracellular $^{22}Na^+$ levels to control values by 45 min.

The experiment shown in Fig. 6 was designed to explore the mechanism whereby *C. perfringens* enterotoxin effects the rapid increase in intracellular $^{22}Na^+$ levels. Hepatocytes were pre-treated for 3 h in medium containing $^{22}Na^+$ and the digitalis glycoside, ouabain, an inhibitor of $(Na^+ + K^+)$-ATPase involved in Na^+ extrusion [18]. Under these conditions intracellular $^{22}Na^+$ increased to about the extracellular concentration. This conclusion is supported by calculations provided in the legend to Fig. 6 and by the fact that enterotoxin had very little effect on the elevated $^{22}Na^+$ level in ouabain-treated hepatocytes (Fig. 6B). *C. perfringens* enterotoxin was then added and at 5 min the cultures were transfered to label-free medium containing ouabain and incubated at 4°C (Fig. 6A). As shown in Fig. 6A, $^{22}Na^+$ levels decreased in cells pre-treated with enterotoxin indicating membrane damage sufficient to permit the passive migration of Na^+. In cultures from the same cell preparation pre-incubated in medium containing $^{22}Na^+$ but lacking ouabain (Fig. 6B), *C. perfringens* enterotoxin effected increased intracellular $^{22}Na^+$ levels at 5 min in agreement with the data of Fig. 5. These experiments indicate that the rapid increase in intracellular $^{22}Na^+$ effected by *C. perfringens* enterotoxin is due principally to membrane damage permitting a rapid migration of Na^+ into the cells.

Fig. 7A shows the uptake of α-aminoisobutyric acid as a function of time (30 s—4 min) in control cells and cells treated for 5 min with *C. perfringens* enterotoxin. A difference in α-aminoisobutyric acid uptake between control and toxin-treated cells is evident at 30 s, a time short enough to approximate uptake rates (influx) (control values at 30 s and 1 min were linear, Fig. 7A).



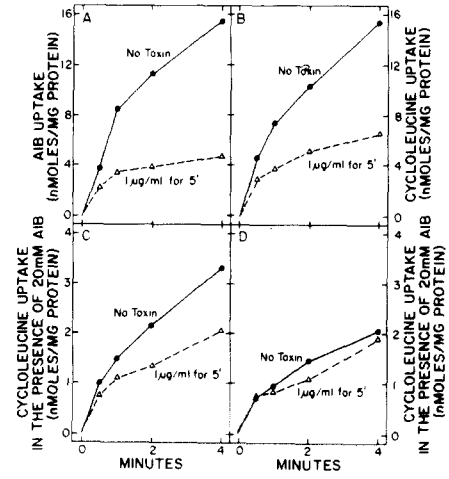


Fig. 6. Effect of *C. perfringens* enterotoxin on exodus of intracellular $^{22}Na^+$. (A) Cultured hepatocytes were pre-incubated for 3 h in Earle's balanced salt solution containing 6 mM ouabain and 1.75 μCi/ml $^{22}NaCl$. Toxin (1 μg/ml) was added and at 5 min later, the medium was removed. The cells were rinsed and reincubated at 4° C in Hanks'/Hepes containing 6 mM ouabain. The amount of $^{22}Na^+$ remaining in the cells was determined at various times thereafter. (B) Cultures were pre-incubated for 3 h in Earle's balanced salt solution containing 1.75 μCi/ml $^{22}NaCl$ plus 6 mM ouabain (closed symbols) or without ouabain (open symbols). Toxin (1 μg/ml) was added and at intervals thereafter the cultures were harvested and analyzed for the amount of $^{22}Na^+$ present in the cells. Values are the means of three replicate plates with S.E. indicated by bars where they exceed the size of the symbol. Assuming complete exchange between extra- and intracellular Na^+, then based on dpm/μg protein and the water content of cultured hepatocytes [8] the concentration of intracellular Na^+ in ouabain-treated cells shown in this figure is approximately that of the extracellular medium (143 mM).

Fig. 7. Effects of *C. perfringens* enterotoxin exposure for 5 min on the uptake of α-aminoisobutyric acid (AIB) and cycloleucine as a function of time. Cultured rat hepatocytes were pre-treated with dexamethasone and glucagon as described under Materials and Methods. Enterotoxin (1 μg/ml) was added 5 min before measuring α-aminoisobutyric acid or cycloleucine uptake. (A) α-Aminoisobutyric acid uptake; (B) cycloleucine uptake; (C) cycloleucine uptake in the presence of 20 mM α-aminoisobutyric acid; (D) cycloleucine uptake in the presence of 20 mM α-aminoisobutyric acid in hepatocytes pre-loaded with α-aminoisobutyric acid (5 mM for 2 h). In cultures from the same liver preparation which were pre-loaded with α-aminoisobutyric acid, enterotoxin effected the following changes in α-aminoisobutyric acid level (nmol/mg protein): 0 min, 63.4 ± 1.7; 5 min, 62.2; 10 min, 26.7.

Moreover, the data of Fig. 2 indicate that at 5 min the intracellular levels of α-aminoisobutyric acid in control and toxin-treated cells pre-loaded with α-aminoisobutyric acid for 2 h were similar, and the same observation was made with cells from the preparation studied in Fig. 7 (legend to Fig. 7). Hence, it appears that α-aminoisobutyric acid influx is depressed by *C. perfringens* enterotoxin.

Fig. 7B—D show the effect of *C. perfringens* enterotoxin on the uptake of cycloleucine, a non-metabolizable amino acid transported by both the sodium-dependent 'A' system and the sodium-independent 'L' system as described for Ehrlich ascites cells [18]. In Fig. 7B, cycloleucine uptake in Hanks'/Hepes buffer was determined as a function of time in both control hepatocytes and hepatocytes treated with enterotoxin for 5 min. Under these conditions where

cycloleucine is transported by both the 'A' and 'L' systems, there is a difference in cycloleucine uptake between control and enterotoxin-treated cultures.

LeCam and Freychet [12] have demonstrated that 'A' and 'L' system-mediated entry of cycloleucine can be separated in freshly isolated hepatocytes by (1) determining cycloleucine uptake in Na^+-free buffer, and (2) employing a Na^+-containing buffer which also contained an excess of unlabeled α-aminoisobutyric acid to compete with cycloleucine for entry via the 'A'-mediated system. We have verified that in Hanks'/Hepes buffer altered to contain choline rather than sodium, and in Hanks'/Hepes buffer containing sodium salts and 10—20 mM unlabeled α-aminoisobutyric acid, cycloleucine uptake is identical for normal control hepatocytes (not shown). In Fig. 7C (Hanks'/Hepes buffer containing 20 mM α-aminoisobutyric acid), cycloleucine uptake appears to be somewhat lower in toxin-treated cells. However, in cells preloaded with α-aminoisobutyric acid prior to determination of cycloleucine uptake in the presence of 20 mM unlabeled α-aminoisobutyric acid, toxin pretreatment had little effect on cycloleucine uptake during the 4 min assay period (Fig. 7D). We originally designed this protocol (Fig. 7D) in an attempt to minimize as much as possible efflux of cycloleucine via 'A' carriers but it appears that influx of cycloleucine in control hepatocytes is reduced under these conditions whereas uptake in toxin-treated cells is altered only slightly if at all (compare Fig. 7C and D). This may be due in part to the fact that in hepatocytes α-aminoisobutyric acid is transported by both systems 'A' and 'L' [19] and thus may compete with cycloleucine for the 'L' as well as the 'A' carriers. Such competition might be most effective under conditions where high extracellular and intracellular concentrations of α-aminoisobutyric acid exist. The data of Fig. 7 support the conclusion that 'L'-mediated entry is less affected than amino acid uptake via the 'A' system in hepatocytes treated for short times with *C. perfringens* enterotoxin. In other experiments (not shown) the effect of toxin on the exodus of cycloleucine from preloaded hepatocytes resembled closely data on the exodus of α-aminoisobutyric acid (Fig. 2).

Discussion

Purified enterotoxin from *C. perfringens* rapidly depresses the uptake of α-aminoisobutyric acid in primary cultures of adult rat hepatocytes (see Ref. 11 and this report). In this investigation we have obtained evidence indicating that at very early times (i.e. 5 min) the depression is due primarily to a direct inhibition of α-aminoisobutyric acid influx. It appears unlikely that loss of intracellular α-aminoisobutyric acid to passive permeation or carrier-mediated efflux contribute significantly to the decrease in α-aminoisobutyric acid uptake observed at 5 min after toxin addition. However, at later times increased passive migration appears to gain in significance (Fig. 3). The uptake of cycloleucine by hepatocytes treated with toxin for 5 min appears slightly depressed when excess unlabeled α-aminoisobutyric acid is present in the assay medium, but unaffected when the liver cells were preloaded with α-aminoisobutyric acid (Fig. 7C and D). Therefore, the rapid decrease in α-aminoisobutyric acid uptake following exposure to *C. perfringens* enterotoxin is limited primarily to the sodium-dependent amino acid transport system 'A'. In other experiments

(not shown) involving short uptake times (30 s) and a range of substrate (α-aminoisobutyric acid) concentrations it appears that the toxin may both decrease V and increase the apparent K_m for transport. However, because of unexplained anomalies in replicate studies we cannot yet state this conclusion with certitude.

Recently Tosteson and Tosteson [20] showed that cholera toxin complexes with artificial lipid bilayers containing gangliosides to form ion-conducting channels which permit the passage of either Na^+ or K^+, but not Cl^-. In other studies Schein et al. [21] have found that a single colicin K molecule can form a voltage-dependent and relatively non-selective ion-conducting channel in artificial phospholipid bilayers, and suggested that the physiological effects of colicin K and functionally related colicins may result from their ability to form ion-permeable channels in the membranes of sensitive bacteria. In our investigation it was found that $^{22}Na^+$ rapidly enters cultured hepatocytes treated with *C. perfringens* enterotoxin (Fig. 5) apparently because of membrane damage (Fig. 6) but it is clear that this effect is not limited to ions since L-glucose and 3-*O*-methylglucose both pass through the membranes of toxin-treated cells (Figs. 3 and 4). McDonel et al. [22] have reported electron microscopic evidence of membrane damage in cells at the tips of villi in rat and rabbit ilea exposed in vitro to *C. perfringens* enterotoxin. High levels of toxin (1—2 ml containing 100 μg/ml) and long exposure times (90 min) were employed so it is not possible at this time to directly compare our results with the report of McDonel et al. [22], but their conclusion that *C. perfringens* enterotoxin acts directly on the cell membrane is in agreement with our findings.

The results of our investigation argue strongly that *C. perfringens* enterotoxin 'attacks' and directly produces changes in the permeability of the hepatocyte membrane. Elsewhere [11] we have demonstrated the existence of both sensitive and resistant hepatoma cell lines, indicating that the cellular 'site' recognized by the toxin is apparently specific and present on only some cells. The finding that decay of α-aminoisobutyric acid transport stops soon after toxin is removed (Fig. 1) indicates that the toxin-cell 'complex' is easily dissociated at early times and this conclusion is supported by experiments involving specific antiserum addition (Figs. 2—5).

We have also obtained evidence (Fig. 1) which indicates that depending upon how long the cells were exposed to toxin a limited recovery of α-aminoisobutyric acid uptake may occur upon incubation in toxin-free medium: following 5 min of exposure recovery can be complete whereas after 20 min of exposure to toxin, α-aminoisobutyric acid uptake can recover to only 40% of the control (fully induced) level. Castellot et al. [23] recently showed that cultured animal cells made permeable to small molecules (including the vital dye trypan blue) by hypertonic treatment for 40—50 min could completely recover and divide when reincubated in isotonic growth medium. Further, we have demonstrated that hepatocytes incur membrane damage during isolation which can be repaired during subsequent incubation in monolayer culture [7]. Hence, the finding that recovery was less than 100% following only 10—15 min of toxin treatment (Fig. 1) suggests that the toxin-cell interaction may involve an early reversible step followed by an irreversible step which cannot be repaired. A two-step toxin-cell interaction has been described for diphtheria toxin [24].

In previous studies [6] it was shown that 40% of the amino acids which compose *C. perfringens* enterotoxin are hydrophobic and that the protein contains clustered hydrophobic regions. It therefore appears likely that the toxin has affinity for lipid. Perhaps the toxin, after encountering a specific recognition site (receptor?), partially 'melts' into lipid in the cell membrane, thereby producing a local perturbation allowing Na^+ (as well as other ions) to cross the permeability barrier. At this point the toxin-membrane complex is active but unstable and easily dissociated by washing or by adding specific antiserum. However, at some subsequent time the toxin reorients in the membrane and becomes fixed. One possible mechanism for fixation is based on the model of Blobel and Dobberstein [25] to explain the loss of 'signal sequences' from proteins which cross membranes: the portion of the enterotoxin facing inside (cytoplasm side) may be enzymatically attacked. This could then result in a change in the shape of the toxin-membrane complex possibly facilitating migration of larger hydrophilic molecules such as L-glucose and 3-*O*-methylglucose. This discussion remains speculative but it is significant that *C. perfringens* enterotoxin appears very sensitive to proteolysis (even the most highly purified preparations are microheterogeneous [6]) and fragments termed 'enterotoxin-like' protein have been isolated from enterotoxin-positive and negative strains of *C. perfringens* [26].

Acknowledgements

The authors express their gratitude to Dr. Nancy Shoptaugh and Dr. Gino Saccone for helpful suggestions, and to Ms. Virginia Garcia for excellent technical assistance. This work was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison; the University of Wisconsin-Madison Graduate School; the Wisconsin Agricultural Experiment Station; Training Grant T32ES07015-04 from NIEHS to the U.W. Center for Environmental Toxicology; Grant 1-R01-AI15693-01 from NIAID; and by the University of Wisconsin-Madison Food Research Institute.

References

1 Stark, R.L. and Duncan, C.L. (1972) Infect. Immun. 6, 662—673
2 Duncan, C.L., Strong, D.H. and Sebald, M. (1972) J. Bacteriol. 110, 378—391
3 Sakaguchi, G.T., Uemura, T. and Reimann, H.P. (1973) Appl. Microbiol. 26, 762—767
4 Stark, R.L. and Duncan, C.L. (1971) Infect. Immun. 4, 89—96
5 Stark, R.L. and Duncan, C.L. (1972) Infect. Immun. 5, 147—150
6 Enders, G.L., Jr. and Duncan, C.L. (1976) Can. J. Microbiol. 22, 1410—1414
7 Kletzien, R.F., Parzia, M.W., Becker, J.E. and Potter, V.R. (1976) J. Biol. Chem. 251, 3014—3020
8 Kletzien, R.F., Pariza, M.W., Becker, J.E. and Potter, V.R. (1975) Anal. Biochem. 68, 537—544
9 Kletzien, R.F., Pariza, M.W., Becker, J.E. and Potter, V.R. (1976) J. Cell Physiol. 89, 641—646
10 Pariza, M.W., Butcher, F.R., Kletzien, R.F., Becker, J.E. and Potter, V.R. (1976) Proc. Natl. Acad. Sci. U.S. 73, 4511—4515
11 Giger, O. and Pariza, M.W. (1978) Biochem. Biophys. Res. Commun. 82, 378—383
12 LeCam, A. and Freychet, P. (1977) J. Biol. Chem. 252, 148—156
13 Wheeler, K.P. and Christensen, H.N. (1967) J. Biol. Chem. 242, 3782—3788
14 Earle, W.R. (1943) J. Natl. Cancer Inst. 4, 165—212
15 Pariza, M.W. and Duncan, C.L. (1977) Fed. Proc. 36, 669
16 Stein, W.D. (1967) The Movement of Molecules Across Membranes, Academic Press, New York
17 LeFevre, P.G. (1961) Pharmacol. Rev. 13, 39—70

18 Christensen, H.N. (1975) Biological Transport, 2nd edn., W.A. Benjamin, Inc., Reading, MA
19 Kelly, D.S. and Potter, V.R. (1978) J. Biol. Chem. 253, 9009–9017
20 Tosteson, M.T. and Tosteson, D.C. (1978) Nature 274, 143–144
21 Schein, S.J., Kagan, B.L. and Finkelstein, A. (1978) Nature 276, 159–163
22 McDonel, J.L., Chang, L.W., Pounds, J.G. and Duncan, C.L. (1978) Lab. Invest. 39, 210–218
23 Castellot, J.J., Jr., Miller, M.R. and Pardee, A.B. (1978) Proc. Natl. Acad. Sci. U.S. 75, 351–355
24 Boquet, P. and Pappenheimer, A.M., Jr. (1976) J. Biol. Chem. 251, 5770–5778
25 Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 835–851
26 Frieben, W.R. and Duncan, C.L. (1975) Eur. J. Biochem. 55, 455–463