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PROTECTIVE EFFECTS OF OSMOTIC STABILIZERS ON MORPHOLOGICAL AND PERMEABILITY ALTERATIONS INDUCED IN VERO CELLS BY *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN

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Summary

Culture medium made hypertonic by the addition of osmotic stabilizers such as sucrose, poly(ethylene glycol), dextran and bovine serum albumin protected against changes in morphology and plasma membrane permeability induced by *Clostridium perfringes* enterotoxin. The protection did not appear to be due to binding inhibition. Results of these studies support an osmotic disruption mechanism for the action of the enterotoxin. A comprehensive model of the enterotoxin's action based on an osmotic disruption mechanism is proposed.

Introduction

Studies of the mode of action of *Clostridium perfringens* enterotoxin have shown it to cause transport alterations [1—4], histological damage [5], inhibition of macromolecular synthesis [6], and permeability alterations [7,8] in various model systems. The enterotoxin induced membrane blebs in cultured Vero (African green monkey kidney) cells [6,9] and intestinal epithelial cells [10]. Additionally, it causes rapid (within 15 min) permeability alterations in Vero cells [7]. It has been proposed [7,10] that the enterotoxin acts via a direct plasma membrane interaction which induces structural and functional changes in the plasma membrane of sensitive cells.

As yet, sequential integration of this membrane-damage theory of enterotoxin action with the various biochemical and physiological alterations

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observed in treated cells has been unclearly defined. One mechanism by which the enterotoxin could cause the observed biological effects would be to disrupt the osmotic barrier of the mammalian cell. Other bacterial toxins, including Staphylococcal alpha-toxin [11] and *Clostridium septicum* toxin [12], are thought to act by this mechanism. These studies have shown that the addition of osmotic stabilizers to medium inhibited the actions of alpha-toxin and *C. septicum* toxin. Osmotic stabilizers [11] are agents which do not penetrate the plasma membrane of eukaryotic cells, but inhibit cytolytic toxins by decreasing colloidal osmotic swelling [11]. This paper reports studies of the effects of various osmotic conditions on the enterotoxin's action and proposes a model for the mechanism by which the enterotoxin acts.

Materials and Methods

Enterotoxin. C. perfringens enterotoxin was prepared as described [13] and the biological activity determined in erythermal units, E.U. * [14]. The specific activity of the enterotoxin preparations varied from 1400—1800 E.U./mg protein. Enterotoxin was dissolved in double-distilled water and 60-μl aliquots were stored frozen at —20°C until used. Aliquots were used within 2 weeks of their preparation. Enterotoxin preparations had no detectable RNAase, protease, or phospholipase C activity [15]. ^{125}I-labeled enterotoxin was prepared as described [16].

Culture of Vero cells. Vero cells were grown in 75-cm^2 tissue culture flasks (Corning) with Medium 199 supplemented with 5% fetal calf serum (Grand Island Biological Company) and 0.75% $NaHCO_3$. When monolayers reached confluency, they were removed by trypsinization with 0.25% trypsin in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution. Trypsinized cells were inoculated into 16-mm wells in tissue culture cluster dishes (Costar). The seeding density was $5 \cdot 10^4$ cells in 3 ml of medium per well. The cluster dishes were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. After 3 days, fresh medium was added. After 6 days, each well contained a confluent monolayer with $6—8 \cdot 10^5$ cells.

Light microscopy of Vero cells. Confluent monolayers were washed once with 1 ml of Medium 199 without serum. To each well were added 2 ml of the desired osmotic test medium containing enterotoxin. Each well received an enterotoxin dose of 4 E.U. (6 μg). Photomicrographs were taken with an American Optical inverted phase microscope.

Nucleotide labeling of Vero cells. Cells were RNA and nucleotide labeled by the methods previously reported [7]. For nucleotide labeling, 6-day-old monolayers were washed with Medium 199 without serum and labeled by the addition of 2 ml of Medium 199 containing 2 μCi [^{3}H]uridine (41.3 Ci/mmol, New England Nuclear). After 2 h, the labeling medium was removed. Previous studies [7] have shown that cells labeled by this method contain approx. 70% of the total cytoplasmic label in a nucleotide form.

RNA labeling of Vero cells. 24 h after inoculation, the cultures were washed with warm Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution and labeled by the addition of 2 ml of Medium 199 containing 2 μCi [^{3}H]uridine. After 24 h,

* The erythermal unit is defined as that amount of enterotoxin which causes a zone of erytherma 0.8 cm in diameter when injected intradermally into a depilated guinea-pig [14].

the labeling medium was removed, the cells were washed with warm Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution, and incubation was continued for 4 days in Medium 199 without label. Previous studies [7] have shown that cells labeled by this method contain about 90% of their cytoplasmic label in the RNA form.

Treatment of cells with enterotoxin. Medium 199 used for washing cells and for enterotoxin treatment of Vero cells did not contain serum.

(a) Determination of the effects of osmotic stabilizers on nucleotide-label release. Nucleotide-labeled cultures were incubated with 4 E.U. of enterotoxin diluted in 2 ml of the specified osmotic test medium (Medium 199 containing 0.3 M sucrose, 5% dextran, 25% poly(ethylene glycol), or 16% bovine serum albumin). After 30 min of incubation, the culture supernatant was gently removed, vortex mixed, and centrifuged at 4°C for 3 min in an Eppendorf microcentrifuge. Radioactivity was measured in the supernatant.

(b) Determination of the effects of sucrose addition following enterotoxin treatment. Nucleotide-labeled cultures were incubated for 5 or 10 min with 4 E.U. of enterotoxin diluted in 2 ml of Medium 199. Following this incubation, the enterotoxin-containing medium was removed, the cells were washed with Medium 199 and 2 ml of either Medium 199 or Medium 199 containing 0.3 M sucrose were added to each culture well. After 30 min of further incubation time, the culture supernatants were removed and assayed as above.

Determination of binding of ^{125}I-labeled enterotoxin. Binding studies were performed with Vero cells in the presence or absence of 0.3 M sucrose. Binding of ^{125}I-labeled enterotoxin to cells in suspension was performed according to the method of McDonel and McClane [16]. Controls consisted of cells that were pretreated with an excess of native enterotoxin followed by treatment with ^{125}I-labeled enterotoxin. The amount of binding that occurred under these conditions was taken to be nonspecific binding. Specific binding was calculated as the difference between total binding in the absence of native enterotoxin and nonspecific binding.

Determination of the effects of hypotonic NaCl on leakage of label from RNA- and nucleotide-labeled cultures. RNA- or nucleotide-labeled cultures were washed with Medium 199 without serum and incubated for 30 min in NaCl of various molarities (5—150 mM). The desired NaCl concentrations were prepared by addition of the appropriate amount of NaCl to double-distilled water. After incubation, the culture supernatant was removed and assayed for the leakage of label as described for the above experiments.

Determination of percent of maximal release. Released radioactivity was calculated as described by Thelestam and Möllby [17]:

$$\% \text{ of maximal release} = \frac{\text{toxin-induced release}-\text{spontaneous release}}{\text{maximal release}-\text{spontaneous release}} \times 100$$

Spontaneous release after 30 min at 37° C was less than 1% for RNA label and less than 5% for nucleotide label. As described previously [7], the maximal release of cytoplasmic label was determined after cell membrane rupture by the addition of 1 ml of 1 M citric acid and 1 ml of a 0.5% saponin buffer per well.

Samples (0.2 ml) from labeled cultures were counted in a Packard Tricarb liquid scintillation spectrophotometer. All experiments were performed in duplicate or triplicate.

Results

C. perfringens enterotoxin in Medium 199 without serum caused morphological alterations of Vero cells which were similar to those noted in previous studies [6–9]. Enterotoxin-treated cells changed from the normal spindle shape (Fig. 1A) characteristic of Vero cells to a spherical shape (Fig. 1B).

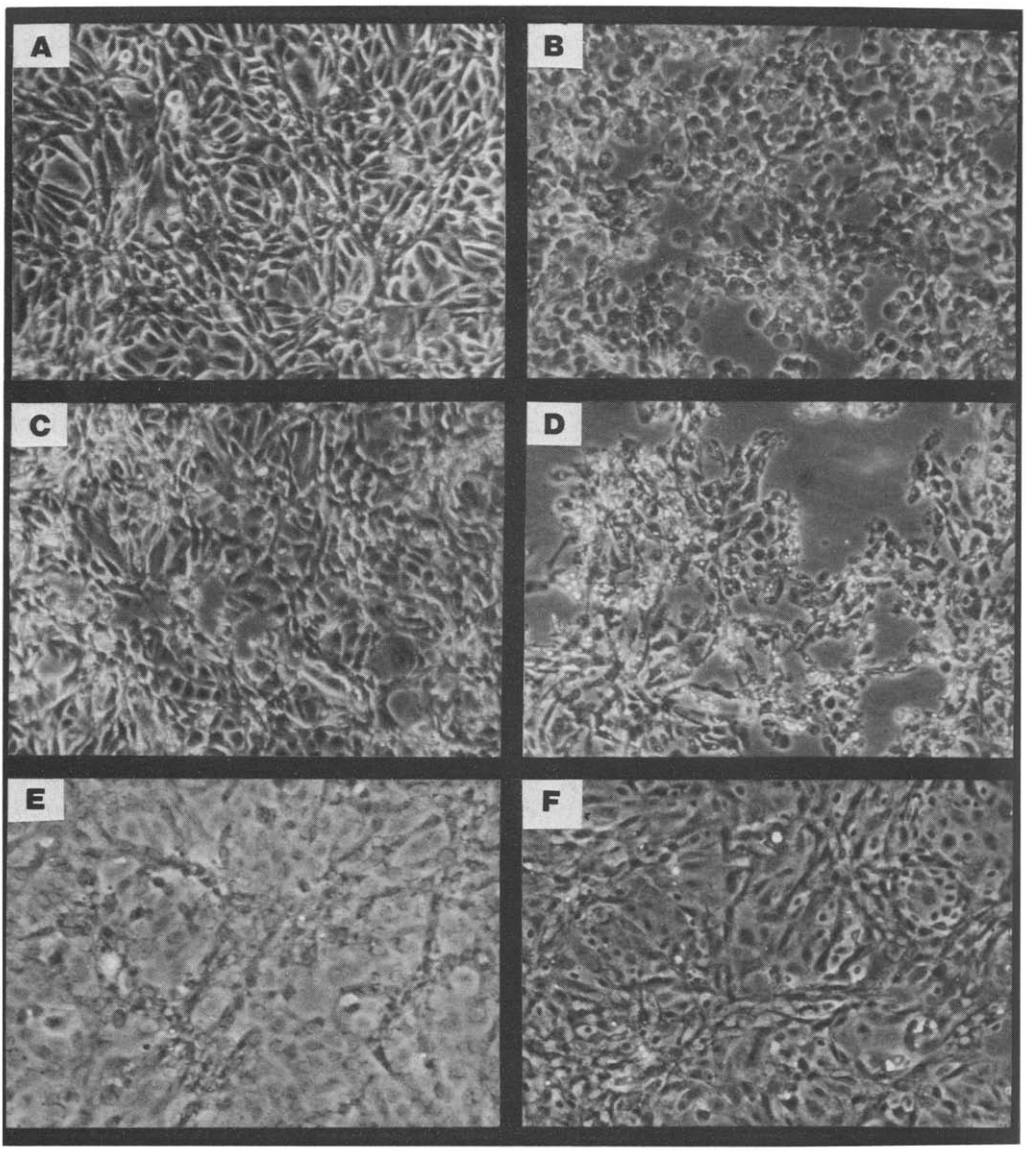

Fig. 1. Protective effects of osmotic stabilizers on enterotoxin-induced morphological alterations in Vero cells. (A) Control culture. (B) Culture treated with enterotoxin. Note rounding of cells and formation of membrane blebs. (C) Culture treated simultaneously with enterotoxin and 0.3 M sucrose. (D) Culture treated simultaneously with enterotoxin and 5% dextran. (E) Culture treated with 25% poly(ethylene glycol). (F) Culture treated simultaneously with enterotoxin and 25% poly(ethylene glycol). All cultures received 2 ml of Medium 199 without serum containing the desired osmotic stabilizer and/or 4 E.U. of enterotoxin. Photomicrographs were made after 30 min of treatment.

Extensive membrane bleb formation and increased cell aggregation were observed in enterotoxin-treated cells.

To investigate whether *C. perfringens* enterotoxin acts by disrupting the osmotic barrier of the cell membrane, osmotic stabilizers such as sucrose (mol. wt. 342), poly(ethylene glycol) (mol. wt. 6000), dextran (mol. wt. 9400), and bovine serum albumin (mol. w. about 66 000) were added to the culture medium concurrently with enterotoxin [11,12,18]. Fig. 1C shows that the addition of 0.3 M sucrose to Medium 199 was highly protective against morphological alterations. After 30 min of enterotoxin treatment in sucrose-containing medium, there was rounding in only a few (less than 20%) cells. Additionally, enterotoxin-induced bleb formation was not apparent in sucrose-treated cultures. After 60 min of enterotoxin treatment in sucrose-containing medium, there was still a noticeable protective effect in terms of morphological alterations (not shown). 5% dextran in Medium 199 was less protective than sucrose against enterotoxin-induced morphological alterations (Fig. 1D). In dextran-containing medium, rounding and cell aggregation were repressed by the addition of dextran to the culture medium. Addition of bovine serum albumin (16% final concentration) to cultures concurrently with enterotoxin produced morphological protection (not shown) apparently similar to that with dextran treatment.

Polyethylene glycol (25%) treatment of Vero cells caused rapid fusion of the cytoplasmic membranes of the cells in the confluent monolayer (Fig. 1E). The ability of poly(ethylene glycol) to induce cell fusion has been widely studied [19]. Interestingly, enterotoxin seemed to disrupt poly(ethylene glycol)-induced cell fusion. In comparison to poly(ethylene glycol) controls, cultures treated simulataneously with enterotoxin and poly(ethylene glycol) exhibited an increased maintenance of the distinct shape of individual cells (Fig. 1F) within the culture monolayer. There was little or no rounding (less than 10% of the cells) of bleb formation in cells treated concurrently with enterotoxin and poly(ethylene glycol).

The results presented above indicate that the addition of osmotic stabilizers to the culture medium concurrently with enterotoxin protects against enterotoxin-induced morphological alterations. It has recently been shown that *C. perfringens* enterotoxin alters plasma membrane permeability [7,8,16]. To study whether osmotic stabilizers protect against enterotoxin-induced permeability damage (as well as morphological damage), leakage assays of [^{3}H]nucleotide label were performed (Table I). These studies show that the addition of osmotic stabilizers concurrently with enterotoxin was protective against leakage effects. After 30 min, sucrose was the most effective treatment for inhibiting leakage, but all four of the osmotic stabilizers significantly reduced enterotoxin-induced leakage. After 60 min there still was protection from enterotoxin-induced leakage with the stabilizers assayed. Cultures treated with osmotic stabilizers, but not enterotoxin, exhibited slightly lower (about 10%) spontaneous release rates than cultures only in Medium 199.

It has been shown [16] that *C. perfringens* enterotoxin binds to Vero cells and, furthermore, that binding is important for the expression of the biological action of the enterotoxin. The protective effects of osmotic stabilizers could have been due to binding interference. Table II shows that addition of sucrose

TABLE I

OSMOTIC PROTECTION OF ENTEROTOXIN-TREATED VERO CELLS

Values are given as mean ± S.E. (n = 4). Spontaneous release in controls without stabilizers was 7 and 11% of maximal release at 30 and 60 min, respectively. SS = spindle-shaped cellular morphology; SP = spherical-shaped cellular morphology, SS-SP = some spindle and rounded cellular morphology.

Incubation with enterotoxin	Percent inhibition of nucleotide-label release			
	10% sucrose [a]	25% poly(ethylene glycol) [b]	5% dextran [c]	16% bovine serum albumin [d]
30 min	84 ± 3 SS	61 ± 8 SS	25 ± 3 SS	32 ± 5 SP
60 min	66 ± 4 SS-SP	67 ± 4 SS	27 ± 8 SP	13 ± 7 SP

[a] 300 mM sucrose in Medium 199.
[b] 42 mM poly(ethylene glycol) in Medium 199.
[c] 5.3 mM dextran in Medium 199.
[d] 2.4 mM bovine serum albumin in Medium 199.

to cells that have been pretreated with enterotroxin still resulted in protection from nucleotide leakage.

^{125}I-labeled enterotoxin binding studies were performed in the presence or absence of sucrose. Specific binding of ^{125}I-labeled enterotoxin to cells suspended in Medium 199 containing 0.3 M sucrose was 2236 cpm per mg protein while cells suspended in Medium 199 without sucrose bound 1900 cpm per mg protein. These values, when recalculated as molecules of enterotoxin bound per cell (average, $1.25 \cdot 10^6$/cell), are comparable to the value for average maximum number of enterotoxin binding sites ($1.42 \cdot 10^6$/cell) reported by McDonel and McClane [16]. Binding studies performed with monolayer cultures showed similar results. Taken collectively, these studies show that the protective effects of sucrose appear not to be due to binding interference.

It was noted that a significant number of cells in the enterotoxin-pretreated cultures rounded up immediately after the addition of sucrose. This may have been due to a membrane effect [11] resulting from rapid expansion and contraction of the plasma membrane of cells exposed first to enterotoxin and, subsequently, to hypertonic medium. This effect could explain the reduced inhibition of leakage by sucrose when added after pretreatment of cells with

TABLE II

PROTECTIVE EFFECTS OF SUCROSE FOLLOWING PRETREATMENT WITH ENTEROTOXIN

The preincubation medium for all experiments was Medium 199 without serum. Values are means ± S. E. (n = 4).

Incubation with enterotoxin	Percent of maximal release		Percent reduction
	Without 0.3 M Sucrose	With 0.3 M Sucrose	
5 min	8.2 ± 1.0	5.2 ± 0.4	37
10 min	10.9 ± 0.4	7.7 ± 0.2	29

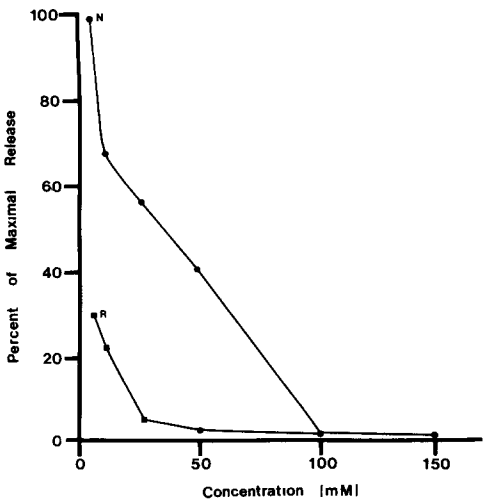

Fig. 2. Effects of hypotectonic conditions on release of RNA label (■———■) and nucleotide label (●———●). The effects of various concentrations (5—150 mM) of NaCl on leakage of RNA and nucleotide label from Vero cells after 30 min of incubation are shown.

enterotoxin. Interestingly, preliminary results (not shown) of these experiments performed with poly(ethylene glycol) in place of sucrose showed that treatment of cells with enterotoxin followed by poly(ethylene glycol) treatment resulted in more than a 2-fold increase in leakage (although morphological damage was still minimal at the light microscope level). This effect could again be a result of membrane damage following rapid expansion and contraction of the plasma membrane or might be a synergistic membrane effect resulting from poly(ethylene glycol)-induced loss of membrane lipids [20].

Earlier studies [7] of enterotoxin-induced membrane permeability alterations in Vero cells showed that after 30 min, 4 E.U. of enterotoxin in Medium 199 caused a maximal release of nucleotide label of about 11%. The current studies show that pretreatment of cultures with enterotoxin for 10 min prior to 30 min incubation in Medium 199 was sufficient to obtain a maximal release of approx. 11%. These results support earlier studies [9,21] which suggested that enterotoxin binds rapidly and its biological action is not reversible by washing.

C. perfringens enterotoxin alters membrane permeability by a mechanism apparently involving a size-dependent, molecular-sieving action [7]. Small molecules leak from treated cells faster than large molecules. This could be a function of membrane stretching due to the enterotoxin-induced formation of membrane blebs. Reductions of the osmolarity of culture medium towards hypotonicity have been reported, for red blood cells [22] and WI-38 cells [23], to result in similar membrane stretching due to water influx, with a subsequent molecular-sieving action. Fig. 2 shows that Vero cells in a hypotonic medium leak nucleotide label preferentially to RNA label. Vero cells placed in a hypotonic medium rapidly formed membrane blebs. These results show that conditions favorable for the rapid entrance of water into Vero cells can result in membrane stretching and a subsequent size-dependent loss of the permeability barrier of the cell.

Discussion

This report supports the concept of involvement of an osmotic mechanism in the mode of action of *C. perfringens* enterotoxin. Osmotic stabilizers offered protection against enterotoxin-induced morphological and permeability alterations. The addition of sucrose after pretreatment with enterotoxin still protected against enterotoxin-induced alterations. The presence of sucrose had no apparent inhibition effects on the binding of ^{125}I-labeled enterotoxin. These findings and the widely diverse nature of the four osmotic stabilizers tested strongly suggest that the protective effects of these agents were due, at least partially, to their osmotic properties. A number of membrane-active agents, including some bacterial toxins [11,12], are known to act by disrupting the osmotic barrier of the eukaryotic cell.

It is now possible to propose a model for the mode of action of *C. perfringens* enterotoxin. Initially, enterotoxin binds to susceptible cells [16] and may be inserted into the plasma membrane (for details see McDonel, J.L., Ref. 25). Soon after binding, the enterotoxin renders the plasma membrane leaky to ions by a still unknown mechanism. Other studies using *C. perfringens* enterotoxin have suggested that Na^+ [8] and/or Ca^{2+} [9] may be involved in this ion imbalance. Preliminary studies (McClane, B.A. and McDonel, J.L., unpublished observations) have shown a 2-fold increase in Na^+ influx after 5 min of enterotoxin treatment. It is important to note [8] that changes in levels of intracellular Na^+ occurred rapidly in enterotoxin-treated hepatocytes and preceded generalized permeability alterations in leakage of glucose, methylglucose or aminoisobutyric acid. With the creation of the enterotoxin-induced ion imbalance, it is conceivable that there is a net influx of ions and water into the treated cells due to a disruption of the colloid-osmotic equilibrium [11]. As a result of rapid water influx, the plasma membrane would be expected to stretch followed by membrane bleb formation [11,18,24]. Support for enterotoxin-induced formation of membrane blebs being due to osmotic disruptions is offered in this paper, following earlier suggestions by others [9] of such action. Upon the onset of expansion of the plasma membrane, the permeability barrier of the cell would be affected [11,18,22,23]. Molecules of increasing size leak with time from enterotoxin-treated Vero cells [7]. It has been shown in this report that similar leakage patterns occurred when the plasma membranes of Vero cells were subjected to rapid expansion under other conditions. With the loss of essential precursors through leakage and/or improper cell electrolyte balance, metabolism would rapidly be affected and vital processes such as macromolecular synthesis would be inhibited [6]. Eventually, many enterotoxin-treated cells die, as revealed by plating-efficiency studies [6,16].

Further studies are needed to verify this model. For example, detailed studies of electrolyte transport in the presence of osmotic agents will be necessary to elucidate better and to verify this model. Additionally, the specific action of the enterotoxin to induce these physiological changes remains unknown. It seems increasingly likely, however, that the action of the enterotoxin involves disruption of the function and/or orientation of some membrane component(s).

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