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CALCIUM-INDEPENDENT AND DEPENDENT STEPS IN ACTION OF CLOSTRIDIUM PERFRINGENS ENTEROTOXIN ON HELA AND VERO CELLS

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SUMMARY. Purified enterotoxin (20-200 ng/ml) of Clostridium perfringens rapidly induced bleb and balloon formation on HeLa and Vero cells in the presence, but not the absence, of Ca^{2+}. The action of the toxin involved two, sequential, temperature-dependent steps:The first was Ca^{2+}-independent and included binding of toxin and the bound toxin after 30-60 sec could no longer be removed by washing. The second step was Ca^{2+}-dependent and eventually led to bleb and balloon formation. On adding Ca^{2+} to cells pretreated with toxin in Ca^{2+}-free medium, bleb and balloon formation started immediately. The ionophore A23187 mimicked the action of toxin. The effects of sucrose (0.2 M), trypsin-treatment of the cells and various pretreatments of the toxin on the action of enterotoxin were studied.

Clostridium perfringens, one of the most common causes of food poisoning produces an enterotoxin that is responsible for the symptoms of diarrhea. The enterotoxin is known to induce alterations of transport (1, 2, 3, 4) and metabolism (5, 6) and tissue damage (1, 7, 8) in various model systems and is believed to act rapidly by direct interaction with membranes (8, 9, 10).

Recent isolation of the toxin as a highly purified protein with a mol. wt. about 35,000 (11, 12, 13) and the use of tissue culture cells (4, 9, 14, 15, 16) have facilitated precise studies on the mechanism of the toxin action under simple defined conditions. Thus recently it has been possible to show that the toxin depresses amino acid transport in cultured rat hepatocytes (4) and that labeled toxin binds specifically to Vero cells, inhibiting macromolecular synthesis, and causing detachment and death of the cells (15). But little is known about the exact mechanism of action of the enterotoxin.

This paper reports studies on the requirements for the action of the purified enterotoxin of C. perfringens on HeLa and Vero cells and demonstration of two steps in its action.

Abbreviations: PBS, phosphate-buffered saline (17); PBS(+), PBS containing Ca^{2+}(0.9 mM) and Mg^{2+}(0.5 mM) (17); PBS(-), PBS free from Ca^{2+} and Mg^{2+}.

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MATERIALS AND METHODS. Enterotoxin was prepared from sporulated cultures of a strain NCTC 8239 of C. perfringens obtained through Dr. G. Sakaguchi. It was purified as described by Sakaguchi et al.(12). Toxin was diluted in PBS (17) free from Ca^{2+} and Mg^{2+}(PBS(-)) and added to the cells at a final concentration of 20-200 ng/ml medium. Rabbit antitoxin serum was prepared as described by Sakaguchi et al.(12). Ionophore A23187(A grade) was purchased from Calbiochem., and cholesterol(guaranteed reagent) from Nakarai Chemicals, Ltd, Kyoto.

HeLa-S_3 cells and Vero (African green monkey kidney) cells were provided by Dr. Y. Sakaue of this Institute and by Dr. R. Murata of the National Institute of Health, Tokyo, respectively. The growth medium was 95% Eagle's minimum essential medium and 5% calf serum(Biken). Cells were grown at 37°C under a water-saturated atmosphere of 5% CO_2 in air. Stock cultures were maintained as monolayers in flasks(5 x 11 cm) containing 15 ml of medium. Subculture every 4-6 days was performed by detaching the cells with 0.25% trypsin-0.02% EDTA mixture (1:1 by volume) after they had become confluent. For experiments, cells were removed from the stock flask with trypsin-EDTA mixture and seeded into 60-mm diameter plastic dishes(Falcon) at densities of 3×10^4 and 1×10^5 cells per dish. With these inocula toxin was added 4 and 2 days later, respectively. Just before adding toxin, the medium was replaced by fresh growth medium or the cells were washed three times with PBS(-) and then an appropriate buffer was added (final volume, 5 ml per dish).

RESULTS AND DISCUSSION

Requirement of calcium ions for the action of enterotoxin on HeLa and Vero cells: In growth medium the toxin(20-200 ng/ml) rapidly(in 10-15 min at 200 ng/ml) induced bleb and balloon formation on HeLa and Vero cells at 37°C, but not at 0-4°C. Similar bleb formation has been observed in rat ileum treated with the enterotoxin (8, 10). We tested the effects of various simpler media in place of the growth medium and found that the toxin acted on these cells in PBS containing Ca^{2+}(0.9 mM) and Mg^{2+}(0.5 mM)(PBS(+)(17)) as well as in growth medium. Further analyses showed that Ca^{2+}, but not Mg^{2+}, in PBS(+) was essential for the action of the toxin (Fig. 1 and 2 A-D); in PBS containing Mg^{2+} but no Ca^{2+} the toxin induced only slow swelling of the cells (Fig. 1C and 2B), even when the concentration of Mg^{2+} was increased to 1 mM. In PBS containing 90 μM Ca^{2+} the toxin had a rapid effect on both HeLa and Vero cells but at lower concentration of Ca^{2+} its effect took longer(about 1 hour at 9 μM Ca^{2+}) and with below 0.9 μM Ca^{2+} the toxin(200 ng/ml) did not have such effect within 120 min. Enterotoxin pretreated with PBS(+) did not produce such morphological alterations of cells in PBS(-)(final concentrations: toxin, 200 ng/ml; Ca^{2+}, 0.9 μM).

These cellular responses are faster and more sensitive to toxin than amino acid transport in cultured hepatocytes (4), which was not depressed within 120 min by 40 or 100 ng of toxin/ml.

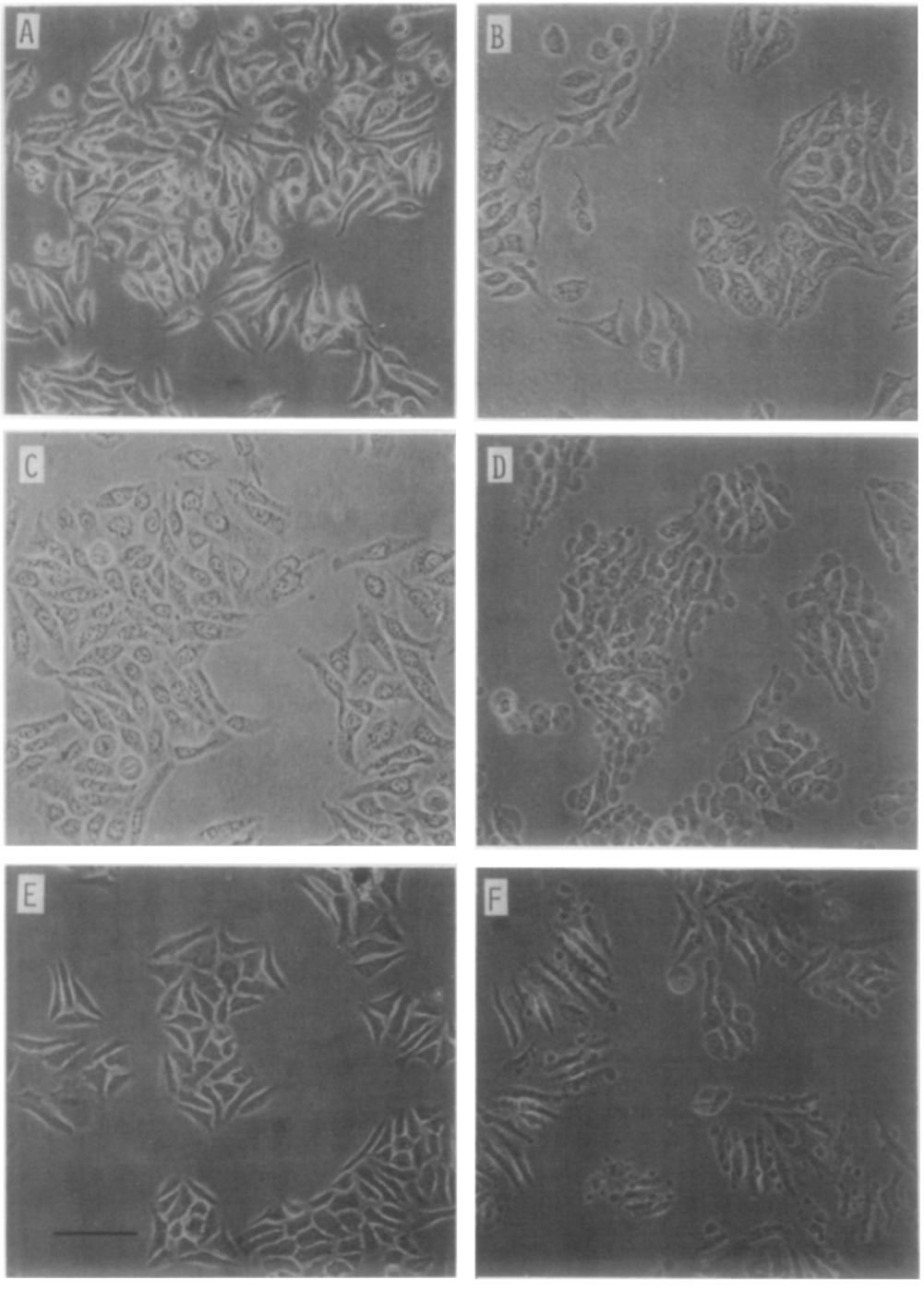

Fig. 1. Phase contrast micrographs of HeLa cells (2-day cultures) treated at 37°C. (A) Control without toxin in PBS(-)(free from Ca^{2+} and Mg^{2+}), 15 min; (B) with toxin (200 ng/ml), in PBS(-), 15 min; (C) with toxin (200 ng/ml), in PBS containing Mg^{2+}(0.5 mM) but no Ca^{2+}, 15 min; (D) with toxin (200 ng/ml), in PBS containing Ca^{2+}(0.9 mM) but no Mg^{2+}, 15 min; (E) with toxin (200 ng/ml) in PBS(+) containing 0.2 M sucrose, 20 min and (F) with ionophore A23187 (2 μg/ml) in PBS(+), 35 min. The scale indicates 100 μm.

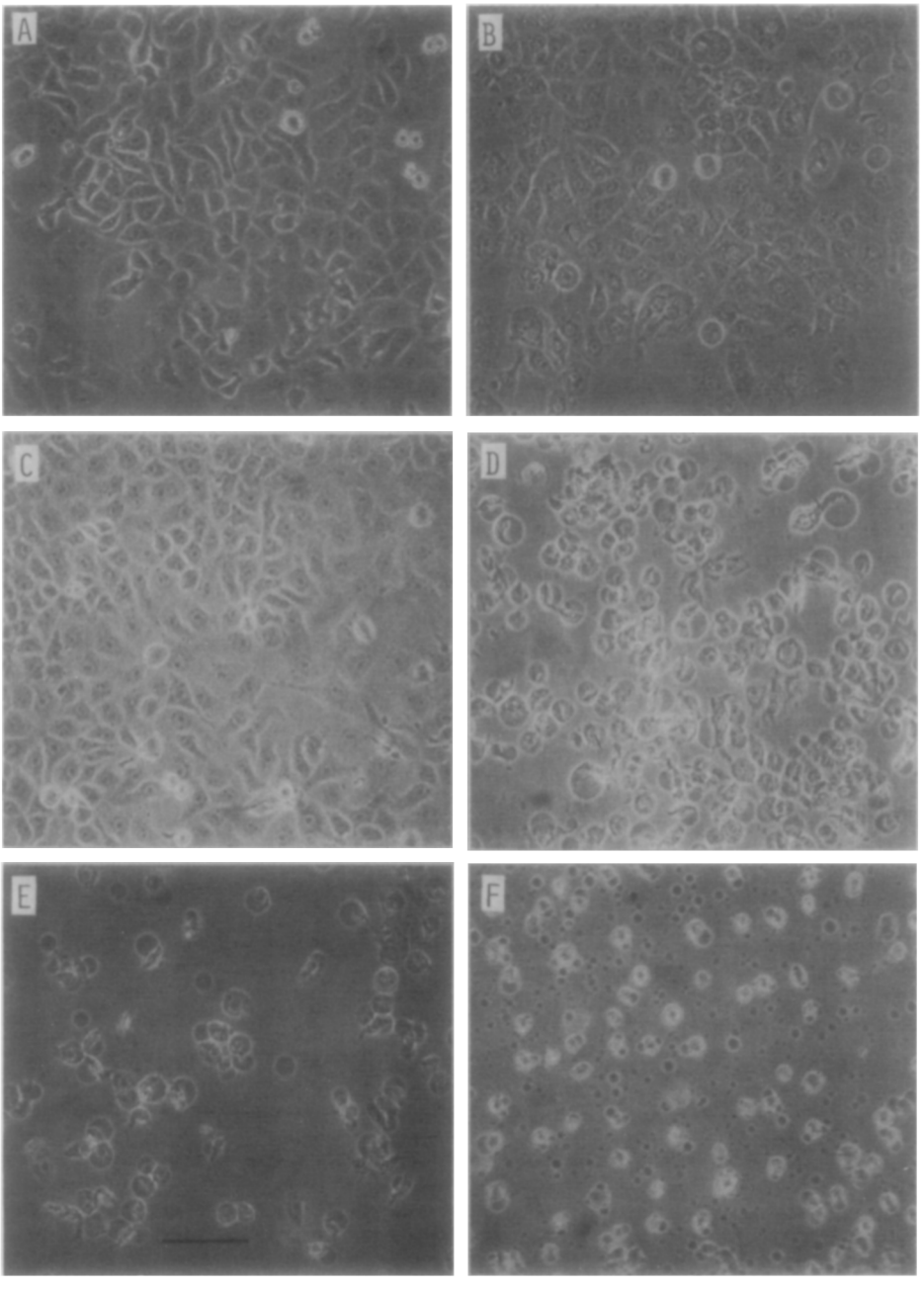

Fig. 2. Phase contrast micrographs of Vero cells (4-day cultures) treated at 37°C. (A) Control without toxin, in PBS containing Mg^{2+}(0.5 mM) but no Ca^{2+}, 15 min; (B) with toxin (200 ng/ml) in the same buffer as for (A), 15 min; (C) control without toxin in PBS containing Ca^{2+}(0.9 mM) but no Mg^{2+}, 15 min; (D) with toxin (200 ng/ml) in the same buffer as for (C), 15 min; (E) pretreated with toxin (200 ng/ml) in PBS(-) for 20 min, then washed and incubated in PBS(+), 5 min and (F) trypsin-EDTA treated cells in suspension, in PBS(+), toxin (200 ng/ml), 15 min. The scale indicates 100 μm.

Dissociation of two steps in the action of enterotoxin on HeLa and Vero cells:

Ca^{2+} was essential for the action of the toxin as described above. However, recently McDonel et al.(15) observed binding of [^{125}I]-labeled enterotoxin to Vero cells in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution. Thus neither Ca^{2+} nor Mg^{2+} must be necessary for toxin binding.

We incubated the cells at 37°C for 20 min with toxin(200 ng/ml) in Ca^{2+}-and Mg^{2+}-free PBS(PBS(-)). Under these conditions the cells did not form blebs or balloons. But when Ca^{2+}(final 0.9 mM) was then added to the cells, with or without washing, they immediately began to show bleb and balloon formation. Figure 2E shows that pretreatment of Vero cells with the toxin in the absence of Ca^{2+} resulted in bleb and balloon formation on subsequent incubation in medium with Ca^{2+} but without toxin as observed when toxin and Ca^{2+} were both present from the beginning of incubation. Similar results were obtained with HeLa cells.

Therefore, there must be at least two steps in the action of the enterotoxin: an initial step that is Ca^{2+}-independent and includes the binding of toxin to the cells, and a second step that is Ca^{2+}-dependent and leads ultimately to bleb and balloon formation. Cells pretreated with the toxin at 0-4°C did not show such morphological alterations when washed and incubated at 37°C in PBS(+).

Rapidity of response of cells to enterotoxin: We incubated the cells with the toxin(40 ng/ml) at 37°C for short periods (0, 30, 60 or 120 sec) and then washed them three times with PBS(-) and incubated them at 37°C in PBS(+). Results showed that washing the cells immediately after adding the toxin (0 sec) rescued them all from the action of the toxin (Fig. 3A), but that washing them after 30-60 sec preincubation with the toxin had no effect on the ultimate morphological alterations of HeLa (Fig. 3B) and Vero cells observed at 37°C after 20 min in PBS(+). After preincubation with toxin for 60 sec, even washing with PBS(-) containing antitoxin(10 μg toxin equivalent), could not prevent the action of the toxin (Fig. 3C and D).

This shows that on 30-60 sec exposure to the toxin, the action of the enterotoxin proceeds to an irreversible stage from which the intoxicated cells cannot be rescued by washing.

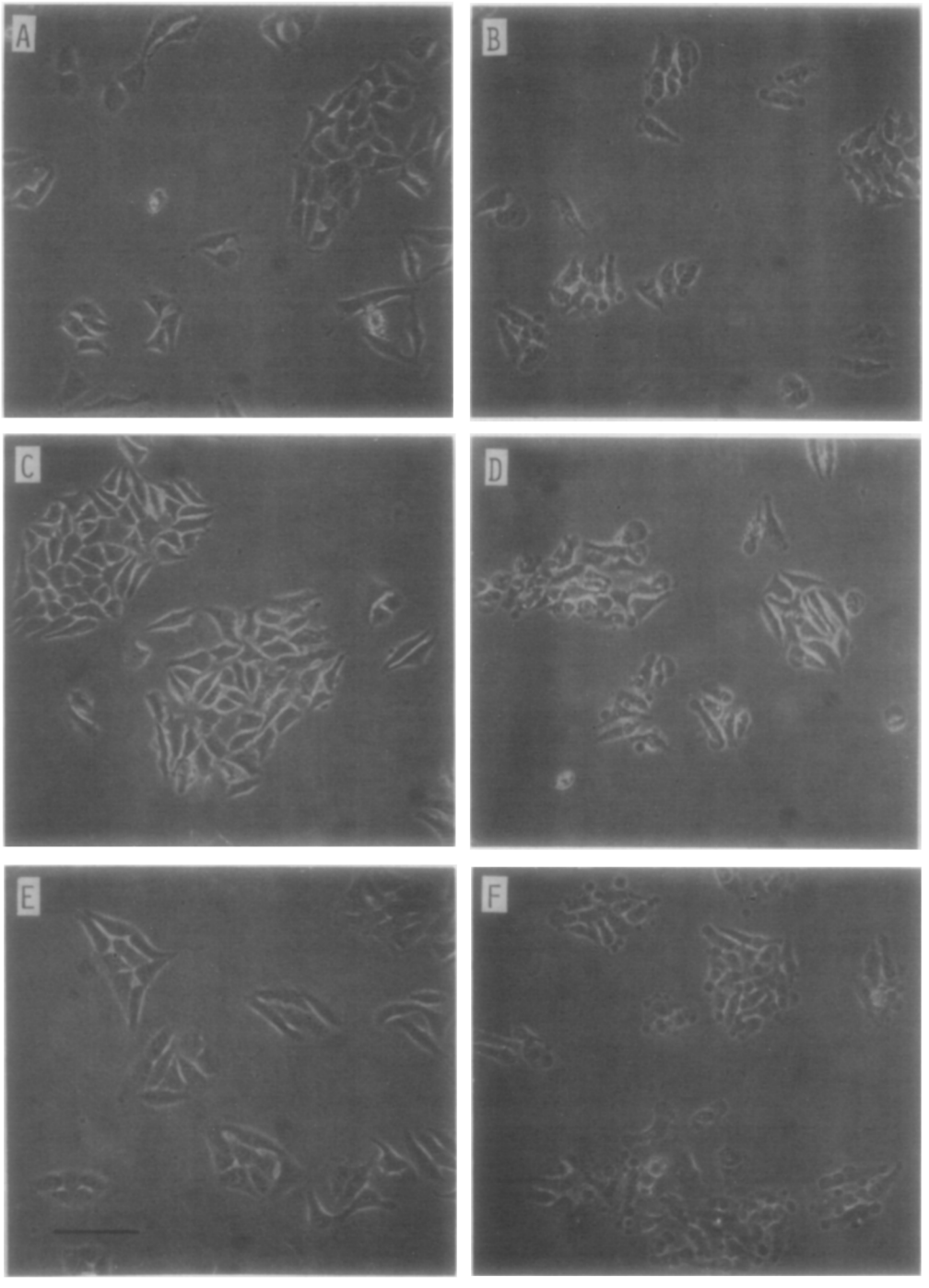

Fig. 3. Phase contrast micrographs of HeLa cells (2-day cultures) treated at 37°C. Cells washed, (A) immediately, (B) and (C) 30 sec, or (D) 60 sec after adding toxin (40 ng/ml) in PBS(-), with PBS(-) (A and B) or with PBS(-) containing antitoxin (C and D), and subsequently incubated in PBS(+), 20 min. Cells in PBS(+) for 15 min with toxin (200 ng/ml) that had been pretreated with (E) 20 mM cysteine at 25°C for 60 min and (F) with cholesterol (200 ng/ml) at 37°C for 60 min. The scale indicates 100 μm.

Effect of ionophore A23187 on HeLa and Vero cells: A23187 was dissolved in ethanol and 25 µl of the solution was added to cells in 5 ml of PBS (final concentration of ethanol, 0.5%). In the presence of Ca^{2+}, but not in its absence, the ionophore (2 µg/ml) at 37°C, but not at 0-4°C, induced bleb and balloon formation on HeLa (Fig. 1F) and Vero cells indistinguishable from that induced by the toxin. Ethanol (0.5%) alone did not produce such morphological alterations. Unlike the toxin, the ionophore produced similar bleb and balloon formation on mouse neuroblastoma C1300 clone N18 and rat glial cells C_6. On a molar basis the toxin is roughly 670 times more effective than the ionophore, possibly because there is some efficient mechanism of interaction of the enterotoxin with specific receptors on the sensitive cells.

Effects of various treatments on the action of the enterotoxin: Cells suspended in PBS(+) immediately after trypsin-EDTA treatment were as sensitive to toxin as cells attached to the dish as monolayers (Fig. 2F). The presence of sucrose (0.2 M in PBS(+)) delayed the response of the cells (Fig. 1E) and partially inhibited the action of the toxin (fine vesicles were formed in 40 min at 37°C).

Heating the toxin at 80°C for 10 min or pretreating it with 0.2% formalin(18) or antitoxin (37°C for 60 min) inactivated its cytotoxic activity. Unlike thiol-activated cytolytic toxins, such as C. perfringens θ-toxin, C. perfringens enterotoxin was inactivated by 20 mM cysteine (25°C for 60 min)(Fig. 3E) but not by cholesterol (200 ng/ml, 37°C for 60 min)(Fig. 3F).

The above data show that the action of C. perfringens enterotoxin proceeds very rapidly, probably through an interaction with trypsin-resistant receptors on the surface of the sensitive cells and that Ca^{2+} is required for the subsequent action leading to morphological alterations. The osmotically sensitive surface alterations induced by the enterotoxin and the similar effect of the ionophore in the presence of Ca^{2+} suggest that the enterotoxin exerts its cytotoxic effect by change in permeability of the membranes and consequent influx of Ca^{2+} ions. On the basis of the above findings studies are now in progress on exact molecular mechanism of the action of enterotoxin.

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