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# CLOSTRIDIUM PERFRINGENS TYPE A ENTEROTOXIN INDUCES RELEASE OF NORADRENALINE FROM THE NEUROSECRETORY PC12 CELL LINE

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SUMMARY: Clostridium perfringens type A enterotoxin(500 ng/ml) induced extensive release of noradrenaline (1/3-2/3 of the total cell content) from PC12 cells in 2-4 min in the presence, but not the absence of extracellular $Ca^{2+}$. Cells treated with toxin in the absence of $Ca^{2+}$ released noradrenaline promptly on subsequent addition of $Ca^{2+}$ to the medium. The amount of noradrenaline released depended on the concentrations of both $Ca^{2+}$ and toxin in the medium(ED50, 0.3 mM and 420 ng/ml respectively). $Ca^{2+}$ could be replaced by $Ba^{2+}$ or $Sr^{2+}$, and $Mn^{2+}$ or $Co^{2+}$, which are $Ca^{2+}$ channel blockers, did not inhibit the release of the transmitter. These findings are discussed in relation to the systemic effects of enterotoxin.


An enterotoxin is produced by certain strains of Clostridium perfringens type A (1,2). It is a simple protein (mol wt ca. 35000) and its amino acid sequence was recently clarified (3). The toxin is known to be the causative agent of diarrhea in food poisoning by this organism (1,2). Besides the symptom of diarrhea, disturbances of the secretory system, such as lacrimation and hyper-salivation, have also been observed in intoxicated animals (4,5). However, the exact mechanism of action of the enterotoxin in the toxic disorder has not been clarified. We have been studying the mechanism of action of the toxin in vitro using tissue culture cells under simple defined conditions. We found previously that the toxin rapidly changed the membrane permeability of HeLa and Vero cells and that if $Ca^{2+}$ was present in the medium, $Ca^{2+}$ influx resulted from the characterisitic changes in membrane permeability (6,7,8). Since $Ca^{2+}$ influx is known to trigger various cellular and subcellular events, including secretory processes, we used clonal PC12 cells, which can secrete catecholamines, to study the effect of the enterotoxin on secretion in

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vitro. This paper reports that the enterotoxin induced $Ca^{2+}$-dependent release of noradrenaline from neurosecretory PC12 cells.

Materials and Methods: C. perfringens type A enterotoxin was highly purified by high performance liquid chromatography as described previously (9). The clonal rat pheochromocytoma PC12 cell line was provided by Dr. K. Hayashi, Gifu Pharmaceutical College. Cells grown to confluence were dispersed by pipetting and seeded at densities of 0.5-1.5 x $10^6$ cells per dish into 35-mm diameter dishes(Falcon) coated with poly-L-lysine. They were cultured in Dulbecco's modified Eagle's minimum essential medium supplemented with 5% fetal calf serum (Microbiol. Assoc., Md.) and 10% horse serum (Biken, Osaka) at 37°C under a water-saturated atmosphere of 5% $CO_2$ in air. Two days later, they were washed four times with Buffer A [50 mM Hepes-Na buffer, pH 7.4, containing 130 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM $NaH_2PO_4$, 5.5 mM glucose, 1.2 mM ascorbic acid and 150 mM sucrose (Buffer B) supplemented with 2 mM EGTA ] and treated with enterotoxin (0-3000 ng/ml) in 1 ml of Buffer B supplemented with various concentrations of $CaCl_2$ (0-3 mM)(Buffer C). For examination of the effect of replacement of $Ca^{2+}$ by $Sr^{2+}$ or $Ba^{2+}$, $NaH_2PO_4$ was omitted and $MgCl_2$ was added in place of $MgSO_4$ in Buffer B (Buffer D). All the buffers were adjusted with sucrose to the same osmotic pressure (ca. 480 mOsm) as that of Buffer A. In studies on release of noradrenaline, buffers were prewarmed to 37°C. All the buffers, media and solutions were prepared with ultrapure water obtained in a Barnstead ROpure-NANOpure system (Barnstead, Boston, Mass.). For measurement of noradrenaline released into the medium, the reaction medium (total volume, 1 ml) was replaced by fresh medium (1 ml) every 2 min, and the medium removed was chilled, and centrifuged. The supernatants obtained were mixed with 10 µl of 200 mM ascorbic acid, 10 µl of 200 mM EDTA (pH 6.5) and 40 µl of 2.5 N $HClO_4$ (final pH, 1.5) and stored at -20°C. At the end of experiments, the cells were harvested with a rubber policeman and suspended in 1 ml of Buffer C supplemented with ascorbic acid, EDTA and $HClO_4$ as described above, sonicated for 10 sec in a Branson sonifier Model 200 in an ice-bath and stored at -20°C. Thawed supernatants and sonicated cells were centrifuged at 0-4°C and the resultant supernatants were diluted appropriately with Diluting Solution(0.18 g of ascorbic acid, 0.37 g of EDTA-$Na_2$ and 20 ml of 60% $HClO_4$ per liter, pH adjusted to 1.5 with NaOH) and their noradrenaline contents were measured by the automated trihydroxyindole method described by Yamatodani and Wada (10) in a fluorescence spectrophotometer, Model F3000 (Hitachi, Japan), using TSK precolumn CA and TSK gel Catechol pak (Toyo Soda Manufacturing, Tokyo) with L-noradrenaline bitartrate monohydrate as a standard. The amounts of noradrenaline released from the cells were expressed as percentages of the total noradrenaline contents of the cells. All chemicals were obtained from Wako Pure Chemicals, Osaka, unless otherwise mentioned.

## RESULTS AND DISCUSSION

1. Enterotoxin induced release of noradrenaline from PC12 cells. Fig. 1 shows that when the cells were incubated with toxin(500 ng/nl) in the presence of extracellular $Ca^{2+}$(2 mM), a sharp increase of noradrenaline in the extracellular medium was observed 2-4 min after adding the toxin. The amount of noradrenaline released into the medium between 2 and 4 min after toxin addition was 40.2% of the total noradrenaline content of the cells.

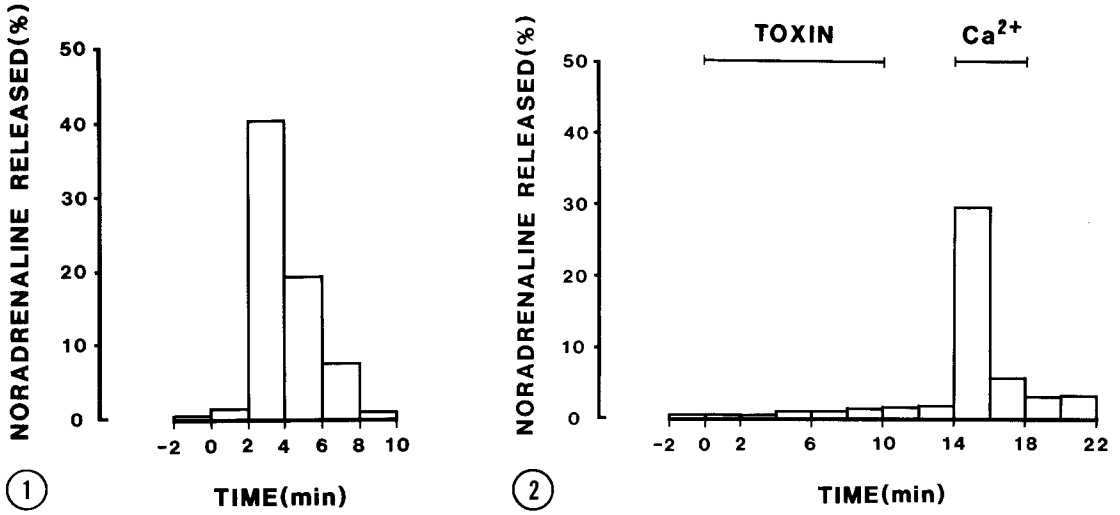


Fig. 1. Induction of release of noradrenaline from PC12 cells by C. perfringens type A enterotoxin. Washed cells (5 x $10^5$ cells per dish) were incubated at 37°C in 1 ml of Buffer C containing $Ca^{2+}$ (2 mM) from -2 min to time 0. Enterotoxin was added at time 0. The medium was replaced by fresh Buffer C (1 ml) containing $Ca^{2+}$(2 mM) and toxin (500 ng/ml) every 2 min. The total cellular noradrenaline content before release was 420.6 pmoles.

Fig. 2. Effect of $Ca^{2+}$ in the medium on the induction of release of noradrenaline from PC12 cells by C. perfringens type A enterotoxin. Washed cells (1 x $10^6$ cells per dish) were incubated at 37°C with 1 ml of Buffer C with or without $Ca^{2+}$(2 mM) and toxin (500 ng/ml) and the medium (1 ml) was changed every 2 min as indicated. The total cellular noradrenaline content before release was 810.2 pmoles.

2. Addition of $Ca^{2+}$ to the medium triggered release of noradrenaline from enterotoxin-treated PC12 cells. When the PC12 cells were incubated with toxin(500 ng/ml) for 10 min in the absence of extracellular $Ca^{2+}$, no significant release of noradrenaline was observed (Fig. 2). However, when these cells were then washed twice with medium containing no toxin and then incubated in medium containing $Ca^{2+}$(2 mM) but no toxin, noradrenaline was promptly released into in the medium (Fig. 2). This result indicates that induction of noradrenaline release by the enterotoxin did not require the continuous presence of both the toxin and $Ca^{2+}$ in the medium, and that addition of $Ca^{2+}$ to the medium triggered release of noradrenaline from cells that had been pre-treated in the absence of extracellular $Ca^{2+}$ with toxin.

3. Effect of extracellular $Ca^{2+}$ concentration on enterotoxin-induced release of noradrenaline. Fig. 3 shows the effect of the $Ca^{2+}$ concentration

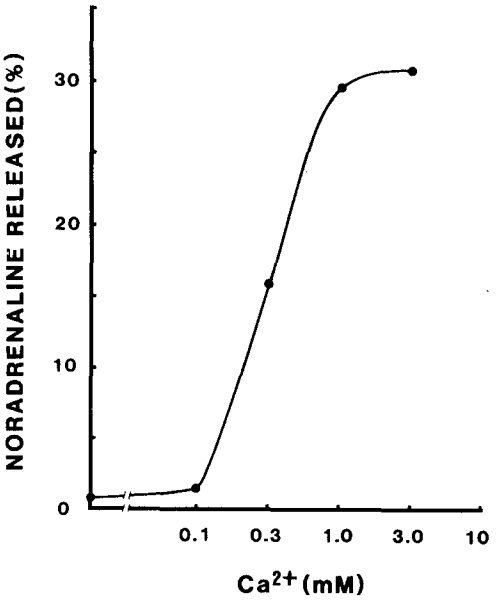


Fig. 3. Effect of the concentration of $Ca^{2+}$ in the medium on the amount of noradrenaline released from PC12 cells treated with C. perfringens type A enterotoxin. Washed cells (1.2 x $10^6$ per dish) were treated with enterotoxin (500 ng/ml) at 37°C for 2 min in Buffer C (1 ml) without $Ca^{2+}$ and then washed twice with buffer containing neither toxin nor $Ca^{2+}$, and incubated at 37°C for 2 min in fresh Buffer C (1 ml) containing various concentrations of $Ca^{2+}$ but no toxin. The total noradrenaline content of the cells (per dish) before release was 976.4 ± 54.4 pmoles.

in a $Ca^{2+}$ pulse for 2 min on the amount of noradrenaline released from PC12 cells treated with enterotoxin (500 ng/ml). The amount of noradrenaline released increased with increase in the extracellular $Ca^{2+}$ concentration (from 0.1 mM to 1.0 mM) to a plateau at a concentration of about 1-3 mM. The $ED_{50}$ was ca. 0.3 mM. This shows that the enterotoxin induced release of noradrenaline at a physiological concentration of $Ca^{2+}$ in the medium. $Ca^{2+}$ could be replaced by $Ba^{2+}$ or $Sr^{2+}$, and $Co^{2+}$ and $Mn^{2+}$, which are $Ca^{2+}$ channel blockers, did not inhibit the induction of $Ca^{2+}$-dependent release of noradrenaline from the toxin-treated PC12 cells (Table 1).

4. Effect of toxin concentration on $Ca^{2+}$-dependent release of noradrenaline from PC12 cells. Fig. 4 shows the amounts of noradrenaline released in response to a $Ca^{2+}$(2 mM) pulse for 2 min from PC12 cells treated in the absence of extracellular $Ca^{2+}$ with various concentrations of toxin. The amount of noradrenaline released increased with increase in the toxin

Table 1. Effects of divalent cations on release of noradrenaline from PC12 cells[a] by C. perfringens type A enterotoxin [e]

(A) Replacement of $Ca^{2+}$ by other divalent cations[b]

| Cation | Noradrenaline released[c] (%) |
|---|---|
| $Ca^{2+}$ 2 mM | 26.9 |
| $Ba^{2+}$ 2 mM | 43.0 |
| $Sr^{2+}$ 1 mM | 20.7 |

(B) Effects of divalent cations on release of noradrenaline in the presence of extracellular $Ca^{2+}$(2 mM)[d]

| Cation | Noradrenaline released[c] (%) |
|---|---|
| None | 23.8 |
| $Mn^{2+}$ 5 mM | 24.4 |
| $Co^{2+}$ 5 mM | 15.5 |

[a] The cells (1.4 x $10^6$ per dish) were treated with enterotoxin (500 ng/ml) in Buffer B for 2 min.

[b] The washed toxin-treated cells were incubated in Buffer D (1 ml) containing the indicated divalent cations for 2 min.

[c] The total noradrenaline content of the cells (per dish) before release was 1171±98.3 pmoles.

[d] The washed toxin-treated cells were incubated for 2 min in the presence of $Ca^{2+}$(2 mM) in Buffer C containing divalent cations as indicated.

[e] The cells were incubated at 37°C.

concentration to a plateau at a toxin concentration of 1000-3000 ng/ml. The $ED_{50}$ was ca. 420 ng/ml.

The data presented above show that enterotoxin of C. perfringens type A induces $Ca^{2+}$-dependent release of noradrenaline from PC12 cells. The release was rapid and extensive, reaching 1/3-2/3 of the total cellular content of noradrenaline. When the cells had been treated with enterotoxin, they were ready for prompt release of noradrenaline in response to influx of extracellular $Ca^{2+}$(Fig. 2). The extensive release of noradrenaline by the enterotoxin suggests prolonged flow of $Ca^{2+}$ into the cells. It is unlikely that increase in the amount of noradrenaline in the medium induced by the enterotoxin was due to mere leakage from the cells resulting from extensive damage of the cell membrane and/or subcellular membrane structures for the following reasons: (i) No morphological

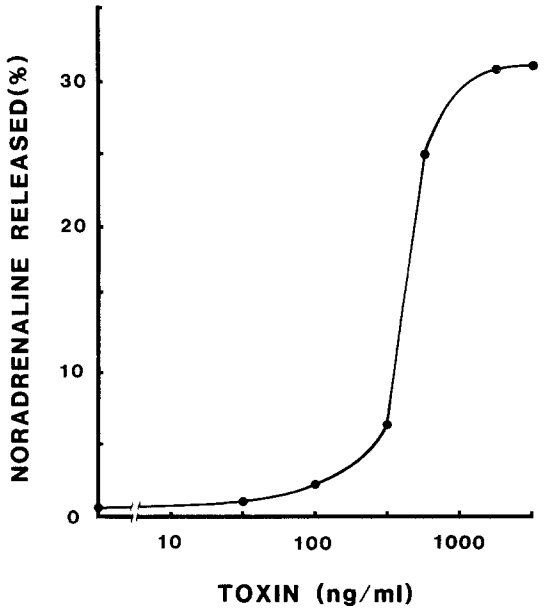


Fig. 4. Effect of toxin dose on release of noradrenaline from PC12 cells by a $Ca^{2+}$-pulse. Washed cells (1.5 x $10^6$ cells per dish) were treated at 37°C with various concentrations of toxin in Buffer C containing no $Ca^{2+}$, washed twice with Buffer C containing neither $Ca^{2+}$ nor toxin and then incubated at 37°C in Buffer C containing no toxin but $Ca^{2+}$ (2 mM) for 2 min. The total noradrenaline content of the cells before release was 1220 ± 110 pmoles.

alterations, bleb and balloon formation, which are observed in toxin-treated HeLa and Vero cells at the final stage of the intoxication (6,7,8), were observed by phase-contrast microscopy in PC12 cells after the release of noradrenaline by the enterotoxin under the experimental conditions (Fig. 5). (ii) The membrane of toxin-treated PC12 cells was not permeable to Trypan blue (mol wt 960.83) at the end of experiments on noradrenaline release. (iii) The enterotoxin did not appear to enter the cells and act within the cells (11). We are now examining whether in toxin-treated PC12 cells noradrenaline is released from secretory vesicles through exocytosis by morphological studies using electronmicroscopy and biochemical studies on the stimultaneous releases of intravesicular materials. In any case, the present finding that enterotoxin of C. perfringens type A induces rapid and massive release of noradrenaline from neurosecretory PC12 cells in vitro strongly suggests that the enterotoxin leads to dis-

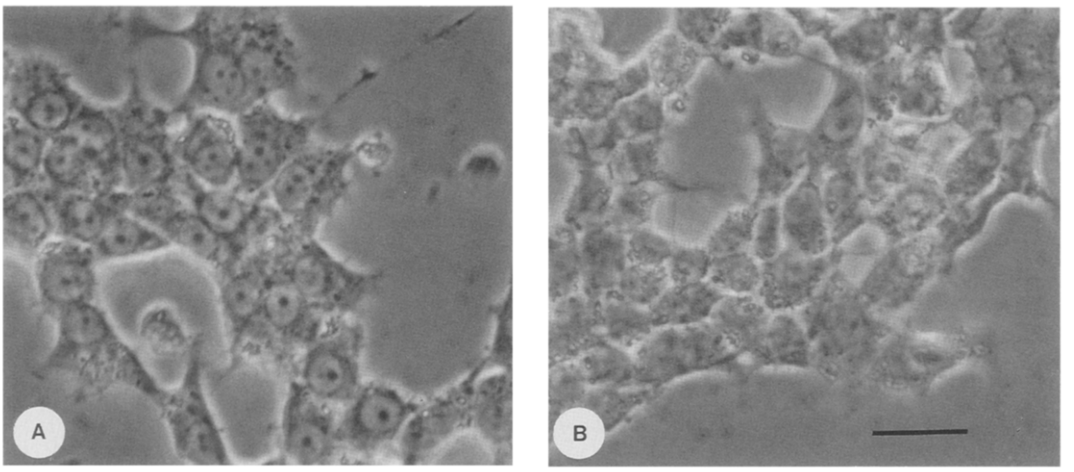


Fig. 5. Phase contrast micrographs of PC12 cells treated with toxin (500 ng/ml) before (A) and after (B) release of noradrenaline by a $Ca^{2+}$ (2 mM) pulse. The bar indicates 25 μm.

functions of the secretory systems and causes symptoms such as lacrimation, hyper-salivation and nasal discharge in enterotoxin-intoxicated animals. The extensive exhaustion of neurotransmitters by the enterotoxin may explain the mechanism underlying the acute systemic effects, including death, observed on intravenous injection of the enterotoxin into animals (4,5,12). Electrophysiological studies on this phenomenon are also in progress in our laboratory.

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