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ENTEROTOXIN OF Clostridium perfringens TYPE A FORMS ION-PERMEABLE CHANNELS IN A LIPID BILAYER MEMBRANE

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SUMMARY: The enterotoxin of Clostridium perfringens type A was found to form ion-permeable channels in a lipid bilayer. A patch clamp technique was used to detect channel activities in an asolectin bilayer with incorporated enterotoxin. About 20% of the lipid bilayer patches examined showed rectangular or stepwise shift of membrane current. The shifts indicated the gating of ion-permeable channels in the patches. The channels showed high conductance (40-450 pS), no rectification in current-voltage curves and occasional long-lasting events. The significance of these findings is discussed in relation to the mechanism of action of the toxin.

The enterotoxin produced by certain strains of Clostridium perfringens type A is a simple protein with a molecular weight of about 35,000 and is a causative agent of food poisoning by this organism. It is cytotoxic to certain cells in culture(1) and causes disturbances of amino acid transportations(2), morphological alterations(3) and leakage of intracellular macromolecules(4) of the cells. The mechanism of the cytotoxic action of the enterotoxin can be divided into two groups of steps on the basis of their requirement for calcium in the extracellular fluid, Ca-independent early steps and Ca-dependent late steps, as we reported previously(5) and as confirmed recently by others(6, 7). Intoxicated cells show influx of Na and efflux of K and Mg(8, 9). Recently, we demonstrated that these ionic fluxes occur in the Ca-independent early steps, and that addition of substances with molecular

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weights of more than about 200 to the extracellular fluid prevented induction of morphological alterations of HeLa and Vero cells by the enterotoxin(10). These results suggested that the enterotoxin formed 'holes' in the cell membrane through which substances with molecular (or atomic) weights of less than about 200, such as Na and K, passed across the cell membrane. However, the molecular mechanism of the formation of 'holes' in the cell membrane by the enterotoxin is unknown.

In this study, we used lipid bilayer membranes of asolectin to examine the action of the enterotoxin on the membrane in a simpler system than cellular membranes. We applied the patch clamp technique, which has been used for analyses of single channel activities, to examine the changes elicited by the enterotoxin. Here we report that the enterotoxin of Clostridium perfringens type A forms ion-permeable gating channels in lipid bilayers of asolectin and discuss the significance of this finding in relation to the molecular mechanism of the action of the enterotoxin.

MATERIALS AND METHODS: The enterotoxin of C. perfringens was produced and purified to homogeneity as described previously(11). The purified preparation of enterotoxin had a lethal activity of about 470 mouse minimum lethal dose per mg of protein and a cytotoxicity of about 320 ng/ml of the 50% effective dose to HeLa S_3 in the trypan blue dye exclusion test.

Asolectin (soybean lecithin; Daigo Eiyo, Osaka, Japan) was suspended at a concentration of 9 mg/ml in 4 mM Tris-Hepes buffer, pH 6.4, containing 130 mM NaCl and 4 mM $CaCl_2$ by sonication under continuous bubbling with N_2 gas. Then 200 µl of the suspension was mixed with an equal volume of a solution of 300 ng/ml of the enterotoxin in the same buffer and sonicated at 37℃ for 2 min. Test mixtures were made by adding 5 µl of the sonicate to 0.2 ml of the buffer. The formation of lipid monolayers on the aqueous surface of the test mixtures was confirmed by observing drops of water running over the surface.

Patch pipettes were made of hard glass capillaries of 1.5 mm diameter and filled with the buffer. The electrical resistance of the patch pipettes was 20-50 MΩ. A lipid bilayer was formed at the tip of the patch pipettes by the method of Suarez-Isla et al.(12) by dipping the tip into the test mixture twice. The formation of a lipid bilayer at the tip of the patch pipette was confirmed by demonstrating increase in the electrical resistance of the patch pipette. The voltage of the inside of the patch pipette against the bath electrode was clamped, and current passed through the lipid bilayer at the tip of the patch pipette was amplified with a patch clamp amplifier (CEZ-2200, Nihonkoden, Tokyo, Japan), monitored with a pen recorder (WR-3701, Graphtec, Tokyo, Japan) and recorded with an FM data recorder (MR-10, TEAC, Tokyo, Japan).

RESULTS AND DISCUSSION

1. Formation of ion-permeable channels in a lipid bilayer by the enterotoxin: In all, 102 lipid bilayer patches of asolectin membranes with incorporated enterotoxin were studied. Of these, 21 lipid bilayer patches showed definite channel activities in records of their membrane currents. Figure 1 shows an example of the current records. The level of the membrane current shifted abruptly rectangularly or stepwise. These abrupt shifts of the membrane current indicate gating of the ion-permeable channel(s) in the lipid bilayer patch. As seen in the figure, the channel activities showed at least 4 conductance levels of 125, 250, 380 and 490 pS, respectively. This multiplicity in conductance levels of channel activities can be explained by supposing that the patch of the lipid bilayer used in this experiment contained 4 ion-permeable channels with similar conductances of about 125 pS. The channels activities of 14 patches of lipid bilayers showed multiple conductance levels. In 11 of these 14 lipid bilayer patches, the values of conductance were integrals of the minimum value in each lipid bilayer patch, and the frequencies and durations of channel events with larger conductances were much less than those of events with smaller conductances. Therefore, we conclude that the multiple conductances of channel activities in these lipid bilayer patches were summations of the activities of several channels that had similar conductances. The minimum conductance value in each lipid bilayer

Figure 1. An example of the membrane current showing channel activities. A patch of lipid bilayer was formed at the tip of a patch pipette as described in Materials and Methods. The resistance of the lipid bilayer was 0.53 GΩ. The membrane potential was clamped at +11.5 mV. Calibration bars indicate 30 sec (abscissa) and 7.5 pA (ordinate).

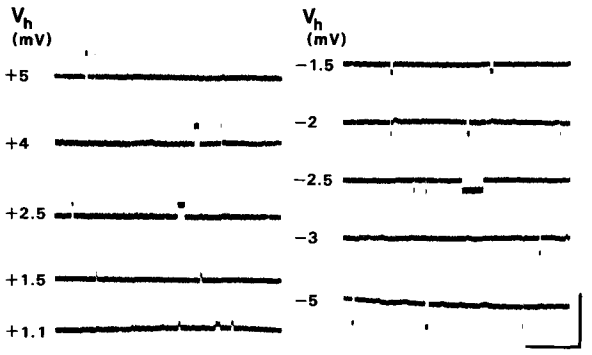

Figure 2. Records of membrane current obtained from a lipid bilayer patch at different holding potentials(Vh). The holding potential of each trace of the current record is shown on the left of the trace. Calibration bars indicate 30 sec (abscissa) and 5 pA (ordinate).

patch ranged from 40 to 450 pS. This variation of the values for minimum conductance may be due to minor differences in experimental conditions. But in all cases, the values for the conductance of ion-permeable channels formed by the enterotoxin are from about ten to several hundred times higher than the values of conductances reported for Na channels(13) and Ca channels(14, 15).

2. Characterization by single channel recording of the 'enterotoxin-channels' formed in the lipid bilayer: For further analysis of the channels formed in the lipid bilayer by the enterotoxin, we selected patches of lipid bilayer with single conductance levels. Figure 2 shows a series of current records obtained at various holding potentials from a patch of this type. The trace at a holding potential of -2.5 mV in Fig. 2 shows an event that lasted as long as 19 sec. Events with durations of at least a few seconds were frequently seen in this experiment. Records from other membrane patches also indicated that the 'enterotoxin-channels' sometimes opened for several seconds, though the numbers of events decreased exponentially with increase of the duration time. The results showed that the events of the 'enterotoxin-channels' sometimes have very long durations, though gating of the channels seemed to occur randomly. The current-voltage curve (Fig. 3) obtained from the same patch as that for Fig. 2 indicates that the conductances of the 'enterotoxin-channels' at different holding potentials give the same value of 415 pS. All the 'enterotoxin-channels' in this study gave linear current-voltage relationships and no rectification of the current was observed. These

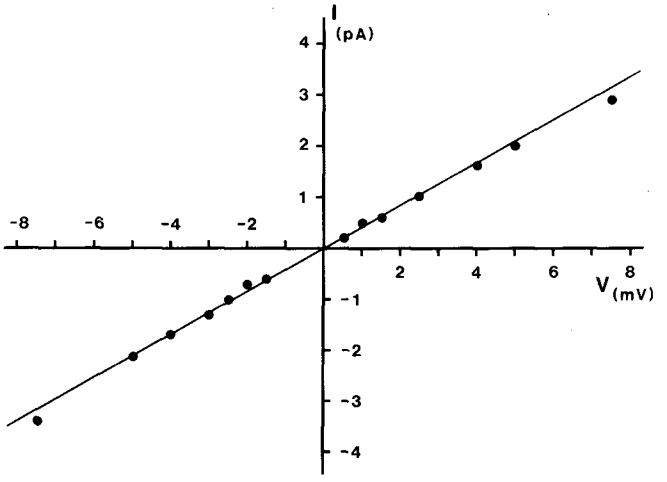

Figure 3. Current-voltage relationship of an 'enterotoxin-channel' obtained from the same lipid bilayer patch as for Fig. 2. Amplitudes of events are plotted against the holding potentials. The line was drawn by eye.

results indicated that the 'enterotoxin-channels' pass ions evenly in both directions, from outside to inside and from inside to outside.

The present results clearly showed that the enterotoxin of Clostridium perfringens type A formed characteristic ion-permeable channels in a lipid bilayer of asolectin. This finding provides a molecular basis for the mechanism of action of the enterotoxin. From our previous results in experiments on whole cells, we expected that the enterotoxin would form a static 'hole'. But on the contrary, the 'enterotoxin-channels' found in this study showed dynamic gating. Nevertheless, the characteristics of the 'enterotoxin-channels' are consistent with the results of previous studies. The large conductance of the 'enterotoxin-channels' may explain the fact that substances with molecular weights of less than ca. 200 appeared to pass through the 'holes' in the cell membrane of enterotoxin-treated cells. The bidirectional characteristic of the 'enterotoxin-channels' in the lipid membrane is consistent with bidirectional movement of cations in enterotoxin-treated cells: influx of Na and Ca, and efflux of K and Mg. The extraordinarilly long-lasting events in the channels of lipid membranes could explain the apparent 'hole' formation in intoxicated cells. Further characterization of the 'enterotoxin-channels' in lipid bilayers and studies

on the memberanes of intoxicated cells by the technique of single channel recording are now in progress to clarify the exact role of formation of ion-permeable channels by the enterotoxin in the mechanism of its cytotoxic action.

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