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MODE OF ACTION OF COLICIN Ib

FORMATION OF ION-PERMEABLE MEMBRANE CHANNELS

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Addition of purified colicin Ib to whole *Escherichia coli* cells or cytoplasmic membrane vesicles inhibits their subsequent ability to generate a membrane potential. In addition, this colicin is shown to bring about a voltage-dependent increase in the conductance of an artificial planar bilayer membrane prepared from soybean phospholipids. This results from the formation of ion-permeable channels. These data provide strong evidence that the depolarization of *Escherichia coli* cells by this colicin results from an Ib-induced increase in membrane permeability to ions.

Introduction

Colicin molecules exhibit three distinct functional specificities: mode of action, immunity, and adsorption to specific receptors on the bacterial outer membrane (for reviews, see Refs. 1 and 2). For example, colicins of different modes of action such as E1 (disrupts energy metabolism), E2 (degrades DNA) and E3 (inactivates ribosomes) adsorb to a common receptor. However, cells producing either E1 or E2 or E3 are immune (insensitive) to the colicin type produced, yet sensitive to the other two colicins. It is also possible for colicins of apparently common mode of action (colicins E1, K and Ia) to adsorb to different bacterial receptors. These same three colicins also exhibit immunity specificity; that is, producing organisms are specifically immune to the colicin produced.

Recently several studies have led to the conclusion that the functional specificities of colicins are reflected in distinct domains of colicin structure [3–11]. Thus, receptor and mode of action specificities have been assigned to N- and C-terminal regions, respectively, for colicins A, Ia, E1, E2, E3 and K [3,4,7–11] as well as for cloacin DF13 [5,6]. No information is available concerning the location of immunity recognition sites.

Colicins Ia and Ib are particularly well suited for studies on immunity. Strains harboring the Col Ia plasmid are immune to colicin Ia, yet sensitive to colicin Ib, and vice versa [12], even though Ia and Ib adsorb to the same bacterial receptor [13], share many physical properties [14], have common antigenic determinants [15], and exhibit extensive sequence homology in primary structure [16]. In addition, early studies suggested a common mode of action [17]. In this communication, we report a more detailed investigation of the mode of action of colicin Ib. We conclude that this colicin disrupts energy metabolism in sensitive cells by inducing the formation of aqueous channels in the bacterial cytoplasmic membrane and, thus, has a mode of action identical to that of colicin Ia.

Abbreviations: $TPMP^+$, triphenylmethylphosphonium cation; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Experimental procedures

Preparation of cells and membrane vesicles. The strains of *Escherichia coli* K-12 used were *trp*$^+$ transductants of JK114 and JK114 *cir* [18]. A mutation in *cir* results in loss of outer membrane receptor activity and, hence, resistance to colicins Ia and Ib. For convenience, these otherwise isogenic strains will be designated I^s and I^r, respectively. Cells were grown at 37°C with required growth supplements in a Tris-based minimal medium (described in Ref. 19, but without added $FeCl_3$) plus 0.2% glucose 6-phosphate to late exponential phase. When required, growing cells were treated with colicin (3 μg/ml) at 37°C for 10 min. Cells were next harvested and treated with 10 mM potassium EDTA as described in Ref. 20. EDTA-treated cells were then harvested, washed once with 50 mM potassium phosphate pH 6.6, and suspended in 1/10 original culture volume of the same buffer (usually 1.0–1.2 mg cell protein per ml).

Membrane vesicles were prepared from I^r cells, grown as described above, by the method of Kaback [21], except that spheroplasts were prepared by treatment with 20 μg per ml lysozyme in 30 mM Tris-HCl (pH 8.0)/30% sucrose. Vesicles were suspended in 100 mM potasium phosphate (pH 6.6) and stored in liquid nitrogen. When required, the pH of the buffer system was changed as described in Ref. 22. The receptorless strain was used in order to prevent specific binding of the I colicins to residual outer membrane in vesicle preparations. This specific binding interferes with colicin Ia activity on vesicle preparations [22].

Solute accumulation. Frozen vesicles were rapidly thawed at 46°C (followed by change of pH if necessary), diluted with one volume of distilled water (final potassium phosphate concentration, 50 mM), and $MgSO_4$ added to 10 mM. Vesicle aliquots (50 μl) were subjected to a freeze/thaw cycle with or without indicated amounts of colicin as previously described [22]. These vesicles or EDTA-treated cells (50 μl aliquots) were incubated at 25°C for 5 min under a stream of water-saturated O_2 prior to initiation of accumulation experiments. In all cases, energization was provided by potassium ascorbate (20 mM) plus phenazine methosulfate (0.1 mM) added just prior to labeled solute. At zero time, labeled solute was added and incubation continued at 25°C under O_2.

Accumulation of the permeant cation triphenylmethylphosphonium ($TPMP^+$) was used as a measure of the membrane electrical potential [23]. The final concentrations of [^{3}H]$TPMP^+$ were 0.4 mM (21.8 Ci/mol) for EDTA-treated cells and 0.1 mM (87.2 Ci/mol) for membrane vesicles. Transport of glucose 6-phosphate in membrane vesicles is driven by ΔpH at 5.5 and thus can be used as an indirect measure of ΔpH [24]. D-[1-^{14}C]Glucose 6-phosphate (8.5 Ci/mol) was added at zero time to a final concentration of 0.1 mM.

At appropriate times after addition of labeled solute, reactions were diluted with 2 ml 0.1 M LiCl and rapidly filtered through Millipore Celotate filters (0.5 μm) under vacuum. Filters were washed with an additional 2 ml LiCl solution, air dried, and counted in toluene plus 4 g/l 2,5-diphenyloxazole. Zero time values, determined by dilution and filtration immediately following addition of labeled solute, were subtracted.

Planar lipid bilayers. Planar phospholipid bilayer membranes (area ≡ 0.36 mm^2) separating symmetrical salt solutions (100 mM KCl, 5 mM dimethylglutaric acid, 5 mM $CaCl_2$, 0.1 mM EDTA, all adjusted to pH 6.1 with KOH) were formed at room temperature by the union of two monolayers [25] of crude soybean phospholipid (lecithin type II from Sigma) [26] from which neutral lipid had been removed [27]. Squalene was used to precoat the hole on which the membrane was eventually formed. Such bilayers were used for determining the response of colicin-treated membranes to voltage. For studies on current fluctuations resulting from the presence of the colicin, the membrane was formed over a small hole (area ≡ 0.01 mm^2) which was precoated with vaseline and separated identical salt solutions containing 3 M KCl, 5 mM dimethylglutaric acid, 5 mM $CaCl_2$, 0.1 mM EDTA all adjusted to pH 6.1.

Other methods and reagent sources. Cellular and vesicular protein was determined by the method of Lowry et al. [28] using bovine serum albumin as standard. Colicins Ia and Ib were purified as previously described [13]. Pure colicins E1 and E3 were kind gifts from W. Cramer, Purdue University, and D.R. Helinski, University of California, respectively. [^{3}H]TPMP bromide was generously provided by H.R. Kaback. [1-^{14}C]Glucose 6-phosphate (disodium salt) was obtained from New England Nuclear.

Results and Discussion

Fig. 1 shows the effect of colicins Ia and Ib on $TPMP^+$ accumulation ($\Delta\psi$ generation) in the I^S strain. Although equilibrium had not been established in this experiment, a minimum membrane electrical potential of −55 mV can be calculated for the untreated cells based on $TPMP^+$ accumulation at 10 min. This potential was calculated using a value of 5.4 μl water-permeable cytoplasmic volume per mg of cell protein [29] and from the Nernst equation, $\Delta\psi = -58.8$ mV ($\log[TPMP^+]_{in}/[TPMP^+]_{out}$), at 25°C. Treatment of sensitive cells with colicins Ia or Ib, or with 100 μM CCCP (data not shown) prevented the generation of a membrane potential. In a similar kind of experiment, treatment with colicin Ib prevented active transport of proline. Neither colicin Ia nor Ib perturbed the ability of I^r cells to generate $\Delta\psi$. Control experiments showed that in both colicin I sensitive and resistant strains, colicin E1 prevented the accumulation of $TPMP^+$, whereas treatment with colicin E3 (known to kill cells by virture of its ability to inactivate ribosomes; see Ref. 30) did not. In all of the experiments described above, treatment of sensitive cells with colicin resulted in greater than 99% killing.

The ability of membrane vesicles to generate an electrical potential after a freeze/thaw cycle, with or without added colicin, is shown in Fig. 2. Using a value of 2.2 μl intravesicular volume per mg protein [31] and the Nernst equation, electrical potentials of −49, −37 and −25 mV were calculated in this typical experiment for untreated, Ia-treated vesicles, and Ib-treated vesicles, respectively. Colicin Ib inhibited proline transport in vesicles to the same extent as it

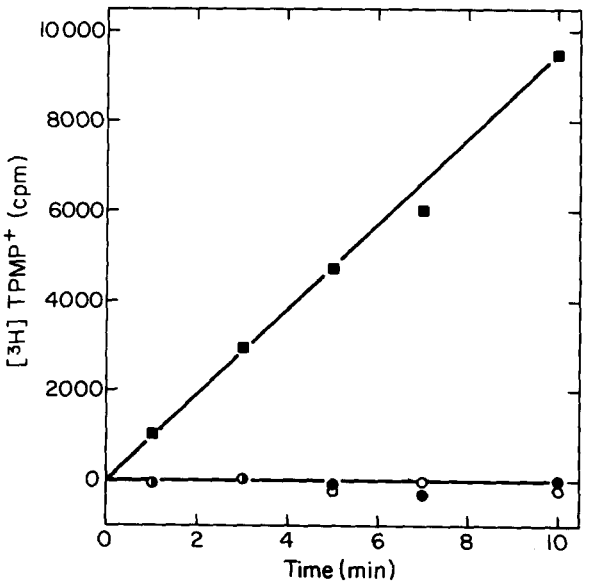

Fig. 1. Effect of colicins Ia and Ib on $TPMP^+$ accumulation in whole cells. $[^3H]TPMP^+$ accumulation was determined as described in Materials and Methods for EDTA-treated whole cells of strain I^S after no colicin pre-treatment (■), pre-treatment with 3 μg/ml colicin Ia (○), or with 3 μg/ml colicin Ib (●). $[^3H]TPMP^+$ accumulation in the presence of 100 μM CCCP (not shown) was essentially identical to the Ia and Ib levels. Cell protein concentration was 1.02 mg/ml.

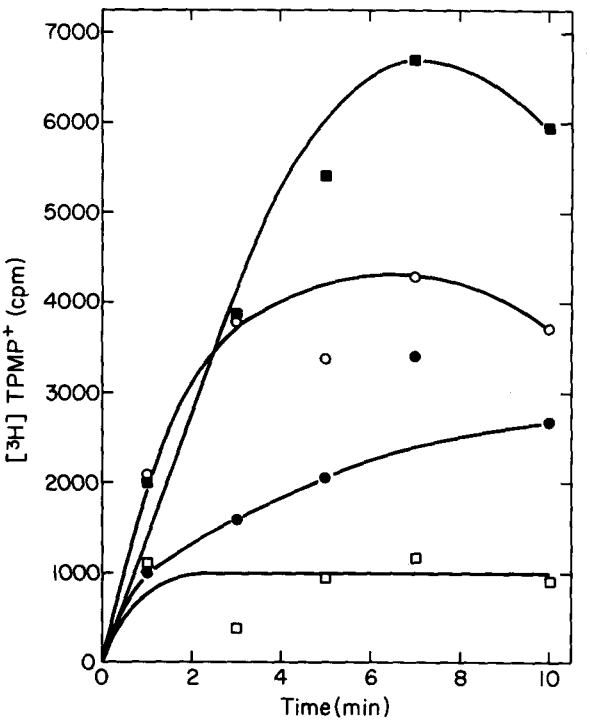

Fig. 2. Effect of colicins Ia and Ib on $TPMP^+$ accumulation in membrane vesicles. $[^3H]TPMP^+$ accumulation was determined exactly as described in Materials and Methods. $\Delta\psi$ was determined from the amount of $TPMP^+$ accumulated at 10 min for Ib-treated and at 7 min for Ia-treated and control vesicles. For calculations each of these values was corrected for the amount of $TPMP^+$ accumulated in the presence of 100 μM CCCP. This amount was assumed to reflect the amount of $TPMP^+$ accumulated at $\Delta\psi = 0$. Under these conditions CCCP completely inhibited proline uptake. Vesicular protein was 2.36 mg/ml; pH was 6.6. Colicins Ia and Ib were added at 1 μg per 50 μl vesicle suspension prior to the freeze/thaw cycle. (■) untreated; (○) Ia; (●) Ib; (□) CCCP.

inhibited $TPMP^+$ accumulation (data not shown). Thus, colicin Ib inhibits the formation of $\Delta\psi$ in membrane vesicles as does colicin Ia. An explanation for the apparent difference in activity between these colicin preparations is unclear at this time.

It has previously been shown that treatment of whole cells [20] or membrane vesicles [22] with colicin Ia resulted in a transient increased ability to generate a transmembrane proton gradient (ΔpH). In whole cells at pH 5.5, this was reflected in increased glucose 6-phosphate transport over non-treated cells. Fig. 3 shows that Ia treatment of membrane vesicles of pH 5.5 led to increased transport of glucose 6-phosphate. In the case of colicin Ib, a small but reproducible transient increase in glucose 6-phosphate was observed. Once again there appears to be qualitative differences between the activities of colicins Ia and Ib, but it is clear that neither initially inhibit glucose 6-phosphate transport and thus do not prevent the initial formation of ΔpH.

The effects of colicin Ib on the conductance of an artificial planar phospholipid bilayer was next examined. Fig. 4 shows the response of a membrane which separates symmetric salt solutions and has been treated with the colicin on one side. Initially the membrane voltage (V) is clamped to zero, and the resulting current (I) is zero. The voltage was next stepped to −70 mV, and after a rapid capacitance transient, the current (and therefore conductance $g \equiv I/V$) rises to a value of 320 pA, which corresponds to a conductance of 4600 pS. This represents about 700 colicin Ib channels in the membrane (see below). After an initial rapid rise, the conductance shows a slower, linear rise over long periods of time. This change may result from entry of channels into the bilayer membrane from the surrounding lipid monolayer (Kagan, B.L. and Finkelstein, A., unpublished observations). When the potential is reversed to +70

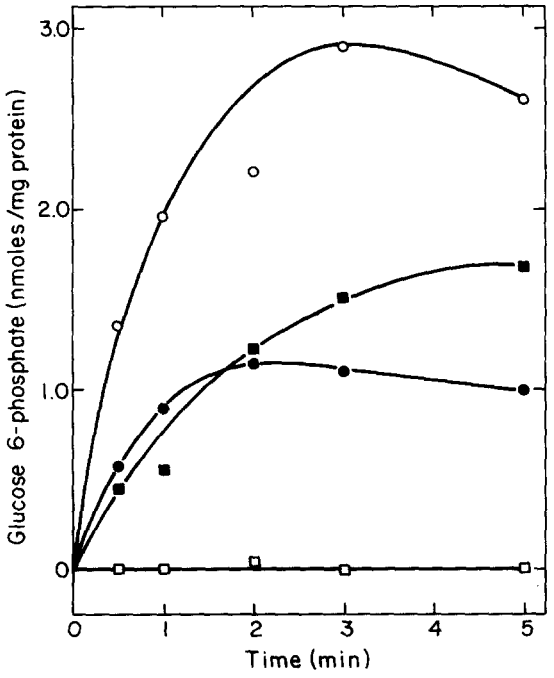

Fig. 3. Effect of colicins Ia and Ib glucose 6-phosphate transport in membrane vesicles. Transport of [1-^{14}C]glucose 6-phosphate in membrane vesicles was determined exactly as described in Materials and Methods. Vesicle protein concentration was 2.36 mg/ml. pH was 5.5. Colicins Ia and Ib were added at 1 μg per 50 μl vesicle suspension prior to the freeze/thaw cycle. (■) untreated; (○) Ia; (●) Ib. No detectable transport occurred in the presence of 100 μM CCCP (□).

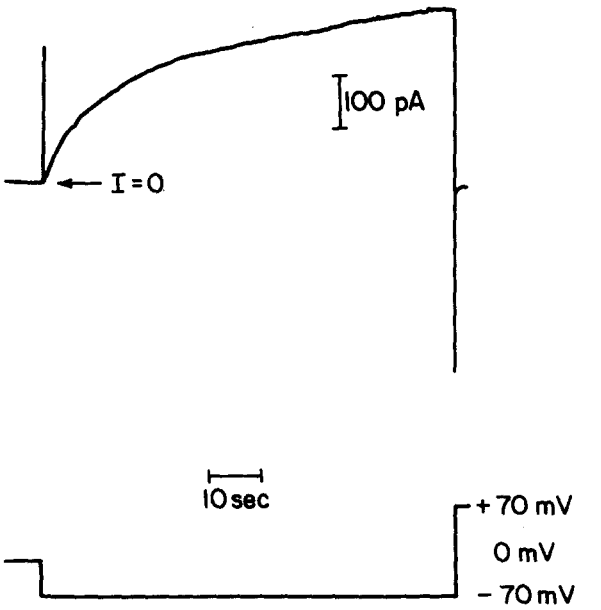

Fig. 4. Current response of colicin Ib-treated membrane to voltage. After membrane formation (see Materials and Methods) colicin Ib was added to the front (or *cis*) compartment to a final concentration of 200 ng/ml. The record was obtained 15 min later. The *cis* compartment was connected to virtual ground; V is the potential of the rear (or *trans*) compartment, analogous to the inside of the target bacterium.

mV, the instantaneous current is of equal magnitude (but of opposite sign) as that at −70 mV, and decays rapidly ($\tau < 1$ s) to a very low value corresponding to a conductance of about 100 pS. In the absence of colicin, the conductance of the membrane is about 50 pS.

In order to determine whether colicin Ib exhibited single channel behavior in such bilayers, a small amount of colicin was added, the voltage held at −30 mV, and the resulting current (conductance) fluctuations monitored. As can be seen (Fig. 5), 'jumps' are observed due to the opening and closing of discrete channels. The conductance of an open channel is about 41 pS (in 3 M KCl) and is ohmic in the range −70 mV to +70 mV. Note that the two channels present in the membrane are not always open at −30 mV which is close to V_0, the switching voltage (see Ref. 32 for details). At −70 mV, they are open all the time, resulting in a measured conductance of 83 pS (data not shown). The size of the conductance fluctuations is generally uniform (mean = 41.4 ± 5.0 pS). Two exceptions are marked with arrows. These events occur just before and after about one-third of the channel openings and closings and may represent another conductance state of the channel.

It is interesting to note that the single channel conductance of colicin Ib in 100 mM KCl (6.5 pS) is indistinguishable from the single channel conductance of colicin Ia in 100 mM KCl (Ref. 32 and unpublished observations). The fact that the single channel behavior of other colicins (e.g. K, E1) can be readily distinguished from one another as well as from Ia, suggests that channels formed by colicins Ia and Ib may be similar if not identical. This suggestion is further supported by the observation that colicins Ia and Ib show similar kinetics and voltage dependence (Kagan, B.L. and Finkelstein, A., unpublished results).

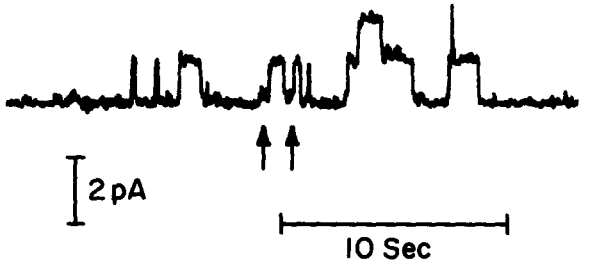

Fig. 5. Current fluctuations due to the presence of two colicin Ib channels in the membrane. Voltage is clamped −30 mV.

We have shown that colicin Ib abolishes the capacity of whole cells and derived cytoplasmic membrane vesicles to generate a membrane potential. This colicin was also shown to form channels in an artificial planar bilayer membrane. These results strongly suggest that killing of sensitive cells by this colicin derives from the formation of aqueous-ion permeable channels in the bacterial inner membrane leading to membrane depolarization and loss of intracellular ions. It is important to point out that such channels can be permeable to protons without abolishing ΔpH [32,33]. Thus, colicins Ia and Ib share two (receptor and mode of action) of the three functional specificities found in colicin molecules and differ only in their patterns of immunity. It therefore seems likely that detailed structural comparisons of these two molecules will yield information pertinent to the mechanism whereby they interact with the immunity systems of Ia- and Ib-colicinogenic cells.

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