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Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes

(voltage-dependent conductance/protein transport across membranes/lysosomes/endocytosis)

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ABSTRACT **The cytotoxic effect of diphtheria toxin requires the entry of its enzymatic A fragment ($M_r \approx 21,000$) into the cytosol of sensitive cells. We show that the B_{45} fragment ($M_r \approx 24,000$) forms, in lipid bilayers, pores that are large enough (diameter $\geqq 18$ Å) to allow the passage of extended fragment A. Pore formation is maximal when the B_{45}-containing side is at low pH (4.7) and the opposite side is at high pH (7.4). These conditions resemble the pH gradient existing across lysosomal membranes. We suggest that fragment A passes through these pores from acidic endocytotic vesicles (lysosomes?) to the cytosol.**

Diphtheria toxin, a protein produced by *Corynebacterium diphtheriae* lysogenic for bacteriophage β carrying the *tox*$^+$ gene, consists of a single M_r 62,000 polypeptide chain. (See ref. 1 for a review of all aspects of diphtheria toxin.) This chain can be split by site-specific proteolytic cleavage and by thiol reduction into an A fragment ($M_r \approx 21,000$) and a B fragment ($M_r \approx 40,000$). The toxin inhibits protein synthesis in sensitive cells by enzymatically catalyzing ADP ribosylation of elongation factor 2, and this enzymatic activity resides entirely with the A fragment. However, fragment A alone is nontoxic to cells, although it is fully active in cell-free extracts; the B fragment, which binds to cell-surface receptors on sensitive cells, is required for the entry of A fragment into the cytosol.

The mechanism of A fragment entry into the cytosol is of considerable interest because of its relevance to the general problem of receptor-mediated transport of toxins, hormones, and other proteins (2). Two classes of mechanisms have been proposed: (*i*) receptor-mediated endocytosis (3) and (*ii*) channel formation (or opening) in the plasma membrane (4). [Mechanism *i* still requires some means for fragment A (or the entire toxin molecule) to exit from endocytotic vesicles.] The report (4) that CRM45, a crossreacting mutant protein of diphtheria toxin, can enter detergent micelles suggested to us that this molecule also might interact with lipid bilayers and that this interaction could be relevant to the mechanism of transport of fragment A.

CRM45 consists of a normal A fragment plus a B fragment lacking a terminal M_r 17,000, hydrophilic amino acid sequence with receptor recognition properties (5) (Fig. 1); both CRM45 (M_r 45,000) and its B_{45} portion ($M_r \approx 24,000$) are water soluble. In this paper we describe the permeability increase induced in planar lipid bilayer membranes and in multilamellar lipid bilayer vesicles (liposomes) by CRM45 and B_{45}. The permeability increase results from the formation by B_{45} (and the B_{45} portion of CRM45) of ion-permeable channels large enough to permit the passage of the octasaccharide, γ-cyclodextrin, and the polyethyleneglycol, PEG 1500. We believe these channels are relevant to the mode of action of diphtheria toxin and to the mechanism of transport of fragment A across membranes. A preliminary report of our work appeared earlier (6).

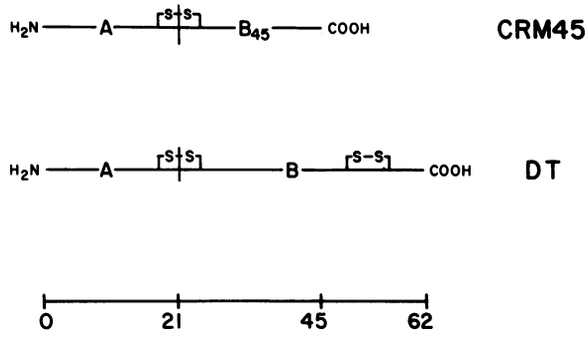

FIG. 1. Diagram of diphtheria toxin (DT) and CRM45. (The disulfide bonds are not drawn to scale.) M_r markers are shown $\times 10^{-3}$.

MATERIALS AND METHODS

Planar Membrane Experiments. Planar lipid bilayer membranes separating two salt solutions were formed at room temperature by the union of two monolayers (7) of crude soybean phospholipid [lecithin type II (Sigma) from which neutral lipid was removed (8)] across a hole (0.1- to 0.5-mm diameter) in a SaranWrap or Teflon partition precoated with Vaseline (9) or squalene (10). After addition of CRM45 or B_{45} to the *cis* compartment to 5–5000 ng ml^{-1} (0.1–100 nM), known voltages (V) were applied across the membrane, and the currents (I) which resulted were measured (9). The conductance (g) of the membrane is defined as I/V; in the absence of CRM45 or B_{45}, $g \approx 10$ pS.

Liposome Experiments. The lipid composition by weight of the multilamellar vesicles (liposomes) in all experiments was 92:8 egg phosphatidylcholine (Sigma)/bovine phosphatidic acid (Avanti Biochemicals). Preparations containing B_{45} consisted of approximately 9 μg of B_{45} per mg of lipid. With the assumption of M_rs for lipid and B_{45} of 800 and 24,000, respectively, this corresponds to about one B_{45} molecule per 3300 lipid molecules.

Intermixing of lipid and B_{45} was accomplished as follows. The phosphatidylcholine/phosphatidic acid lipid mixture ($\approx$11 mg), dissolved in chloroform/methanol, was flash evaporated to dryness in a round-bottom flask and then suspended in 2.0 ml of distilled water. To this, 50 μl of B_{45} stock solution ($\approx$2 mg ml^{-1}

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Abbreviations: PEG, polyethylene glycol; Mes, 4-morpholineethanesulfonic acid.

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in 50 mM sodium phosphate, pH 7.2) was added, and the mixture was sonicated under N_2 for about 30 sec to clarity in a cup-horn attachment to a Branson sonifier. It then was divided into two samples of 1 ml each, lyophilized, and stored dessicated at −20°C until needed. Liposomes were prepared simply by taking up a lyophilized sample in 1 ml of 20 mM KCl/1 mM EDTA, pH 7.2, and mixing until the material was completely suspended in solution.

Aliquots of this liposome preparation were suspended at 0.5 mg ml^{-1} in 20 mM KCl/1 mM EDTA/5 mM 4-morpholineethanesulfonic acid (Mes), pH 5.5, and placed in a cuvette; the final pH of the suspension was 6.1. (Note that the liposomes underwent a pH change from 7.2.) After a stable OD (turbidity) was obtained, the osmolarity of the medium was increased 5–10 mM by the addition of a small volume of concentrated nonelectrolyte solution in 20 mM KCl, and the change in OD was recorded over time. The permeability of B_{45}-containing liposomes to nonelectrolytes was determined from the volume changes (as measured by changes in OD) after these osmotic shocks (11). If a nonelectrolyte is impermeant, the liposomes merely shrink (OD increases); if a nonelectrolyte is permeant, initial shrinkage is followed by reswelling, as nonelectrolyte enters the liposome. The more permeant the solute, the less is the degree of initial shrinkage, the sooner is the point of minimum volume reached, and the faster is the rate of reswelling. OD measurements were made at 400 nm with a Hitachi Perkin–Elmer UV spectrophotometer; the output of the spectrophotometer was displayed on a strip-chart recorder.

Stock solutions of B_{45} (≈2 mg ml^{-1}) in 50 mM sodium phosphate (pH 7.2) were stored at −20°C. CRM45, obtained as a suspension in 75% saturated $(NH_4)_2SO_4$, was centrifuged and the pellet was dissolved in 100 mM KCl/5 mM $CaCl_2$/1 μM EDTA/5 mM Tris, pH 7.5, and then stored at −20°C. B_{45} and CRM45 stored in this way were indefinitely stable (>1 yr); repeated freezing and thawing at these concentrations had no deleterious effects on their bilayer activity.

RESULTS

Planar Membrane Experiments. *CRM45 and B_{45} increase membrane conductance.* When a step of positive voltage (i.e., *cis* side positive) was applied across a membrane with CRM45 or B_{45} on the *cis* side, the conductance rose throughout the duration of the voltage step, never reaching a steady-state value (Fig. 2*a*). The rate of rise increased *e*-fold for about every 8 mV up to 50 mV (not shown) and increased with approximately the square of the protein concentration at a given voltage (Fig. 2*b*). After the voltage was returned to zero, the conductance remained at the value attained prior to termination of the step. At large negative voltages (≤ −80 mV), the conductance almost completely turned off (Fig. 2*a*). CRM45- and B_{45}-treated membranes were cation selective, giving potentials of about 47 mV per 10-fold difference of KCl or NaCl activity.

At very low concentrations of CRM45 and B_{45}, or during the early stages of conductance turn-on and the late stages of conductance turn-off, single-channel activity was observed (Fig. 3). In 100 mM KCl, channel conductances were approximately 10 pS. The merging of single-channel activity with macroscopic conductance states indicates that the latter result from summation of large numbers of channels. We observed no dramatic voltage dependence of channel lifetime at positive voltages; the effect of positive voltages was primarily on the step(s) involving channel turn-on.

Essentially identical results were obtained with CRM45 and B_{45}. In contrast, fragment A was without effect on membrane conductance, even at a concentration of 1 μg/ml. Thus, the channels formed by CRM45 are entirely attributable to the B_{45} fragment of the molecule.

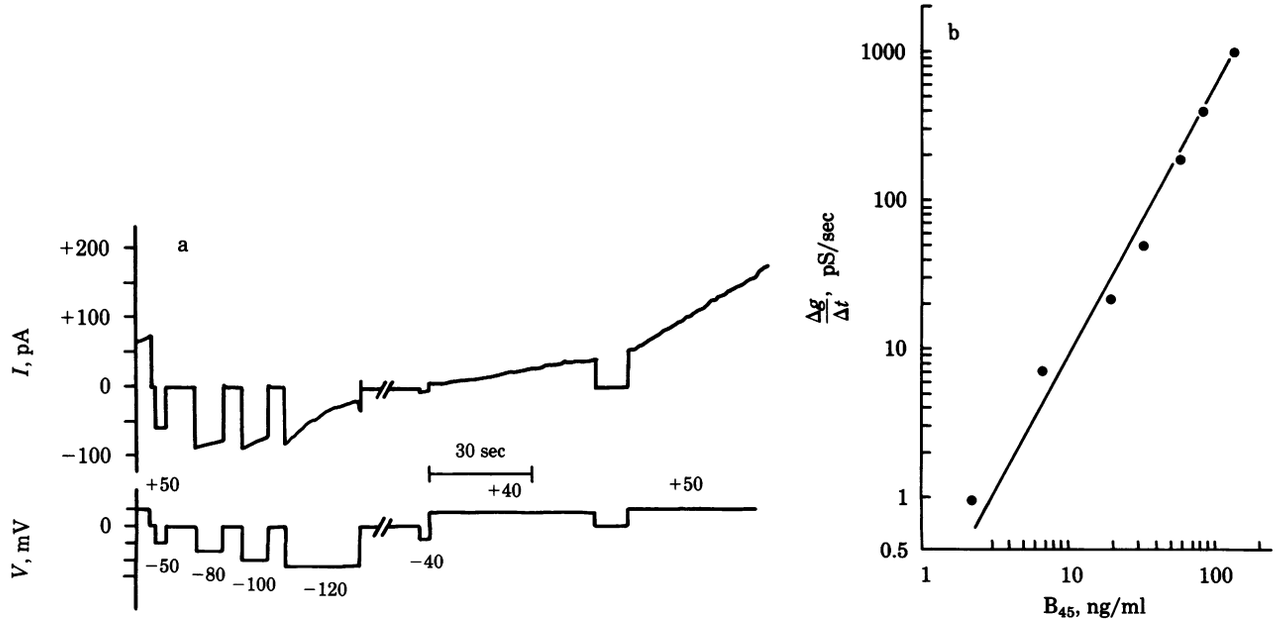

FIG. 2. (*a*) Current responses of B_{45}-treated membrane to voltage steps. After formation of the membrane (in 100 mM KCl/2 mM $MgCl_2$/0.1 mM EDTA/5 mM Mes, all adjusted to pH 5.5), B_{45} was added to the *cis* compartment to a concentration of 44 ng ml^{-1}. The *trans* compartment was connected to virtual ground; *V* is the potential of the *cis* compartment. The record shown was taken several minutes after the B_{45} addition. Note the upward concavity of the current responses (which after about 5 sec become linear) to positive voltages and the faster rate of current rise (i.e., conductance rise) at +50 mV than at +40 mV. Also note that there is a progressively faster rate of current decrease (i.e., conductance decrease) as the stimulating voltage is made more negative. Similar records are obtained with CRM45. (*b*) Double logarithmic plot of the constant rate of conductance rise at a given voltage against B_{45} concentration. The data were obtained on a single membrane. After each addition of B_{45}, the compartments were stirred for 1 min, and 5 min later the constant rate of conductance rise at +50 mV was recorded. The slope of the drawn line is 1.8. Composition of the aqueous solutions is the same as for Fig. 2*a*.

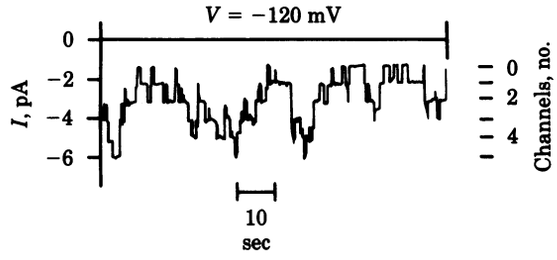

FIG. 3. Current (conductance) fluctuations due to the lingering presence of five CRM45 channels in the membrane at an applied voltage of −120 mV. The membrane separates identical solutions consisting of 100 mM NaCl/2 mM $MgCl_2$/0.1 mM EDTA/5 mM Mes, pH 5.5; CRM45 concentration in the *cis* compartment = 12 ng ml^{-1}.

Effect of pH on cis *and* trans *sides.* All of the results described above were obtained at pH 5.5. At symmetrical pH 7.1 and above, both CRM45 and B_{45} were ineffective in producing channels, but if the pH was subsequently lowered symmetrically to 5.5, full activity appeared. When the *trans* pH was raised to 6.2, the rate of conductance rise at a given positive voltage increased about 10-fold (Fig. 4); on the other hand, when the *cis* pH was raised to 6.2, the conductance declined (Fig. 4). When the *cis* pH was lowered from 5.5 to 4.7, the rate of conductance rise increased about 3-fold. With pH 4.7 on the *cis* side and 7.4 on the *trans* side, the conductance rose even in the absence of an applied positive voltage (and even at small negative voltages). In addition to high pH on the *cis* side inhibiting activity, we also found that there was little if any activity in the absence of EDTA (100 μM). Because the EDTA requirement for activity occurred even in the presence of millimolar amounts of Mg^{2+}, we attribute the inhibition in its absence to trace amounts of multivalent cation(s).

Regardless of whether the channels were turned on or turned off, CRM45 was irreversibly associated with the membrane at pH 5.5. Perfusion of the *cis* compartment with CRM45-free solution under all voltage conditions at this pH failed to wash out channel activity from the membrane. CRM45 clearly spanned the membrane, as shown by complete destruction of channel activity after addition of Pronase to the *trans* compartment.

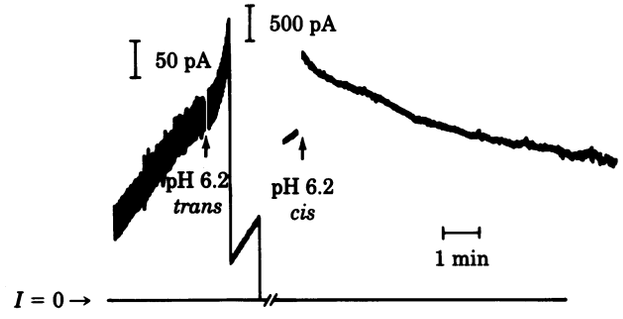

FIG. 4. Effects of raising *trans* and *cis* pHs on the rate of conductance rise (at a given voltage) of a B_{45}-treated membrane. Initially the membrane separated identical solutions consisting of 100 mM KCl/2 mM $MgCl_2$/1 mM EDTA/5 mM Mes, pH 5.5. B_{45} was added to the *cis* compartment to a final concentration of 10 ng ml^{-1}. The record begins about 11 min later and shows the current (conductance) rising linearly in response to a maintained +50 mV stimulus. At the first arrow, the pH of the *trans* compartment was raised to 6.2 (by addition of Tris to a concentration of 20 mM). The rate of the current rise immediately begins to increase, and we see that after about 30 sec it attains a constant rate, which is about 10-fold greater than at pH 5.5 (note the change in current scale). Various voltage maneuvers (not shown) intervened during the next 17 min, after which we see that the current still rises rapidly (although at a somewhat smaller rate) in response to a +50mV stimulus. At the second arrow, the pH of the *cis* compartment was raised to 6.2 (by addition of Tris to a concentration of 20 mM) and, despite the maintained +50 mV stimulus, the current (conductance) continuously decreases. [The initial jump in current when the *cis* pH was raised to 6.2 results from an increase (≈50%) in channel conductance.] The *cis* and *trans* solutions were stirred continuously during the experiment.

Membrane composition requirements. Most of our experiments were with soybean phospholipid (asolectin) membranes formed by the union of two monolayers; such membranes contain negatively charged phospholipids (12). In contrast, we were unable to obtain B_{45} activity on membranes formed from lipids having no net charge; these included bacterial phosphatidylethanolamine membranes, glycerylmonooleate/phosphatidylethanolamine membranes, 1:1 (wt/wt), and diphytanoylphosphatidylcholine membranes. B_{45} was also inactive when added to asolectin membranes formed from decane solutions (13). If, however, such membranes were formed with B_{45} already present in the aqueous solution, good activity was obtained.

Liposome Experiments (Channel Size). Our primary interest in the B_{45} channel was to determine if it is large enough to allow the A fragment to pass through it. However, we could not obtain B_{45}-treated planar membranes of sufficiently high conductance (i.e., with sufficiently large numbers of channels) and stability for nonelectrolyte sieving experiments (e.g., ref. 14). Consequently, we employed liposomes to size the B_{45} channel.

In the channel-sizing experiments (Fig. 5), the B_{45} channel showed a graded permeability, P, to nonelectrolytes in the order: P(glucose) > P(sucrose) > P(raffinose) > P(stachyose) > P(γ-cyclodextrin) ≈ P(PEG 1500) > P(carbohydrate moiety of fetuin) = P(inulin) = P(PEG 6800) = 0. Thus, molecules the size of γ-cyclodextrin ($M_r \approx 1300$) and PEG 1500 can pass through the channel, whereas somewhat bulkier molecules, such as the carbohydrate moiety of fetuin ($M_r \approx 2000$), are impermeant.

As with the planar membranes, B_{45} does not appear to form channels in liposome bilayers at high pH. Thus, at pH 7.5, B_{45}-treated liposomes were impermeable to glucose, but if the pH

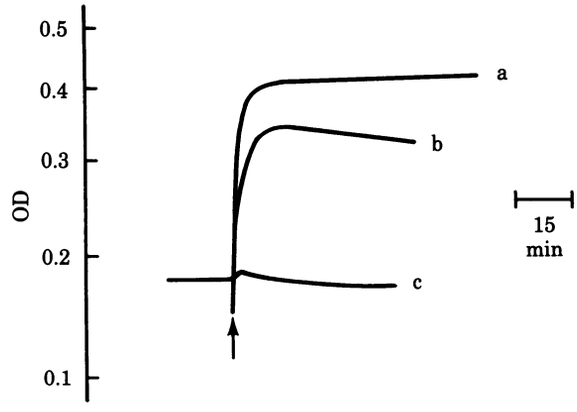

FIG. 5. Response of B_{45}-containing liposomes to osmotic shocks (5 mM) with nonelectrolytes. Liposomes were suspended at a concentration of 0.5 mg ml^{-1} in 20 mM KCl/1 mM EDTA/5mM Mes; the pH of the suspension was 6.1. After a stable OD (turbidity) was obtained, the osmolarity of the medium was increased 5 mM by the addition (at the arrow) of a small volume of a concentrated solution of glucose (c), stachyose (b), or PEG 6800 (a) in 20 mM KCl, and the change in OD was recorded over time. The records are superimposed in the figure. We see (*i*) a very small, early peak in OD and a rapid return to base line with glucose; (*ii*) a much larger, later peak in OD and a much slower decline with stachyose; and (*iii*) a continuous rise in OD with PEG 6800. From records such as these, the permeability order given in the text for nonelectrolytes was obtained. (Control liposomes not containing B_{45} gave responses similar to that seen with PEG 6800 for all nonelectrolytes tested.)

was subsequently lowered to 6.2, they displayed the permeability characteristics listed in the previous paragraph and shown in Fig. 5. The reported absence of nonelectrolyte leakage from CRM45-treated liposomes (15) probably resulted from those experiments being done at pH 7.5.

DISCUSSION

Voltage Dependence of B_{45} Channels. We have demonstrated that the B_{45} fragment of CRM45 forms channels in lipid bilayers and that these channels are large enough to allow passage of γ-cyclodextrin and PEG 1500. These conclusions result from complementary studies of the permeability effects of B_{45} and CRM45 on planar lipid bilayer membranes and multilamellar lipid vesicles (liposomes). In both systems, low pH (<6.3) is a requirement for channel activity. In the planar bilayers there is, under symmetrical conditions at pH 5.5, also a voltage requirement (*cis* side positive) for activity. Because channel incorporation occurred in the liposomes with a pH gradient present across their bilayers, we cannot say if the voltage requirement (under symmetrical pH conditions) is also present there. However, we have found that B_{45}-induced K^+ release from single-walled asolectin vesicles (diameter ≈ 1000 Å) does *not* require a transmembrane voltage, although it still requires a low pH (unpublished results). This difference between vesicular and planar bilayers is perplexing. The voltage dependence of colicins K, E_1, and Ia in planar membranes (16) apparently also is absent in vesicle membranes (17, 18). "Hydrocarbon-free" planar bilayers (7) are possibly thicker than vesicle bilayers, and conceivably this is responsible for the voltage requirement in the former; the inactivity of B_{45} (and the poor activity of the colicins) when added to the even thicker decane-containing membranes is consistent with this explanation.

Molecularity of B_{45} Channel. There are two indications that the B_{45} channel is *not* formed by a single B_{45} molecule. One is the shape of the current vs. time records when a positive voltage is applied (Fig. 2*a*); the initial upward concavity suggests a cooperative interaction among subunits. The other is the dependence of the final constant rate of conductance rise (achieved at a given voltage) on approximately the *square* of the B_{45} concentration (Fig. 2*b*), which suggests that two molecules come together to form a channel.

Mechanism of Fragment A Entry into Cytosol. More data are needed to further characterize channel formation and properties, topics of some interest in their own right. However, our primary focus at this juncture is on the possible relevance of these channels to the mechanism by which fragment A of diphtheria toxin enters the cell cytosol. Our principal finding in this regard is that the B_{45} channel is large enough to allow γ-cyclodextrin and PEG 1500 to pass through it. This means that the channel diameter is ≥18 Å. We have determined with space-filling models that, in extended form, the "fattest" segment of the A fragment (amino acids 44 through 55, which contain one tryptophan, one phenylalanine, and two tyrosines) can fit through a cylinder of this diameter. Thus, the B_{45} channel is large enough to allow a fully extended A fragment to pass through; it remains to be demonstrated that the A fragment actually does so.

Assuming that the A fragment of diphtheria toxin enters the cytosol through a channel formed by B fragment(s), several biological scenarios can be envisioned. The binding of B fragment to cell receptor could lead to an enzymatic cleavage of part of the B chain, leaving something similar to CRM45 that could now insert into the plasma membrane and form the channel (4). A more appealing idea comes from the finding that agents which raise lysosomal pH (e.g., chloroquine, NH_4Cl) and inhibit lysosomal functions block the cytotoxic action of diphtheria toxin (19, 20). Leppla *et al.* (19) suggest that the delivery of active toxin to the cytosol involves lysosomal processing after adsorptive endocytosis of receptor-bound toxin. Proteolysis within the lysosome could then lead to formation of a CRM45-like molecule that would form a channel in the lysosomal membrane through which A fragment would pass into the cytosol. The stretch of arginines and lysines at about M_r 8000 from the COOH terminus of fragment B might be the region in which this proteolysis occurs (21). Alternatively, even without proteolysis, diphtheria toxin might form the channel. Donovan *et al.* (22) have reported that at low pHs diphtheria toxin creates in planar bilayer membranes channels resembling somewhat those described here for B_{45}. However, their conclusion that ". . .the low channel conductance implies that the diameter of the channel is quite small (≈ 5 Å), rendering this possibility [that fragment A goes through the channel] unlikely" is unwarranted. It is not possible to infer channel diameter from channel conductance, as witness the amphotericin B channel, with a conductance 1/40th that of the gramicidin A channel (23, 24) and a diameter twice that of the latter (14, 25). Furthermore, as we have shown, despite its relatively small conductance, the B_{45} channel is permeable to a molecule the size of γ-cyclodextrin.

Our finding that low pH is necessary for channel formation is particularly relevant to and consistent with entry of fragment A into the cytosol from acidic vesicles (lysosomes?); it is also consistent with the report that this fragment may cross the plasma membrane of cells incubated with diphtheria toxin at low pH (26). It is noteworthy that the conditions under which we get maximal rate of channel formation, low pH (4.7) in the *cis* compartment and high pH (7.4) in the *trans*, emulate conditions existing across lysosomal membranes. Of course, these same conditions may exist across the membranes of other intracellular vesicles in which toxin may find itself. The pH gradient across the membrane of an acidic vesicle may provide the driving force for translocation of fragment A into the cytosol. Thus, the refolding of fragment A at cytosolic pH (7.4) from an extended form at lysosomal-like pH (4.7) (27) could drive the translocation of the extended form through the channel. The ability of fragment A to regain complete enzymatic activity at neutral pH after exposures to low pH (28) is in keeping with this proposed mechanism of translocation.

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