Channels formed by botulinum, tetanus, and diphtheria toxins in planar lipid bilayers: Relevance to translocation of proteins across membranes

(voltage-dependent channels/pH-gating/channel-sizing)

DAVID H. HOCH*, MIRIAM ROMERO-MIRA*, BARBARA E. EHRLICH*, ALAN FINKELSTEIN*, BIBHUTI R. DASGUPTA†, and LANCE L. SIMPSON‡

*Departments of Physiology and Biophysics and of Neuroscience, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461; †Food Research Institute, University of Wisconsin, 1925 Willow Drive, Madison, WI 53706; and ‡Department of Pharmacology, College of Physicians and Surgeons, Columbia University, 630 West 168 Street, New York, NY 10032.

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ABSTRACT The heavy chains of both botulinum neurotoxin type B and tetanus toxin form channels in planar bilayer membranes. These channels have pH-dependent and voltage-dependent properties that are remarkably similar to those previously described for diphtheria toxin. Selectivity experiments with unionized and cations show that the channels formed by the heavy chains of all three toxins are large; thus, these channels could serve as "tunnel proteins" for translocation of active peptide fragments. These findings support the hypothesis that the active fragments of botulinum neurotoxin and tetanus toxin, like that of diphtheria toxin, are translocated across the membranes of acidic vesicles.

Diphtheria toxin (1), botulinum neurotoxin (2), and tetanus toxin (3) are proteins that are similar in origin and macrostructure. All three toxins are synthesized by bacteria (Corynebacterium diphtheriae, Clostridium botulinum, and Clostridium tetani) as single polypeptide chains (diphtheria toxin, ~60 kDa; clostridial neurotoxins, ~150 kDa). When exposed to trypsin or trypsin-like enzymes, they are cleaved to yield two-chain molecules in which a heavy-chain polypeptide is linked by a disulfide bond to a light-chain polypeptide. The two-chain structure is the active form of the three toxins.

Various techniques have been used to generate polypeptide fragments from the toxins. The most straightforward of these is disulfide-bond reduction, which releases the heavy chain from the light chain. Alternatively, the clostridial neurotoxins have been exposed to limited proteolysis (e.g., with papain) to generate a fragment B and fragment C. Finally, traditional techniques have been used to select mutant organisms that synthesize incomplete toxins, such as the CRM45 fragment of diphtheria toxin, from which the B45 fragment can be formed. The various toxins and their fragments are illustrated in Fig. 1.

Diphtheria toxin is the best characterized of the three toxins. The entire molecule has been sequenced (4, 5), and three separate domains mediating binding activity (6, 7), channel-forming activity (8), and enzymatic activity (9) have been characterized. The carbohydrate terminus of the heavy chain mediates binding to cell surface receptors, and the light chain is an enzyme that catalyzes ADP-ribosylation of elongation factor 2. The heavy chains of the clostridial neurotoxins also mediate binding (10–12), but the nature of the putative enzymatic activity of their light chains has not been established.

An important question that remains unanswered for all three toxins is the mechanism by which they reach the cytosol. There is evidence that diphtheria toxin is internalized by receptor-mediated endocytosis (13); there is also evidence that the endocytic vesicles become acidified and that the active fragment of diphtheria toxin requires this acidic environment for its translocation across the vesicle membrane (14). There are findings that suggest that botulinum and tetanus neurotoxins follow a similar pathway into the cytosol (15–17). These observations leave open the question of whether the active fragments from the three toxins use the same mechanism for crossing the vesicle membrane.

Previous studies have shown that the amino terminus of the heavy chain from diphtheria toxin (18) and whole diphtheria toxin (19) form channels in lipid bilayers, and it has been proposed (18) that these channels provide the pathway for the light chain to cross membranes. Here we report that the heavy chains of both botulinum neurotoxin type B and tetanus toxin also form channels in lipid bilayers. Furthermore, for all three toxins, channel formation is maximal when the protein-containing (cis) side of the artificial membrane is at low pH (~4.0) and the opposite (trans) side is at pH ~7.0, a pH gradient comparable to that across the membranes of acidic vesicles in cells. The channels for all three toxins are very large, as determined by selectivity experiments with large anions and cations, and this finding is compatible with the idea that the channels function as "tunnel proteins" for translocation of fully extended active fragments. In addition, tetanus toxin channels display a voltage dependence similar to that of diphtheria toxin channels, opening when positive voltages are applied to the cis side of artificial membranes and closing when negative voltages are applied. We discuss these findings in the context of current views on protein translocation across membranes.

MATERIALS AND METHODS

Planar lipid bilayer membranes separating two salt solutions were formed at room temperature from the union of two monolayers (20) across a hole (100 μm diameter; pretreated with squalene) in a Teflon partition. The composition of the salt solutions is described below and in the figure captions; the membranes were made of asolectin, a crude mixture of soybean phospholipids (lecithin type II; Sigma) from which neutral lipids were extracted (21). Various proteins were applied to the asolectin membranes, including diphtheria toxin (R. J. Collier, Department of Bacteriology, Univ. of California, Los Angeles), the B45 fragment of diphtheria toxin (A. M. Pappenheimer, Jr., Department of Biology, Harvard Univ.), tetanus toxin and its heavy and light chains (P. Boquet, Department of Microbiology, Pasteur Institute), and fragment B of tetanus toxin (O. Zwiesler and K. D. Hungerer, Behring Institute). Botulinum neurotoxin type B and its heavy and light chains were prepared by one of us (B.R.D.);
the details of the isolation will be reported elsewhere.

After addition of toxins or fragments to the cis compartment, known voltages were applied across the membrane and the resulting current responses were measured. The membrane conductance \( g \) in symmetric salt solutions is defined as current \( I \) divided by voltage \( g = I/V \), where \( V \) is the potential of the cis compartment. In the absence of toxins or fragments, \( g \) was \( \approx 5 \) picosiemens (pS).

**RESULTS**

**pH Gradient and Channel Formation.** The addition of fragments possessing the amino terminus of the heavy chains of clostridial neurotoxins (i.e., fragment B of tetanus toxin or heavy chains of botulinum and tetanus toxin) to one side of an asolectin membrane separating solutions at a symmetric pH of 7.0 resulted in modest single-channel activity (Figs. 2a and 3a). There was considerable dispersion in single-channel
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Conductances; in 1 M KCl, the predominant conductance levels were 45 pS for heavy chain of tetanus toxin and for fragment B and 15 pS for heavy chain from botulinum neurotoxin. Within seconds after lowering the pH of the cis compartment to 4.5, there was a tremendous increase in channel activity, resulting in a rapid rise in macroscopic membrane conductance (Figs. 2a and 3a).

At a symmetric pH of 4.0, there was more single-channel activity than at a symmetric pH of 7.0; this was especially true for fragments obtained from tetanus toxin (Figs. 2b and 3b). In 1 M KCl, the predominant single-channel conductances were 15 pS for heavy chain of tetanus toxin and for fragment B and 20 pS for heavy chain of botulinum neurotoxin. This channel activity, which led to a slow rise in macroscopic conductance, was dramatically enhanced when the pH of the trans compartment was raised to 6.5, leading to a several hundred-fold increase in the rate of conductance rise (Figs. 2b and 3b).

These results show that fragments containing the amino terminus of heavy chains from clostridial neurotoxins form channels in lipid bilayer membranes, confirming in part an earlier report (16). These fragments are more active on membranes separating solutions at low symmetric pH (4.0 to 5.0) than at neutral symmetric pH (7.0), and they are dramatically more active on membranes separating a pH gradient (cis, pH 4.0–5.0; trans, pH 6.0–7.0). The intact tetanus toxin molecule behaved similarly to its fragments, particularly with respect to formation of pH-dependent channels (Fig. 2). Under the conditions used in this study, the intact botulinum neurotoxin type B molecule did not form pH-dependent channels.

The light chains of botulinum and tetanus toxins, which are presumed to be the pharmacologically active fragments, were tested for channel-forming activity. Whether tested at symmetric low pH (4.0), symmetric neutral pH (7.0), or in the presence of a pH gradient (cis, pH 4.0; trans, pH 7.0), the light chains were devoid of channel-forming activity.

These findings indicate that the channel-forming properties of clostridial neurotoxins and their fragments are analogous to those previously described for diphtheria toxin and its fragments (18, 19). In addition to functional similarities, there are also structural similarities. For both diphtheria toxin and tetanus toxin, the entire heavy chain is not essential for channel activity. (We have not yet investigated this possibility for botulinum neurotoxin.) The carboxyl terminus of the heavy chain, which is presumably involved in receptor recognition, could be removed, and the remaining amino-terminal end of the heavy chain still retained full channel-forming activity. Thus, fragment B, an incomplete form of tetanus toxin, and CRM45, an incomplete form of diphtheria toxin, both form channels in artificial membranes (Fig. 2; ref. 18).

There is a recent report (22) that tetanus toxin forms channels in planar lipid bilayers at a symmetric pH of 7.0 and that these channels are formed only when mixed gangliosides are present in the membrane. As described above, we have observed similar low levels of toxin-induced channel activity in asolectin membranes at a symmetric pH of 7.0. This activity was not affected by the presence of ganglioside GT2b (asolcetin to ganglioside ratio 9:1), a putative receptor for tetanus toxin (23).

Voltage-Gating of Channels. Channels formed by tetanus toxin and its fragment B showed voltage-dependent behavior remarkably similar to that seen with channels formed by diphtheria toxin (18, 19). At symmetric low pH, positive voltages applied to the cis compartment opened channels and negative voltages closed them; the rate of opening and closing increased with the absolute magnitude of the voltage. In the presence of a pH gradient (cis, 4.0; trans, 7.0), there was significant opening of channels at zero voltage, and larger negative voltages were required to close them.

The voltage dependence of channels formed by the heavy chain of botulinum neurotoxin was investigated mainly in the presence of a pH gradient. Under these conditions the channels opened with both positive and negative voltages. However, in the presence of concentration gradients (10:1) formed with glucosamine chloride or potassium glutathionate, the channels could be closed with large negative voltages applied to the cis compartment.

Size of Toxin-Induced Channels. An important question that must be addressed before postulating that channels provide a tunnel for their respective active fragments is whether the channels are large enough to accommodate movement of peptide chains. Earlier work (18) with multilamellar vesicles indicated that the channels formed by the B45 fragment of diphtheria toxin allowed the nonelectrolytes cyclodextrin and polyethylene glycol 1500 to pass through, which suggested a diameter of at least 18 Å. A channel of this size is large enough to accommodate the extended light chain of the diphtheria toxin molecule (18).

From reversal-potential measurements made in the presence of gradients formed with salts of large anions or cat-
ions, we confirmed with planar lipid bilayers that the diphtheria toxin channel is indeed large (Table 1). The ion selectivity of the diphtheria toxin channel is a sensitive function of pH, with the ratio of K⁺ to Cl⁻ permeability switching from 1:3 at pH 3.5 to 15:1 at pH 5.5. These values were obtained by applying the Goldman–Hodgkin–Katz equation to the data in Table 1. The permeability of a channel depends on both the size and charge of the permeant ion. The possible complications that charge effects can have on studies to deduce channel size were emphasized in experiments with glucosamine, a cation whose diameter is 7–8 Å. At pH 3.5, where the diphtheria toxin channel prefers cations, glucosamine was almost totally excluded. At a pH of 5.5, where the channel prefers cations, the ratio of glucosamine to Cl⁻ permeability was comparable to the value in free solution, indicating that the channel did not discriminate between the two ions (Table 1). These findings show that charge is a critical determinant in the selectivity properties of the diphtheria toxin channel. The data (Table 1) also show that the diphtheria toxin channel is sufficiently large to allow penetration by NAD (12–16 Å), a result that is compatible with the initial sizing experiments done on vesicles and non-electrolytes (18).

Over the pH range 3.5–7.0, channels formed by tetanus toxin, fragment B, and the heavy chains from tetanus toxin and botulimum neurotoxin preferred cations, though only weakly (Table 1). Their permeability to glucosamine, the largest cation tested, was even greater than that shown by the diphtheria toxin channel (Table 1). Thus, these channels, too, are very large. The size of the channels formed by the three toxins are compatible with their proposed function as tunnel proteins to allow the passage of fully extended active fragments.

**DISCUSSION**

There is strong evidence that the active fragment of diphtheria toxin enters the cytosol from acidic vesicles (13, 14), and there is suggestive evidence that the same is true for botulimum neurotoxin (15) and tetanus toxin (16). The results reported in the present study are in complete accord with this view and, furthermore, suggest an explanation for the acidic vesicle requirement. The data indicate that a portion of the heavy chains from all three toxins interacts with lipid bilayer membranes to form channels and that this activity is particularly marked in the presence of a pH gradient comparable to that found across intracellular acidic-vesicle membranes. In addition, channel formation is enhanced in the presence of a voltage gradient that may be equivalent to that which is found across acidic-vesicle membranes.

There are two possible conclusions that could be drawn based on the data. The first is that the pH-dependent and voltage-dependent channels formed by the toxins are epi-phenomena that accompany the interaction of toxin fragments with the membrane. Indeed, recent experiments in which the light chain of diphtheria toxin was labeled by an intramembranous photoreactive probe appear to indicate that the light chain does not traverse the membrane through the channel formed by the heavy chain (24). This conclusion is predicated on the assumption that the photoreactive probe used in these experiments did not have access to the interior of the channel. However, the channel wall of the toxin may not present an impenetrable barrier to the reactive group of the probe, just as the wall of the monazomycin channel does not prevent alkyl chains from passing through it (25). Dismissing these channels forces one to assume that there is some other as yet unexplained mechanism that accounts for toxin translocation. Alternatively, the data can be interpreted to mean that the pH-dependent channels are tunnel proteins through which the active fragments pass. This conclusion does not require one to invoke additional membrane-related mechanisms; to the contrary, it is entirely compatible with our ion-permeability experiments which suggest that the pH-dependent channels are large enough to allow translocation of toxin fragments. As previously suggested (18), the pH gradient across acidic vesicles can also act as a driving force for the translocation of the light chain through the channel into the cytosol, by causing an unfolding of that chain at the lower vesicular pH and its subsequent refolding at cytosolic pH.

It is important to stress the presumed role of the pH-dependent channels in the mechanisms of action of the toxins. The data in this paper do not suggest that these channels are formed in the plasma membrane or that pH-dependent channels cause depolarization of excitable membranes. The fact that channel activity is pronounced only when the toxins (or relevant fragments) are in a low pH environment indicates that channel formation cannot occur under physiological conditions.

**Table 1. Reversal potentials for 10:1 concentration gradients under various pH conditions**

<table>
<thead>
<tr>
<th></th>
<th>K⁺Cl⁻</th>
<th>Glucosamine⁺Cl⁻</th>
<th>K⁺NAD⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cis = pH 4.0</td>
<td>pH 7.0</td>
<td>cis = pH 4.0</td>
</tr>
<tr>
<td><strong>pH 3.5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ideal toxin</td>
<td>-22</td>
<td>+40</td>
<td>-20</td>
</tr>
<tr>
<td>Ideal toxin</td>
<td>-51 (Cl⁻)</td>
<td>+51 (K⁺)</td>
<td>-45 (Cl⁻)</td>
</tr>
<tr>
<td><strong>Tetanus toxin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fragment B</strong></td>
<td>+25</td>
<td></td>
<td>+8</td>
</tr>
<tr>
<td><strong>Ideal</strong></td>
<td>+51 (K⁺)</td>
<td></td>
<td>-45 (Cl⁻)</td>
</tr>
<tr>
<td><strong>Botulimum toxin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Heavy chain</strong></td>
<td>+30</td>
<td></td>
<td>-12</td>
</tr>
<tr>
<td><strong>Ideal</strong></td>
<td>+51 (K⁺)</td>
<td></td>
<td>-25</td>
</tr>
<tr>
<td><strong>Open hole</strong></td>
<td>0</td>
<td>0</td>
<td>-25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-25</td>
<td>+32</td>
</tr>
</tbody>
</table>

K⁺Cl⁻ and glucosamine⁺Cl⁻ (Sigma) gradients were 1 M vs. 0.1 M; K⁺NAD⁻ (Sigma, Grade III-S) gradients were 0.7 M vs. 0.07 M. Diphtheria toxin measurements were done at the symmetric pHs indicated. Tetanus toxin fragment B and botulimum toxin heavy chain experiments were done in the presence of a pH gradient [cis = 4.0; trans = 7.0]. Positive potentials indicate cation selectivity; negative potentials indicate anion selectivity. (All measurements were within ±1 mV of the recorded entries.) The "ideal" rows indicate the potentials for a membrane exclusively permeable to K⁺ or Cl⁻. Thus, for example, if the diphtheria toxin entry under K⁺NAD⁻ had read +46 (the ideal value) instead of +39, this would have indicated that NAD⁻ did not go through the toxin-induced channel. For K⁺NAD⁻ and K⁺Cl⁻, the ideal K⁺ value was determined on membranes treated with nonactin (1 µg/ml), a cyclic antibiotic that is a carrier for univalent cations. For glucosamine⁺Cl⁻ and K⁺Cl⁻, the ideal Cl⁻ value was determined with a Ag/AgCl electrode. The "open hole" row represents the diffusion potential measured across the hole in the partition with no membrane present. The diffusion potential is independent of pH. Thus the entries under K⁺NAD⁻ show that NAD⁻ is restricted somewhat by the diphtheria toxin-induced channel (+39) as compared to an open hole (+32) but is certainly not completely excluded (+46). The channels formed by tetanus toxin and the heavy chain of botulimum toxin are more permeable to glucosamine than is the channel formed by diphtheria toxin, suggesting that the former channels may be larger.
conditions at the cell membrane. A more plausible conclusion is that the pH-dependent channels are formed in acidified vesicles and the role of these channels is to permit translocation of the pharmacologically active fragments into the cytosol.

The findings and conclusions on transmembrane movement of toxins may be important to the general issue of protein translocation across membranes. There are two basic models that have been proposed for this process, one of which can be called the channel model and the other of which can be called a non-channel model. A well known example of the former is Blobel and Dobberstein’s signal hypothesis (26). In this model the hydrophobic leader peptide, or signal sequence, recruits several membrane proteins that, together with the signal sequence, form a transmembrane channel through which a protein can penetrate (or in the case of integral membrane proteins insert into) the endoplasmic reticulum membrane.

Prominent examples of non-channel models are the direct-transfer model of Von Heijne and Blomberg (27) and Wickner (28) and the helical hairpin hypothesis of Engelman and Steitz (29). In these models, the hydrophobic contributions of the leader sequence and subsequent amino acids can overcome the energy barrier associated with inserting charged and polar residues into a low dielectric constant medium, and this in turn leads to direct extrusion of secreted proteins through (and the insertion of integral proteins into) the lipid bilayer.

Proponents of the various models point out that the existence of channels that allow for the passage of proteins has never been directly demonstrated. In this context, we suggest that the toxin-induced channels we have described may provide a good model for endogenous proteins that form tunnels for the passage of other proteins or polypeptides. We point out, however, that tunnel-forming proteins may not be characteristic of all multicomponent toxins that have active fragments. For example, we have not found conditions under which abrin or ricin form channels in lipid bilayer membranes. This suggests that tunnel proteins such as those formed by botulinum, tetanus, and diphtheria toxins may represent only one of several mechanisms by which proteins achieve translocations.

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