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Structure-Function Relationships in Diphtheria Toxin Channels: I. Determining a Minimal Channel-Forming Domain

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Abstract. Diphtheria Toxin (DT) is a 535 amino acid exotoxin, whose active form consists of two polypeptide chains linked by an interchain disulphide bond. DT's N-terminal A fragment kills cells by enzymatically inactivating their protein synthetic machinery; its C-terminal B chain is required for the binding of toxin to sensitive cells and for the translocation of the A fragment into the cytosol. This B fragment, consisting of its N-terminal T domain (amino acids 191–386) and its C-terminal R domain (amino acids 387–535) is responsible for the ion-conducting channels formed by DT in lipid bilayers and cellular plasma membranes. To further delineate the channel-forming region of DT, we studied channels formed by deletion mutants of DT in lipid bilayer membranes under several pH conditions. Channels formed by mutants containing only the T domain (i.e., lacking the A fragment and/or the R domain), as well as those formed by mutants replacing the R domain with Interleukin-2 (Il-2), have single channel conductances and selectivities essentially identical to those of channels formed by wild-type DT. Furthermore, deleting the N-terminal 118 amino acids of the T domain also has minimal effect on the single channel conductance and selectivity of the mutant channels. Together, these data identify a 61 amino acid stretch of the T domain, corresponding to the region which includes α-helices TH8 and TH9 in the crystal structure of DT, as the channel-forming region of the toxin.

Key words: Diphtheria toxin — Site-directed mutagenesis — Planar lipid bilayers — Ion channels — T-domain — Channel-forming peptides

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Introduction

Diphtheria toxin (DT, 535 amino acids) is a potent protein toxin, secreted as a single polypeptide by *Corynebacterium diphtherae,* that is easily cleaved by proteolysis into two fragments, joined by an interchain disulfide bond. The N-terminal A fragment (190 amino acids) kills cells by enzymatically inactivating their protein-synthetic machinery (*for review, see* Collier, 1990). The toxin binds to a cell-surface receptor via its C-terminal B fragment, the toxin-receptor complex is internalized via receptor-mediated endocytosis, and the A fragment is then translocated across the endocytic membrane into the cytosol. This last process, again mediated by the B fragment, is triggered by the acidification of the endocytic vesicle; the mechanism of translocation, however, remains elusive (for general reviews of diphtheria toxin entry into cells, *see* London, 1992; Madshus & Stenmark, 1992).

The recently published crystal structure of DT (Choe et al., 1992) reveals two folding domains within the B fragment, an organization long suspected from functional results. The C-terminal domain, referred to by Choe et al. (1992) as the R (Receptor-binding) domain (residues 387–535, entirely β-structure), mediates toxin-receptor binding. Removing this region abolishes binding to cell-surface receptors (Uchida et al., 1972, 1973), whereas replacing it with another ligand, such as Interleukin-2 (Il-2), shifts the toxin's specificity to cells with receptors for the new ligand (Williams et al., 1990).

The N-terminal region of the B fragment, which consists of a bundle of nine α-helices, is referred to as the T (transmembrane) domain. Several of these helices are hydrophobic or amphipathic, and are long enough that they might span a lipid bilayer membrane. Muta-

tions in the T domain generally affect delivery of the A fragment to the cytosol without changing the toxin's affinity for its receptor (O'Keefe et al., 1992; J.A. Silverman, J.A. Mindell, A. Finkelstein, R.J. Collier, *in preparation*). This domain (under the alias "B_{45}") also forms ion-conducting channels in planar lipid bilayers (Kagan, Finkelstein & Colombini, 1981), and the channels created in plasma membranes by whole toxin and isolated B fragment are presumably also formed by this domain (Papini et al., 1988; Sandvig & Olsnes, 1988). These channels form most readily when the pH conditions across the membrane mimic those found across the membrane of an acidified endocytic vesicle: approximately pH 5 on the toxin-containing side and pH 7 in the opposite solution. Results from several groups suggest that these channels are associated with the translocation of the A fragment into the cytosol, since mutations that reduce the toxin's channel-forming activity in lipid bilayers similarly affect its toxicity for mammalian cells (Falnes et al., 1992; J.A. Silverman, J.A. Mindell, A. Finkelstein, R.J. Collier, *in preparation*).

The channels formed by DT in lipid bilayers are highly pH dependent. Not only does their rate of formation depend sensitively on pH (on both sides of the membrane), but so too do their single channel properties. Both single channel conductance and ion selectivity are functions of pH; in fact, the DT channel shifts from nearly ideal cation selectivity at neutral pH to a mild preference for anions at pH 4 in a 10:1 KCl gradient (Hoch & Finkelstein, 1985). This change in selectivity, however, does not result from a change in the actual size of the pore. Hoch (1985) showed that the channel is about equally permeable to glucuronate at low pH as to glucosamine at high pH; since these molecules are about the same size, we may infer that the pore is *sterically* constant as a function of pH. Thus, the changes in selectivity with pH probably reflect changes in electrostatic interactions of permeant ions with titratable groups within the channel.

In this paper we examine a series of natural and engineered truncation and deletion mutants of DT in order to define the minimal peptide required for channel formation. We find that a 61 amino acid region within the T domain is sufficient to form ion-conducting channels nearly identical to those formed by the whole toxin.

Materials and Methods

Mutants

TH8-9 was originally constructed via oligo-directed mutagenesis in the p*tac* F2 expression vector (Tweten, Barbieri & Collier, 1985). F2 is a DT fragment truncated after residue 382 (at the junction between the T and R domains), and contains an additional 9, vector-coded amino acids at its C-terminus. TH8-9 is a precise deletion removing residues 202–321 from F2. Expression of this plasmid in *Escherichia coli* failed to produce a stable, full-length cross-reacting species. The NsiI-HindIII fragment encoding the TH8-9 T domain was subsequently cloned into the same restriction sites in pMal-c2-DT, creating a fusion between the *E. coli* maltose-binding protein (MBP; Riggs, 1990) and TH8-9. Whole-cell sonicates of induced cultures contained a stable cross-reacting species corresponding to the predicted molecular mass (~71 kD) for the MBP-TH8-9 fusion. This protein was purified by passage over an amylose resin, and the MBP domain was removed by treatment with factor Xa protease (the linker between MBP and TH8-9 contained a sequence cleaved by this protease). Cleavage was verified by SDS-PAGE and Western blotting, and TH8-9 was used without further purification. CRM45 and B_{45} were gifts from Dr. A.M. Pappenheimer; 386-Il-2 and Δ203–262 were gifts from Dr. J. vanderSpek. All proteins were stored at −20°C; working dilutions were prepared in 25 mM Tris, 1 mM EDTA and stored at 4°C for no more than two weeks.

Lipid Bilayer Experiments

Lipid bilayer membranes were formed at room temperature, using a modification of the folded film method of Montal (1974), across a hole (40–80 μm) in a polystyrene cup (Wonderlin, Finkel & French, 1990). This system enabled stable bilayers with low noise (500–900 fA between 300 Hz and 3 kHz) and low capacitance (15–30 pF) to be formed, allowing for recordings with high time resolution. To form a membrane, the hole was precoated on each side with 5 μl of a 1% hexane solution of asolectin [lecithin type IIS (Sigma Chemical, St. Louis, MO) from which neutral lipids were removed by the method of Kagawa and Racker (1971)], allowed to dry, then precoated on the outside (of the cup) with 5 μl squalene (1% in petroleum ether); 50 μl of the same asolectin solution was layered on buffered salt solution outside the cup, which (like the solution in the cup) was above the level of the hole, and the hexane was allowed to evaporate; the solution was then lowered and raised to form a bilayer. The volumes outside and inside the cup were 1 and 0.5 ml, respectively. Bridges containing 3 M KCl in 3% agar connected the solutions inside and outside the cup to Ag/AgCl electrodes in 3 M KCl baths. Membrane formation was monitored by measuring the system capacitance, using the voltage clamp with a triangle wave input; membrane conductances were less than 1 pS. Salt solutions were: 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA and pH buffer as noted in the figure legends. For selectivity experiments the *trans* compartment contained 0.1 M KCl to create a 10:1 (concentration) salt gradient. After the membrane formed, DT or its derivatives were added to one compartment (defined as the *cis* compartment) to a final concentration of 1–100 ng/ml and known voltages applied across the membrane. Voltages are those of the *cis* compartment; the potential of the *trans* compartment is taken as zero. The solutions on one or both sides of the membrane were stirred with miniature magnetic stir-bars when necessary.

Membranes were voltage clamped using an EPC-7 patch-clamp amplifier (List Medical Systems, Darmstadt, West Germany) and current records digitally recorded on video tape at a 44 kHz sampling rate through an A/D converter (Instrutech, Mineola, NY). Tape recorder output was filtered (10 Hz–1 kHz) with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and monitored on an oscilloscope, a chart recorder, or a PC data acquisition system. For these experiments, single channel currents were measured by hand from chart recorder records. Although this method is simplistic compared with more rigorous approaches to current determination, we found that manual analysis yielded comparable numbers to those provided by computer-generated amplitude histograms applied to the same data. We always measured single channel current as the amplitude of a transition between closed and open states. At the end of every experiment in a salt gradient, the electrode offset was determined (0.1–2.2 mV), and any reversal potential determined during that experiment was corrected by this value.

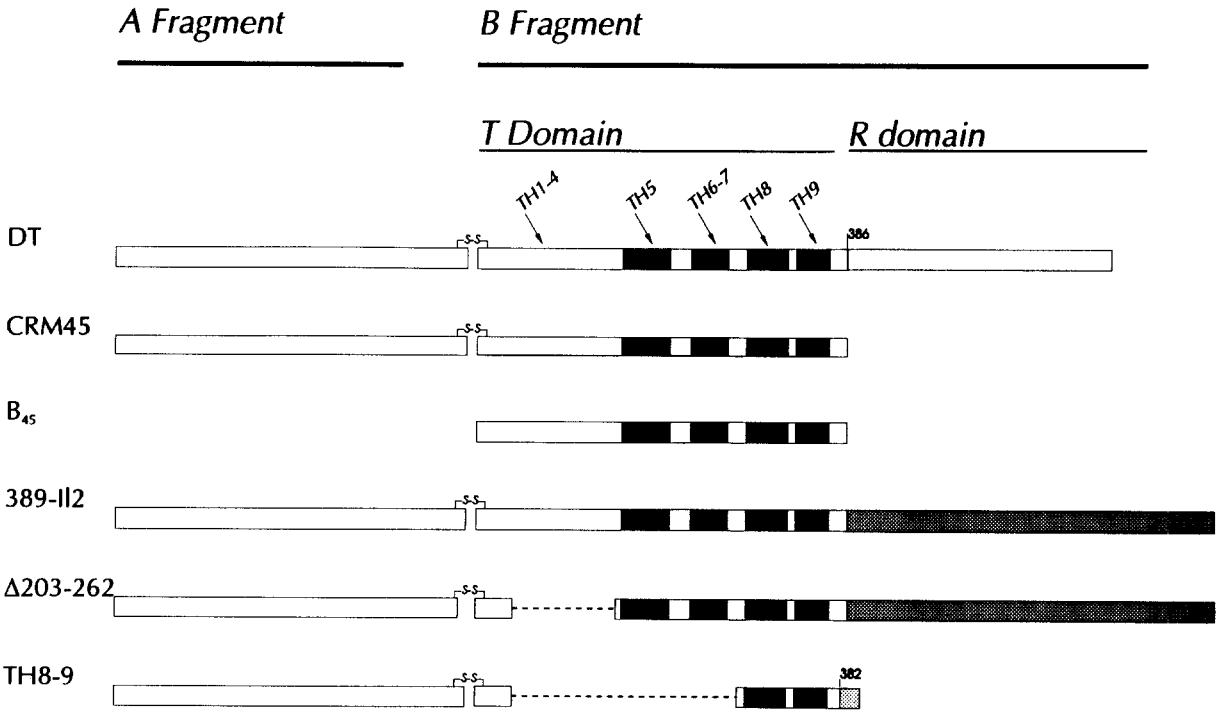

Fig. 1. Schematic representation of the mutants used in this study. This figure schematically depicts DT and several DT derivatives: CRM45, B_{45}, 386-Il2, Δ203–262, and TH8-9. The N-terminal A fragment lies on the left; the C-terminus of the protein is on the right. Black regions denote hydrophobic stretches within the T domain (hydrophobic stretches outside the T domain are unmarked). The grey stretches in 386-Il2 and Δ203–262 represent the interleukin-2 protein, which is fused to the C-terminus of the T domain in these proteins, replacing the R domain of DT. Dotted lines in Δ203–262 and TH8-9 correspond to stretches deleted from these proteins. The light grey region at the C-terminus of TH8-9 indicates the 10 amino acid tail on this protein (*see* Results: Mutants); its sequence is ISFNAVVYHS. Regions of the T domain corresponding to α-helices in the crystal structure are labeled TH1 through TH9.

Results

Mutants

Figure 1 schematically illustrates the primary structures of the proteins studied. *DT* refers either to naturally occurring diphtheria toxin or to the nontoxic mutant DT-E148S, with an active site glutamate replaced by serine to satisfy cloning safety regulations; this mutant forms channels indistinguishable from those of true wild-type DT. CRM45 is a naturally occurring mutant with a C-terminal truncation after position 386 (Pappenheimer, Uchida & Harper, 1972), corresponding exactly to removal of the R domain. B_{45} is the B fragment portion of CRM45 and corresponds to the T domain. 386-Il2 is a construct in which the R domain is replaced with Interleukin-2 (Williams et al., 1990)[1]; the last DT residue in this protein is Thr 386. Δ203–262 is derived from 386-Il2 by the deletion of residues 203–262; this removes residues corresponding to TH1 and TH2, the two amphipathic helices in the N-terminus of the T domain in the crystal structure (vanderSpek et al., 1993).[2]

TH8-9 is derived from F2, a genetic construct mimicking CRM45. This protein has 10 residues (ISFNAVVYHS) after residue 382, instead of the GHKT at the C-terminus of CRM45; these were introduced for ease of cloning and seem to make no difference in channel formation. In TH8-9, the bulk of the T domain has been deleted: namely, residues 203–321, corresponding to helices TH1 through TH7.[3] This leaves the entire A fragment and seventy-four residues of the B fragment (191–203 and 322–382). Thus, TH8-9 basically contains the two hydrophobic helices, TH8 and TH9, and a linker to the A fragment. We included the A fragment in this construct because of the hydrophobicity of TH8 and TH9; the hydrophilic A region helps keep the protein water soluble. (Residues 191–203 of B were left to include Cys 201, which participates in the disulphide

[1] The numbering system used in Williams et al. (1990) is different from ours. Subtract 1 from residue numbers in their paper to find the appropriate number for this work. The toxin we call 386-Il-2 is denoted DAB_{389}-Il-2 in Williams et al. (1990).

[2] In this reference, Δ203–262 is referred to as $DAB(\Delta 204\text{-}263)_{389}$-Il-2.

[3] The notation for helices in the T domain is the one used by Choe et al. (1992). There are nine helices in this domain, denoted TH1 through TH9.

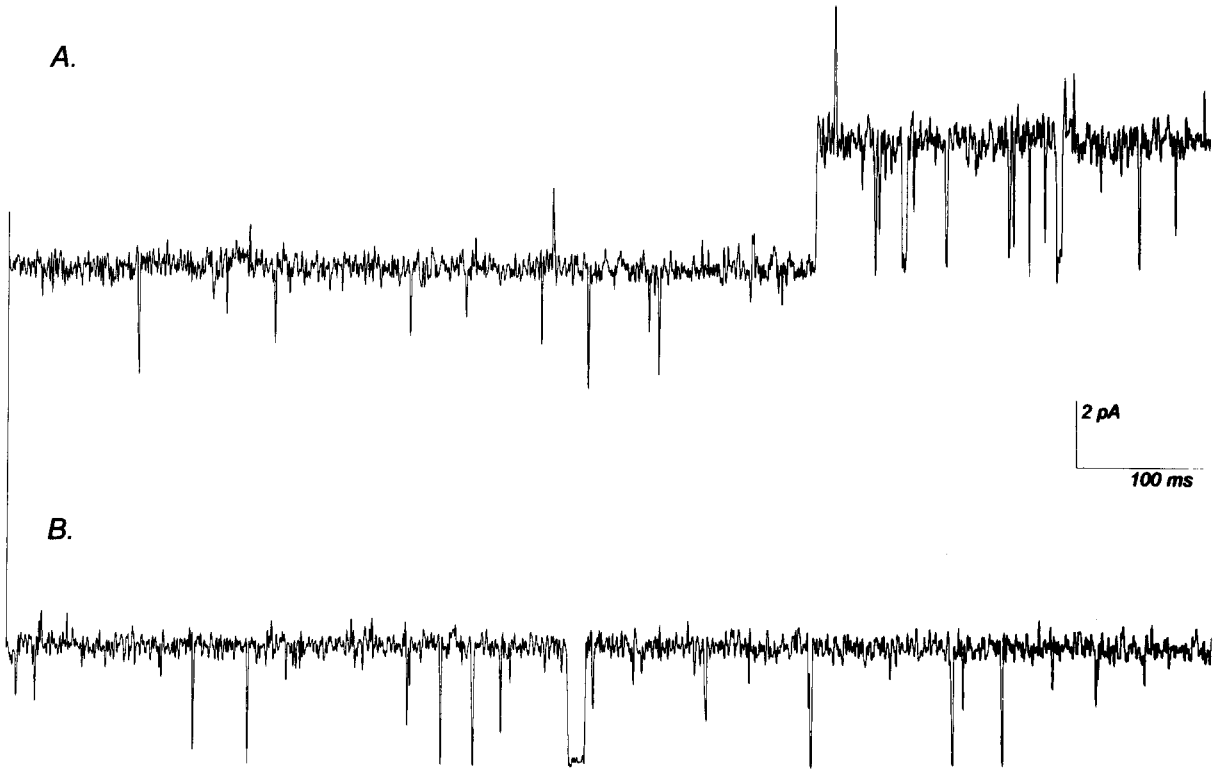

Fig. 2. Currents records, at +100 mV, for single channels formed by wild-type DT (*A*) and by the deletion mutant TH8-9 (*B*). The upper current level represents the open channel (in *A* the prolonged presence of a second channel is seen in the last third of the record); the channels are mostly open with brief closures. Solutions on both sides of the membrane contained 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA. The *cis* compartment was buffered at pH 5.3 with 30 mM MES; the *trans* at pH 7.2 with 5 mM HEPES. 3 ng (wild-type) or 50 ng (TH8-9) of protein were added to the 1 ml volume of the *cis* compartment (the *trans* side is considered ground). The records were filtered at 1 kHz and digitized at 3.3 kHz; a four point running average was used on the displayed traces to improve clarity.

bridge linking B to A.) To facilitate purification, TH8-9 was expressed as a fusion to maltose-binding protein, which was subsequently specifically cleaved, yielding intact TH8-9 and free maltose-binding protein. This mixture was used directly in our lipid bilayer experiments.

Role of the A Fragment and the R Domain

The R Domain

We probed the contribution of the R domain to DT's single channel properties by examining single channels formed in lipid bilayer membranes by DT and by mutants with either no R domain (CRM45) or the R domain replaced by Interleukin-2 (386–Il2). To address the concern that channels formed by mutant proteins might coincidentally overlap in a particular property with those formed by wild-type toxin, we examined not one but a series of single channel properties. Both the conductance and the selectivity of the channels are strongly pH dependent, and we determined each under several pH conditions. Furthermore, the current-voltage (*I-V*) relationship of the wild-type channel is nonlinear at some pHs; thus, any mutant which forms similar channels should also reproduce these nonlinearities. Since most of the intact channel's dependence on *trans* pH is associated with one residue (Mindell et al., 1992, 1994*a*), we focus here on its *cis* pH dependence.

Samples of single channel current recordings are shown in Fig. 2; single channel *I-V* relations determined from such records for wild-type DT, CRM45, and 386-Il2 channels (among others) in 1 M KCl with pH 5.3 *cis*, pH 7.2 *trans* are illustrated in Fig. 3. These pH conditions were chosen initially because they mimic the situation across the endosomal membrane when channel insertion occurs; for DT, similar pH conditions are necessary for biological activity (Draper & Simon, 1980; Sandvig & Olsnes, 1980). The *I-V* relations for all these channels are virtually superimposable. In addition, approximately equal amounts of DT, CRM45, and 386-Il2 were added to the *cis* compartment in each case; the R domain does not noticeably affect the channel-forming activity of these proteins in lipid bilayer membranes. We also measured ion selectivity, in the form of single channel reversal potentials in 10:1 KCl gradients (*cis*/*trans*), for the three toxins at pH 5.3 *cis*/7.2 *trans* (e.g., Fig. 4). The results of these measurements, illustrated in the Table, again show that the channels formed by these three proteins have similar values.

Raising the *cis* pH from 5.3 to 7.0 (while keeping

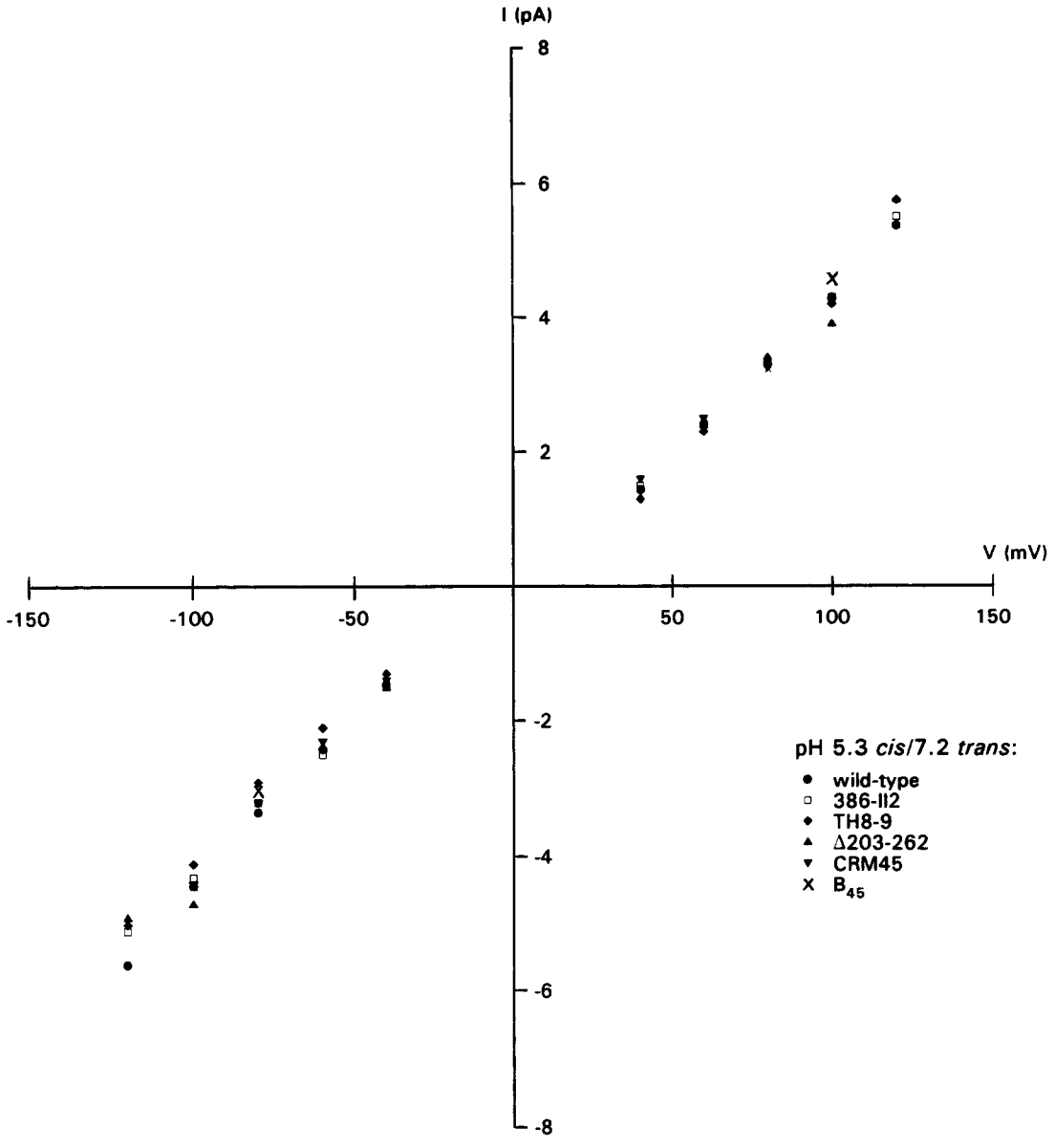

Fig. 3. Single channel current-voltage relations for mutant and wild-type channels at pH 5.3 *cis*/7.2 *trans*. This plot displays single channel current-voltage (*I-V*) relations obtained under the same experimental conditions described for Fig. 2. Current values were measured by hand from records like those in that figure. The current values illustrated for the wild-type channel are averages from four separate experiments; those shown for the mutant channels are representative and were always within 10% of the wild-type value.

the *trans* pH at 7.2) causes the single channel conductance of wild-type channels to increase at all voltages and introduces significant rectification in the *I-V* relation, with positive voltages producing larger currents than corresponding negative voltages. [Since channel formation is extremely poor at symmetric neutral pH, the membranes in these experiments were formed at pH 5.3 *cis*/7.2 *trans,* toxin added, and the *cis* pH then raised to 7.0 by addition of HEPES buffer (*see legend to* Fig. 5).] Figure 5 compares the *I-V* relations for wild-type DT, CRM45 and 386-II2 channels at symmetric pH 7. Again, the curves are superimposable, implying that both conductance and rectification are preserved in the absence of the R domain. The selectivites at pH 7.0 *cis*/7.2 *trans* are also identical for DT and 386-II2 channels (Table).

Lowering the *cis* pH also affects the conductance of the wild-type channel. When the *cis* pH is lowered from 5.3 to 4.1 (again having started the experiment at pH 5.3 *cis*/7.2 *trans*), the single channel current is reduced by as much as 40% (depending on voltage). Once again the mutant CRM45 and 386-II2 channels yield *I-V* relations similar to those observed for the wild-type channel (Fig. 6).

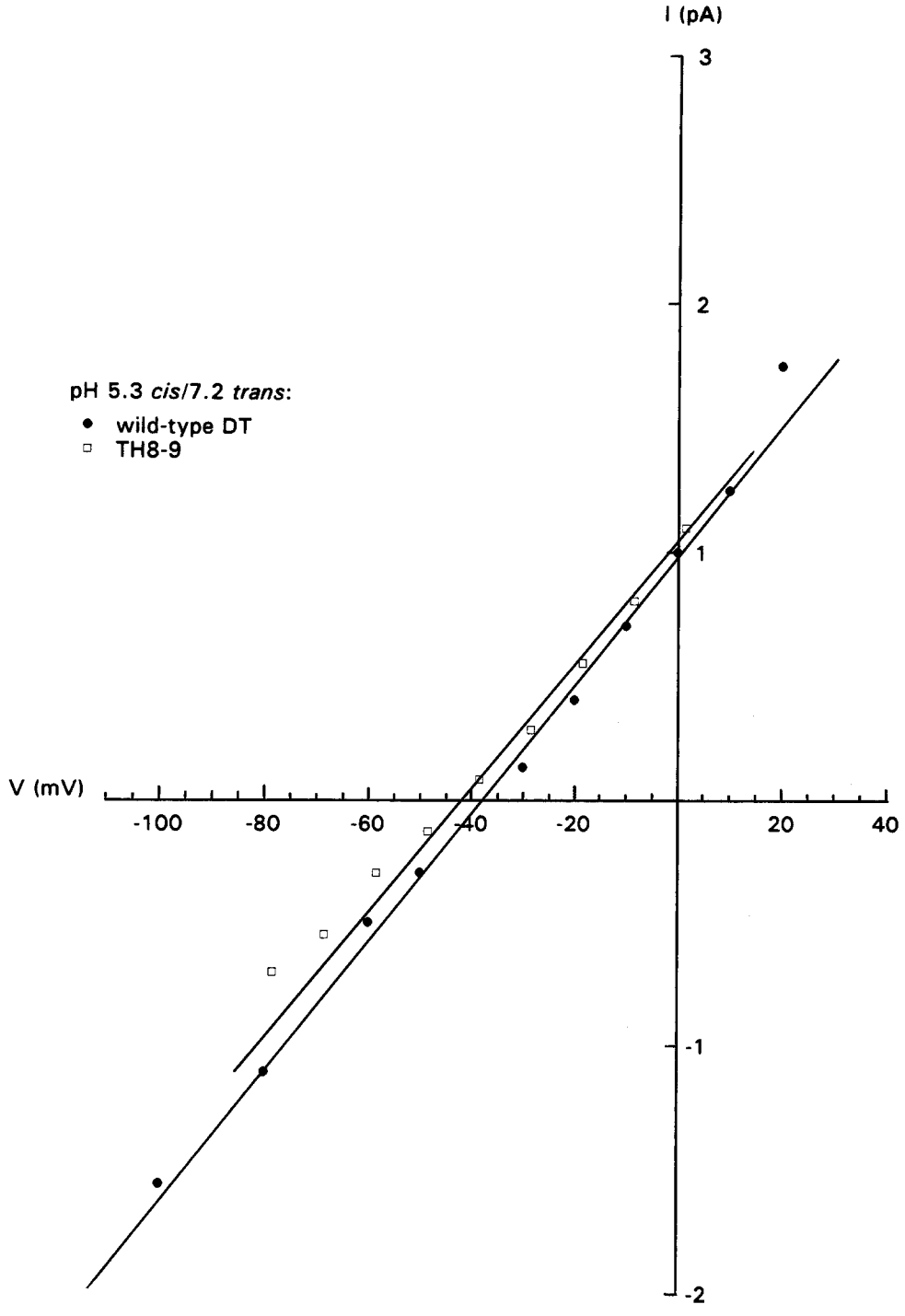

Fig. 4. Determination of reversal potentials from single channel current-voltage relations in a KCl gradient. Single channel *I-V* relations for wild-type and TH8-9 channels in a 10:1 KCl gradient (pH 5.3 *cis*/7.2 *trans*). Reversal potentials are −38 mV for the wild-type channel and −42 mV for the TH8-9 channel (an ideally selective cation channel would yield −51 mV). Solutions contained 1 M KCl *cis* and 0.1 M KCl *trans*, with 2 mM $CaCl_2$ and 1 mM EDTA on both sides. The *cis* compartment was buffered at pH 5.3 with 30 mM MES; the *trans* at pH 7.2 with 5 mM HEPES. Voltages are corrected for measured electrode offset at the end of each experiment (<3 mV). Straight lines were fit to the linear regions of data by eye, and reversal potentials read as the *V*-intercepts of these lines.

Table 1. Reversal potentials of DT and mutant channels at pH 5.3 *cis*/7.2 *trans* and at pH 7.0 *cis*/7.2 *trans*

Toxin	E_{rev} (in mV, 5.3/7.2)	E_{rev} (in mV, 7.0/7.2)
Wild-type	−38	−38
CRM45	−40	
386-Il2	−38	−40
B_{45}	−39	−39
Δ203–262	−37	−39
TH8-9	−43	−44

Reversal potentials determined as shown in Fig. 4 for channels formed by wild-type DT and by various deletion mutants in 1 M/0.1 M (*cis/trans*) KCl gradient. For experiments with pH 5.3 *cis*/ 7.2 *trans*, conditions were exactly as described in Fig. 4. For experiments at symmetric neutral pH, membranes were formed as above, toxin protein added, and channel activity observed. The *cis* pH was then raised to 7.0 by adding 1 M HEPES pH 7.5 (final concentration 100 mM). This addition had a minimal effect on the KCl gradient. Representative E_{rev} values are illustrated here: repeated determinations for a given protein always produced reversal potentials within a 2 mV range.

The A Fragment

The contribution of the A fragment of DT to single channel conductance and selectivity can be assessed by comparing channels formed by B_{45} to those formed by wild-type toxin and CRM45. Again we see that the *I-V* characteristics for the B_{45} channel are similar to those for the parent toxins at pH 5.3 *cis*/7.2 *trans* (Fig. 3) and at pH 4.1 *cis*/7.2 *trans* (Fig. 6). (We did not examine B_{45} channels at pH 7.0 *cis*/7.2 *trans.*) This similarity also holds for selectivity: the reversal potential measured for B_{45} channels in a 10:1 KCl gradient is essentially the same as those measured for wild-type and the other mutant channels discussed above (Table). We therefore find that the conductance and ion selectivity of DT channels are indifferent to the presence or absence of the A fragment.

In summary, under several different *cis* pH conditions, mutants with deletions or alterations of the A fragment and of the R domain form channels that behave very much like those formed by wild-type toxin, allowing us to conclude that the A fragment and the R domain do not contribute significantly to the conductance and selectivity properties of the DT channel.

Effects of Deletions within the T Domain

Having eliminated the A fragment and the R domain as major contributors to DT's ion-conducting channel, we now focus on sequences within the T domain, i.e., the sequences within the B_{45} peptide. As seen in Fig. 1, the mutants Δ203–262 and TH8-9 both lack sections of the T domain. (Both also contain the A fragment, and Δ203–262 contains Il2 in place of the R domain, but we have just seen that these regions have no significant effect on the channels formed by these proteins.) The deletion in Δ203–262 removes four hydrophilic helices from the N-terminus of the T domain, leaving all of the predicted hydrophobic regions intact. TH8-9, on the other hand, removes not only these four helices but also the first two long (potentially membrane-spanning) hydrophobic regions (corresponding to TH5-TH7), leaving only two hydrophobic stretches: TH8 and TH9. This region has been implicated in channel formation by the work of Kayser et al. (1981) on the peptide CB1 [which includes most of this region (and parts of the R domain)], as well as by that of Mindell et al. (1992, 1994*a*) which showed that a residue in this region is associated with the channel.

Both Δ203–262 and TH8-9 form ion-conducting channels in lipid bilayers (Fig. 3). For both, however, a significantly higher protein concentration is necessary for channels to incorporate into the membrane; e.g., whereas 1–2 ng/ml wild-type DT is sufficient to cause a channel to appear within one or two minutes, about 50 ng/ml of either of these mutants is required for a similar incorporation rate. Despite this lower activity, channels formed by Δ203–262 and TH8-9 are very similar to those formed by wild-type toxin.[4] For example, the single channel *I-V* relations for these two deletion mutants at pH 5.3 *cis*/7.2 *trans* closely correspond to that of the wild-type curve, preserving the slight nonlinearities seen for that toxin as well as the numerical values for the currents (Fig. 3). Furthermore, these deletions preserve the pH dependence of single channel currents observed for the wild-type channel (Figs. 5 and 6); that is, the currents are smaller at pH 4.1 *cis*/7.2 *trans* and are larger at pH 7.0 *cis*/7.2 *trans;* under these two conditions, the single channel currents for the two mutants are again comparable to those for the wild-type at all voltages tested. The situation is slightly different when one examines ion selectivity (Table). Both at pH 5.3 *cis*/7.2 *trans* and pH 7.0 *cis*/7.2 *trans,* Δ203–262 channels have exactly the same selectivity as that of wild-type channels in a 10:1 KCl gradient; TH8-9 channels, however, are somewhat more cation selective than those formed by the parent toxin under both these conditions.

Discussion

All of the DT derivatives considered in this paper form ion-permeable channels in lipid bilayer membranes. We have shown that the A fragment and the R domain

[4] The reader should note that these mutants were constructed and expressed in *E. coli* in the absence of a wild-type gene; despite the lower channel-forming activity, there is no possibility that the channels observed with the mutants are a consequence of wild-type contamination.

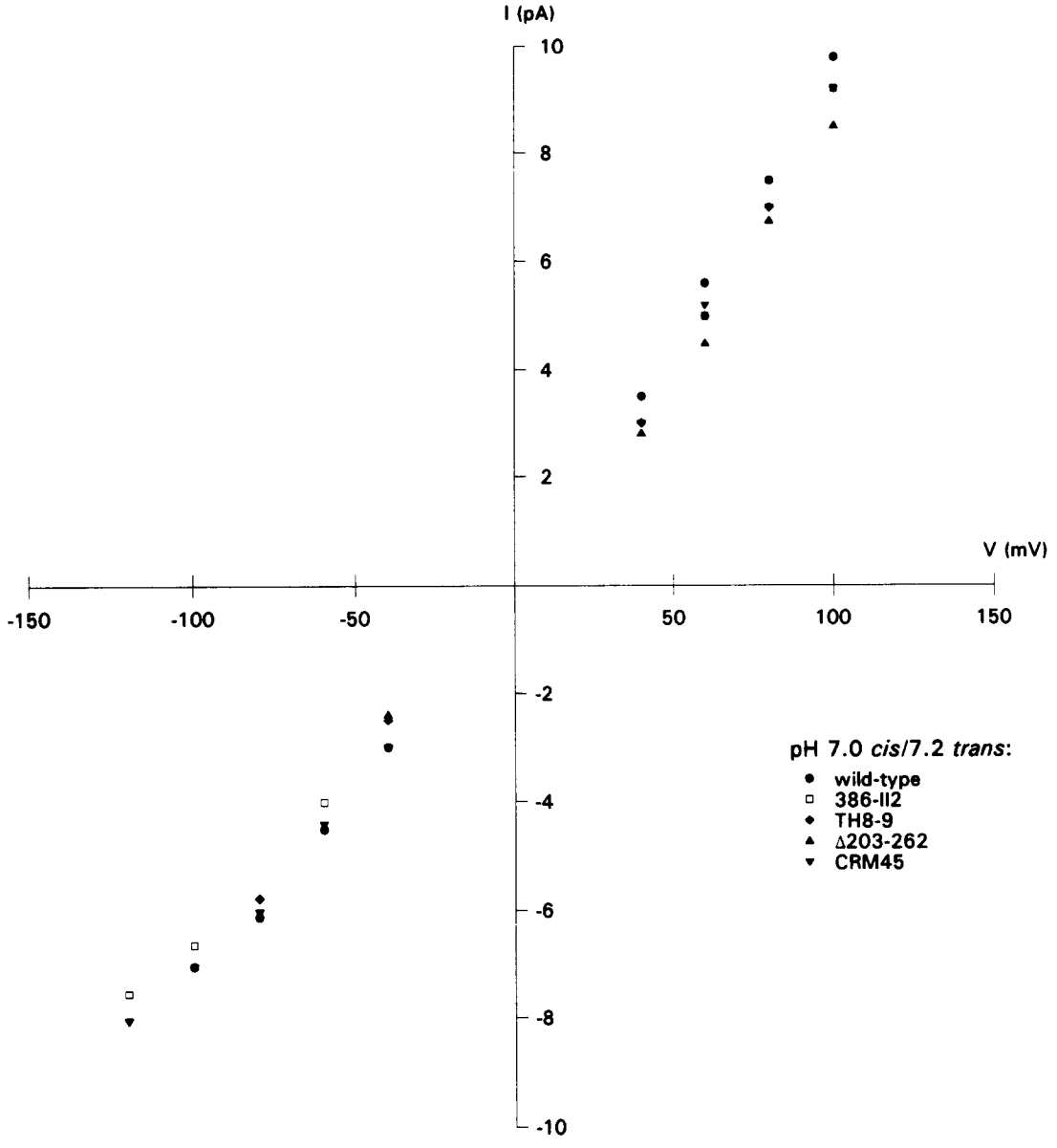

Fig. 5. Single channel current-voltage relations for mutant and wild-type channels at pH 7.0 *cis*/7.2 *trans*. Membranes were formed in 1 M KCl, pH 5.3 *cis*/7.2 *trans*. (The solutions were identical to those for the experiments in Fig. 2.) After the addition of protein (3–100 ng) and the appearance of channel activity, the *cis* pH was raised to 7.0 by addition of 1 M HEPES pH 7.5 to a final concentration of 100 mM. Currents were determined as in Fig. 3; illustrated data are from representative experiments.

do not contribute significantly to the conductance and selectivity of the channel. [This was indicated earlier from experiments with B_{45} (Kagan et al., 1981; Misler, 1983), but it has been examined here more carefully under a variety of conditions.] This leaves the T domain (B_{45}), and we have further found that only a small region of it, TH8-9, consisting of 61 amino acids, is required for formation of a channel largely identical to that formed by the wild-type toxin.

This conclusion is limited in one significant respect. We have considered here only properties of the "permeation pathway," i.e., the path ions take through the open channel; we have ignored the channels' kinetics—their opening and closing. These processes clearly could involve regions of the protein not directly associated with the pore, and are not addressed by our measurements of open channel properties. In fact, certain aspects of channel formation are indeed altered in some of the mutants, most notably the protein concentration required for channel formation by Δ203–262 and TH8-9, which is roughly an order of magnitude higher than that required for wild-type toxin. There al-

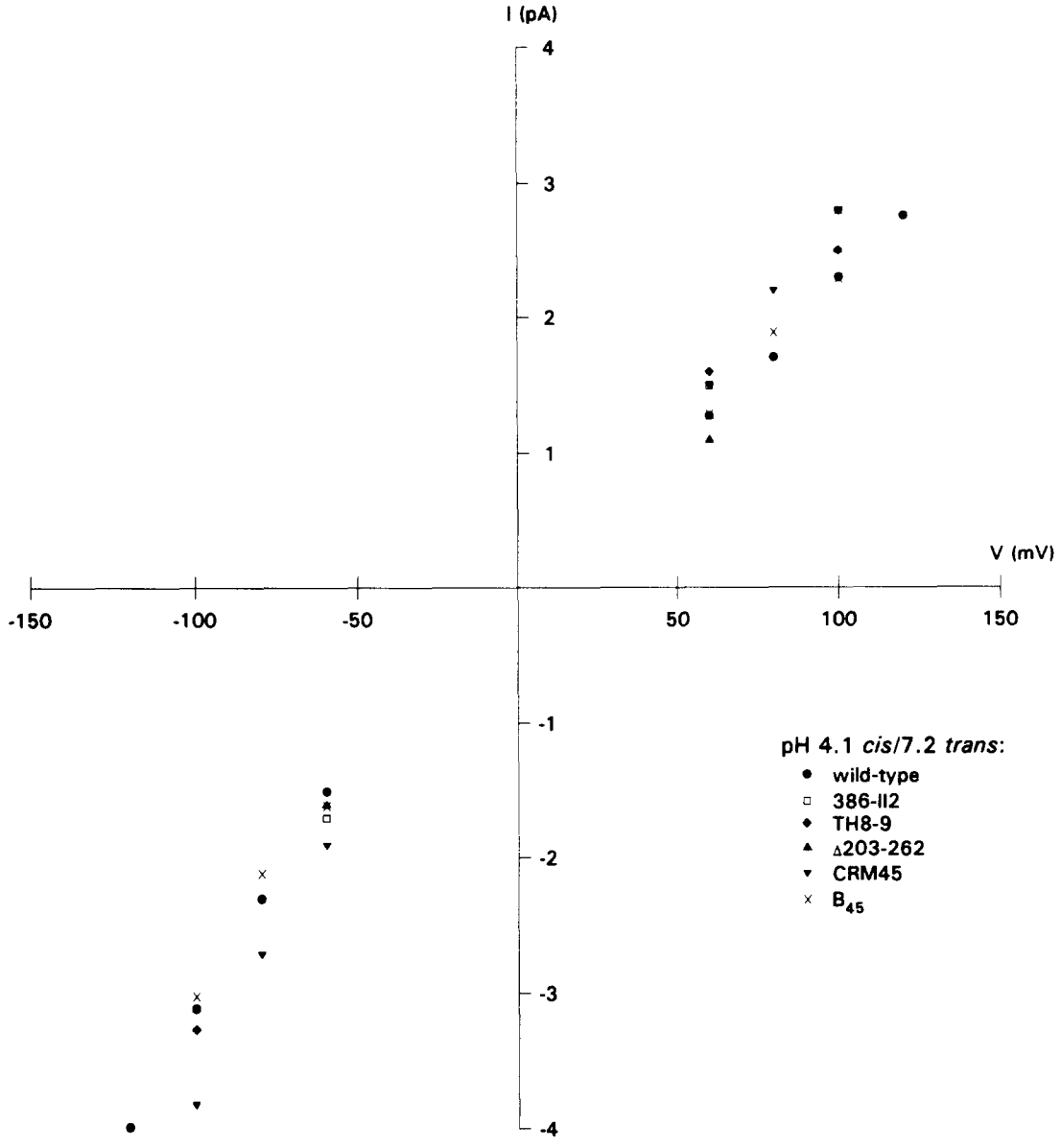

Fig. 6. Single channel current-voltage relations for mutant and wild-type channels at pH 4.1 *cis*/7.2 *trans*. Membranes were formed in 1 M KCl, pH 5.3 *cis*/7.2 *trans*. (The solutions were identical to those for the experiments in Fig. 2.) After the addition of protein (3–100 ng) and the appearance of channel activity, the *cis* pH was lowered to 4.1 by addition of 1 M glycerate pH 4.0 to a final concentration of 75 mM. Again, the data illustrated are representative; repetitions of a given experiment yielded values within about 20% of each other. The spread of current values may reflect real differences among the channels; our only concern, however, is that all these channels have similar dependences on *cis* pH, as is clearly illustrated by comparing the data in this figure with those in Figs. 3 and 5.

so may be more subtle effects, such as indications that the TH8-9 channel manifests voltage dependence different from that of the parent toxin channel (J.A. Mindell, *unpublished observations*).

The slightly greater cation selectivity of the TH8-9 channel compared to that of the wild-type channel (Table) is not inconsistent with the two channels being nearly identical. The groups responsible for this selectivity shift have two possible sources: either they come from the residues 263–321 (present in Δ203–262 but deleted in TH8-9) or from the short, 10 amino acid, vector tail left over from the construct used to make TH8-9. Residues 263–321 comprise the helices TH5, TH6 and TH7, which have been predicted to form a membrane-spanning helical hairpin (Choe et al., 1992). Perhaps these helices are so arranged that Lys299 is near enough to the channel opening to influence the permeation pathway; its removal in TH8-9 could then allow

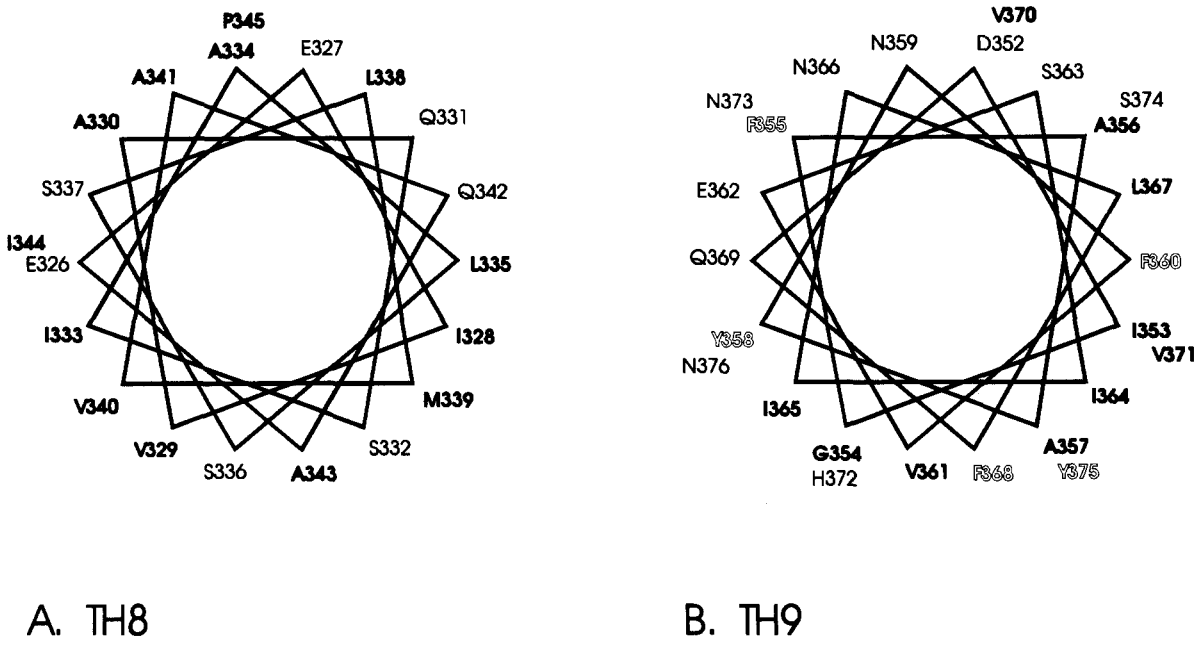

Fig. 7. Helical wheel representation of helices TH8 (*A*) and TH9 (*B*). Residues 326–346 are included in TH8; 353–378 in TH9. These correspond roughly to the helices listed by Choe et al. (1992). Polar residues are lightfaced, nonpolar residues, boldfaced; phenylalanine and tyrosine, which are nonpolar but polarizable, are shown in outline.

more cations through the channel, thereby increasing its cation selectivity. A similar electrostatic effect could be produced by the two adjacent positive residues, His384 and Lys385, present in all the other mutants but absent from TH8-9. Alternatively, a slight structural rearrangement caused by the deletion of TH5–TH7 might also alter selectivity, or conceivably the 10 amino acid tail, present in TH8-9 but not in the other constructs in this paper, is located near the channel lumen and causes the selectivity shift.

The results of these investigations imply that the residues in TH8-9 are the only ones contributing to the lining of the DT channel. This strongly suggests that the channel consists of a toxin multimer, since no arrangement of just two helices could form an aqueous pore completely surrounded by protein. The number of subunits involved in a channel is yet unclear, but the minimum is severely constrained by the known pore diameter of around 15 Å (Kagan et al., 1981; Hoch et al., 1985). Apparently, there is enough information within the TH8-9 fragment to cause the proper oligomerization even with this small fragment, since the channels it forms display the same conductance and selectivity as that formed by the wild-type toxin.

Helices TH8 and TH9 in the DT crystal structure are good candidates for the lining of the DT pore. Spectroscopic data (Cabiaux et al., 1989) suggest that secondary structures in the B fragment are largely preserved in the transition from solution to the membrane. With this in mind, we assume that TH8 and TH9 remain intact in the membrane.

A helical wheel representation of TH9 (Fig. 7) reveals its strikingly dualistic nature: it has a clearly polar face (that could line the pore) and a clearly apolar face (that could face the hydrophobic core of the membrane. Our work indicates that Asp 352, at the end of TH9, and Glu 362, on the polar face of this helix, are intimately associated with the channel pore (Mindell et al., 1992, 1994*a,b*). Furthermore, there is evidence that Ile 364 is not facing the pore (Cabiaux et al., 1993).

TH8, however, is more problematic; a helical wheel representation of it indicates no clear polar face (Fig. 7). We do, however, have data suggesting that TH8 is associated with the pore: point mutations of the groups at the N-terminal end of this helix (Glu 326 and Glu 327) to neutral residues affect the channel conductance under pH conditions where these groups are charged (Mindell et al., 1994*b*). The role of TH8 in the channel could be addressed by mutating polar residues in this helix to charged groups and analyzing the channels formed by such mutants.

Deelers et al. (1983) reported that a cyanogen bromide fragment of DT, called CB1, can form ion-conducting channels in lipid bilayers. This fragment, which contains a portion of the R domain, is missing the N-terminal half of TH8. These channels appear to be similar to those formed by wild-type toxin, but they were studied in membranes made from a neutral lipid (glyc-

erol mono-oleate) in decane, conditions under which wild-type channel activity is extremely poor. Furthermore, Deelers et al. (1983) found that the single channel conductance of CB1 is independent of pH (between pH 4.2 and 7.2), whereas Hoch and Finkelstein (1985) found significant pH dependence for wild-type DT channels in this range. Thus, the CB1 channel is at least somewhat different from that formed by the wild-type toxin. Finally, our data on residues 326 and 327 (Mindell et al., 1994*b*) suggest that TH8-9 is nearly a true minimal channel-forming domain, since mutating these residues affects the high pH behavior of the channel (the N terminus of the TH8-9 region is at His 322). These two glutamate residues could at least partially account for the differences between CB1 and the wild-type channel; because they are responsible for at least some of the *cis* pH dependence of channel conductance (Mindell et al., 1994*b*), their absence in CB1 would result in reduced pH dependence.

In this paper we have established that a 61 amino acid peptide, TH8-9, is sufficient to form the DT ion-conducting channel. Because of its brevity, this peptide is an ideal substrate for analysis of channel behavior using site-directed mutagenesis. By defining the channel-forming region, we have also delimited, by implication, the regions of the T domain with other functions. For example, the two amphiphilic helices at the N-terminus of the T domain have been implicated in the initial association of the toxin to a membrane (vanderSpek et al., 1993). In the following two papers (Mindell et al., 1994*a,b*) we present the results of experiments on point mutations of charged residues in the channel-forming region, showing that the pH-dependent properties of the DT channel can be explained in terms of the titration of a limited number of charged amino acids.

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