J. Membrane Biol. 137, 45–57 (1994)

The Journal of
Membrane
Biology

Structure-Function Relationships in Diphtheria Toxin Channels: III. Residues which Affect the *Cis* pH Dependence of Channel Conductance

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Received: 3 June 1993/Revised: 22 September 1993

Abstract. The conductance of channels formed by diphtheria toxin (DT) in lipid bilayer membranes depends strongly on pH. We have previously shown that a 61 amino acid region of the protein, denoted TH8–9, is sufficient to form channels having the same pH-dependent conductance properties as those of whole toxin channels. One residue in this region, Aspartate 352, is responsible for all the dependence of single channel conductance on *trans* pH, whereas another, Glutamate 349, has no effect. Here, we report that of the seven remaining charged residues in the TH8–9 region, mutations altering the charge on H322, H323, H372, and R377 have minimal effects on single channel conductance; mutations of Glutamates 326, 327, or 362, however, significantly affect single channel conductance as well as its dependence on *cis* pH. Moreover, Glutamate 362 is titratable from both the *cis* and *trans* sides of the membrane, suggesting that this residue lies within the channel; it is more accessible, however, to *cis* than to *trans* protons. These results are consistent with the membrane-spanning topology previously proposed for the TH8–9 region, and suggest a geometric model for the DT channel.

Key words: Diphtheria toxin — Site-directed mutagenesis — Planar lipid bilayers — Single channel conductance — Ion selectivity — pH dependence

Introduction

This is the third in a series of papers analyzing structure-function relationships in the ion-conducting channel formed by Diphtheria Toxin (DT). In the first paper (Silverman et al., 1994), we examined the channels formed by a series of natural and genetically engineered deletion mutants of DT and found that a short region, consisting of 61 amino acids in the B fragment, is sufficient to form a channel essentially identical to that formed by the intact toxin. This region, denoted TH8–9, corresponds to a pair of α-helices, TH8 and TH9, within the T (transmembrane) domain[1] of the recently solved crystal structure of DT (Choe et al., 1992). In the solution form of DT, these helices form a "helical hairpin" connected by a short loop. Given the evidence that there are relatively minimal changes in secondary structure as DT inserts into the membrane (Cabiaux et al., 1989), we suggested that the structure of this hairpin is conserved in the membrane.

In the second paper (Mindell et al., 1994), we compared channels formed by wild-type DT with those formed by point mutants in which the charges on one or both of the acidic amino acids in the loop connecting helices TH8 and TH9 were changed. Mutations of Glutamate 349 (to glutamine or lysine) had no effect on the single channel conductance or ion selectivity of the channel; in contrast, both of these properties were dramatically altered in channels with similar mutations of the other acidic residue, Aspartate 352. All of the effects of changing the charge at residue 352 appeared consistent with this group's interacting electrostatically with channel-permeant ions. By titrating Aspartate 352 with *trans* pH, we demonstrated that this residue

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The T domain contains 9 α-helices, numbered TH1 through TH9; TH8 and TH9 are at the C-terminus of the T domain.

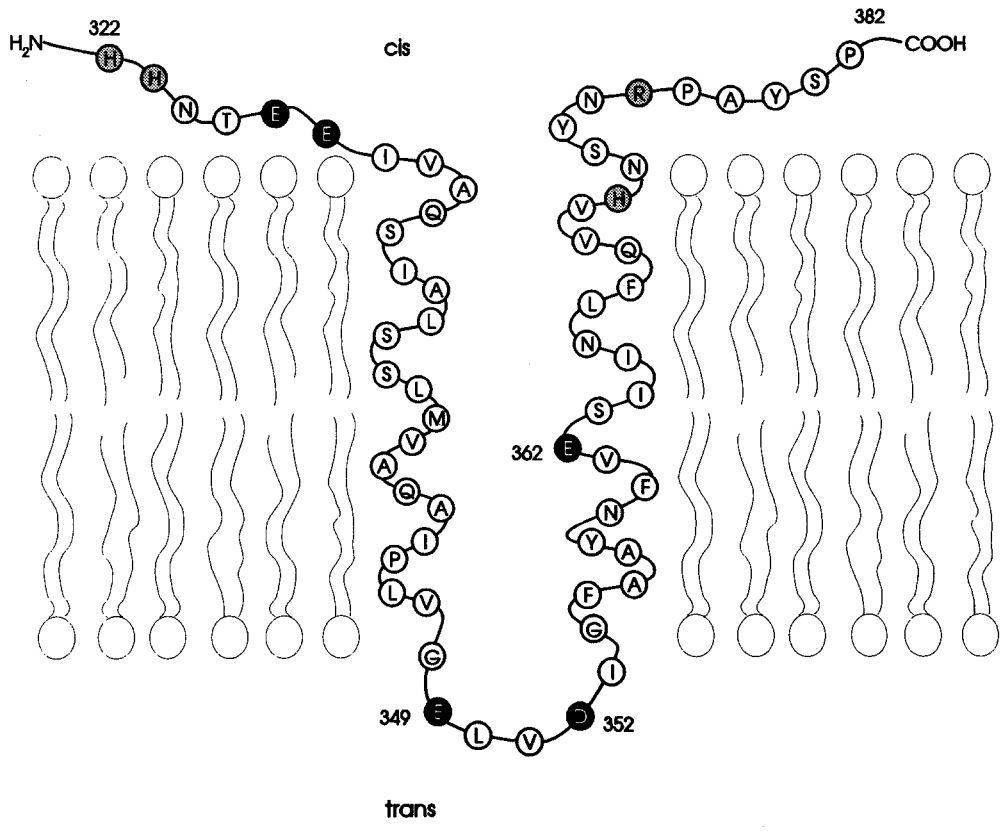

Fig. 1. Proposed transmembrane topology of the diphtheria toxin channel-forming domain. Membrane topology proposed in the previous paper (Mindell et al., 1994). Note that the N-terminus of the region is on the *cis* side; a helix corresponding roughly to TH8 (Choe et al., 1992) spans the membrane from *cis* to *trans;* a short loop, including E349 and D352 projects into the *trans* compartment; helix TH9 again spans the membrane; and the C-terminus of the region lies on the *cis* side. This model predicts that of the charged residues, H322, H323, E326, E327, H372, and R377 lie on the *cis* side of the membrane, E349 and D352 lie on the *trans* side, and E362 lies within the membrane, presumably projecting into the channel lumen. Black = negatively charged residues; Grey = positively charged residues.

lies on or near the *trans* side of the membrane. In fact, under the conditions used in Mindell et al. (1994), Aspartate 352 accounts for *all* of the dependence on *trans* pH of the DT channel's conductance. The location of residue 352 led us to propose a membrane topology for the channel-forming region of DT (Fig. 1): the N-terminus of TH8 on the *cis* side; TH8 itself spanning the membrane; the loop connecting TH8 and TH9 (and containing D352) on the *trans* side; TH9 returning across the membrane; and the C-terminus of TH9 back on the *cis* side.

In this paper, we describe the results from mutating the remaining charged residues in the TH8–9 region. We find that titration of the three acidic amino acids (Glutamates 326, 327, and 362) seems to account for much of the substantial *cis* pH dependence of DT's single channel conductance. This finding is consistent with the proposed membrane topology of the TH8–9 region. In contrast, none of the positively charged groups in TH8–9 appears to play a major role in determining single channel conductance properties.

Materials and Methods

Preparation of mutant toxins is described in detail elsewhere (J.A. Silverman, J.A. Mindell, A. Finkelstein, R.J. Collier, *in preparation*). Toxins were stable indefinitely at −20°C; working dilutions were stored at 4°C for no more than one week, with no change in activity.

Lipid bilayers were prepared as described in the previous paper (Mindell et al., 1994). Toxin was always added to the *cis* compartment. All solutions contained 1 M or 0.1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA, and an appropriate pH buffer. For experiments at pH 7.0 *cis*/7.2 *trans,* or at pH 4.1 *cis*/7.2 *trans,* membranes were formed at pH 5.3 *cis*/7.2 *trans* (30 mM MES *cis,* 5 mM HEPES *trans*), toxin protein added, channel activity observed, and the *cis* pH brought to its final value by adding either 1 M HEPES pH 7.5 (to a final concentration of 100 mM, pH 7.0 *cis*) or 1 M glycerate pH 4.0 (to a final concentration of 75 mM, pH 4.1 *cis*). All voltages refer to that of the *cis* solution (to which protein was added); the potential of the *trans* solution is taken as zero.

Results

The DT channel's ion permeability is highly pH dependent; both its single channel conductance and its

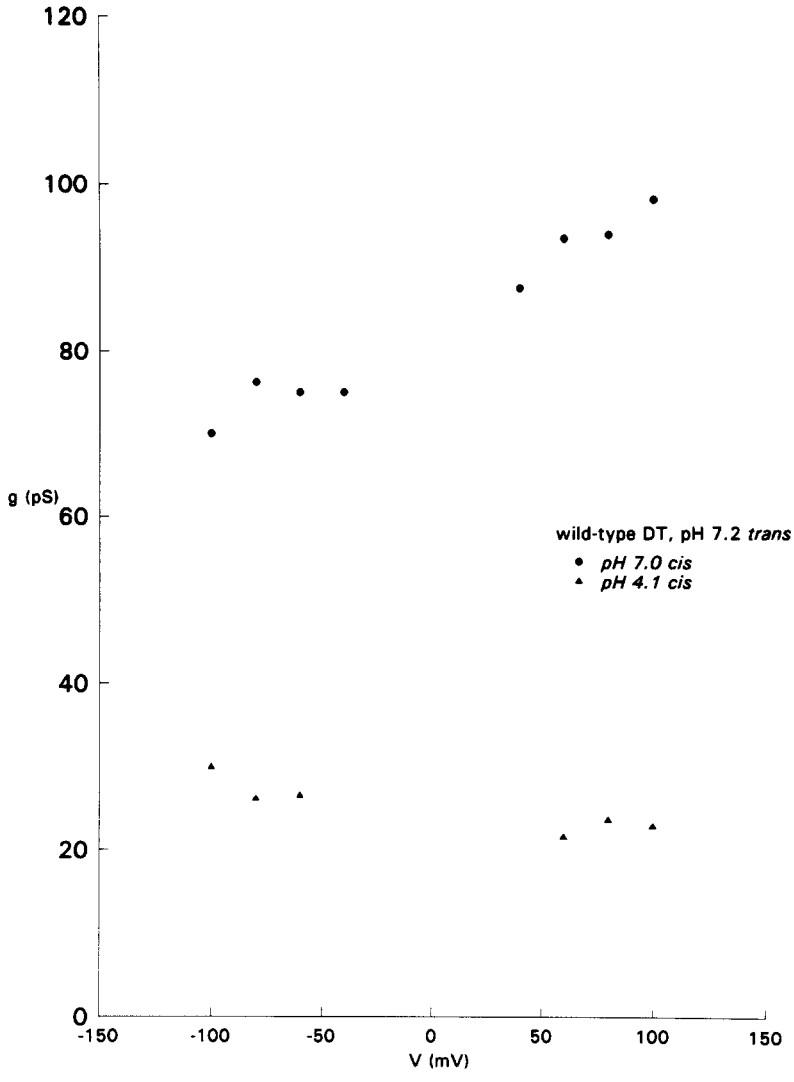

Fig. 2. *Cis* pH dependence of conductance in the wild-type DT channel. Single channel conductance-voltage (g-V) relations of the wild-type DT channel at pH 4.1 *cis*/7.2 *trans* and at pH 7.0 *cis*/7.2 *trans*. This figure demonstrates that when the pH of the *cis* compartment is raised from 4.1 to 7.0 (at constant *trans* pH 7.2), the channel's conductance increases between two- and fourfold, with larger differences at more positive voltages. Membranes were formed in 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA; 30 mM MES *cis* pH 5.3, and 5 mM HEPES *trans* pH 7.2. After toxin addition and the appearance of channels, the *cis* pH was raised to 7.0, 100 mM HEPES (by adding an appropriate amount of 1 M HEPES pH 7.5) or lowered to pH 4.1, 75 mM glycerate (by adding an appropriate amount of 1 M glycerate pH 4.0). This figure presents data from representative experiments; deviations about the mean at any voltage were within 10%.

ion selectivity are functions of proton concentrations on both sides of the membrane. In the previous paper (Mindell et al., 1994), we found that one amino acid, Aspartate 352, is responsible for all of the DT channel's *trans* pH dependence; here, we search for the residues responsible for the DT channel's *cis* pH dependence. Figure 2 illustrates this dependence, plotting conductance *vs.* voltage for the wild-type DT channel with *cis* pH either 4.1 or 7.0, and the *trans* pH constant at 7.2 (in 1 M KCl). Between *cis* pH 4.1 and 7.0, the single channel conductance increases as much as fourfold (at +100 mV), although the increase is smaller at negative voltages. This effect must be due to the titration of charged amino acids, of which aspartate, glutamate, and histidine are the likely candidates.

DT's channel-forming region consists of 61 amino acids (Silverman et al., 1994), which includes helices TH8 and TH9, as well as a short loop connecting the helices, and their N-terminal and C-terminal ends, respectively (*see* Fig. 1). Within this region reside nine charged and titratable amino acids. We examined the effects of Asp 352 and Glu 349 in the previous paper (Mindell et al., 1994), leaving seven residues potentially responsible for the DT channel's *cis* pH dependence: His 322, His 323, Glu 326, Glu 327, Glu 362, His 372, and Arg 377.

Roles of the Positively Charged Residues in the TH8–9 Region

Figure 3 compares the g-V relation of the wild-type channel with those of channels with mutations of the four positively charged residues: DT-H322N/H323N, neutralizing the two adjacent histidines early in the sequence; DT-H372R, replacing a titratable positive charge with a permanent one (at least in the pH range of interest); and DT-R377Q, neutralizing a positively charged residue at the C-terminus of TH9. (Note that

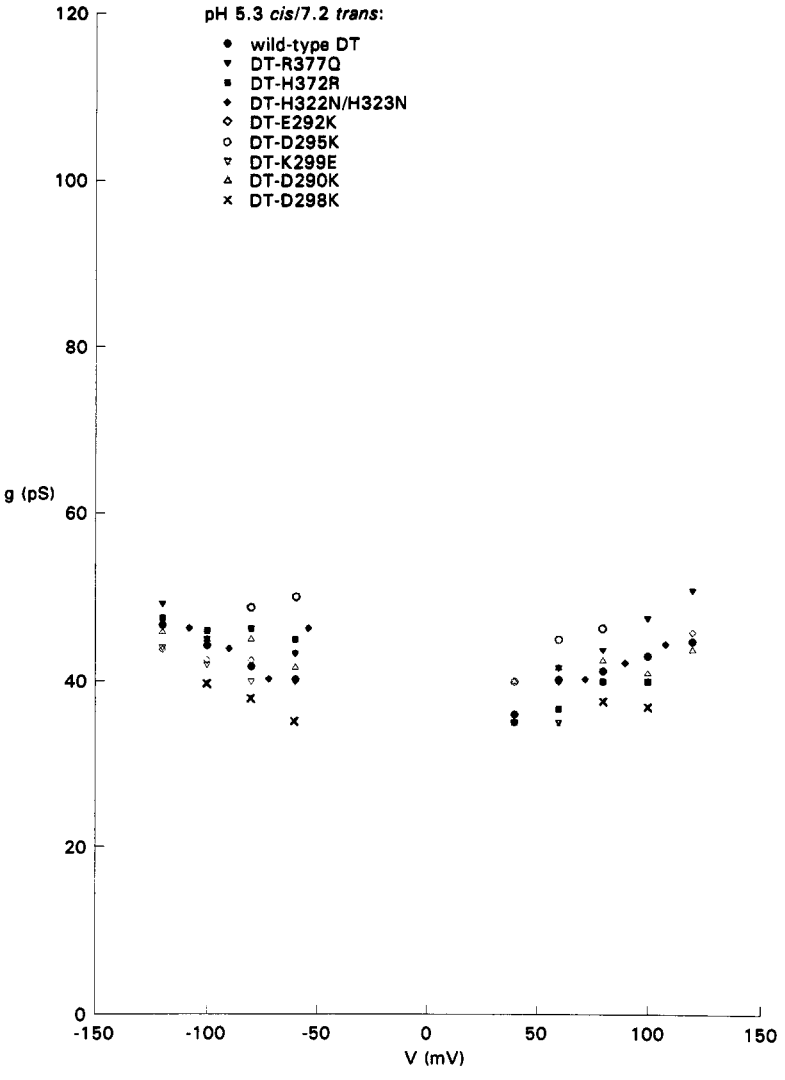

Fig. 3. Effects on DT channel conductance of mutations at charged residues. Single channel conductance-voltage relations for DT-R377Q, DT-H372R, DT-H322N/H323N, DT-D290K, DT-E292K, DT-D295K, DT-E298K, and DT-K299E channels are compared to that of the wild-type channel at pH 5.3 *cis*/7.2 *trans*. (In the last five of these proteins, the mutations are outside of the channel-forming TH8–9 region.) All of these toxins produce similar data, implying that none of the charges at these positions is a major determinant of channel conductance in DT. The increased conductances of DT-D295K and DT-R377Q may be real, but are too small to pursue in depth; that of the former, in any case, is of the wrong sign to be accounted for by a simple electrostatic mechanism. Solutions for these experiments contained: 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA; 30 mM MES *cis* pH 5.3, 5 mM HEPES *trans* pH 7.2. Wild-type data are the mean of four experiments; the standard deviations of these data are illustrated in Fig. 5. For mutant channels, data from representative experiments are illustrated; variations among experiments were within 10%.

several other mutants shown in Fig. 3 are discussed later.) Clearly none of these mutations significantly affects single channel conductance under the pH conditions illustrated (pH 5.3 *cis*/7.2 *trans*). For each mutant, we also compared its single channel conductance to that of wild-type at a *cis* pH where its difference from the potentially charged wild type should be maximal (low pH where the mutation neutralizes a positively charged group and high pH for DT-H372R, which replaces a titratable positive charge with a permanent one); none of these mutant channels is significantly different from the wild-type channel at any tested pH (*data not shown*).

Roles of the Negatively Charged Residues (Glutamates) in the TH8–9 Region

Effect of Glutamate 362

Helix TH9 is predicted to line the pore of the DT channel (Mindell et al., 1994); Glutamate 362, which lies on the polar face of TH9 and about halfway along its length, should therefore form part of the channel lining. Figure 4 presents conductance-voltage relations for channels formed by wild-type DT and by two mutants in which Glutamate 362 is replaced by a neutral amino acid: DT-E362Q and DT-E362A. These data are for experiments at pH 7.0 *cis*/7.2 *trans* in 1 M KCl—pH conditions where the glutamate in the wild-type channel should be charged, maximizing any difference between wild-type and 362-neutral channels. The conductances of both mutant channels are significantly reduced from that of the wild-type channel at large positive voltages; at negative voltages these differences are smaller. Note that the mutant channels formed by DT-E362Q and DT-E362A behave similarly; although there may be a real difference between them, it is relatively small compared with the difference between either mutant and the wild-type channel. Thus, the feature of residue 362 most relevant to channel conductance is its charge; the

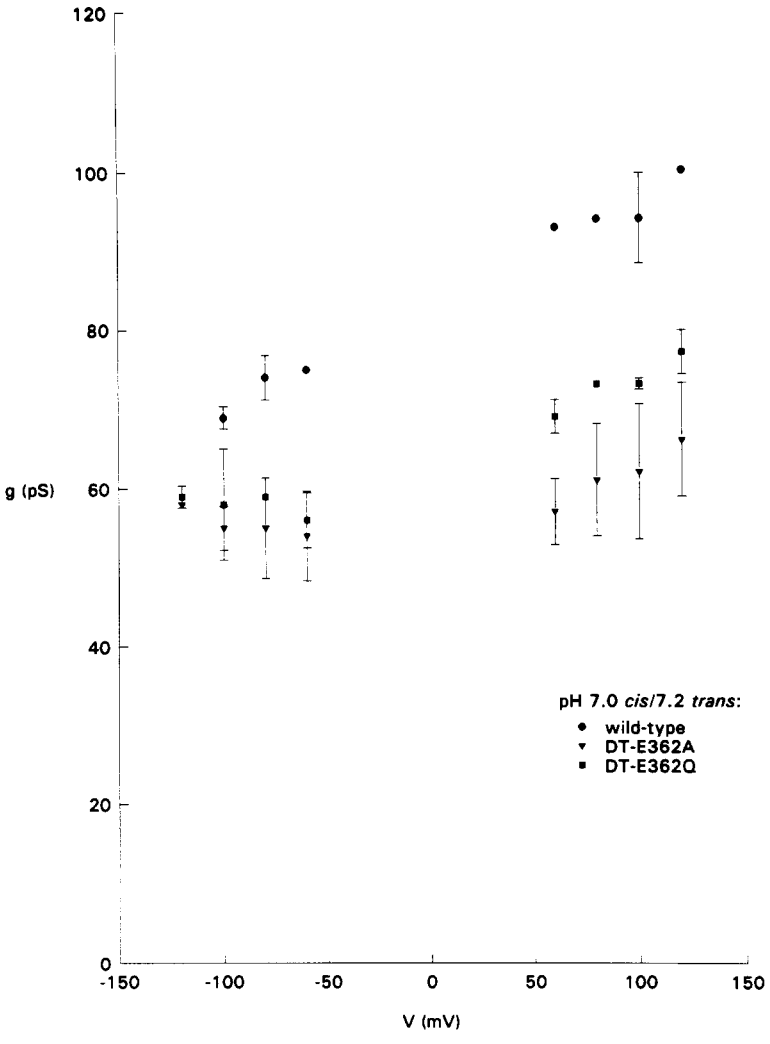

Fig. 4. Mutations at position 362: pH 7.0 *cis*/7.2 *trans*. The *g-V* relations for channels formed by DT-E362A and DT-E362Q are compared to that of the wild-type channel at pH 7.0 *cis*/7.2 *trans*. Note that both mutant channels display conductances significantly reduced from that of the wild type over the entire voltage range. As described in Fig. 2, the membranes were formed in 1 M KCl with pH 5.3 *cis*/7.2 *trans*, toxin added, and after channel appearances the *cis* pH raised to 7.0 by adding 1 M HEPES. Because of relatively large scatter in these experiments, data points are the means of 2–3 experiments, with error bars corresponding to the standard deviations. Points without error bars represent voltages at which currents were determined in only one experiment.

detailed chemistry of the side chain is of second-order importance.

At lower *cis* pH, the conductances of wild-type and 362-neutral channels converge. Figure 5 illustrates conductance voltage relations for wild-type, DT-E362Q and DT-E362A channels at pH 5.3 *cis*/7.2 *trans* (in 1 M KCl). Under these conditions, the channels formed by the three toxins yield essentially identical conductance values over the entire voltage range. The sole difference between these experiments and those illustrated in Fig. 4 is the pH of the *cis* compartment. These data are consistent with the charge on Glutamate 362 interacting electrostatically with permeant ions at high pH, but becoming neutral (protonated) at *cis* pH 5.3.

Effects of Glutamates 326 and 327

The only remaining negative charges in the channel-forming region of DT are the two adjacent glutamates, residues 326 and 327, at the N-terminus of helix TH8. Both of these acidic groups are neutralized in the mutant DT-E326Q/E327Q. Conductance-voltage relations for channels formed by this protein and wild-type DT at pH 7.0 *cis*/7.2 *trans* are compared in Fig. 6; as with the mutants at residue 362, the conductance of the DT-E326Q/E327Q channel is significantly reduced from that of the wild-type channel at positive voltages, and less so at negative voltages.

DT-E326Q/E327Q channels, in contrast to those formed by DT-E362Q, remain somewhat distinct from wild-type channels when the *cis* pH is lowered to 5.3; at pH 5.3 *cis*/7.2 *trans* the conductance of DT-E326Q/E327Q channels is slightly lower at positive voltages compared to that of wild-type channels (Fig. 7). The cation selectivity of this mutant channel is also slightly lower; in a 1 M: 0.1 M (*cis:trans*) KCl gradient, the reversal potential (E_{rev}) equals −32 mV for the DT-E326Q/E327Q channel, as opposed to −38 mV for the wild-type channel (E_{rev} = −51 mV for an ideally cation-selective channel). Thus, Glutamates 326 and 327 appear to still carry some negative charge at *cis* pH 5.3.

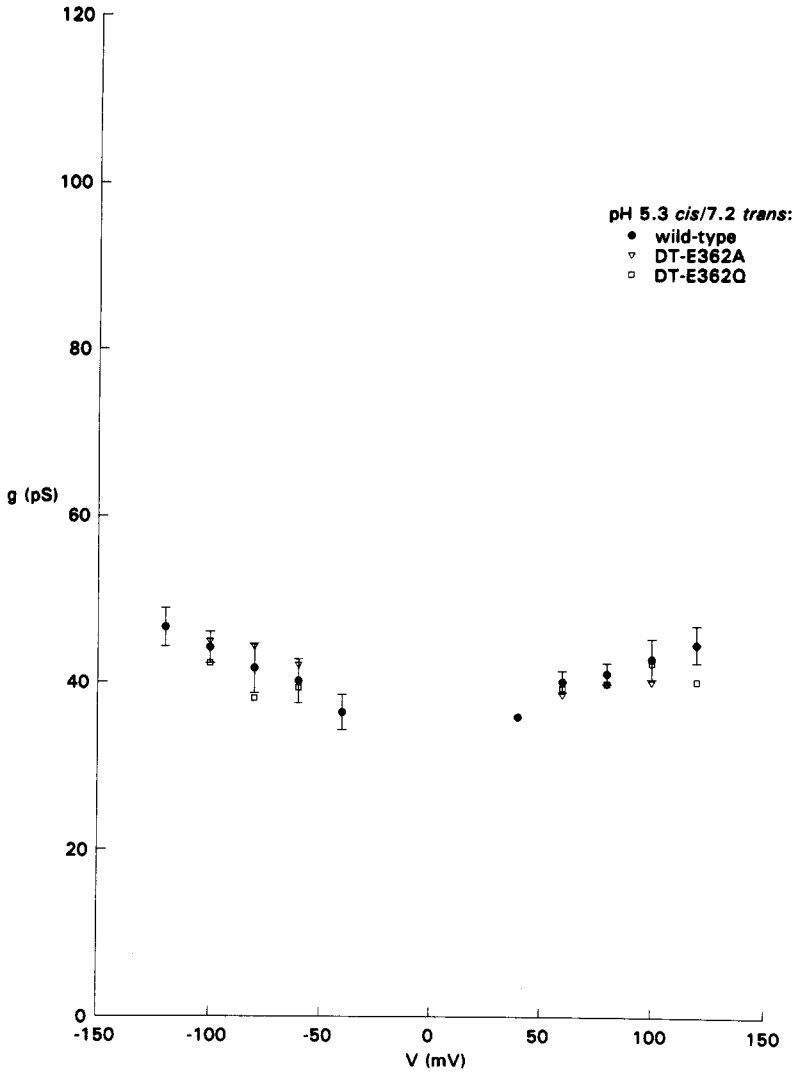

Fig. 5. Mutations at position 362: pH 5.3 *cis*/7.2 *trans*. The *g-V* characteristics of channels formed by DT-E362A and DT-E362Q are compared to that of the wild-type channel at pH 5.3 *cis*/7.2 *trans*. Under these pH conditions, differences between these mutants and the wild-type channel are minimal. Conditions for these experiments were identical to those for the experiments illustrated in Fig. 3. Wild-type data are the mean of four experiments, with error bars indicating standard deviations. Mutant data are averages of three (DT-E362Q) or five (DT-E362A) experiments. Standard deviations of mutant data are similar to those of wild type.

At *cis* pH 4.1, however, the conductance of the DT-E326Q/E327Q channel is essentially indistinguishable from that of the wild-type channel (Fig. 8), suggesting that both these groups are fully titrated at low pH.

Each of these glutamates appears to contribute to the *cis* pH dependence of the DT channel; note that the *g-V* relation of the single mutant DT-E327Q channel at pH 7.0 *cis*/7.2 *trans* lies between those of the wild-type and the double mutant DT-E326Q/E327Q channel at positive voltages (Fig. 6). Similarly, at pH 5.3 *cis*/7.2 *trans* the DT-E327Q channel's conductance is intermediate between those of the wild-type and the double mutant channels (*data not shown*).

We have seen that only three residues in the channel-forming region of DT make significant contributions to the *cis* pH dependence of conductance. As expected, when all three (326, 327, and 362) are mutated to neutral groups, the resulting channel displays a much reduced dependence of single channel conductance on *cis* pH (Fig. 9). Thus, in going from *cis* pH 7.0 to 4.1, the single channel conductance (at +100 mV) of the wild-type channel decreases by more than a factor of four, whereas that of the triple mutant decreases by less than a factor of two.

Conductances of E362 Mutants in pH Gradients

Thus far, we have described conductance differences only at symmetric neutral pH between wild-type channels and those formed by mutants with uncharged groups at position 362. In this section, we examine the effects of these mutations on channel conductance in pH gradients, with an eye toward establishing the position of residue 362 with respect to the membrane.

Raising the *trans* pH to 8.0, with *cis* pH 5.3, induces a difference in conductance between wild-type and DT-E362A channels over the entire voltage range (Fig. 10), not seen with the *trans* pH at 7.2 (Fig. 5). Interestingly, this difference is independent of *trans* buffer capacity for $Tris^+$ between 5 and 55 mM (i.e., the conductance of the wild-type channel is independent of *trans* buffer capacity), even though $Tris^+$ is known to be permeant

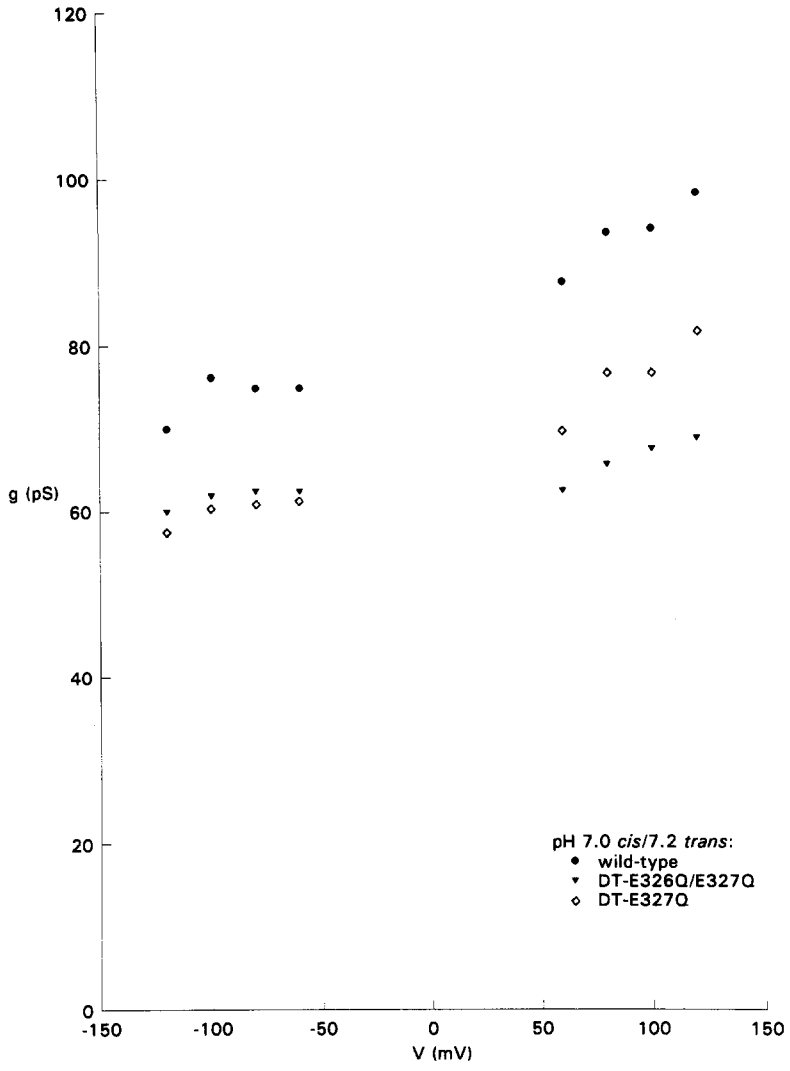

Fig. 6. Mutations at positions 326 and 327: pH 7.0 *cis*/7.2 *trans*. Comparison of the *g-V* characteristics of channels formed by DT-E326Q/E327Q and DT-E327Q to that of the wild-type channel at pH 7.0 *cis*/7.2 *trans*. Representative experiments are illustrated. Conditions as described in the legend to Fig. 2.

(Mindell et al., 1994). We were unable to examine the effect of varying the permeant buffer concentration in the *cis* compartment, because the membranes were unstable at pH 4.0 *cis*/8.0 *trans,* the conditions necessary for such an experiment.

Wild-type and DT-E362A channels also have different conductances in high *cis* pH (7.6) low *trans* pH (5.5), as shown in the Table for +100 mV. Thus, Glutamate 362 is accessible from both sides of the membrane; high pH in either the *cis* or *trans* compartment can titrate this group. However, it appears more difficult to titrate Glutamate 362 from the *trans* compartment than from the *cis;* that is, a higher pH is required in the *trans* compartment to achieve a smaller effect.

Groups Outside TH8–9

Although we have focused on the roles played by charged residues in the minimal channel-forming region of DT, which we determined in a previous paper (Silverman et al., 1994), we have also mutated several charged residues outside this region: in particular, Aspartate 318, in the loop connecting TH7 and TH8 (the same loop as His's 322 and 323, and Glu's 326 and 327), and the five charged residues in the loop connecting helices TH5 and TH6; these are all the remaining charged groups in the hydrophobic region of the T domain of diphtheria toxin.

Most of these residues do not influence the single channel conductance of the DT channel. Figure 3 includes *g-V* data for the mutants D290K, E292K, D295K, E298K, and K299E at pH 5.3 *cis*/7.2 *trans*. Clearly, the conductance of none of these mutants is significantly altered from the wild-type value at any voltage[2], a not surprising result considering that the absence of all these groups in the deletion mutant TH8–9 (Silverman et al.,

[2] The channel formed by the mutant DT-D295K may have a slightly larger conductance than that of the wild-type channel, but this difference is too small to pursue further; it is inconsistent with a simple electrostatic effect.

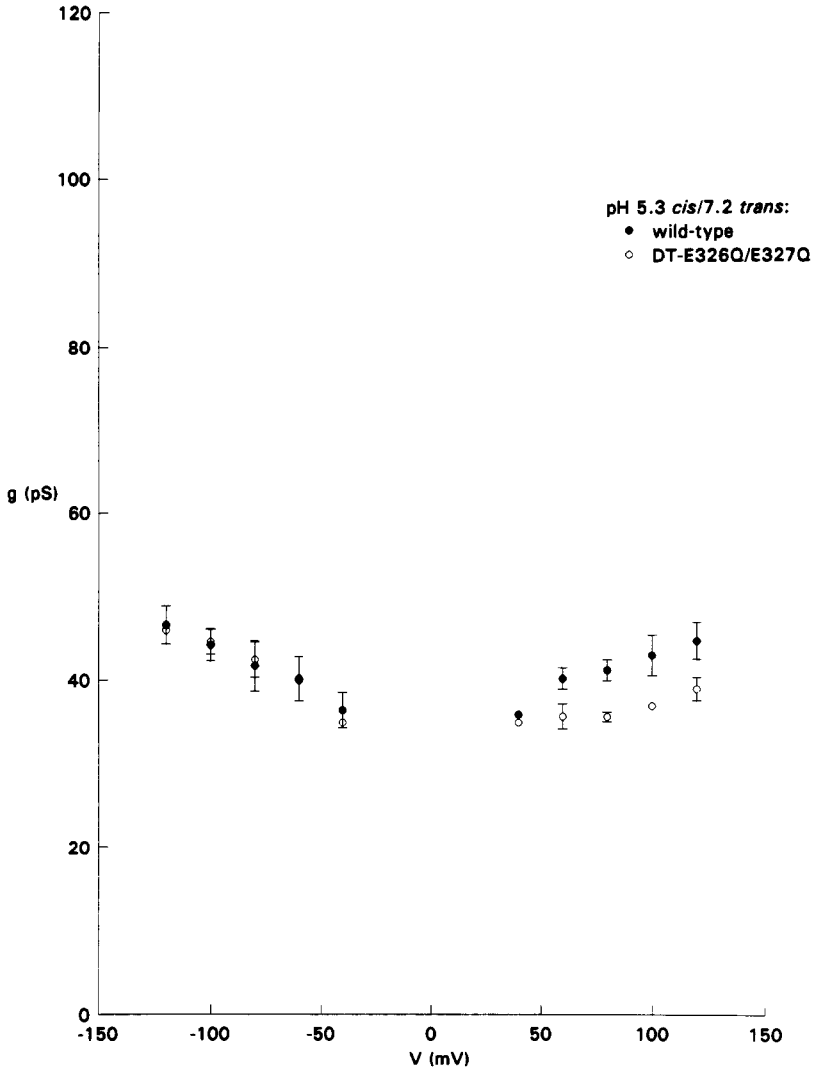

Fig. 7. Mutations at positions 326 and 327: pH 5.3 *cis*/7.2 *trans*. Comparison of the *g-V* relation of the channel formed by the double neutral mutant DT-E326Q/E327Q to that of the wild-type channel at pH 5.3 *cis*/7.2 *trans*. Wild-type data are the same as shown in Fig. 5; mutant data are averages of four experiments, with error bars indicating standard deviations. Experimental conditions as described in Fig. 3.

1994) has no significant effect on DT's single channel properties.

Interestingly, one negatively charged residue outside the TH8–9 region, Asp 318, when mutated to a neutral or positively charged group, affects single channel conductance. Changing this residue to asparagine or to lysine lowered the channel conductance relative to that of the wild type at pH 5.3 *cis*/7.2 *trans,* but not at pH 7.0 *cis*/7.2 *trans* (*data not shown*). This result is inconsistent with a simple electrostatic effect.

Discussion

With this paper we complete a mutagenic analysis of the roles played by charged residues in ion permeation through the DT channel. We have now mutated all 15 of the charged residues in the hydrophobic region of the DT "transmembrane" domain; that is, in its C-terminal 120 amino acids, including helices TH5–TH9. Of these, only four amino acids appear to interact electrostatically with ions in or near the channel. In the previous paper (Mindell et al., 1994) we demonstrated that one of these, Aspartate 352, is located on the *trans* side of the membrane and accounts for all of the *trans* pH dependence of DT's single channel conductance. In this work, we found that three glutamates, at positions 326, 327, and 362, also affect ion permeation; together, these three groups account for most of the *cis* pH dependence of the DT channel's conductance. None of the other mutations we introduced discernably affects single channel conductance; their individual contributions (if any) to the *cis* pH effect are within the scatter of our data (≈10%). Therefore, any portion of the *cis* pH dependence not due to Glutamates 326, 327, and 362 is probably distributed among the remaining groups, since no individual one has a substantial effect.

The larger conductance manifested by the wild-

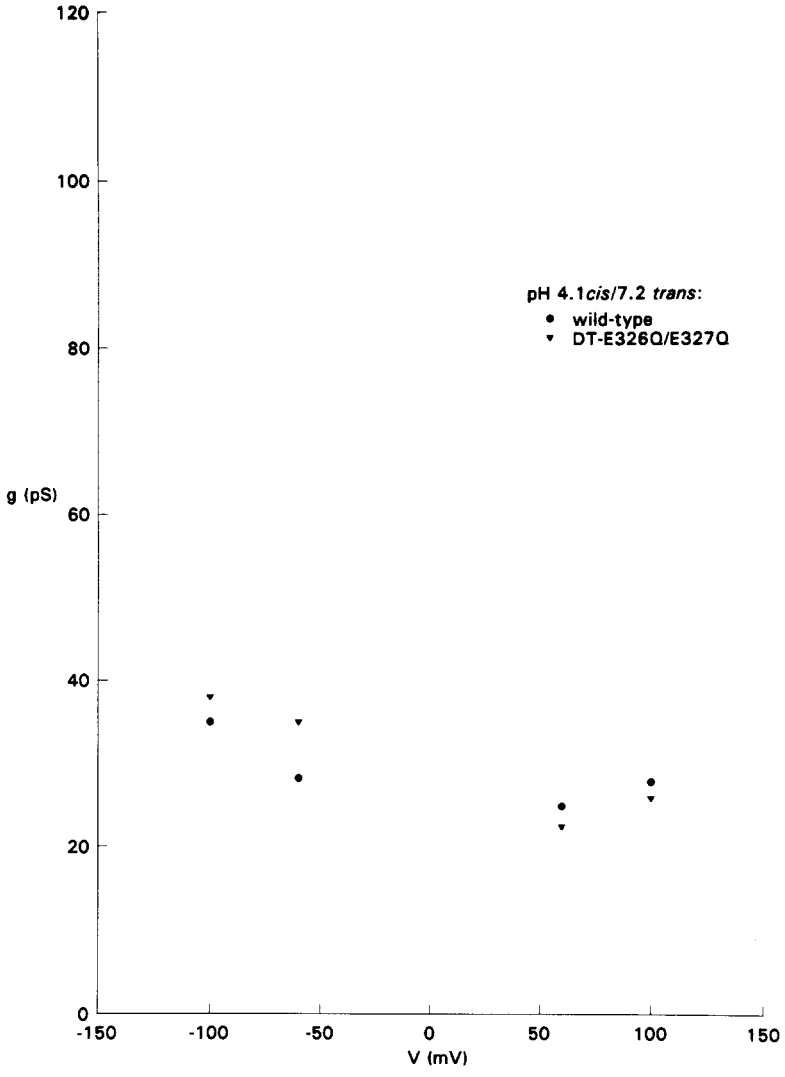

Fig. 8. Mutations at positions 326 and 327: pH 4.1 *cis*/7.2 *trans*. Demonstration that at *cis* pH 4.1, the conductances of DT-E326Q/E327Q and wild-type channels are experimentally indistinguishable. Representative data are illustrated. Experimental conditions as described in Fig. 2.

type channel at *cis* pH 7.0 compared to that at *cis* pH 4.1 (*trans* pH 7.2) reflects the contributions of charged groups titrated by *cis* pH (Fig. 2). Therefore, the ratio of wild-type channel conductance at *cis* pH 7.0 to that at *cis* pH 4.1 (*trans* pH 7.2), at a given voltage, is a measure of the effect on conductance of *cis* pH at that voltage. This ratio is plotted as a function of voltage in Fig. 11. Similarly, the ratios of DT-E362Q, DT-E326Q/E327Q, and DT-E326Q/E327Q/E362Q channel conductances at pH 7.0 *cis* to those at pH 4.1 *cis* reflect the contributions of remaining charged groups to *cis* pH dependence in these channels (since the conductances of the wild-type and these three mutant channels converge at pH 4.1 *cis*, we use data from wild-type channels in the denominator for all three). Figure 11 illustrates the reduction in *cis* pH dependence of conductance in these mutant channels compared to that of wild type.

The shape of the wild-type curve in Fig. 11 suggests that the groups contributing to the *cis* pH dependence are indeed located toward the *cis* side of the membrane. Note that for the wild-type channel, the ratio of its conductance at high *cis* pH (7.0) to its conductance at low *cis* pH (4.1) is smallest at negative voltages, and more pronounced at larger positive voltages. Since the channel is relatively cation selective under both pH conditions, the major current carrier is K^+. A charged group near the *cis* compartment will have a greater effect on K^+ moving from *cis* to *trans*, as it does at positive voltages, than it will have on K^+ moving from *trans* to *cis*, as it does at negative voltages. Thus, even if we did not know that these groups were accessible to protons from the *cis* compartment, the shape of this curve is consistent with the titratable charges being closer to the *cis* side of the membrane than to the *trans*. Contrast these data with those shown in the previous paper (Mindell et al., 1994; Fig. 7) for the ratio of wild type to DT-D352N and DT-D352K channel conductance. Those ratios are large (>4:1 for DT-D352K) at negative voltages, converging to 1 at large positive potentials, consistent with

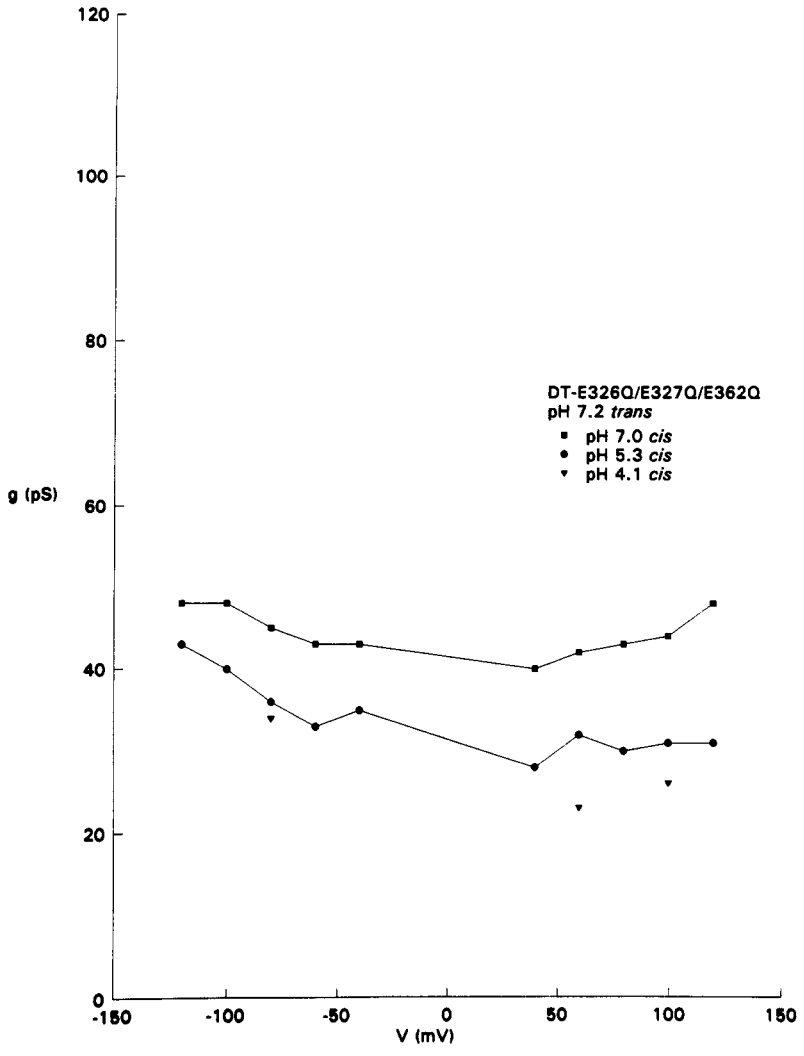

Fig. 9. *cis* pH dependence of the triple neutral mutant DT-E326Q/E327Q/E362A. Single channel conductance-voltage (g-V) relations are shown for the triple neutral mutant at three different *cis* pH values (4.1, 5.3, 7.2; all with *trans* pH 7.2). Compare the range of conductances observed for this mutant with those obtained for the wild-type channel (Fig. 2). Note that the conductance of the channel formed by the triple neutral mutant is significantly less pH dependent than that of the wild type. Conditions as described in Figs. 6, 7, and 8, respectively.

residue 352 lying in or near the *trans* compartment. In Fig. 11, the shapes of the curves for DT-E362Q and DT-E326Q/DT-E327Q channels are similar; the illustrated data provide no means to determine the (presumably different) relative locations of glutamates 326, 327, and 362. If, as predicted, Glutamate 362 is inside the pore and Glutamates 326 and 327 are at its edge, these groups may have different effects on permeant ions, which might become apparent at higher potentials or under different pH conditions.

Data presented in this paper do permit us to locate residue 362 with respect to the pore of the DT channel. Judging from its position in helix TH9, we predicted that Glutamate 362 should line the channel, about halfway across the membrane. If this is so, then it ought to be titratable from either side of the membrane. Such is the case; the data in Fig. 10 and the Table show differences between wild-type channels and DT-E362Q (or DT-E362A) channels at pH 5.3 *cis*/8.0 *trans* as well as at 7.6 *cis*/5.5 *trans*. Thus, high (neutral) pH on either side of the membrane, with the other side at low pH, can deprotonate Glu 362, thereby inducing a difference in conductance between the wild-type channel and those without a titratable group at position 362. This is good evidence that Glu 362 lies within the lumen of the DT channel.

The fine structure of the titration data for residue 362 may tell us even more about the location of this group. Comparing the data in Fig. 5 with those in the Table, we find that with roughly the same pH gradient, there is a difference between wild-type and DT-E362Q channel conductances with the high pH on the *cis* side (pH 7.5 *cis*/5.5 *trans*, Table) but not with the high pH on the *trans* (pH 5.3 *cis*/7.2 *trans*). In fact, the difference in conductance between wild-type and DT-E362A channels only emerges when the *trans* pH is raised to 8.0 (Fig. 10). Furthermore, the difference in conductance between the two channels is larger at pH 7.5

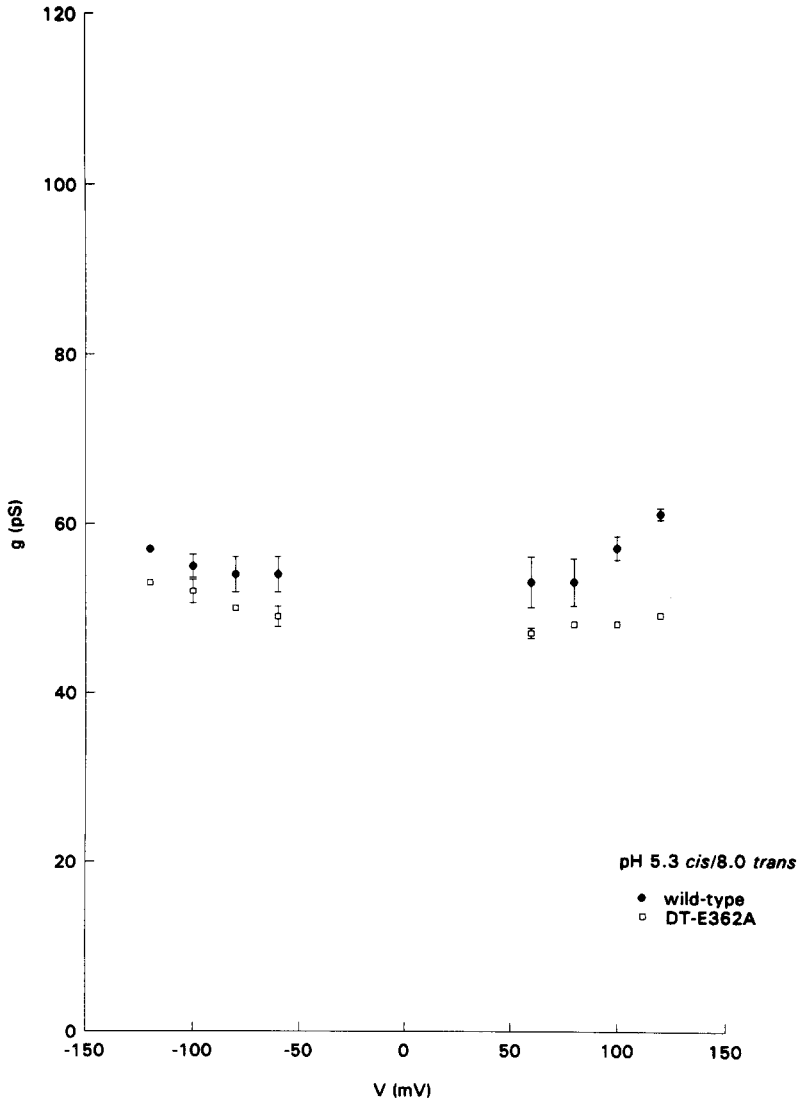

Fig. 10. Comparison of g-V relations for wild-type and DT-E362A channels at pH 5.3 *cis*/8.0 *trans*. Discernable differences in conductance emerge between wild-type and DT-E362A channels when the *trans* pH is 8.0. Compare these data to those in Fig. 5, where the pH conditions are similar, except that the *trans* pH is 7.2. Solutions contained 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA; *cis* solutions were buffered with 5 mM MES pH 5.3; *trans* solutions were buffered with 5 mM Tris pH 8.0. Data points are averages of 2–3 experiments, with error bars indicating standard deviations; points with no error bars indicate voltages at which currents were determined in only one experiment.

Table. Single channel conductances at +100 mV of wild-type DT and DT-E362Q channels at pH 7.6 *cis*/5.5 *trans*

Toxin	Conductance (pS): (at +100 mV; pH 7.6 *cis*/5.5 *trans*)
Wild type	108 ± 11
DT-E362A	75 ± 0

Membranes were formed in 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA; 1 mM citrate *cis* pH 5.4, 1 mM HEPES *trans* pH 7.5. Toxin was added (30 ng), and 10–20 channels were observed to incorporate into the membrane; the *trans* pH was then lowered to pH 5.5 by adding 1 M citrate (to a final concentration of 7 mM), and the *cis* pH raised to 7.6 by adding 1 M Tris (to a final concentration of 60 mM). Most of the channels closed under these new pH conditions, and we obtained single channel current values at only one or two voltages before the last channels were lost. Conductances shown are the mean of two experiments ± SD.

cis/5.5 *trans* (Table) than at 5.3 *cis*/8.0 *trans* (Fig. 10). Together, these observations suggest that it is easier to titrate Glutamate 362 by raising the *cis* pH than by raising the *trans* pH; that is, Glu 362 is "more accessible" from the *cis* compartment than from the *trans*.

All of the data presented for residues 326, 327, and 362 are consistent with the topological model proposed in the previous paper. As predicted by that model, Glutamate 362 lies within the pore of the DT channel. Similarly, the limited evidence presented for Glutamates 326 and 327 suggests that they are near the *cis* side of the channel. The lack of a change in single channel conductance for the mutant H372R is more surprising, but the helical wheel representation of helix TH9 suggests a possible explanation. Assuming that TH9 retains its helical character when inserted in the membrane (*see* Discussion, Mindell et al., 1994), Histidine 372 lies at

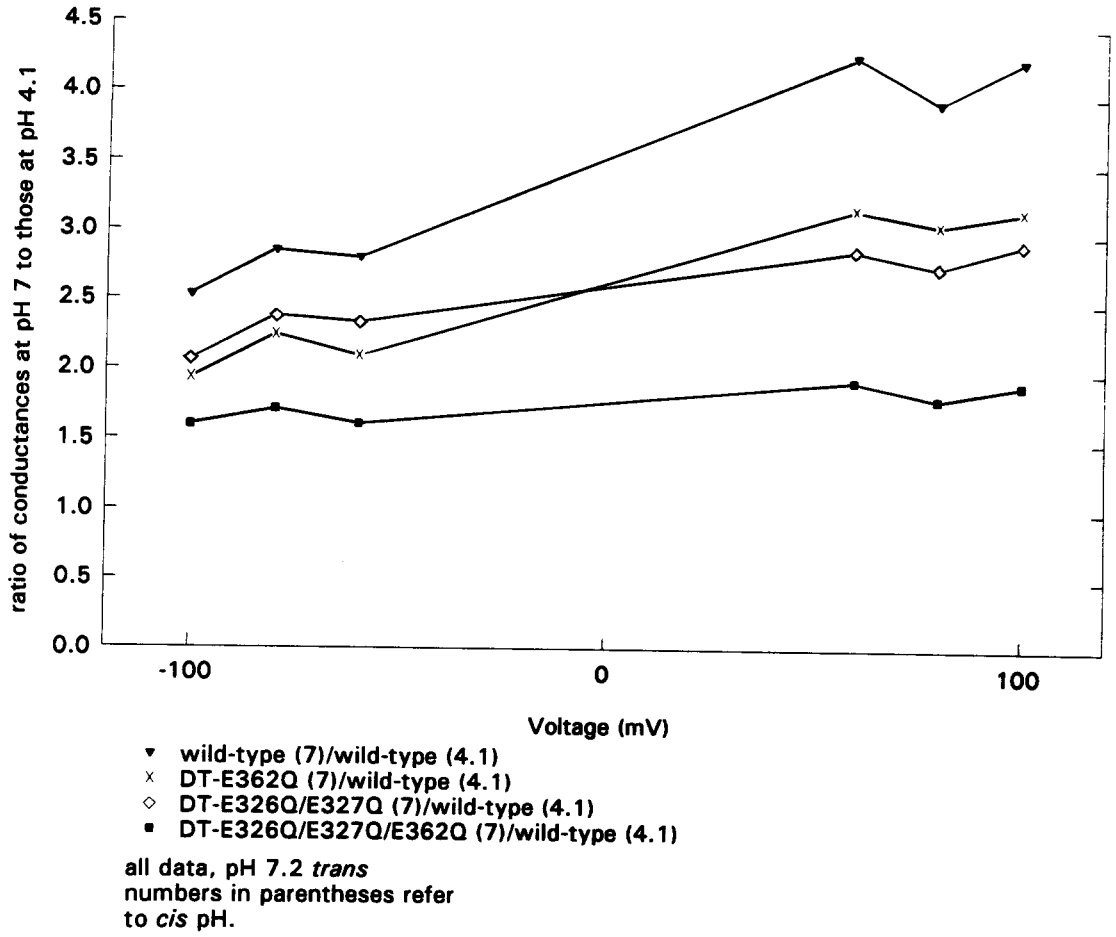

Fig. 11. Effect of *cis* pH on wild-type and mutant DT channels. Ratios of wild-type or mutant (DT-E362Q, DT-E326Q/E327Q, and DT-E326Q/E327Q/E362Q) channel conductance at pH 7.0 *cis* to wild-type channel conductance at pH 4.1 *cis* (all points have pH 7.2 *trans*). Since all four channels have the same conductance at pH 4.1 *cis*/7.2 *trans*, these ratios represent the contribution of charged groups, titrated between pH 4.1 and 7.0 (*cis*), to the conductance of the DT channel. These ratios are calculated from the data in Figs. 2, 4, 6, and 9.

the edge of the helix's nonpolar face; thus, this group may not be directly exposed to the interior of the pore. In this case, channels formed by its mutants would not be expected to differ from that of the wild-type channel in conductance properties.

Comparing the effects, at symmetric neutral pH (where negative residues are fully charged), of mutations at residues 326, 327, 352, and 362 reveals that these groups are not equally important in determining the conductance of the DT channel. The most important group is clearly D352. Figure 7 in the previous paper (Mindell et al., 1994) demonstrates that neutralizing this group causes more than a twofold reduction in channel conductance (at negative voltage), whereas neutralizing E362 causes only a 30% decrease in conductance (Fig. 4), and the depression caused by neutralizing E327 is only about 15% (Fig. 6). Furthermore, we showed in that paper that D352 is an important determinant of the DT channel's ion selectivity; in contrast, neutralization of neither Glutamate 362, nor Glutamates 326 and 327 has a significant effect on ion selectivity (*data not shown*). In fact, the DT channel's ion selectivity is similar at pH 4.1 *cis*/7.2 *trans* to that at pH 7.0 *cis*/7.2 *trans* [−36 mV (pH 4.1 *cis*), −38 mV (pH 7.0 *cis*) in a 1 M:0.1 M (*cis/trans*) KCl gradient], arguing that none of the charges involved in the *cis* pH dependence of conductance greatly influences the channel's ion selectivity. Any structural or functional model of the DT channel should account for these differences.

The differential effects of charges at positions 326/327, 352, and 362 might be explained if the pore of the DT channel is conically shaped, with the narrow end toward the *trans* side of the membrane. If Aspartate 352 lies near this constriction, it would be expected to interact strongly with permeant ions coming from both sides of the membrane. Glutamate 362 could be positioned at a wider region, more distant from ions in the center of the pore; Glutamates 326 and 327 would then be at the widest point of the cone, with relatively weak influence on permeant ions passing through the channel.

A conically shaped channel might also explain the greater sensitivity of Glutamate 362 to *cis* pH than to *trans* pH; in such a channel, groups in the lumen would likely, for steric reasons, be more accessible to protons in the *cis* compartment than to those in the *trans* compartment.

As with residue 352, the apparent pK_a (with respect to bulk pH) for the side chain of Glutamate 362 is significantly shifted upward from the pK_a measured in free solution. Although we have not determined the pK_a of Glu 362, the identity of *g-V* relations for wild-type and DT-E362Q channels at pH 5.3 *cis*/7.2 *trans* (Fig. 5), suggests that Glu 362 is mostly protonated by pH 5.3. Its apparent pK_a is therefore likely to be greater than 6, corresponding to a shift of about two units from the free solution value (Lehninger, 1982), similar to the shift obtained for Aspartate 352 (Mindell et al., 1994).

This work was supported by NIH grants AI22021, AI22848 (R.J.C.), T32 GM07288 (J.A.M.) and GM29210 (A.F.).

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