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Structure Function Relationships in Diphtheria Toxin Channels: II. A Residue Responsible for the Channel's Dependence on *Trans* pH

J.A. Mindell[2], J.A. Silverman[3], R.J. Collier[3], A. Finkelstein[1,2]

[1]Department of Neuroscience, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461
[2]Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461.
[3]Department of Microbiology and Molecular Genetics, and the Shipley Institute of Medicine, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115

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Abstract. Ion-conducting channels formed in lipid bilayers by diphtheria toxin are highly pH dependent. Among other properties, the channel's single channel conductance and selectivity depend on proton concentrations on either side of the membrane. We have previously shown that a 61 amino acid fragment of DT is sufficient to form a channel having the same pH-dependent single channel properties as that of the intact toxin. This region corresponds to an α-helical hairpin in the recently published crystal structure of DT in solution; the hairpin contains two α-helices, each long enough to span a membrane, connected by a loop of about nine residues. This paper reports on the single channel effects of mutations which alter the two negatively charged residues in this loop. Changing Glutamate 349 to neutral glutamine or to positive lysine has no effect on the DT channel's single channel conductance or selectivity. In contrast, mutations of Aspartate 352 to neutral asparagine (DT-D352N) or positive lysine (DT-D352K) cause progressive reductions in single channel conductance at pH 5.3 *cis*/7.2 *trans* (in 1 M KCl), consistent with this group interacting electrostatically with ions in the channel. The cation selectivity of these mutant channels is also reduced from that of wild-type channels, a direction consistent with residue 352 influencing permeant ions via electrostatic forces. When both sides of the membrane are at pH 4, the conductance difference between wild-type and DT-D352N channels is minimal, suggesting that Asp 352 (in the wild type) is neutral at this pH. Differences observed between wild-type and DT-D352N channels at pH 4.0 *cis*/7.2 *trans* (with a high concentration of permeant buffer in the *cis* compartment) imply that residue 352 is on or near the *trans* side of the membrane. Comparing the conductances of wild-type and DT-D352K channels at large (*cis*) positive voltages supports this conclusion. The *trans* location of position 352 severely constrains the number of possible membrane topologies for this region.

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

Introduction

Previous workers have demonstrated that the channels formed by Diphtheria Toxin (DT) in lipid bilayers are of large diameter (Kagan, Finkelstein & Colombini, 1981; Hoch et al., 1985), and that their conduction properties are strongly pH dependent (Hoch & Finkelstein, 1985). Hoch (1985) also showed that although the ion selectivity of the DT channel varies with pH, glucuronate at low pH and glucosamine at high pH are roughly equally permeant, suggesting that the physical dimensions of the pore are not altered by pH. This result implies that ion transport through the DT channel is largely controlled by coulombic forces between permeant ions and charged (titratable) residues within or near the channel wall. In this view, changing the pH of the bathing solutions alters the charge on these groups, thereby modulating the energetics of ion channel interactions.

In the previous paper (Silverman et al., 1994), we found that a very short, 61 amino acids region of DT (TH8-9) is sufficient to form ion-conducting channels

Correspondence to: A. Finkelstein

nearly identical in their ion-permeation properties to those formed by whole toxin. Importantly, the channels formed by this mutant seem to retain the pH-dependent single channel properties of the intact toxin. Thus, we must conclude that this fragment contains all (or most of) the residues whose titration affects ion permeation through the channel. The recently solved crystal structure of DT reveals that the TH8-9 region forms a single "helical hairpin" in the crystal (Choe et al., 1992). This "hairpin" consists of two antiparallel α-helices (TH8 and TH9 in the crystal), each long enough to span a membrane, connected by a short (9 amino acids) loop. Spectroscopic data (Cabiaux et al., 1989), indicate that the secondary structure of the DT B fragment is largely preserved when it inserts into liposomes, suggesting that the TH8-9 hairpin may insert as such into membranes.

In this paper we report the effects on single channel conductance and ion selectivity of mutating, to neutral and positively charged groups, the two negatively charged residues (Glu 349 and Asp 352) in the loop connecting helices TH8 and TH9, and find that although single channel properties are invariant to the charge at position 349, they are profoundly affected by that at position 352. The differences in single channel conductance between wild-type channels and those with mutations at position 352 are pH dependent; by comparing the effects on these channels of changing the *cis* or *trans* pH, we conclude that Asp 352 is located on or very near the *trans* side. Some of the data presented here were reported previously in abbreviated form (Mindell et al., 1992).

Materials and Methods

Construction and Preparation of Mutant Proteins

These methods will be described in detail elsewhere (J.A. Silverman, J.A. Mindell, A. Finkelstein, R.J. Collier, *in preparation*). Briefly, point mutants were constructed via oligo-directed mutagenesis in an M13 derived single-stranded vector, and subcloned into a derivative of pDO1 (O'Keefe et al., 1992). This plasmid contains DT-E148S under the control of the IPTG-inducable *tac* promoter, and results in periplasmic expression of the encoded toxin, since the DT gene contains a bacterial signal sequence. For cytoplasmic expression, a similar vector, from which the signal sequence had been deleted, was used.

Mutant toxins were expressed in *Escherichia coli* induced with IPTG. For toxins secreted into the periplasm (those with signal sequences), extracts were prepared by osmotic shock followed by centrifugation of cells, and concentrated prior to further use. For toxins in the cytoplasm (those without signal sequences), extracts were similarly prepared from cells lysed by sonication. Toxins were nicked using trypsin, and levels of cross-reacting material were quantitated using densitometric scans of Western blots. Extracts containing stable DT derivatives were used directly in lipid bilayer experiments. Such lysates prepared from *E. coli* lacking a gene for DT caused no channel activity in lipid bilayers. Protein preparations were stable indefinitely at −20°C. Before use, extracts were diluted in 25 mM Tris, 1 mM EDTA; these dilutions were stable for up to two weeks at 4°C.

Lipid Bilayers

Asolectin lipid bilayers were formed using the method described in the previous paper (Silverman et al., 1994). For experiments in KCl, the solutions contained 1 M or 0.1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA, and an appropriate pH buffer. In experiments with KCl gradients, we ensured the magnitude of the gradient by replacing both *cis* and *trans* solutions immediately before forming the membrane. Reversal potentials were always corrected for electrode offset, which was never more than 2.5 mV. Tetraethylammonium chloride (TEACl, Fluka Chemical, Ronkonkoma, NY) stock solutions were twice filtered after treatment with activated charcoal. Solutions for experiments in 1 M TEACl contained 2 mM $CaCl_2$, 1 mM EDTA and pH buffer; these were brought to their final pH's with Bis-Tris-Propane or HCl to avoid adding monovalent cations. Agar bridges contained 1 M TEACl instead of 3 M KCl for experiments in TEACl. Solutions for experiments in potassium glycerate contained 1 M or 0.1 M K glycerate, 2 mM Ca glycerate, and 1 mM EDTA. Solutions for experiments in TrisCl contained 1 M or 0.1 M TrisCl, 1 mM EDTA, and were titrated with HCl or TrisOH. For membranes in TrisCl and K glycerate gradients, bridges contained 1 M of the appropriate salt *cis*, and 0.1 M of the appropriate salt *trans*.

Reversal potentials in K glycerate and TrisCl gradients were measured by zeroing the current for membranes containing many channels, since single channel currents were unresolvable at the small voltages necessary for determining reversal potentials. Equilibrium potentials for potassium were measured by adding the K^+ carrier valinomycin (final concentration 1 μg/ml) and zeroing the current. The valinomycin-induced conductance was at least 100-fold greater than that pre-existing from the DT channels. Open hole potentials (diffusion potentials) were determined at the end of each experiment.

Channel-forming proteins were added in all experiments to the *cis* compartment to a final concentration of 1–60 ng/ml, depending on the toxin and the pH conditions. Voltages are those of the *cis* compartment with respect to that of the *trans*, which is taken as zero. The recording system was the same as described previously (Silverman et al., 1994). For some of these experiments, manual analysis was impossible because the channels could not be resolved on the chart recorder. The data for those experiments were analyzed with a computer data acquisition system (Axon Instruments, Foster City, CA), using either manual adjustment of screen cursors, all points amplitude histograms, or both, depending on the situation.

Results

Mutations of Aspartate 352: Conductance Effects

pH 5.3 cis/pH 7.2 trans

We have compared channels formed by wild-type DT to those formed by the mutants DT-D352N and DT-D352K, which change the negatively charged residue at position 352 to a neutral and a positively charged group,

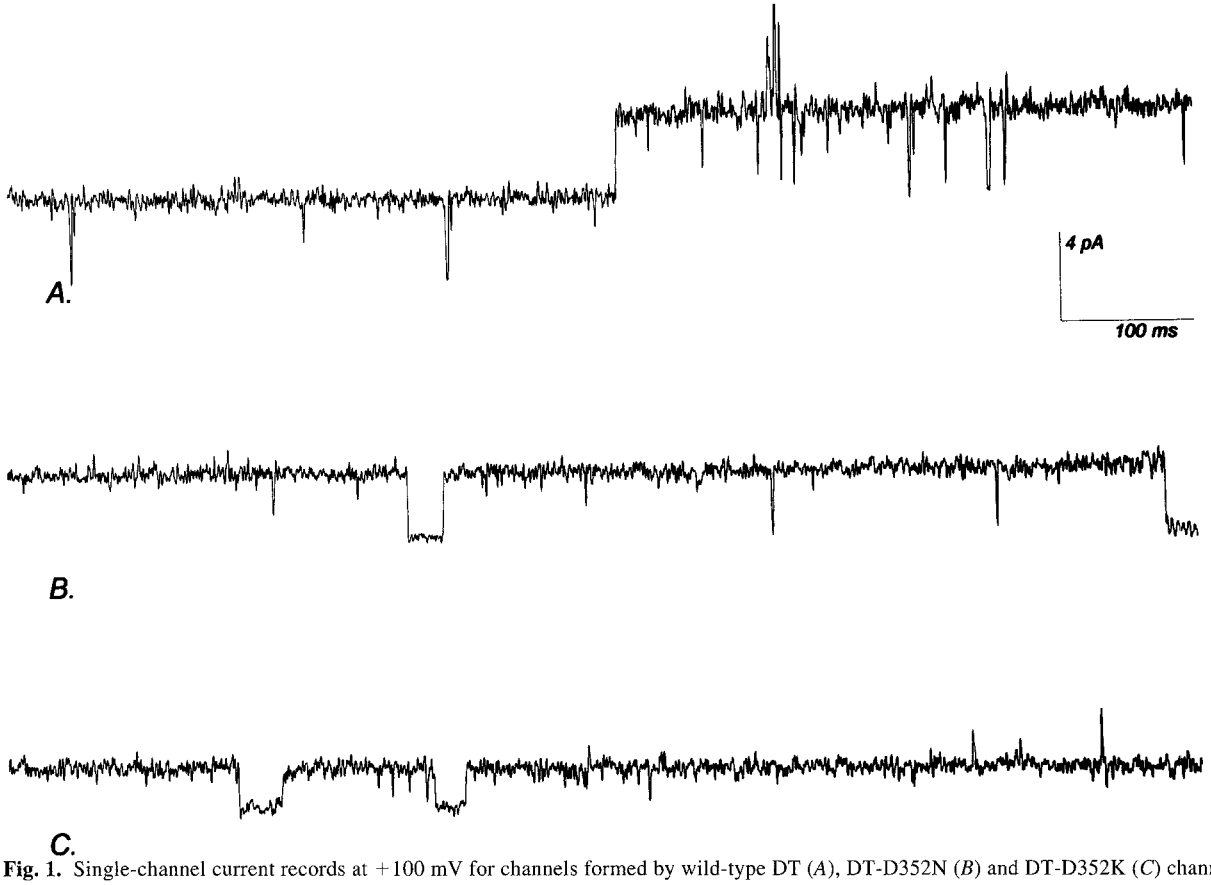

Fig. 1. Single-channel current records at +100 mV for channels formed by wild-type DT (*A*), DT-D352N (*B*) and DT-D352K (*C*) channels in 1 M KCl at pH 5.3 *cis*/7.2 *trans*. The upper current level represents the open channel; the channels are mostly open with brief closures. Note the sequential reduction in single channel current from wild-type to DT-D352N to DT-D352K. Transitions which appear as incomplete closures are actually full closures which are unresolved by the recording system. A second channel opens about halfway through the current trace for the wild-type channel. Solutions on both sides of the membrane contained 1 M KCl, 2 mM $CaCl_2$, and 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), 5 ng (DT-D352N), or 24 ng (DT-D352K) of protein were added to the 1 ml volume in the *cis* compartment (the *trans* side is considered ground). These 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.

respectively. The conductances of DT channels are highly pH dependent; for our initial study the pH conditions were pH 5.3 *cis*/7.2 *trans,* proton concentrations similar to those which stimulate the translocation of the A fragment across the endocytic membrane. Figure 1 illustrates single channel current records for wild-type DT, DT-D352N, and DT-D352K channels under these pH conditions in 1 M KCl. Note that the current through both mutant channels is significantly reduced from the wild-type value. This observation is extended in Fig. 2, where single channel currents and conductances are plotted as a function of voltage; current through the DT-D352N channel is reduced from that through the wild type by 30–40% at all voltages; current through the DT-D352K channel is reduced a further 40% throughout the voltage range. The channels are not perfectly ohmic; the slight superlinearities of the current-voltage (*I-V*) curves at both positive and negative voltages are especially evident in the conductance-voltage (*g-V*) plot. The effects on channel conductance of these Asp 352 mutations are consistent with this residue interacting electrostatically with ions traversing the pore, given that under the pH conditions of these experiments the DT channel is reasonably cation selective (37 mV for the wild-type channel in a 10:1 KCl concentration gradient; *see later section*). Thus, as the charge on residue 352 changes from negative to neutral to positive, it becomes progressively more difficult for cations to get through the channel.

Symmetric pH 4.0

The side chains of aspartate and asparagine are similar, with the carboxyl group in aspartate replaced by an amide in asparagine. At low pH both are uncharged, but at neutral pH aspartate's side chain is negatively charged, whereas asparagine's remains uncharged. Thus, if a charge at residue 352 is exerting an electro-

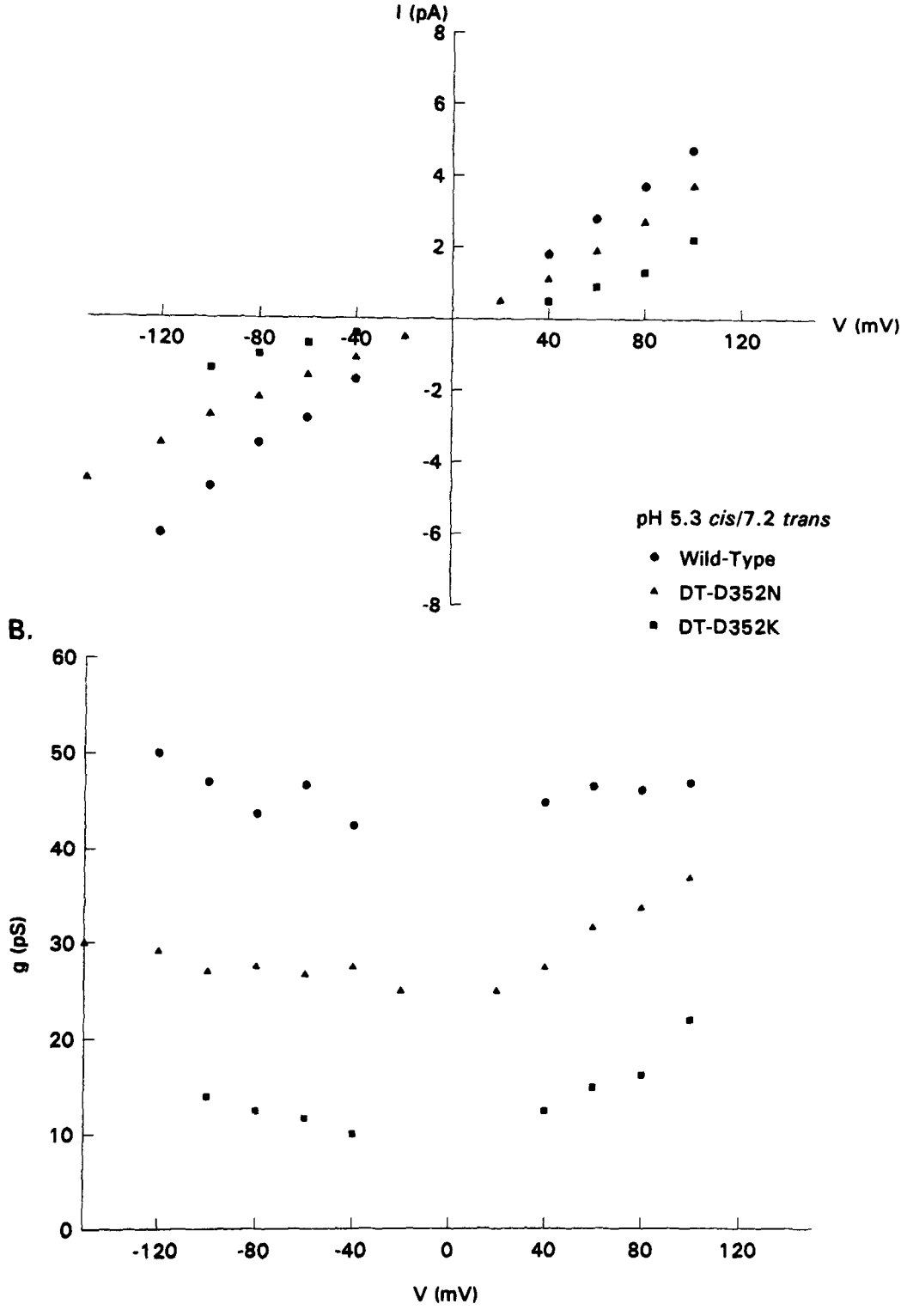

Fig. 2. Single channel current-voltage (*A*) and conductance-voltage (*B*) relations for wild-type, DT-D352N and DT-D352K channels in 1 M KCl at pH 5.3 *cis*/7.2 *trans*. These data are from the experiments illustrated in Fig. 1; currents were measured by hand from current records like those shown in that figure. The values shown here (and in all figures) are representative; measured values under given salt and pH conditions varied less than 10% among experiments. Conductance (g) values in *B* were calculated by dividing the corresponding current (I) in *A* by the applied voltage (V); i.e., $g \equiv I/V$.

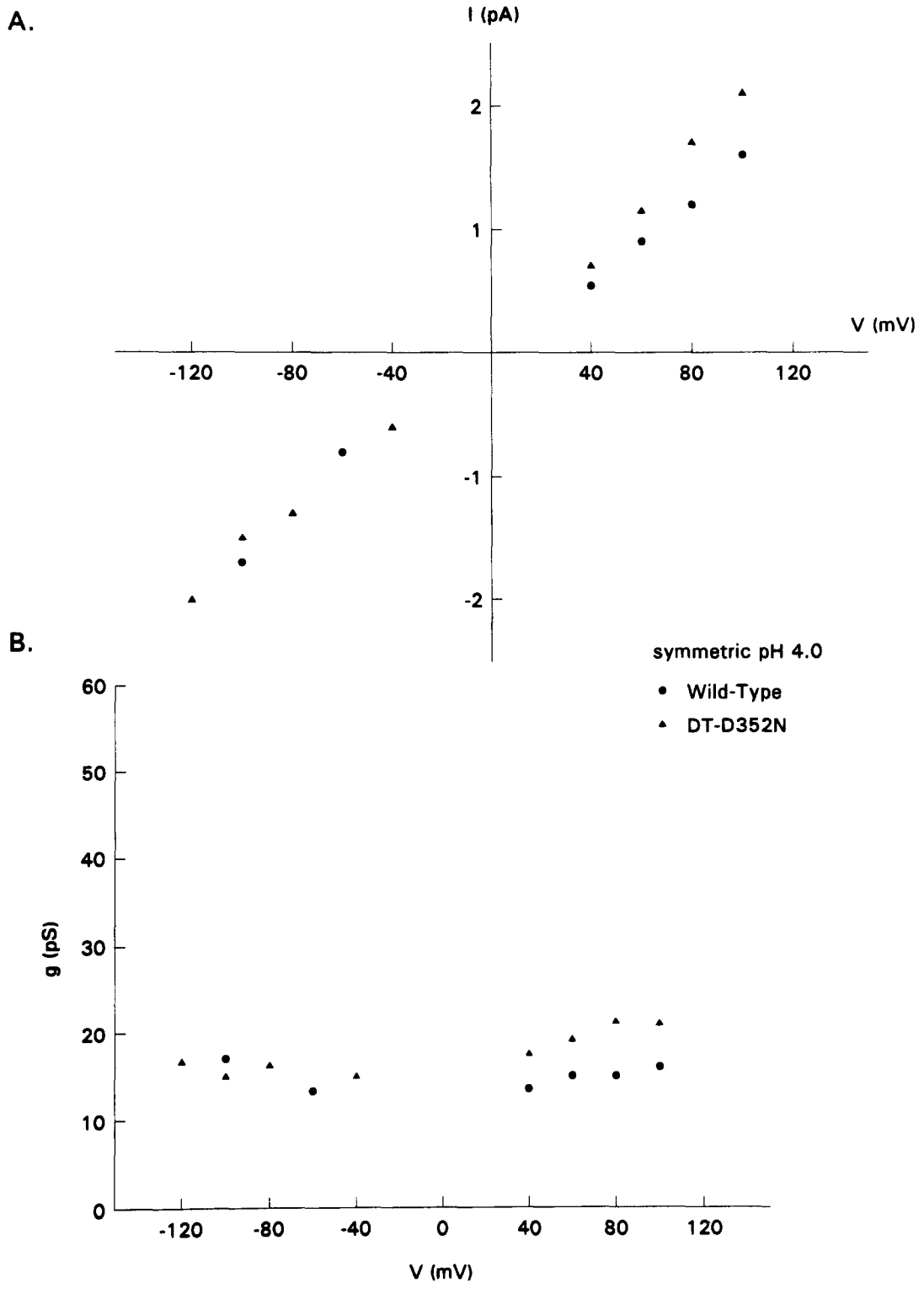

Fig. 3. Single channel current-voltage (*A*) and conductance-voltage (*B*) relations at symmetric pH 4.0. The conductances of wild-type and DT-D352N channels converge at low pH, indicating that at low pH the aspartate at position 352 in the wild-type channel and the asparagine at this position in DT-D352N have similar effects on permeant ions. Although the conductance of the DT-D352N channel is slightly larger than that of the wild type at positive voltages, this difference never exceeds 15% [as opposed to the almost twofold larger conductance of the wild-type channel at pH 5.3 *cis*/7.2 *trans*, (Fig. 2)]. Solutions in both compartments contained 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA, and 10 mM citrate buffer, pH 4.0. 33 ng (wild type) or 20 ng (DT-D352N) of toxin were added to the *cis* compartment to induce channel activity. Note that the channel conductances under this condition are much smaller than those shown in Fig. 2.

static influence on channel conductance, then lowering the pH, thereby protonating the wild-type aspartate's carboxyl group, should cause the conductances of wild-type and DT-D352N channels to converge. Figure 3 illustrates *I-V* and *g-V* relations for wild-type and DT-D352N channels at symmetric pH 4.0; they are nearly identical, suggesting that the aspartate in wild-type DT is indeed protonated at this low pH and exerts an electrostatic influence on channel conductance at higher pH's.

Conductance in TEACl

We further the electrostatic nature of residue 352's interaction with permeant ions by comparing single channel currents through wild-type and DT-D352K channels in 1 M TEACl. Since TEA^+ is much less permeant than K^+, even at pH 5.3 *cis*/7.2 *trans* the DT channel conducts mostly anions in this salt (Romero, 1988). Figure 4 presents current records from these experiments. Although the measured currents are very small, we see that the single channel currents through the DT-D352K channel are actually slightly *larger* than those through the wild-type channel! Again, this implies that the amino acid at position 352 primarily interacts electrostatically with permeant ions.

Locating Residue 352

We have just seen that the charge on Aspartate 352 can be titrated; by changing the pH independently on either side of the membrane, we can find the location of this group within the pore. If the *cis* compartment is at pH 4.0 with a high concentration (55 mM) of the permeant buffer glycerate (*see below*) and the *trans* compartments is at pH 7.2 with a low concentration of (presumably) impermeant buffer, then the permeant buffer in the *cis* compartment should also diffuse into the pore, clamping the channel lumen at about pH 4.0 throughout most of its length. Thus, if residue 352 is on the *cis* side or within the channel lumen, the pH it "sees" should be about 4, and the aspartate in wild-type toxin will be neutral; there will then be no difference in conductance between the wild-type and DT-D352N channels. If, on the other hand, residue 352 lies near the *trans* compartment, it will "see" pH 7.2, and the aspartate should be negatively charged (whereas asparagine is still neutral); the channels formed by wild-type and DT-D352N will then have different conductances. Figure 5 presents *I-V* (and *g-V*) curves under these pH conditions for wild-type and DT-D352N channels. Note that the conductance of the wild-type channel is larger than that of the DT-D352N channel (particularly at negative voltages), thereby indicating that residue 352 of the DT channel resides in or near the *trans* compartment. This result is independent of buffer choice for the *trans* compartment; the same result was obtained using positively charged Bis-Tris-Propane, negatively charged HEPES, or even EDTA (which has a charge of about -3 at this pH). Conversely, if the *trans* compartment is held at pH 4.0 and the *cis* at pH 7.2 under similar buffering conditions, the wild-type and DT-D352N channels have indistinguishable conductances (*data not shown*). Thus, since a difference arises between the two channels if, and only if, the *trans* compartment is at high (neutral) pH, residue 352 must be on, or near the *trans* side of the membrane.

Titration Curve of Aspartate 352

Comparison of the *g-V* curve for the DT-D352N channel at pH 4.0 *cis*/4.0 *trans* (Fig. 3) with that at pH 4.0 *cis*/7.2 *trans* (Fig. 5) indicates that the conductance of this mutant channel is independent of the *trans* pH between pH 4.0 and 7.2, implying that Aspartate 352 is responsible for *all* of the *trans* pH effect on the wild-type channel's conductance. This is strikingly demonstrated in Fig. 6, in which conductance at -100 mV (where the difference between wild-type and DT-D352N channel conductances is maximal) is plotted against the pH of the *trans* compartment (*cis* and lumen held at pH 4.0). Clearly, the conductance of the DT-D352N channel is independent of *trans* pH between pH 4.0 and 7.0, whereas the conductance of the wild-type channel changes by a factor of 2 over this pH range; its pH dependence is fit by a standard titration curve, with $pK_a = 5.5$.

Behavior at Large Voltages

If position 352 is indeed on or near the *trans* side of the membrane, then under conditions where current is carried primarily by ions from the *cis* compartment, we would predict that the conductances of mutant DT-D352N and DT-D352K channels should converge to that of the wild-type channel. This situation is realized at large positive voltages in symmetric neutral pH; since channels formed by all three toxins are relatively cation selective under these pH conditions, cations traveling from *cis* to *trans* will be the major current carrier at large positive voltages. Figure 7*A* compares *g-V* data for wild-type, DT-D352N and DT-D352K channels at pH 7.0 *cis*/7.2 *trans*. Although channels formed by these toxins exhibit as much as a fourfold difference in conductance at negative voltages, their conductances converge at large positive voltages. This behavior is dramatically displayed in Fig. 7*B*, where the ratio of the wild-type channel conductance to that of DT-D352N and DT-D352K channels is plotted as a function of voltage and is seen to approach 1 at $+200$ mV. Such convergence of conductances supports the conclusion

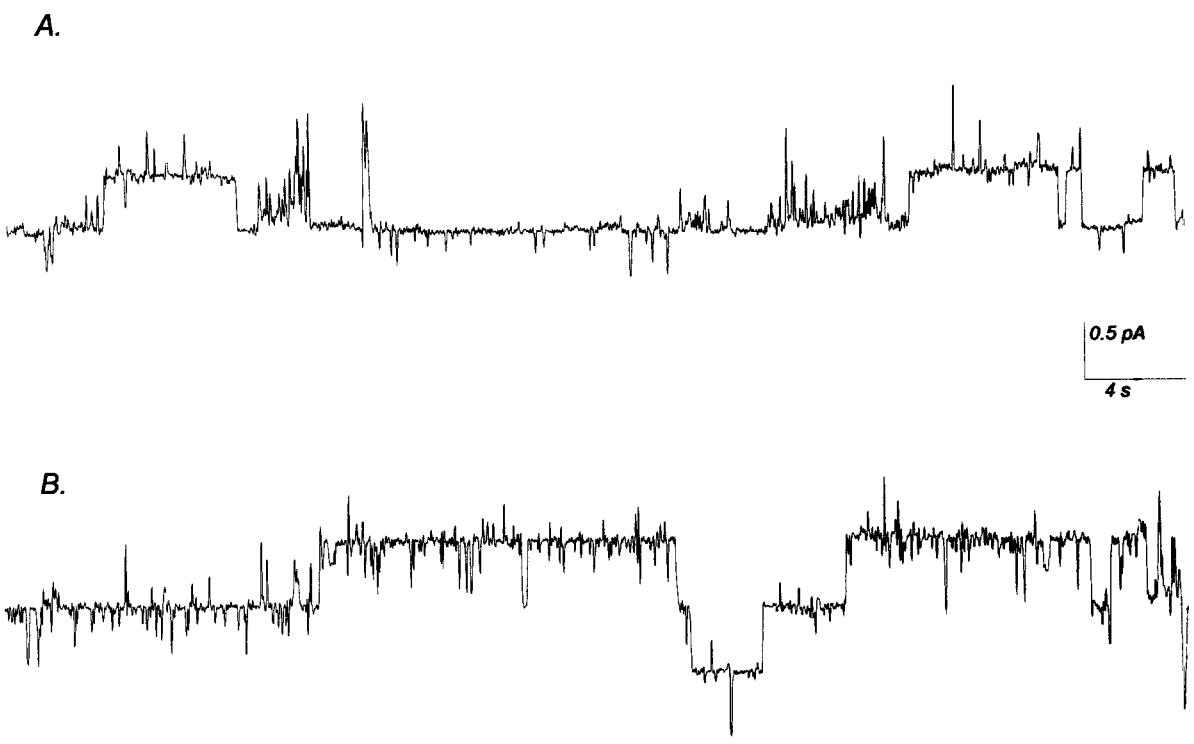

Fig. 4. Single channel current records for wild-type (*A*) and DT-D352K (*B*) channels in 1 M TEACl. In 1 M TEACl, the current through the DT-D352K channel is slightly larger than that through the wild-type channel. The wild-type current corresponds to a conductance of 4.0 pS; the DT-D352K current corresponds to 4.7 or 5.1 pS. Since the DT channel prefers anions in TEACl, this observation is consistent with residue 352 interacting electrostatically with permeant ions. Voltage was clamped at +150 mV. Solutions on both sides of the membrane contained 1 M TEACl, 2 mM $CaCl_2$, and 1 mM EDTA. The *cis* compartment was buffered at pH 5.3 with 30 mM MES; the *trans* at pH 7.2 with 10 mM HEPES. Precautions were taken to avoid contaminating monovalent cations (*see* Materials and Methods). 0.2 ng wild-type toxin or 5 ng DT-D352K were added to the *cis* compartment. Records were filtered at 10 Hz. All points histograms of amplitude data were fit well with Gaussian curves. (Peaks at 0 and 0.6 pA for wild-type channels and peaks at 0, 0.70, and 1.46 pA for DT-D352K channels, of which there were two in the current record shown. Standard deviations were about 0.1 pA.)

that residue 352 is located on or near the *trans* side of the DT channel.

Mutations of Aspartate 352: Selectivity Effects

The effects of mutations at residue 352 on channel ion selectivity support the "electrostatic" role of this residue. At pH 5.3 *cis*/7.2 *trans* with 1 M KCl *cis* and 0.1 M KCl *trans*, the reversal potential (E_{rev}) is −38 mV for the wild-type channel (E_{rev} = −51 mV for an ideally cation-selective channel), −33 mV for the DT-D352N channel, and −11 mV for the DT-D352K channel[1] (Fig. 8). All of these channels are cation selective, but as residue 352 goes from negative to neutral to positive, this preference diminishes. Again, these are the qualitative effects expected from the replacement of the negative aspartate in the wild-type channel with neutral and positively charged groups, if these groups interact electrostatically with permeant ions.

pH Dependence of Selectivity

Table 1*A* presents reversal potentials for channels formed by wild-type and DT-D352N channels in a 10:1 KCl gradient at a series of pH conditions. Note that these channels have similar reversal potentials at symmetric pH 4.0, suggesting that protonated aspartic acid and asparagine are chemically equivalent, at least as far as permeant ions are concerned. The reversal potential for the DT-D352N channel, as opposed to its conductance, does manifest some *trans* pH dependence; this is apparent when we compare E_{rev} measured for the DT-D352N channel at symmetric pH 4.0 (−15 mV) with that measured for pH 4.0 *cis*/5.1 *trans* (−27 mV); its values are near those for the wild-type channel in this range. All of the DT-D352N channel's *trans* pH de-

[1] When the KCl gradient is reversed, i.e., 0.1 M KCl *cis*, 1 M KCl *trans*, the reversal potentials are of similar magnitude to those shown for 1 M KCl *cis*, 0.1 M KCl *trans* (for a given pH gradient).

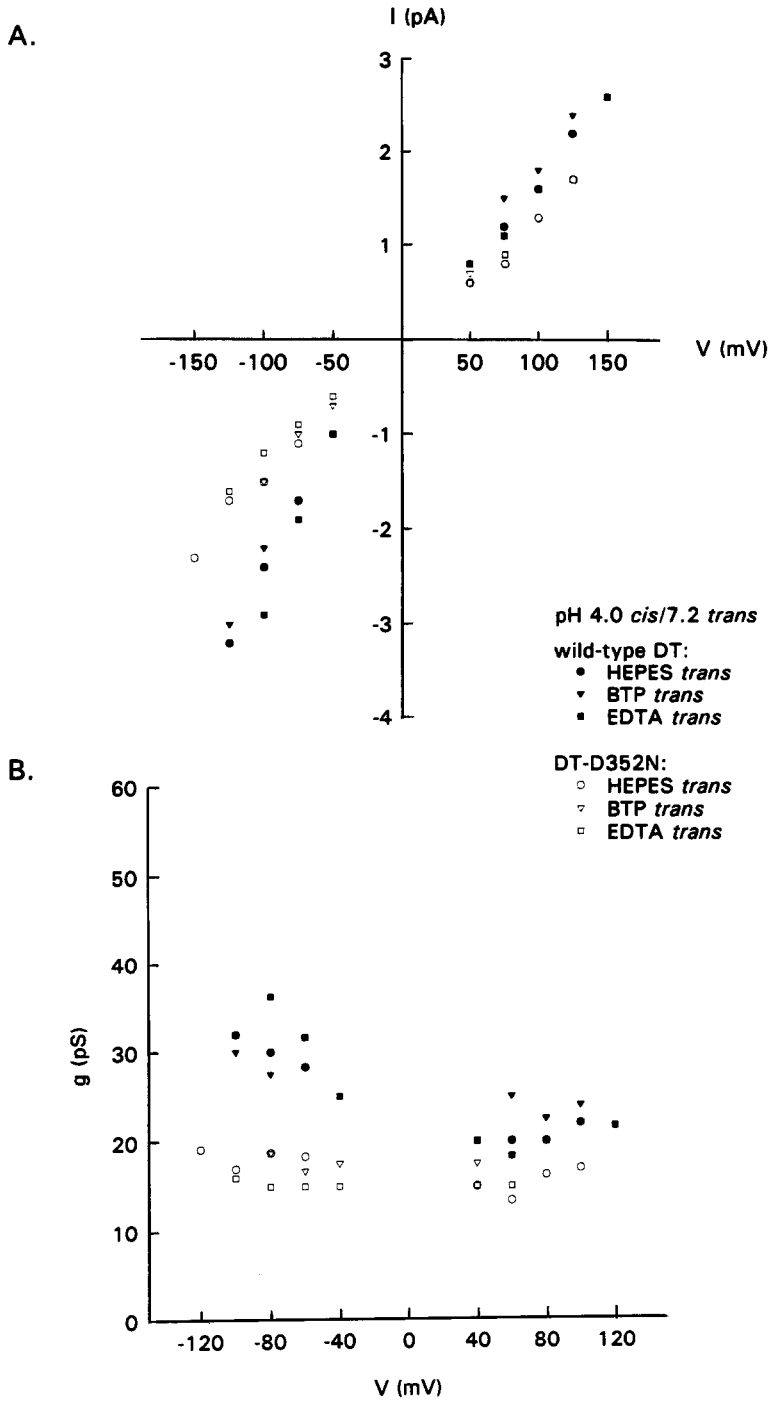

Fig. 5. Demonstration that residue 352 is on or near the *trans* side of the membrane: "lumenal pH clamp." The *cis* compartment and channel lumen are held at pH 4.0, and the *trans* compartment at pH 7.2. Note the divergence, at negative voltages, between wild-type and DT-D352N channels. Current *vs.* voltage (*A*) and conductance *vs.* voltage (*B*). Experiments were done with three different buffer molecules in the *trans* compartment; the result is clearly independent of buffer choice. Membranes were formed with 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA on both sides and 5 mM glycerate pH 4.0 *cis;* on the *trans* side there was either 5 mM HEPES, 5 mM Bis-Tris-Propane (BTP), or an additional 9 mM EDTA, pH 7.2. After membrane formation, 1 M glycerate pH 4.0 was added to the *cis* compartment to a final concentration of 55 mM; 0.1–0.25 ng of wild-type or DT-D352N protein were subsequently added to the *cis* compartment. After the experiment, pH was measured in both compartments; it never differed from the intended pH by more than 0.05 units. Because single channel currents in these membranes became obscured with time by aggregate channels (Romero, 1988), voltages were rapidly changed using a computer program which pulsed a range of voltages for 0.5 sec each, and single channel currents were measured from digitized data using manual adjustment of cursors on a computer screen.

pendence of selectivity occurs between pH 4.0 and 5.1; E_{rev} at *trans* pH 7.2 is the same as that at *trans* pH 5.1. Differences between wild-type and DT-D352N channel selectivity largely emerge between *trans* pH 5.1 and 7.2 (Table 1), consistent with the wild-type aspartate's pK_a of 5.5.

What groups are titrated between pH 4.0 and 5.1 in the DT-D352N channel? Since the channel formed by the deletion mutant TH8-9, containing primarily the TH8 and TH9 helices and their connecting loop, has the same selectivity as that formed by wild-type toxin at pH 5.3 *cis*/7.2 *trans* (Silverman et al., 1994), conditions where all acidic groups in the *trans* compartment should be charged, there are only a limited number of contenders. The most obvious of these is Glu 349, the second negatively charged group in the TH8-9 loop. Selectivity data for channels formed by DT-E349Q and the double mutant DT-E349Q/D352N, however, reveal that

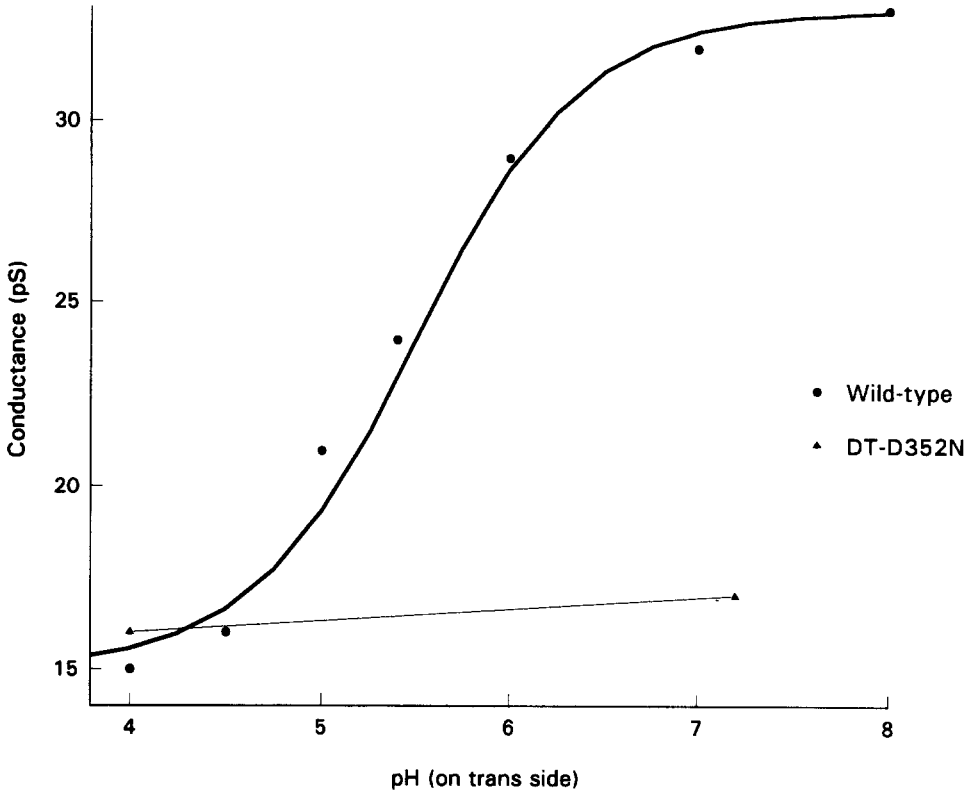

Fig. 6. "Titration curve" of aspartate 352. Channel conductance (at −100 mV) for wild-type and DT-D352N channels plotted against *trans* pH for experiments similar to those illustrated in Fig. 5. Although the DT-D352N channel's conductance is essentially independent of *trans* pH, the wild-type channel's conductance is highly pH dependent. The heavy unbroken line is a standard titration curve with $pK_a = 5.5$; the light unbroken line is straight. Solutions on both sides contained 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA. Except at symmetric pH 4.0, the *cis* compartment contained 55 mM glycerate (added after membrane formation to avoid its diffusion through the hole into the *trans* compartment). The *trans* compartment contained buffer appropriate to the desired pH: 5 mM succinate (pH 4.5, 5.0, 5.5), 5 mM MES (pH 6.0), 5 mM HEPES (pH 7.2, 8.0). For experiments at symmetric pH 4, both compartments contained 10 mM citrate. 0.3 ng–32 ng of wild-type DT were added to the *cis* compartment. The points shown for DT-D352N channels are taken from Figs. 3 and 5.

the charge at position 349 has no effect on channel selectivity: DT-E349Q channels have essentially the same selectivity as that of the wild-type channel (−36 mV in a 1 M/0.1 M KCl gradient, pH 4.0 *cis*/7.2 *trans*), and DT-E349Q/D352N channels have roughly the same selectivity as that of DT-D352N channels (−31 mV in a 1 M/0.1 M KCl gradient, pH 5.3 *cis*/7.2 *trans*). Thus, Glutamate 349, the one remaining negatively charged group thought to be on the *trans* side of the DT channel, is not responsible for the *trans* pH dependence (between pH 4.0 and 5.1) of selectivity found in the DT-D352N channel.

An alternative explanation for the *trans* pH dependence of selectivity in DT-D352N channels is partial titration of the negative charges on the lipid phosphate groups, but this is unlikely given their low pK_a's [about 1.5 (Tocanne & Teissié, 1990)]. A more likely explanation is titration of the carboxyl groups of fatty acids contaminating the lipid used in our experiments. Whatever the explanation, it is possible to screen this effect by increasing the salt concentration in the *trans* compartment. Table 1*B* presents selectivity data for channels formed by wild-type DT and DT-D352N at a series of *trans* pH values (*cis* pH 4.0) in a 1 M/0.3 M (*cis*/*trans*) KCl gradient. At pH 4.0 *trans* the wild-type and DT-D352N channels have reversal potentials of −10 and −11 mV, respectively, substantially identical values. At pH 5.1, however, the selectivities for the two channels diverge, with the DT-D352N channel remaining essentially constant (−12 mV) and the wild-type channel becoming increasingly cation selective (−16 mV). At pH 7.2 *trans* the difference is still larger: E_{rev} for the DT-D352N channel is −14 mV, whereas E_{rev} for the wild-type channel is −22 mV. Thus, by increasing the KCl concentration of the *trans* compartment, we virtually eliminate the *trans* pH dependence of selectivity of the DT-D352N channel but not that of the wild-type channel. This is consistent with the suggestion that the negative charges responsible for the *trans* pH dependence of selectivity observed for DT-D352N channels in 0.1 M KCl (*trans*) can be screened by increasing the *trans* [KCl] to 0.3 M.

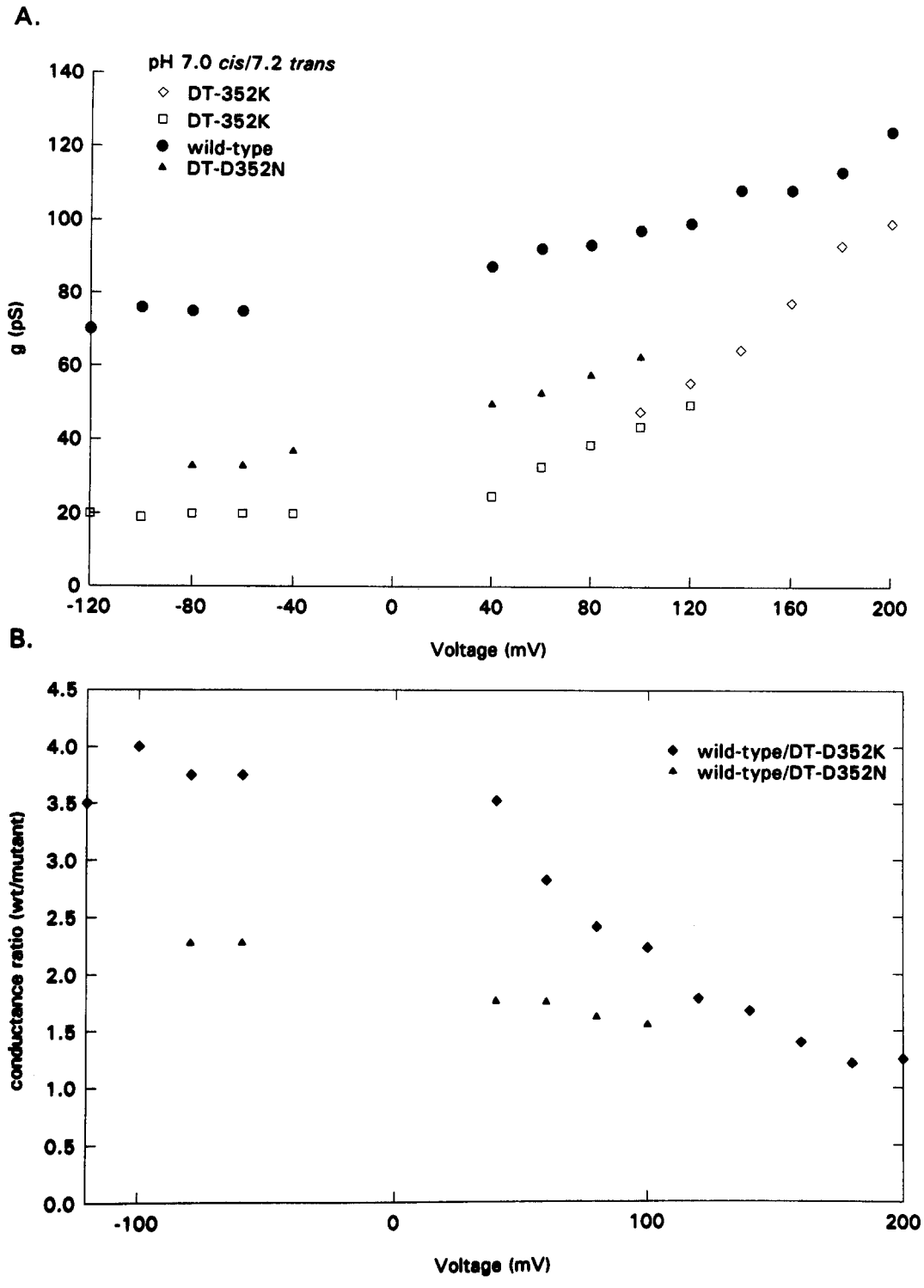

Fig. 7. Convergence of wild-type, DT-D352N, and DT-D352K channel conductances at large positive voltages. Conductance-voltage relations of wild-type, DT-D352N, and DT-D352K channels (*A*) and the ratio of wild-type channel conductance to that of DT-D352N and DT-D352K channels as a function of voltage (*B*) in 1 M KCl at symmetric pH 7 (where both channels are relatively cation selective). Although the conductances of these channels differ by as much as a factor of four at negative voltages, they converge to near equality at large positive voltages. Note that the two different symbols shown for DT-D352K represent the two experiments required to obtain data for this mutant over the entire voltage range. Membranes were formed and toxin added under the same conditions as in Figs. 1 and 2: 1 M KCl, pH 5.3 *cis*/7.2 *trans*. After several channels had opened, the *cis* pH was raised to 7.0 by the addition of 1 M HEPES to a final concentration of 100 mM, since DT channels rarely form spontaneously at symmetric neutral pH.

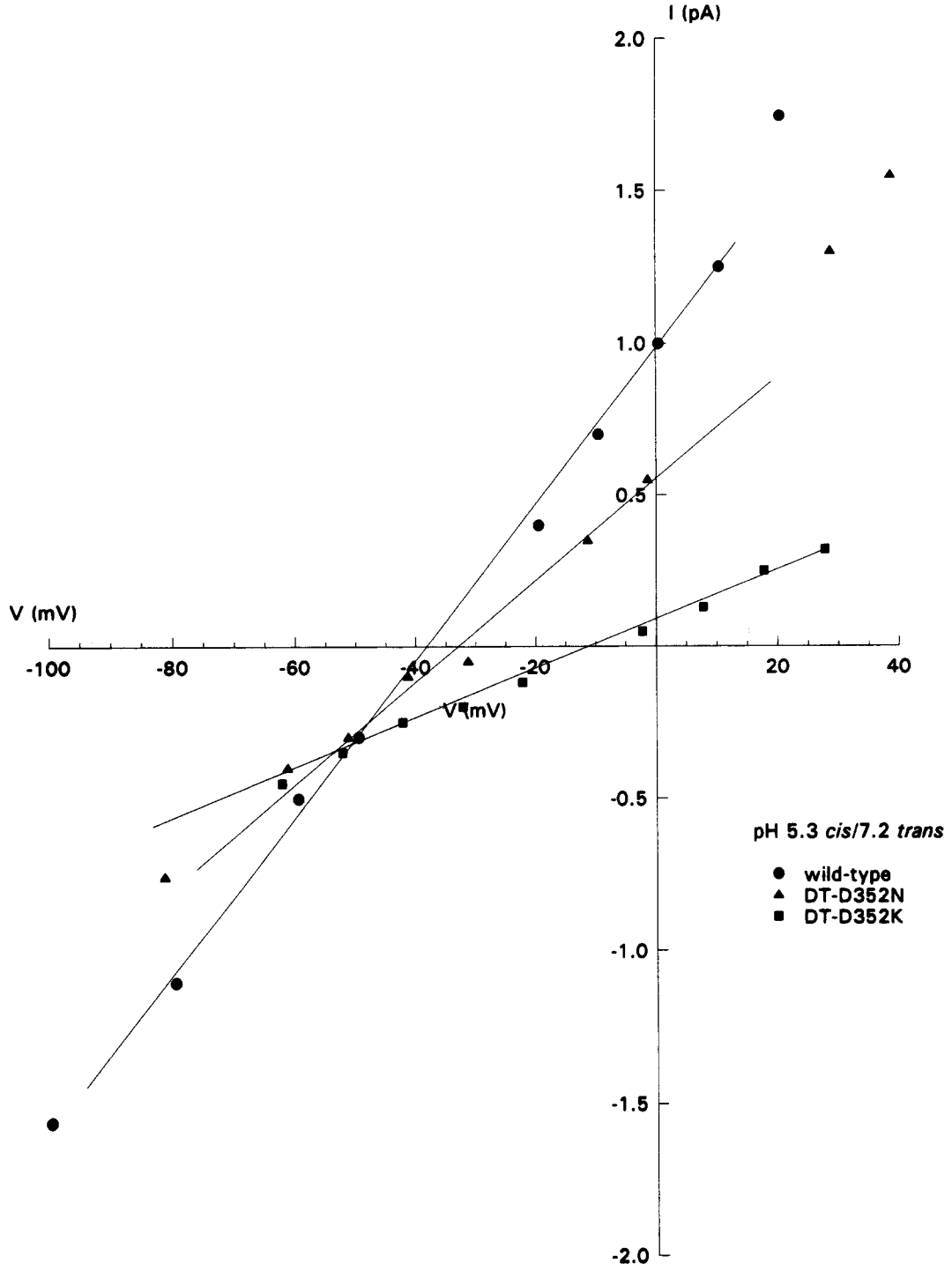

Fig. 8. Single channel current-voltage relations in 10:1 KCl gradient: Reversal potentials. Single channel currents for DT, DT-D352N, and DT-D352K channels at pH 5.3 *cis*/7.2 *trans* in a 1 M:0.1 M (*cis*/*trans*) salt gradient. As residue 352's charge becomes progressively more positive, the channel becomes progressively less cation selective: for wild-type DT, $E_{rev} = -38$ mV; for DT-D352N, $E_{rev} = -33$ mV; for DT-D352K, $E_{rev} = -11$ mV (for an ideally cation selective channel, $E_{rev} = -51$ mV). The *cis* solution was 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA, 30 mM MES, pH 5.3; the *trans* solution was 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA, 5 mM HEPES, pH 7.2. Voltages were corrected for electrode offset, measured at the end of every experiment (<3 mV). Straight lines were fit by eye to points corresponding to the linear part of the *I-V* curves, and reversal potentials read from the *V*-intercepts of these lines.

Table 1. Reversal potentials as a function of *trans* pH (*cis* pH 4.0) in different KCl gradients

KCl Gradient	*trans* pH	E_{rev} (mV)	
		Wild type	DT–352N
A. 1 M KCl/0.1 M (E_K = −51 mV)	4.0	−18	−15
	5.1	−30	−27
	7.2	−37	−28
B. 1 M KCl/0.3 M (E_K = −31 mV)	4.0	−10	−11
	5.1	−16	−12
	7.2	−22	−14

Reversal potentials of both wild-type and DT-D352N channels depend on the *trans* pH in 0.1 M KCl, but only that of the wild-type channel depends on *trans* pH in 0.3 M KCl *trans*. Note that when $[KCl]_{trans}$ = 0.1 M (*A*), both channels have similar values of E_{rev} at pH 4.0 *trans* and at pH 5.1 *trans* but, whereas E_{rev} for the wild-type channel is significantly larger at pH 7.2 *trans*, that for the mutant channel is unchanged. In contrast, when $[KCl]_{trans}$ = 0.3 M (*B*), the reversal potential of the DT-D352N channel is nearly independent of *trans* pH, whereas that of the wild-type retains its pH dependence.

Solutions contained 1 M, 0.3 M, or 0.1 M KCl, 2 mM $CaCl_2$, and 1 mM EDTA. For experiments at symmetric pH 4.0, both sides were buffered with 10 mM citrate. Otherwise, the *cis* solution contained 55 mM glycerate and the *trans* contained 10 mM citrate (pH 5.1) or 10 mM HEPES (pH 7.2). Voltages were corrected for electrode offset, measured at the end of every experiment. Reversal potentials were determined as described in the legend to Fig. 8, by manually fitting a straight line to the linear region of the channel's *I-V* curve and reading the *V*-intercept as the reversal potential.

Permeability to Large Ions

The location of Asp 352 on or near the *trans* side of the membrane rests on the assumption that the DT channel is permeable to glycerate, the *cis* compartment buffer. This is confirmed by the fact that although the wild-type channel in potassium glycerate is cation selective, it is far from ideal (Table 2); thus, glycerate must be permeant. Table 2 similarly demonstrates that $Tris^+$ is also permeant. Interestingly, although the wild-type channel is more permeable to $Tris^+$ than to Cl^- at pH 5.5 *cis*/7.5 *trans*, the DT-D352K channel is nonselective under these conditions (Table 2). Its shift away from cation selectivity is again consistent with the proposed electrostatic role of residue 352.

Mutations of Glutamate 349

Changing the charge at residue 349 from the negative glutamate to either a neutral glutamine (DT-E349Q) or a positive lysine (DT-E349K) has no effect on channel conductance at any voltage (pH 5.3 *cis*/7.2 *trans*); i.e., the *I-V* relations for wild-type, DT-E349Q, and DT-E349K channels are superimposable (Fig. 9). Similarly, the selectivity of the DT-E349Q channel is unchanged from that of the wild-type channel, at least at pH 4.0 *cis*/7.2 *trans*. The double mutant DT-E349Q/D352N, in which both negative charges in the TH8-9 loop have been neutralized, forms channels with conductances and selectivities indistinguishable from those formed by DT-D352N at pH 5.3 *cis*/7.2 *trans* (*data not shown*). Thus, the charge on residue 349, in marked contrast to the charge on residue 352, has no effect on ions moving through the DT channel. Note that although the mutant E349K has reduced toxicity on cells (O'Keefe et al., 1992), as well as reduced channel-forming activity in lipid bilayers (J.A. Silverman, J.A. Mindell, A. Finkelstein, R.J. Collier, *in preparation*), there is no possibility in these experiments (or in any others in this paper) that we are seeing contaminating wild-type channels, since the mutant toxins are expressed in *E. coli*, in the absence of a gene for the wild-type protein.

Discussion

In this paper we have presented evidence, from the effects of pH on single channel conductance, that residue 352 is located on or near the *trans* side of the membrane. We have also argued that, via electrostatic forces, this amino acid is a major determinant of ion permeation through the DT channel and indeed have shown, using measurements of single channel conductance and ion selectivity, that it is the only determinant on the protein that is titratable from the *trans* side. In contrast, residue 349 seems to exert no detectable influence on permeant ions.

The differences in effects between mutations at residue 349 and those at residue 352 are surprising at first glance. Glu 349 and Asp 352 are separated by only two amino acids in the linear sequence, yet mutations of Asp 352 to uncharged asparagine or to positively charged lysine produce dramatic changes in the conductance and selectivity properties of the channel, whereas alterations of Glu 349 to glutamine or lysine yield no detectable effects on ion permeation. The configuration of the TH8-9 loop in the crystal structure of DT, however, suggests a plausible explanation. [We must take care, of course, in extrapolating from the crystal structure, since clearly it represents the molecule in solution, whereas we are obviously studying the membrane-inserted form of the toxin. This said, we assume that the general structure of the helical hairpin seen in the crystal (Choe et al., 1992) is maintained in the channel.] The structure of the short loop connecting the two α-helices (TH8 and TH9) constrains the side chains of these two residues to face different directions. In fact, the distance between the charged portions of

Table 2. Reversal potentials of wild-type and DT-D352K channels in 10:1 gradients of large buffer ions

Toxin	Salt	pH (*cis/trans*)	E_{rev}	E_K	$E_{Diffusion}$
Wild-type	K glycerate	4.0/4.0	−37 mV	−54 mV	−21 mV
Wild-type	TrisCl	5.5/7.5	−23 mV		+25 mV
DT-D352K	TrisCl	5.5/7.5	0 mV		+25 mV

Since the wild-type channel is not ideally cation selective in a 10:1 K glycerate gradient, it must be permeable to glycerate as well as to potassium. Similarly, we deduce that the channel is permeable to $Tris^+$. Interestingly, the DT-D352K channel is less cation selective than the wild-type in the TrisCl gradient, consistent with residue 352 interacting electrostatically with permeant ions.

Solutions for experiments in K glycerate: 1 M K glycerate *cis*, 0.1 M *trans;* both solutions: 2 mM Ca glycerate, 1 mM EDTA, pH 4.0. For experiments in TrisCl: 1 M TrisCl *cis*, 0.1 M TrisCl *trans;* both solutions: 1 mM EDTA; pH 5.5 *cis*/7.5 trans. E_K was measured using the K^+ carrier, valinomycin. Diffusion potentials were measured as the potentials across the open hole in each experiment.

these groups in the crystal structure is about 15 Å. Considering that the Debye length is about 3 Å in 1 M salt, and even only about 10 Å in 0.1 M KCl, we begin to see how these residues could exert such different effects on ion movement through the channel. That is, residue 352 could lie very close to the channel entrance, whereas residue 349 would be (electrostatically) very far removed.

It is logically impossible, in any study involving site-directed mutagenesis, to rule out the possibility that the changes observed in the mutant channels are due to gross conformational changes of the protein rather than to specific effects. However, such an alternative appears highly unlikely in our experiments for a number of reasons. First, measurements of several distinct channel properties yield results consistent with an electrostatic model (e.g., Fig. 2 and Table 1); such consistency from independent experiments would be surprising in the context of a significant conformational change. Second, the properties of the wild-type channel converge with those of the DT-D352N channel at low pH (e.g., Fig. 3). Thus, as the aspartate in the wild-type becomes protonated, it behaves exactly like the asparagine in DT-D352N. Third, differences between properties of the wild-type and mutant channels are relatively conservative, consistent with the induction of relatively small perturbations on a stable structure. We know, for example, that the DT-D352K channel still has a large pore, since it passes $Tris^+$, and that the *I-V* and *g-V* characteristics of the three channels are compellingly similar in shape to one another. Finally, the conductance changes in the wild-type channel are a *smooth* function of *trans* pH (Fig. 6), suggesting that the conformation of the wild-type channel is independent of pH; a large scale conformational change might be expected to yield a more precipitous change.

The method used here to locate residue 352 on the *trans* side of the membrane is generally useful for finding titratable charges in large channels. The major assumption of this method is that the environment inside the pore is similar to that in bulk solution. This in turn implies that pH, a bulk property, is defined within the pore, and that glycerate in the channel buffers as it does outside. Given the large diameter of the DT channel, this assumption seems reasonable. Even if it is incorrect, our conclusion is probably valid, since in a worst case, with a linear gradient of proton concentration through the pore (from *cis* pH 4 to *trans* pH 7), over 90% of the channel would have pH <5).

We presented an apparent pK_a (=5.5) for aspartate 352. The degree to which this represents the true pK_a of this aspartate depends on there being a linear relationship between channel conductance and the charge on residue 352. The existence of such a relation is by no means obvious *a priori,* though the nearly linear effects (on conductance) of changing Asp 352 to Asn and Lys, respectively (Fig. 2), imply that it may. Ironically, the best evidence that the charge on residue 352 is linearly related to conductance is the quality of the fit of the conductance *vs. trans* pH relation by a standard titration curve (Fig. 6); this equation has only one free parameter, yet fits the data nicely. Insofar as the pK_a of the fit represents the true pK_a of Asp 352, its value is shifted by almost two units from the aspartate side chain's free solution pK_a value of 3.86 (Lehninger, 1982). Such a shift is not unusual for an acidic side chain in a protein, as is well known.

Together, all the results presented in this paper argue strongly that ion permeation through the DT channel is strongly influenced by electrostatic interactions. Interestingly, not all of these are due to charges on the protein: the *trans* pH dependence of selectivity observed in DT-D352N channels seems to result from interactions between permeant ions and some charge on the lipids, a notion with some precedent in this field. For example, Hoch (1985) found that the DT channel is 20 mV more anion selective in neutral membranes than in asolectin (in a 10:1 KCl gradient). We were unable to do similar experiments because of the extremely low channel-forming activity of DT in uncharged membranes; since we had limited quantities of mutant proteins, we could not compensate for this by increasing the amount of toxin added.

The location of Asp 352 on the *trans* side of the

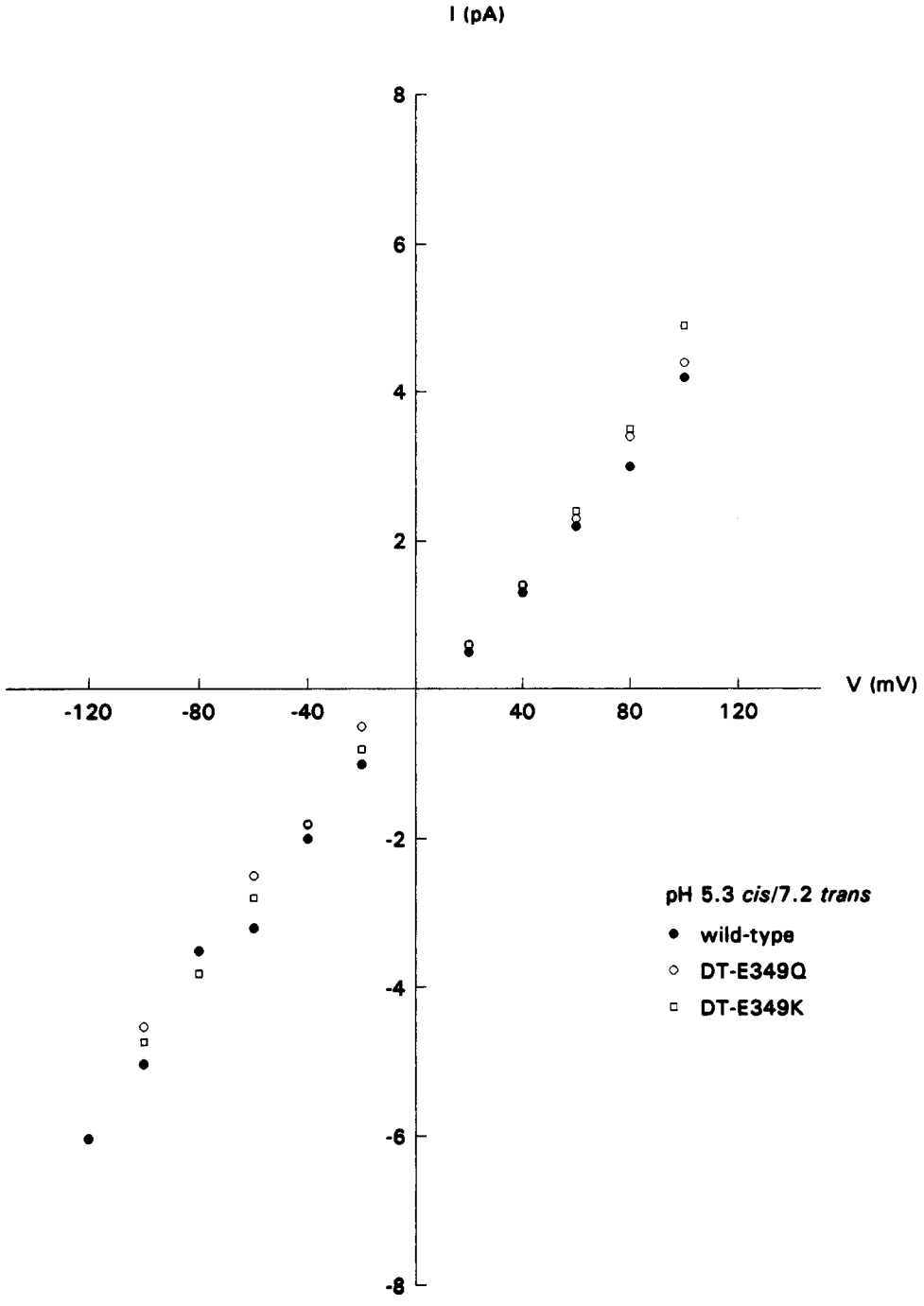

Fig. 9. Single channel *I-V* relations of wild-type, DT-E349Q and DT-E349K channels in 1 M KCl (pH 5.3 *cis*/7.2 *trans*). Note that the *I-V* relations for DT-E349Q and DT-E349K channels are indistinguishable from those of the wild-type channel over the entire voltage range. Conditions were the same as in Fig. 2: 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA; 30 mM MES pH 5.3 *cis*, 5 mM HEPES pH 7.2 *trans*. After membrane formation, 3 ng (wild type), 8 ng (DT-E349Q), or 50 ng (DT-E349K) of toxin were added to the *cis* compartment; 1–10 min later, a channel opened, and was pulsed to a series of voltages.

membrane restricts the possible topologies of the DT channel-forming region. In the previous paper (Silverman et al., 1994) we demonstrated that a limited region of the B fragment (61 amino acids) is sufficient to form normal channels in lipid bilayers. This region corresponds to a "helix-loop-helix" motif in the DT crystal structure, which Choe et al. (1992) suggest inserts into the membrane. Spectroscopic data (Cabiaux et al., 1989) imply that there are no major changes in secondary structure in this region as the B fragment goes

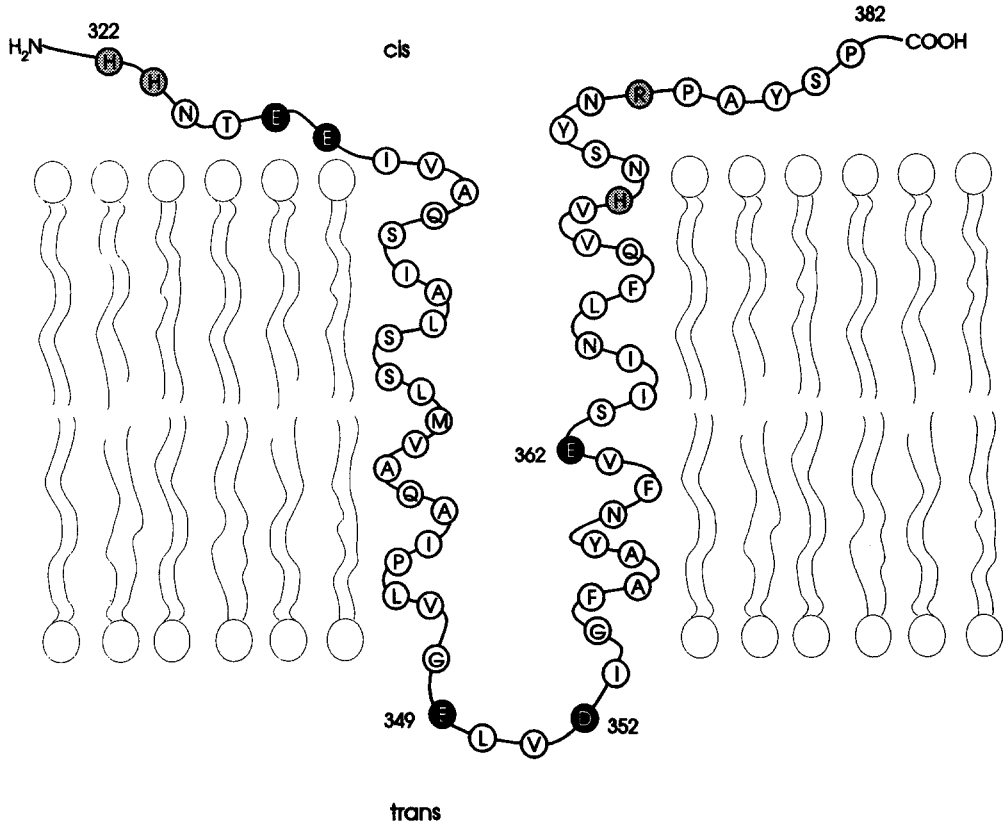

Fig. 10. Hypothetical membrane topology of the DT channel-forming region. Possible membrane topology for the TH8–9 region of DT. Asp 352 and Glu 349 are located on the *trans* side of the membrane, and regions corresponding roughly to helices TH8 and TH9 span the hydrophobic portion of the membrane. Positively charged residues, H322, H323, H372, and R377 may anchor the N-terminal loop of TH8 and the C-terminal loop of TH9 on the *cis* side of the membrane. This model predicts that E362 lies in the middle of the membrane, probably facing the channel lumen. Grey = positively charged residues; Black = negatively charged residues. This figure indicates which residues are in the membrane, which are in the *cis* compartment, and which are in the *trans* compartment; it is not meant to distinguish pore-lining residues from those exposed to the hydrophobic interior of the membrane.

from solution to its membrane-bound form. Now, our results show that Asp 352, in the short loop connecting helices TH8 and TH9, is on the *trans* side of the membrane. This supports Choe et al.'s (1992) insertion hypothesis and leads us to the membrane topology illustrated in Fig. 10. In this model both the N-terminus of TH8 and the C-terminus of TH9 remain on the *cis* side of the membrane, while the hairpin loop projects slightly into the *trans* solution. This model is also supported by activity data on mutants at positions 349 and 352 (O'Keefe et al., 1992; J.A. Silverman, J.A. Mindell, A. Finkelstein, R.J. Collier, *in preparation*): namely, mutants having a positively charged residue at either of these positions are much less active both biologically and in channel formation, consistent with the presumed difficulty of translocating a positive charge (lysine) across the bilayer. In the next paper (Mindell et al., 1994), we examine the roles of residues predicted by the model to line the pore and to project on the *cis* side.

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