Single-Channel Currents Produced by the Serotonin Transporter and Analysis of a Mutation Affecting Ion Permeation

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ABSTRACT Single-channel activities were observed in outside-out patches excised from oocytes expressing a mammalian 5-hydroxytryptamine (5-HT) transporter. Channel conductance was larger for a mutant in which asparagine^{177} of the third putative transmembrane domain was replaced by glycine, suggesting that this residue lies within or near the permeation pathway. The N177G mutant enables quantitative single-channel measurements; it displays two conducting states. One state, with conductance of ~6 pS, is induced by 5-HT and is permeable to Na^+. The other state (conductance of ~13 pS) is associated with substrate-independent leakage current and is permeable to both Na^+ and Li^+. Cl^- is not a major current carrier. Channel lifetimes under all conditions measured are approximately 2.5 ms. The single-channel phenomena account for previously observed macroscopic electrophysiological phenomena, including 5-HT-induced transport-associated currents and substrate-independent leakage currents. The channel openings occur several orders of magnitude less frequently than would be expected if one such opening occurred for each transport cycle and therefore do not represent an obligatory step in transport. Nevertheless, single-channel events produced by neurotransmitter transporters indicate the functional and structural similarities between transporters and ion channels and provide a new tool, at single-molecule resolution, for detailed structure-function studies of transporters.

INTRODUCTION

Ion-coupled transporters for monoamine neurotransmitters play important roles in neurotransmission (Amara and Kuhar, 1993; Lester et al., 1994). Neurotransmitter transporters also serve as targets for many drugs of therapy and abuse; for instance, modern antidepressant drugs inhibit the 5-hydroxytryptamine (5-HT) transporter (Fuller and Wong, 1990). Despite important progress in understanding transporter pharmacology, localization, and physiology, little is known about the molecular nature of the transport mechanism. Recent cloning and heterologous expression of various neurotransmitter transporters (Guastella et al., 1990; Blakely et al., 1991; Hoffman et al., 1991) enable detailed study of these aspects.

Mammalian 5-HT transporters expressed in *Xenopus* oocytes display several distinct macroscopic conductances, including a leakage current in the absence of 5-HT, a transient current induced by voltage jumps to high negative potentials in the absence of 5-HT, and a substrate-induced current in the presence of 5-HT. Furthermore, 5-HT-induced currents are 5- to 12-fold larger than the influx of [^{3}H]5-HT, as though ions flow in excess of those that stoichiometrically accompany the 5-HT molecules (Mager et al., 1994). Excess currents have also been observed for glutamate transporters (Fairman et al., 1995) and a norepinephrine transporter (Galli et al., 1995). We report that these macroscopic phenomena have their basis in elementary currents resembling single-channel events of ion channels. These elementary currents are mainly carried by cations and regulated by extracellular Cl^-. Similar elementary events also occur at the norepinephrine transporter (Galli et al., 1996) and, in the absence of organic substrate, for the γ-aminobutyric acid (GABA) transporter GAT1 (Cammack and Schwartz, 1996). In addition, substrate-induced noise was observed for glutamate transporters in cone photoreceptors of tiger salamander (Picaud et al., 1995; Larsson et al., 1996), for the norepinephrine transporter expressed in the HEK-293 cell line (Galli et al., 1995), and for the GABA transporter GAT1. The noise is believed in some cases to arise from channel-like activity (DeFelice and Blakely, 1996).

Knowledge about ion channels has benefited greatly from studies of site-directed mutants with altered conduction properties. As briefly reported elsewhere (Lin et al., 1995), we also extend the new single-channel measurements by showing that a site-directed mutant of the 5-HT transporter has altered single-channel conductance, suggesting that the residue in question is within or close to the permeation pathway. Single-channel recordings from the 5-HT transporter indicate the similarity between ionic channels and transporters, lead to additional understanding of the transport process, and provide a sensitive assay for structure-function studies.

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MATERIALS AND METHODS

Expression of 5-HT transporter in *Xenopus* oocytes

The *Xba*I fragment from pBS5HTT (Gift of Dr. Beth Hoffman) was subcloned into pAlter (Promega, Madison, WI). The *Nco*I site was introduced at the first ATG of the coding region by oligonucleotide-directed mutagenesis. The *Nco*I-*Xho*I fragment containing the coding region was

subcloned into pAMV-PA, a modified pBluescript (SK^+) vector containing an alfalfa mosaic virus (AMV) region directly upstream from the coding region and an A_{50} sequence downstream from the insert. The construct was named pAMV5HTT. 5HTT in pAlter was further mutated at asparagine[177]. Mutations were verified by sequencing. The *Eco*RI fragment containing the mutation was then transferred into pAMV5HTT. cRNA was synthesized in vitro with T7 RNA polymerase. Fifteen nanograms of cRNA was injected into stage V or VI oocytes (Quick and Lester, 1994). The injected oocytes were incubated 5–20 days at 19°C for translation.

Electrophysiology

For single-channel recordings, the extracellular solution contained (in mM) NaCl, LiCl, *N*-methyl-D-glucamine (NMDG) chloride, or sodium gluconate 135; HEPES 10; $MgCl_2$ 1; $CaCl_2$ 0.1. The elevated monovalent ion concentration was intended to render the single-channel currents as large as possible; Ca^{2+} was added to increase patch stability. The pipette solution contained (in mM) KCl or potassium gluconate 100, HEPES 10, EGTA 10, $MgCl_2$ 1. Patch pipettes had a tip diameter of ~1.5 μm. Recordings were obtained with a standard patch-clamp amplifier (Axopatch-1D, Axon Instruments). Single-channel recording data were sampled at 20 kHz and analyzed with pCLAMP 6.0 (Axon Instruments, Foster City, CA) after low-pass filtering at 1 kHz.

The outside-out patches were obtained in the usual way, as follows. After the pipette contacted the membrane of a devitellinized oocyte, negative pressure was applied to obtain a gΩ seal, followed by suction to break the patch (indicated by a sudden increase in the capacitance). The pipette was then withdrawn to form the outside-out patch (indicated by the reforming of a gΩ seal). Outside-out patches obtained in this fashion were always destroyed by passage through the air-water interface, but inside-out patches (obtained by withdrawing the pipette after obtaining the giga-seal) often survived a brief exposure to air.

Macroscopic currents were measured with a two-electrode voltage clamp, and the chamber was perfused continually with an external solution containing (in mM) NaCl, LiCl, or NMDG chloride 96; HEPES 5; $MgCl_2$ 1; KCl 2. For Cl^--free solutions, Cl^- was replaced by acetate.

For all solutions, pH was adjusted to 7.4 with NaOH, KOH, LiOH, or HCl.

Uptake of [^{3}H]5-HT was measured as described previously (Mager et al., 1994). Fluoxetine was a gift from Eli Lilly and Co (Indianapolis, IN). Desipramine was purchased from Research Biochemicals International (Natick, MA). All experiments were performed at 20–22°C.

RESULTS

Single-channel currents from the wild-type transporter in the absence of 5-HT

We begin with a description of single-channel currents in the absence of 5-HT; we term these openings the leakage state. In the absence of 5-HT, single-channel events were observed in 24 of 29 patches from 24 injected oocytes expressing the wild-type rat 5-HT transporters (Fig. 1 *A*). Open frequencies were reversibly decreased more than 75% (to frequencies so low that positive identification of the 5-HT transporter channel became impossible) either by the specific 5-HT uptake blocker fluoxetine (10 μM, 8 patches) or by desipramine (25–50 μM, 4 patches; data not shown), which, although less specific, was employed in previous two-electrode clamp experiments because it is more rapidly reversible. Cations were required to maintain the channel opening,

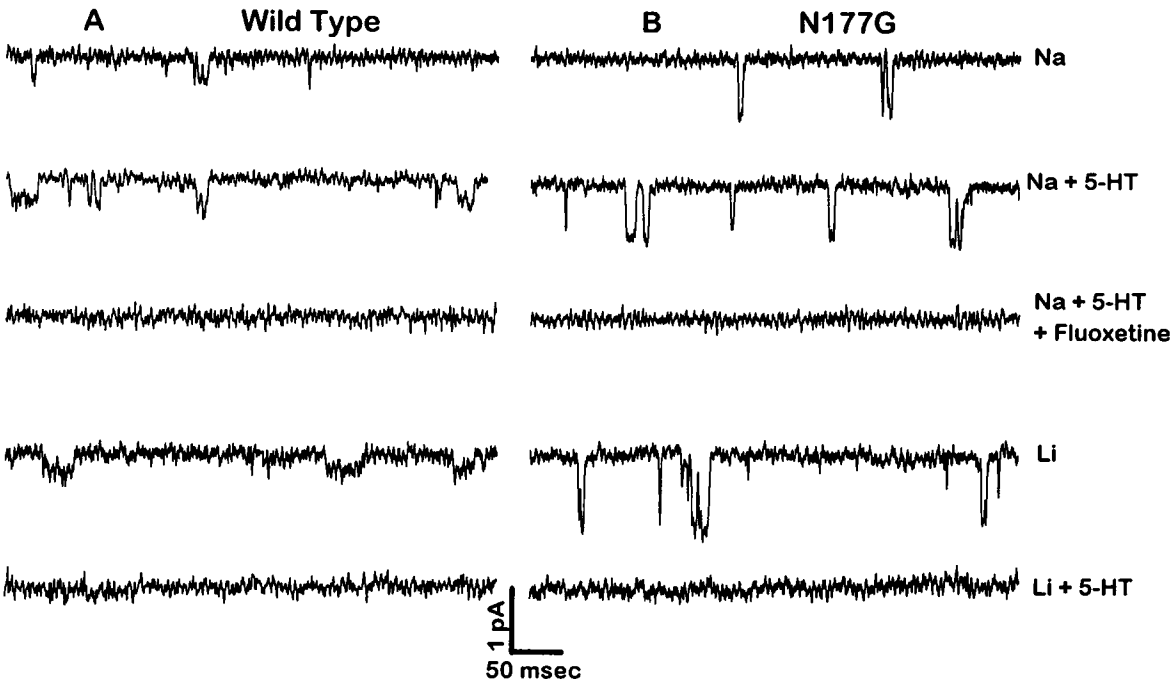

FIGURE 1 Channel activities from oocytes expressing the wild-type (*A*) and N177G mutant (*B*) rat 5-HT transporter in outside-out patches at −100 mV. Traces labeled Na and Li are recorded in the absence of 5-HT, showing the leakage states. Traces labeled Na + 5-HT represent the channel activities induced by 5-HT (10 μM), showing the 5-HT-induced state. The channel activities are blocked by the uptake inhibitor fluoxetine (10 μM; see also Fig. 5 *A*) 5-HT-induced channel activities require Na^+ as permeable ions; only a few openings are observed in the presence of Li^+ plus 5-HT (see also Fig. 4 *B*). All traces in *A* are from a single patch; those in *B* are from another patch. Both experiments were performed with normal Cl^- concentrations at both sides of the membrane.

because the inward single-channel openings were abolished when external Na^+ was replaced by NMDG (NMDG chloride, 7 patches), and no additional outward openings were observed. The possible role of Cl^- will be discussed below.

The N177G mutant displays much larger single-channel currents

We sought to construct mutant 5-HT transporters with altered functional properties, because such differences might reveal residues that play key roles in transporter function. We identified several residues that are conserved among all monoamine transporters and that are different but also conserved among the GABA and related transporters. Our initial survey experiments focused on asparagine^{177} in the third putative transmembrane domain. At this position, asparagine is conserved for all amine transporters, and isoleucine is conserved for the GABA transporter subfamily. Both N177I (unpublished data) and N177G mutants were constructed and tested. We chose the N177G mutant for detailed single-channel analysis, because 1) oocytes were more viable after injection of N177G cRNA, 2) macroscopic currents were larger than for wild type, and 3) most importantly, single-channel current amplitudes were approximately twofold larger than for the wild-type transporter (Fig. 1 *B*). The N177G mutant displayed single-channel activities (39 of 50 patches from 46 injected oocytes from 18 different batches). The N177G mutant channels displayed the same pharmacological properties and ionic dependence as the wild-type channels: reversible suppression by fluoxetine (10 μM, 26 patches; see also Fig. 5 below) and by desipramine (25–50 μM, 8 patches, data not shown) and disappearance when cations were replaced by NMDG (13 patches).

The single-channel currents for the wild-type transporter are, frankly, so small that they challenge the limits of quantitative analysis. On the other hand, the dramatically increased N177G single-channel currents provide a robust set of signals for extensive study. Nonetheless, where possible, we also present analyses of the wild-type transporter.

The single-channel events described here are not endogenous channels. We tested outside-out patches from 14 uninjected oocytes from the same batches that received RNA injections. Under our conditions, these control patches showed no major class of channels. The various endogenous channel activities can readily be distinguished from the 5-HT-transporter-related channel activity by several criteria. 1) 5-HT transporter channels have distinct amplitudes. Endogenous channel amplitudes are usually >2 pA at -100 mV, and all of the transporter-related channel amplitudes are <1.5 pA at -100 mV. 2) 5-HT transporter channels have a distinct pharmacological specificity. Endogenous channels are insensitive to the presence of 5-HT or to the 5-HT uptake inhibitors fluoxetine and desipramine, but as described below, 5-HT transporter channels are affected by each of these reagents. 3) 5-HT transporter channels have a distinct Li^+ sensitivity. Li^+ potentiates the single-channel open probability for the leakage state (see Fig. 4 below), whereas Li^+ has no effect on the open probability of the endogenous channels observed. 4) Finally, the N177G mutant displayed dramatically increased single-channel amplitudes at all of the potentials tested (Fig. 2 *A*). This increase in single-channel conductance by the mutation provides the most clear and direct evidence that the channels described above are formed by the transporter protein itself.

Li^+ is often used in studies of transporters for 5-HT (Rudnick, 1977; Talvenheimo et al., 1979; Talvenheimo et al., 1983; Wood, 1987; O'Reilly and Reith, 1988) and other substrates (Kanner and Schuldiner, 1987) because it does not support coupled transport. Although wild type and N177G had different single-channel conductances, channel conductance was indistinguishable in Na^+ and Li^+ (Fig. 2 *B*). However, the open probability was considerably greater in Li^+ (Fig. 4), as discussed in greater detail below.

We also measured single-channel mean open time by constructing open-time histograms, as shown in Fig. 3 *A*. There is no significant difference in the mean open time for both wild-type and N177G channels under all conditions described here (Fig. 3 *B*).

Single-channel currents in the presence of 5-HT

When 5-HT (10 μM) was added to the chamber perfusing the excised outside-out patch, single-channel opening frequency increased by factors of $\sim$2 and $\sim$4 for wild type and N177G, respectively. For the N177G mutant, we were able to identify channel amplitudes that are somewhat smaller in the presence of 5-HT than in the absence of 5-HT. Fig. 2 *A* compares the single-channel amplitudes for different conducting states of both wild type (upper panel) and N177G mutant (lower panel) at a holding potential of -100 mV. For the N177G mutant, the amplitude peak of the 5-HT-induced state is clearly shifted toward smaller currents, compared with those of Na^+ or Li^+ leakage states. This shift was observed at all membrane potentials tested, and the N177G displays a smaller 5-HT-induced channel conductance than does the leakage state (average values were 6.2 pS vs. 13.4 pS, Fig. 2 *B*).

The current-voltage relations raise additional points that cannot be directly addressed at present. First, the two conducting states of the N177G transporter seem to extrapolate to different reversal potentials, but because endogenous channels complicate the recordings at positive potentials, we have not directly investigated this possibility. Second, for the wild-type transporter, the available data suggest 1) no apparent change in channel conductance (average value, 2.4 pS) in the presence of different substrates and 2) a rather more positive extrapolated reversal potential than for either N177G state, but we emphasize again that we have limited confidence in quantitative measurements on the wild-type transporter.

In the presence of Li^+, 5-HT decreased the open probability by two- to fivefold, compared with that measured in Li^+ alone

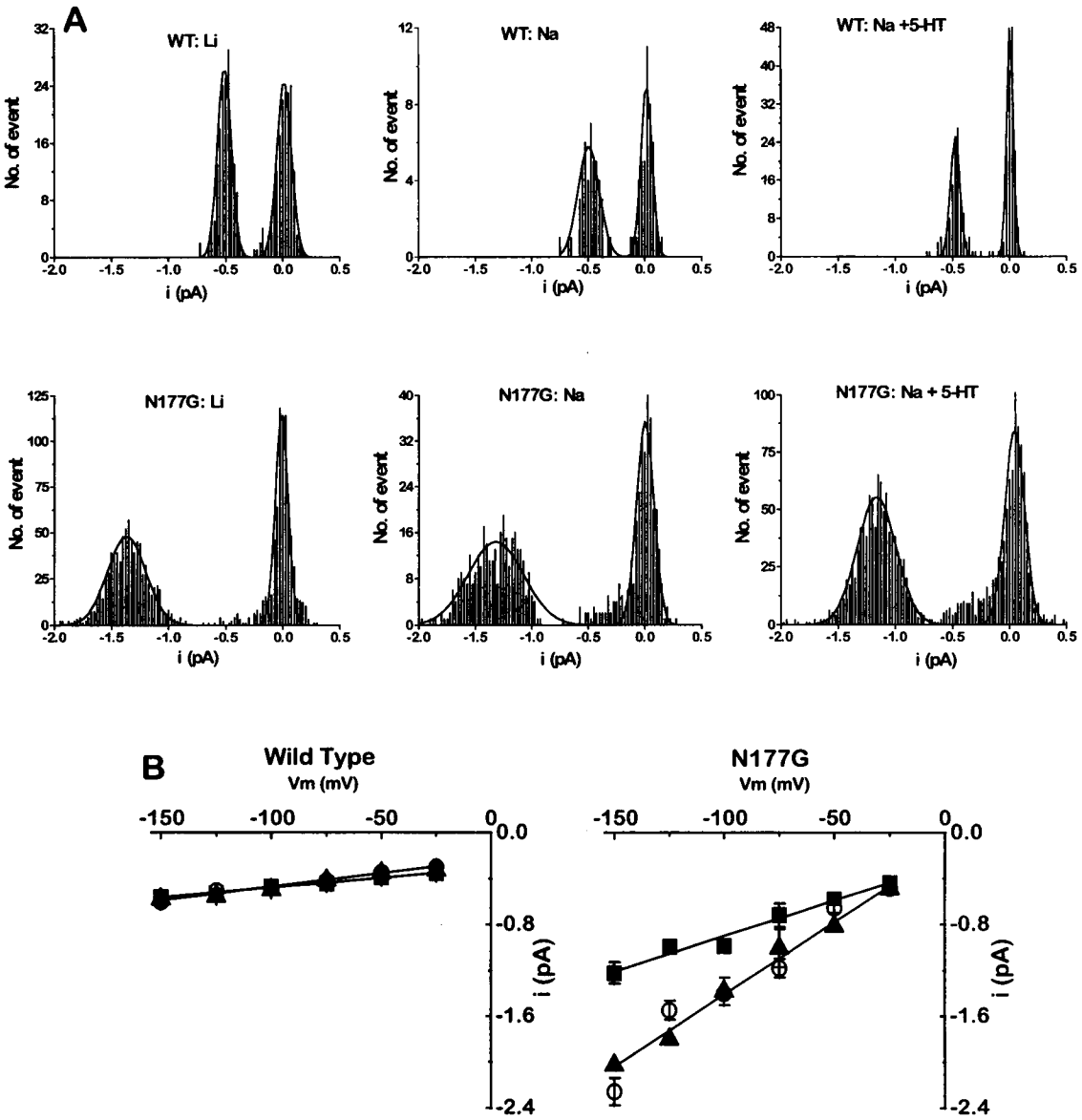

FIGURE 2 (*A*) Amplitude histograms for Li^+ leakage states (left), Na^+ (center) leakage states, and 5-HT-induced states (right) at −100 mV. The upper panel shows the data obtained from a patch containing wild-type 5-HT transporters. The lower panel shows the data obtained from another patch containing N177G mutant transporters. (*B*) Single-channel amplitude versus membrane potential. The left panel shows the data for wild type; the right panel shows the data for the N177G mutant. ■, 5-HT-induced states; ○, leakage states in NaCl solution; ▲, leakage states in LiCl solution. Each symbol represents mean ± SEM (n = 2–11 patches). Lines are least-square fits to the data and correspond to conductances of 13.4 pS for N177G in the absence of 5-HT, 6.2 pS for N177G in the presence of 5-HT, and 2.4 pS for wild type.

(Fig. 4). Because both the single-channel conductance and the effect of Li^+ are different in the presence and absence of 5-HT, we believe that 5-HT induces a distinct channel-like conducting state of the transporter. Like the leakage channel, the 5-HT-induced channel displayed average open times of 2–3 ms for both wild type and N177G mutant (Fig. 3 *B*).

Single-channel currents underlie macroscopic currents

We begin this section by comparing the macroscopic functional properties of the N177G and wild-type transporters, measured at −40 mV. The N177G mutant displayed larger 5-HT-induced currents than did the wild-type transporter at 71 ± 13 vs. 24 ± 3 nA (mean ± SEM, n = 17 and 11, respectively), whereas the [^{3}H]5-HT uptake level of N177G was comparable to that of the wild type. At 10 μM 5-HT, uptake of [^{3}H]5-HT was 690 ± 117 and 942 ± 278 fmol/oocyte/min for wild type and N177G, respectively (n = 5 in each case). The discrepancy between ionic current and uptake of [^{3}H]5-HT, described previously (Mager et al., 1994), reached factors of 52 and 15 for N177G and wild type, respectively (10 μM 5-HT; 5 oocytes for each measurement). Importantly, the EC_{50} for the 5-HT-induced cur

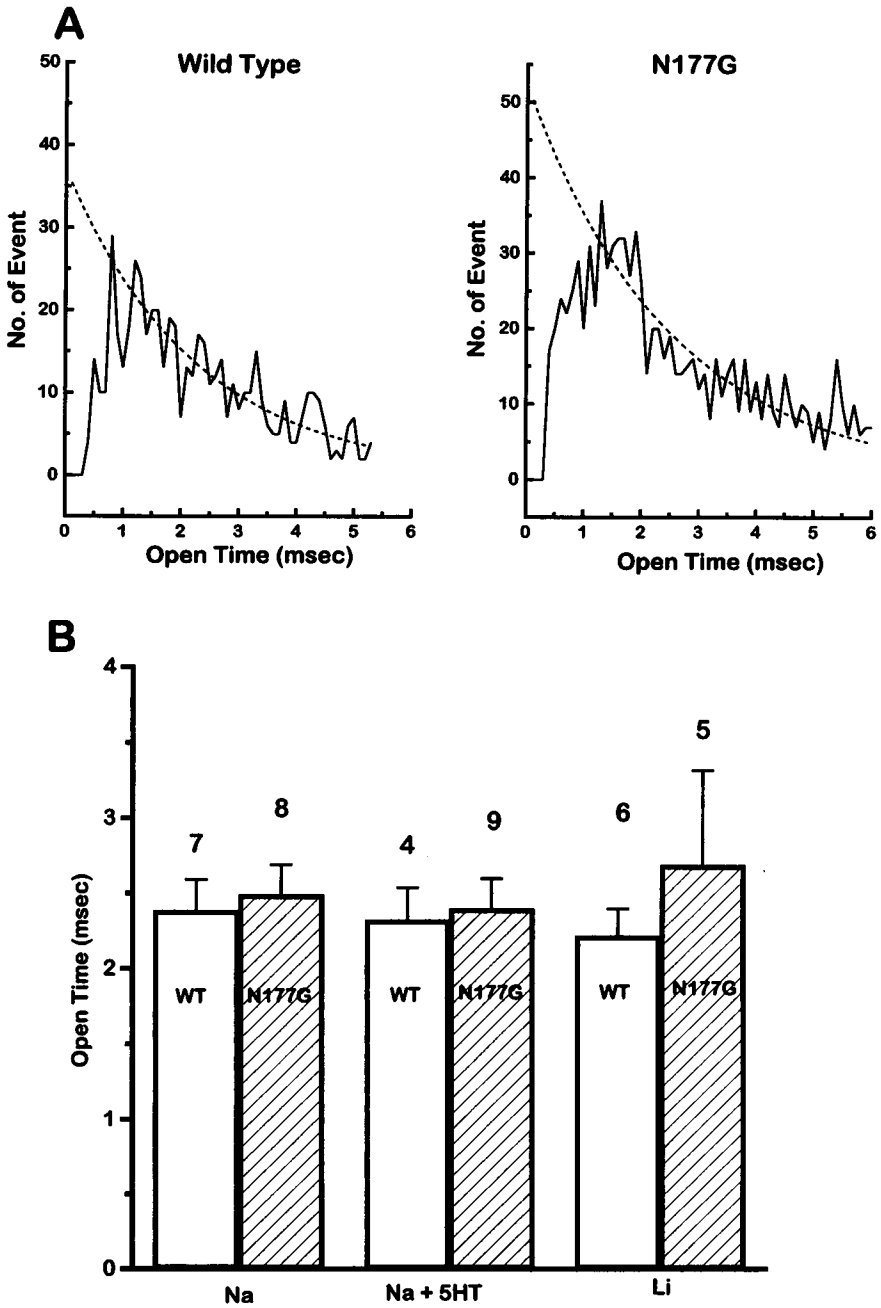

FIGURE 3 (*A*) Single-channel open-time histograms for wild-type (*left*) and N177G mutant (*right*). Both histograms were constructed from the channel activities in Li^+ leakage states. (*B*) Single-channel mean open times for different conducting states for wild type (*open bars*) and N177G mutation (*striped bars*). Each bar represents data from four to nine patches (mean ± SEM). All data were measured at −100 mV, with normal Cl^- concentration.

rents was 0.63 ± 0.04 μM for wild type compared with 2.73 ± 0.38 μM for N177G mutant (mean ± SEM, n = 3 and 4, respectively). Thus, 10 μM represents a saturating 5-HT concentration for both mutant and wild-type 5-HT transporters. Mutation N177G also increased the leakage current, especially the Li^+ leakage current (160 ± 35 nA (n = 20) vs. 93 ± 7 nA, (n = 13) for mutant and wild type, respectively). We demonstrate here that most of the macroscopic electrophysiological properties have counterparts at the single-channel level.

Li^+ does not support the normal function of neurotransmitter transporters (Kanner and Schuldiner, 1987); instead, at the 5-HT transporter (Mager et al., 1994) and GABA transporter GAT1 (Mager et al., 1996), macroscopic measurements show that Li^+ carries large leakage current and does not support the substrate-induced current. Fig. 4 makes two points by comparing macroscopic currents (Fig. 4 *A*) and single-channel currents (Fig. 4 *B*). First, there are qualitative parallels between these Li^+ and 5-HT effects on the macroscopic currents and on the single-channel currents. Li^+ potentiated the macroscopic leakage current (Fig. 4 *A*) and increased the frequency of single-channel opening in the leakage state (Fig. 4 *B*); and second, 5-HT induced a macroscopic current (Fig. 4 *A*) as well as single-channel openings (Fig. 4 *B*).

Comparison of the data for wild type (white bars) and for N177G (striped bars) in Fig. 4 shows that there are qualitative parallels between the effect of the N177G mutation on

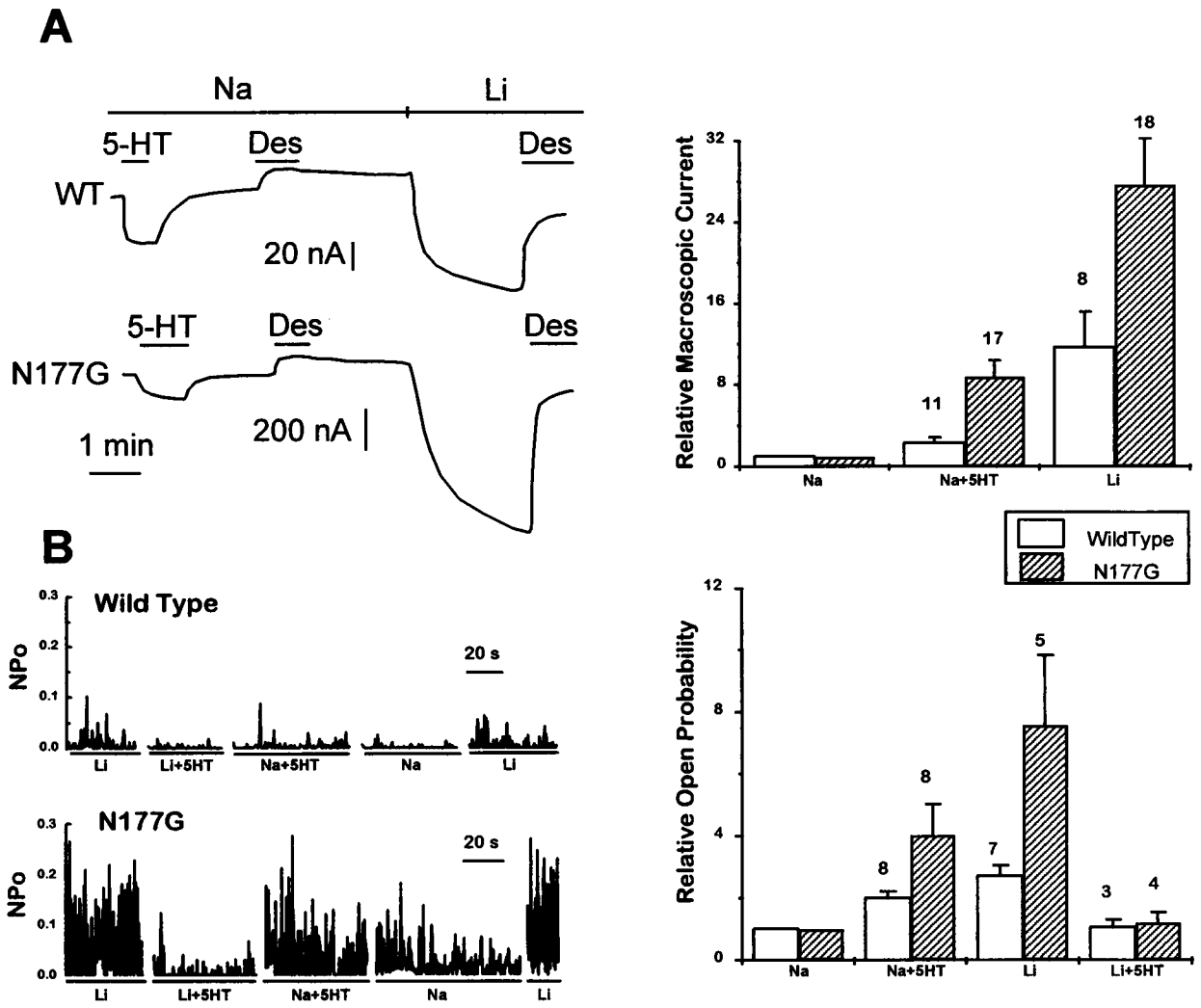

FIGURE 4 Correlation of single-channel and macroscopic conducting states of N177G mutant and wild-type rat 5-HT transporters. (*A*) Macroscopic voltage-clamp currents (*left*), showing typical traces from oocytes expressing each transporter; membrane potential was held at −40 mV. 5-HT-induced current was measured in response to the application of 10 μM 5-HT. Application of the transport inhibitor desipramine (Des, 100 μM) produced an outward deflection, which revealed a small sustained inward Na^+ leakage current. This leakage current increased in the Li^+ solution. Note the different current scales for the wild type and mutant; 1-min time bar applies to both traces. 5-HT-induced currents and leakage currents are normalized to Na^+ leakage currents for each oocyte (*right*). Bars represent data from 8–17 different oocytes (mean ± SEM) expressing the wild type (*open bars*) or the N177G mutation (*striped bars*). Data were obtained at −40 mV. (*B*) Single-channel open probabilities for outside-out patches (*left*). Data for two different patches from oocytes were injected with wild-type (*upper traces*) and N177G mutant (*lower traces*) 5-HT transporter cRNA. Membrane potentials were held at −100 mV. The indicated solutions were applied to the external face of the patch. The leakage state was measured in LiCl and NaCl solutions. The 5-HT-induced state was measured in the additional presence of 5-HT. Breaks indicate 30- to 60-s washing periods between each substrate change. Single-channel open probabilities for the conducting states were first calculated as the mean of open probabilities $NP_o(t)$ (single-channel open probability per patch), such as those shown in the left panel, and then normalized to the mean open probability of the NaCl leakage state obtained from the same patch to correct for different transporter numbers per patch (*right*). The normalized open probabilities for different conducting states were compared for wild type (*open bars*) and N177G mutation (*striped bars*). Each bar represents data from four to nine patches (mean ± SEM).

the macroscopic currents and on the single-channel currents. Li^+ enhanced the macroscopic N177G leakage currents more than the wild-type current (Fig. 4 *A*), and the openings to the N177G leakage state increased more than the openings to the wild-type leakage state (Fig. 4 *B*). For the N177G mutant, there were both more 5-HT-induced current (Fig. 4 *A*) and more openings to the 5-HT induced state.

The absolute amplitude of macroscopic currents is also consistent with the single-channel recordings. We provide a sample calculation here. The NP_o (measured single-channel open probability per patch) for the N177G 5-HT-induced channels was 3.9 ± 1.1% (mean ± SEM, $n = 8$), implying a time-averaged current of 20 fA (−40 mV) in a patch of assumed area of ~6 μm^2 (we assume that the invaginations of the oocyte membrane increase the area by approximately threefold). The typical oocyte has a surface area of approximately 3×10^7 μm^2, leading to an expected macroscopic current of approximately 100 nA, which is in reasonable agreement with the measured 5-HT-induced currents for

N177G (71 ± 13 nA, n = 17) at −40 mV, given the uncertainties about patch area. The data summarized in Fig. 4 allow satisfactory agreements to be calculated for other transport states as well. The major kinetic factor affected by Li^+, Na^+, and the N177G mutation appears to be the frequency of opening, because the average open time was 2–3 ms for all of the states studied (Fig. 3).

Cl^- is not the major current carrier

Extracellular Cl^- is required for 5-HT uptake by the 5-HT transporter (Nelson and Rudnick, 1982) as well as for the macroscopic inward 5-HT-induced current (Mager et al., 1994). The macroscopic current, and/or the single-channel currents measured here, could be due either to influx of cation(s) or to efflux of anion(s). To address this issue, we carried out single-channel recording in nominal intracellular Cl^--free solution. Fig. 5 *A* presents single-channel open probabilities obtained in various extracellular solutions with Cl^--free internal solutions from a patch containing N177G mutant transporters. Importantly, channel activities were observed in the absence of intracellular Cl^-. These channel activities were enhanced by 5-HT and were inhibited by the addition of fluoxetine. This finding strongly suggests that these unitary currents are carried by Na^+ influx.

Nevertheless, 5-HT-induced channel openings greatly decreased in frequency when extracellular Cl^- was removed (Fig. 5 *A*), which further suggests that extracellular Cl^- is required to maintain the channel opening of the 5-HT-induced state. The role of extracellular Cl^- was explored further. Fig. 5 *B* depicts single-channel open probabilities obtained in various solutions with another patch; the intracellular Cl^- concentration was normal 100 mM. Removing extracellular Cl^- slightly reduced the channel open probability for the Na^+ leakage state. However, in the absence of extracellular Cl^-, we did not observe 5-HT-induced channel openings. Rather, 5-HT reduced the opening frequency of the Na^+ leakage state.

We further correlated Cl^- effects on macroscopic and single-channel currents in experiments on oocytes from the same batch. For N177G, there was no detectable current (<2 nA) induced by 5-HT (10 μM, −40 mV) in extracellular Cl^--free solution, compared with an average of 31 ± 2 nA (n = 5) at normal Cl^-. A similar Cl^- dependence was noted for the wild-type transporter in a previous study (Mager et al., 1994). Extracellular Cl^- also affects the leakage state. Li^+ current was reduced to 46.6 ± 3% (n = 10) for N177G and to 49.5 ± 4.8% (n = 5) for wild type in extracellular Cl^--free solution. The decrease of macroscopic currents in extracellular Cl^--free solution is mainly due to a decrease in the single-channel opening frequency, as shown in Fig. 5, because there was no significant influence on single-channel amplitude and mean open time. At −100 mV, the single-channel amplitude for the Na^+ leakage state was 1.12 ± 0.06 pA (n = 6) in extracellular Cl^--free solution compared with 1.21 ± 0.04 pA (n = 5) in the presence of both intracellular and extracellular Cl^-, whereas the mean open times were 1.8 ± 0.1 ms (n = 6)

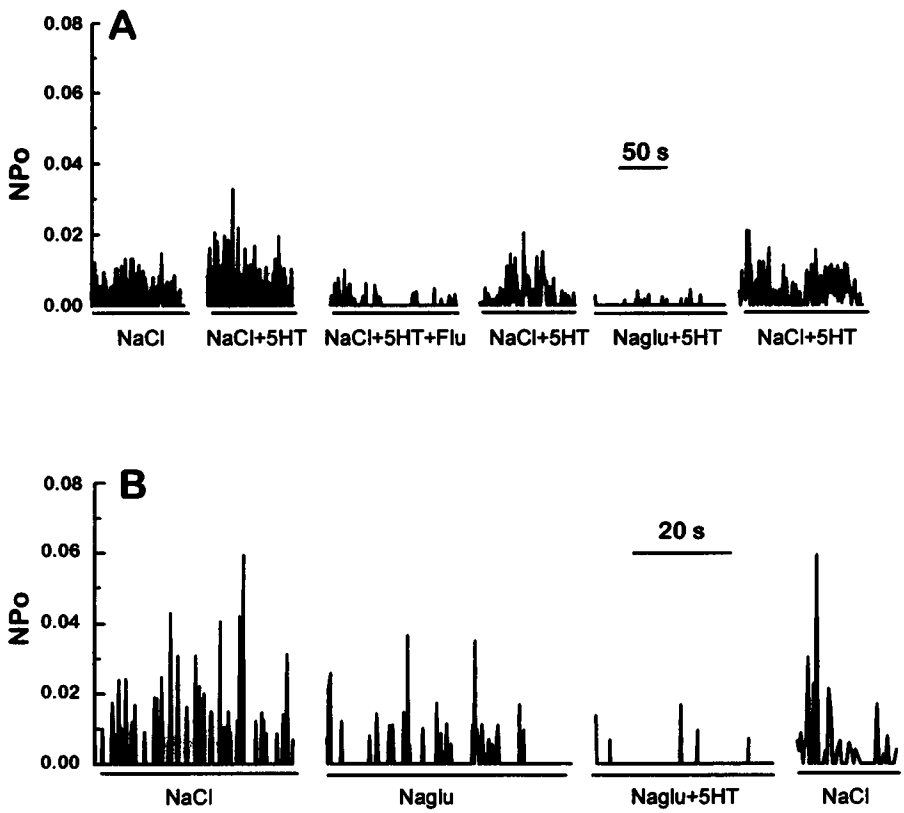

FIGURE 5 Single-channel open probabilities in nominal intracellular Cl^--free situation (*A*) and extracellular Cl^--free solution (*B*). Each panel represents data from a single outside-out patch from an oocyte injected with N177G transporter cRNA. Indicated solutions were applied to the external face of the patch. Breaks indicate 30- to 60-s wash periods.

compared with 2.1 ± 0.2 ms (n = 5). We conclude that Cl^- is not the major current carrier but does participate in regulating the 5-HT-induced current by influencing the channel opening rate.

DISCUSSION

Ion-coupled transporters were previously thought to operate at a level of one or a few ions per cycle. Yet the observed single-channel activities were on the order of approximately 10^4 ions per event. Our findings, like other recent studies cited in the Introduction and below, provide an explanation for previous reports of two phenomena concerning ion-coupled transporters: 1) substrate-independent leakage currents and 2) excess currents during coupled transport (transport-associated currents). More generally, the observations provide a new tool, at single-molecule resolution, with which to study transport proteins.

The following evidence shows that the single-channel recordings arise directly from the transporter itself. 1) Activity was recorded only in oocytes injected with 5HTT RNA. 2) Channel activity was induced by 5-HT, blocked by two uptake inhibitors, and enhanced by substitution of Li^+, all as expected from macroscopic current recordings. 3) Single-channel conductance was altered by a mutation in the transporter.

Channel behavior at neurotransmitter transporters appears to be a widespread phenomenon (DeFelice and Blakely, 1996). In addition to the data reported here, channel behavior also appears to explain fluctuations induced by organic substrates for norepinephrine transporters (Galli et al., 1996) and glutamate transporters (Picaud et al., 1995; Wadiche et al., 1995; Larsson et al., 1996). Unitary events account for substrate-independent leakage currents for GABA transporters (Cammack and Schwartz, 1996). Furthermore, both flux and electrophysiological measurements have provided indirect evidence for the existence of a channel-like permeation pathway within Na,K pumps (Gadsby et al., 1993; Hilgemann, 1994).

Single-channel events do not occur with each transport cycle

In this study, the frequency of opening to the 5-HT-induced conducting state for the wild-type transporter may be calculated as follows. We assume that all of the functional transporters in the surface membrane contribute equally to the single-channel recordings, because nearly all patches had channels (unlike the case for the GABA transporter (Cammack and Schwartz, 1996)). Therefore the frequency of channel opening f can be calculated as $f = t_{open}/P_o$, where t_{open} is mean open time (~2.5 ms in Fig. 3 *B*). P_o is the single-channel open probability and is calculated as $P_o = NP_o/N$, where NP_o is measured-channel open probability with a mean of ~2% for the wild-type 5-HT-induced state. N is the number of channels per patch and can be estimated from the transporter density (~1000 μm^{-2} (Mager et al., 1994)). For a patch area of 6 μm^2 and mean NP_o of 2 and 5% for wild-type and mutant transporters, the estimated channel opening frequency is one opening per 750 s for wild type and one per 300 s for the N177G mutant. Clearly, the transporter opens to the 5-HT-induced state much less frequently than the ~1 s required to transport a single 5-HT molecule (Mager et al., 1994). If, contrary to our assumption, single-channel events are associated with only a subpopulation of functional transporters, the frequency would be correspondingly greater for that subpopulation. Similar, infrequent single-channel events associated with uptake of the organic substrate were reported for the norepinephrine transporter (Galli et al., 1996).

Single-channel events and the alternating-access model

For one simple interpretation of channel events produced by transporters, we work within the context of the alternating-access model (Lester et al., 1994). In this scheme, the transporter protein resembles a channel with gates at each end. The single-channel currents arise when both gates open simultaneously, whereas sequential openings produce normal transport. The low open frequency calculated above suggests that simultaneous opening of the two gates does not occur during each transport cycle. Nevertheless, the single-channel events may provide information about conformational changes that also occur during normal transport. If these events accurately mirror the function of the native transporter in intact cells, the frequency of channel openings may represent a compromise between transport speed and energy cost under physiological conditions. This frequency, as modified by substrate binding, would be high enough to allow the gates to open rapidly for coupled transport, but it would also be low enough to render spontaneous openings, for instance, in the absence of 5-HT, so infrequent that they constitute an insignificant metabolic burden or electrical shunt for most cells. The present data provide a mechanism for previously described instances of slippage in ion-coupled transporters (Lauger, 1991).

For the normal transporter, the open probability of the 5-HT-induced state is 8.1×10^{-7}; if the two gates function independently and with roughly equivalent open probabilities, then each opens with a probability of 0.09%, corresponding to a free energy difference of ~4.1 kcal/mol between the closed and open state. Similar calculations give a value of ~4.3 kcal/mol for the spontaneous openings to the leakage state. The EC_{50} for Na^+, Cl^-, and 5-HT are approximately 30 mM, 5 mM, and 0.5 μM, respectively; therefore, standard free energies for binding of these substrates are 2.0, 3.1, and 8.4 kcal/mol, respectively.

The smaller apparent single-channel conductance of the N177G 5-HT-induced state could arise from one of two phenomena. First, there could be a multi-ion interaction involving 5-HT and Na^+ or a direct channel blockade by

5-HT itself. The kinetics of this interaction would be determined by the 5-HT concentration of 10^{-5} M and a forward binding rate as high as 10^{8} M^{-1} s^{-1}, or 1000 s^{-1}, which is near the limits of our present resolution. In the second possibility, the interaction would involve a 5-HT-induced change in channel conformation that restricts Na^+ flow and completely blocks Li^+ flow.

Chloride ion is required for substrate transport at many Na-dependent neurotransmitter transporters (Nelson and Rudnick, 1982; Rudnick and Clark, 1993; Lester et al., 1994), including the 5-HT transporter. We tested the role of Cl^- in transporter single-channel currents by removing Cl^- from either intracellular or extracellular solutions or both. From our data, it is clear that 1) Cl^- is not the major carrier for both leakage and 5-HT-induced currents, unlike the case for excess currents at some glutamate transporters (Fairman et al., 1995) and that 2) extracellular Cl^- is required to maintain 5-HT-induced channel activities. Cl^- is believed to be co-transported with 5-HT during normal transport, but we cannot presently decide whether the Cl^- requirement for 5-HT-induced macroscopic (Mager et al., 1994) and single-channel currents arises because Cl^- is acting as a co-transported substrate or as a modulator.

Previous experiments have disclosed three conducting states of the 5-HT transporter expressed in oocytes (Mager et al., 1994). The present single-channel recordings provide good, albeit semiquantitative, explanations for two of these currents: the 5-HT-induced transport-associated currents and the substrate-independent leakage current. We have also attempted to find a single-channel basis for the third state, a transient current activated by voltage jumps to large negative potentials, but our data on this point remain inconclusive.

N177 lies within or close to the permeation pathway

Because the pharmacological and ionic dependence of the 5-HT-induced currents and of the underlying single-channel events closely matches that of 5-HT uptake, we propose that the ionic conductance pathway is at least partially congruent with the 5-HT permeation pathway in the 5-HT transporter. The single-channel activities described in this paper provide important information about the protein's conformational changes and the permeation pathway within the 5-HT transporter and allow us to study transporters at the single-molecule level. Although the complete transport cycle and channel activities reflect different molecular events, both phenomena may share the same structural components, such as gates, binding site, and permeation pathway. Therefore, the effect of the N177G mutation on single-channel conductance strongly suggests that the mutated residue lies within or close to the permeation pathway. Nonetheless, it remains formally possible that the residue we studied is distant from the conduction pathway and that the changed conductance reflects a propagated structural change. The combination of single-molecule functional measurement and mutagenesis has been applied with great success to map the pore of traditional ion channels. It is now possible to envision similar progress on mapping the permeation pathway of ion-coupled transporters.

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