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Channel behavior in a γ-aminobutyrate transporter

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ABSTRACT **Current produced by a γ-aminobutyrate (GABA) transporter stably transfected into a mammalian cell line was observed in cell-attached and excised membrane patches. When GABA was absent, a fraction of the transporters produced cation-permeable channels. When GABA plus Na^+ was on either side of the membrane, the majority of transporters produced a high-frequency current noise attributed to the movement of ions in an occluded pore.**

Channels, pumps, and transporters span cell membranes and provide paths for ion movement. While channels allow ions to move down an electrochemical gradient, pumps use the hydrolysis of a chemical bond to move substrate against a concentration gradient. Transporters, combining features of both channels and pumps, couple the dissipative movement of ions to the unfavorable movement of substrate. Although channels, pumps, and transporters were originally assumed to have very different mechanisms for ion translocation, the distinction has recently blurred. The opening of some channels is coupled to the hydrolysis of a chemical bond; for example, Cl^- channels formed by the cystic fibrosis transmembrane conductance regulator (CFTR) require the hydrolysis of ATP (1). On the other hand, pumps can have channel properties: the Na^+,K^+-ATPase has a pore that penetrates deep into the membrane (2, 3). Thus, channels and pumps can share structural features. Relatively less is known about transporters. They have often been modeled as tightly coupled, reversible carriers (for example, see ref. 4). However, the electrophysiological study of cloned transporters has indicated that substrate and ion movement are not always tightly coupled. Some transporters have an uncoupled mode that operates in the absence of substrate (5–7). Moreover, the stoichiometry between charge and substrate movements can be variable (6, 7). Similar observations have also been made for native transporters expressed in their normal host cell (8, 9). Now we report that a γ-aminobutyrate (GABA) transporter, GAT-1 (10), forms channels. The ability of a single molecule to form both a channel and a transporter can provide insight into the transport mechanism. The formation of channels provides an alternative path for current flow and may explain why current is not always proportional to substrate flux. In addition, the ability to form channels may allow transporters to produce large electrical signals and have a cellular function beyond the uptake and release of substrate.

METHODS

Experiments were performed on human embryonic kidney 293 (HEK293) cells stably transfected with GAT-1 cDNA (7). Membrane currents were recorded with an Axopatch 200A voltage clamp (Axon Instruments, Burlingame, CA). Patch pipettes were covered with an undercoat of silicone plastic (Sylgard 184; Dow) and an overcoat of dental wax (Shur Wax; Miles Dental Products, South Bend, IN). The resistance of a fire-polished pipette was 3–4 MΩ; access resistance during whole-cell recording was usually 5–10 MΩ. The intracellular solution contained (in mM) X-gluconate, 140; magnesium gluconate, 1; EGTA, 11; and Hepes, 10, adjusted to pH 7.2. X^+ could be Li^+, Na^+, Cs^+, or *N*-methylglucamine (NMG^+) as indicated in the text and tables. The extracellular solution contained (in mM) X-gluconate, 140; calcium gluconate, 1.0; magnesium gluconate, 2.0; and Hepes, 10, adjusted to pH 7.4. K^+- and Cl^--free solutions minimized the contribution of endogenous channels. Cl^- replaced gluconate as described in the text and tables.

A low recording noise was achieved by studying patches with a seal resistance of 50–100 GΩ. Records for spectral analysis were low-pass filtered at 5 kHz and digitized at 12.5 kHz with a 16-bit analog-to-digital converter. Preliminary spectra were calculated on data blocks containing 1024 points; 400–1200 preliminary spectra were averaged to produce a final spectrum. Spectra were corrected for the attenuation produced by low-pass filtering. The spectrum of a 500-MΩ test resistor was flat between 10 Hz and 5 kHz.

RESULTS AND DISCUSSION

The GAT-1 transporter has three modes of operation (7): an uncoupled current produced in the absence of GABA, an in-gated current produced by intracellular GABA, and an ex-gated current produced by extracellular GABA. First, we describe single channel openings associated with the uncoupled current. Afterwards, we describe a high-frequency current noise associated with the in-gated and ex-gated currents.

Channels that contribute to the uncoupled current were observed during cell-attached (Fig. 1*A*) and excised-patch (Fig. 1*B*) recording. Channel openings occurred in bursts, and openings were usually longer after excision. Two different stable amplitudes were observed. In 19 excised patches, channel amplitude was -0.7 ± 0.2 pA at -50 mV with Na^+ in the extracellular solution (as in Fig. 2); in 7 patches, channel amplitude was -1.4 ± 0.2 pA (as in Fig. 1). The cytoplasmic face of an excised patch could be superfused with different solutions. Table 1 summarizes the results. Channels shared properties with the previously observed whole-cell, uncoupled current (7): (*i*) Inward current was carried by any group Ia cation present in the extracellular solution. (*ii*) Channel opening required either a group Ia cation (as in Fig. 1*C*) or Cl^- in the intracellular solution. (*iii*) A linear current–voltage relation was seen only when Na^+ plus Cl^- was present in the intracellular solution (and a group Ia cation was in the extracellular solution); otherwise, the current–voltage relation was rectified and only inward current was observed. We observed this type of channel in 36 of 119 patches excised from transfected cells, but we never observed this type of channel in 17 patches excised from untransfected cells. In addition, channel opening stopped when 3 patches excised from transfected cells were superfused with 1 μM SKF89976A, a potent inhibitor of GABA transport (11). Because (*a*) channel opening depended on exactly the same ions as the whole-cell, uncoupled current, (*b*) channels were seen only in transfected cells, and (*c*) channels were blocked by a specific pharmaco-

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Abbreviations: GABA, γ-aminobutyrate; NMG, *N*-methylglucamine.

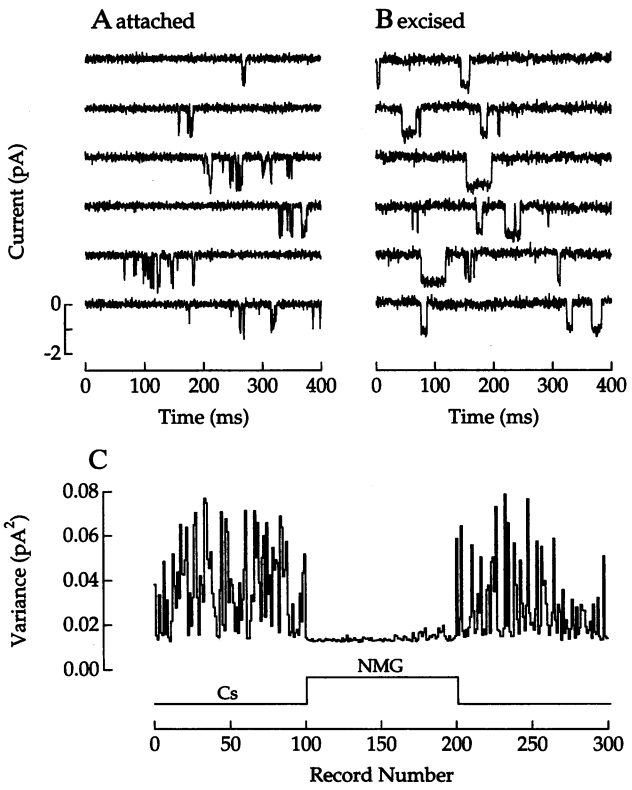

FIG. 1. Channels observed in cell-attached and excised patches. (*A*) Cell-attached recording. The voltage across the patch was the cell resting membrane voltage. (*B*) An excised patch. The cytoplasmic face was superfused with cesium gluconate. The voltage was maintained at −50 mV. (*C*) Channel opening depends upon the identity of ions at the cytoplasmic face. The variance was measured in consecutive traces and plotted as a function of time. The timing trace indicates when the cytoplasmic face was superfused with cesium gluconate or NMG gluconate. The patch pipette contained sodium gluconate. Recordings were low-pass filtered at 1 kHz. Individual records in *C* contained 1024 points and spanned 410 ms. The data in *A–C* are from the same patch.

logical agent, we conclude that channels are produced by the expression of GAT-1.

GABA had different effects depending on whether it was added to the intracellular or the extracellular solution. The addition of GABA (1 mM) to the intracellular solution stopped channel opening (Fig. 2). In contrast, channel openings were still observed when a patch pipette contained GABA. We were not able to change the solution in a patch pipette and record channel behavior before and during exposure to extracellular GABA. Instead, we recorded in the whole-cell configuration. The variance of the whole-cell current increased when GABA was added to an extracellular solution containing NaCl (Fig. 3*A*). Thus, intracellular GABA decreases channel opening, and extracellular GABA may increase channel opening.

Table 1. Ions required to observe channels

Extracellular solution	Intracellular solution	*n*	Voltage (−50, +50 mV)
Na gluconate	Na gluconate	4	− only
Li gluconate	Na gluconate	2	− only
Cs gluconate	Na gluconate	4	− only
Na gluconate	NMG-Cl	3	− only
NaCl	CsCl	4	− only
M^+	**M^+ or Cl^-**		**− only**
NaCl	NaCl	7	+ and −
CsCl	NaCl	4	+ and −
M^+	**NaCl**		**+ and −**

The cytoplasmic face of every patch was superfused first with NMG gluconate and then a solution as listed in the table. While exposed to NMG gluconate, channel openings were not observed. Every patch was tested at −50 and +50 mV. While exposed to a test solution, channel openings were observed at the potential indicated. Inward currents were observed at −50 mV; outward currents were observed at +50 mV. The ions required for channel opening are summarized in bold type. M^+ indicates any group Ia cation. *n* is the number of patches.

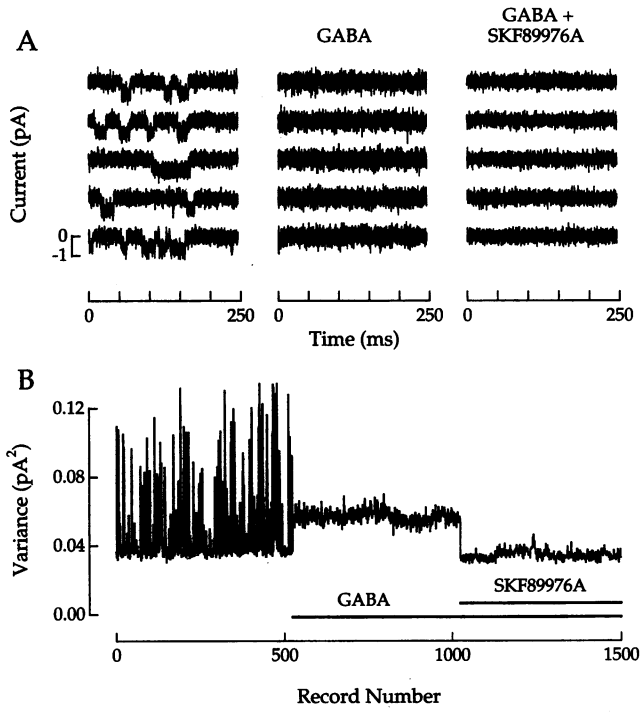

FIG. 2. Cytoplasmic GABA stops channel opening and produces a high-frequency fluctuation. Recordings were acquired at 12.5 kHz and low-pass filtered at 5 kHz. The patch pipette contained NaCl. Membrane voltage was −50 mV. (*A*) Individual traces were recorded while the cytoplasmic face was superfused with NaCl (left set of traces), NaCl plus 1 mM GABA (center set of traces), and NaCl plus GABA plus 1 μM SKF89976A (right set of traces). (*B*) A history of current variance. The timing bars indicate periods when the cytoplasmic face was superfused with GABA or GABA plus SKF89976A. Individual records in *B* contained 1024 points and spanned 81.92 ms. The data in *A* and *B* are from the same patch.

Two calculations allow a rough estimate of channel density. The first calculation uses the observation that 36 of 119 patches had channels. If channels are randomly distributed in the cell membrane, then the probability of channels being captured in a patch is given by the Poisson distribution and the fraction of patches without channels is ke^{-k}, where k is the average number of channels per patch. Because channels were not observed in 70% of the patches, k was ≈1 channel per patch. The number of channels can also be estimated from the open probability, mean whole-cell current, and single channel amplitude. Four cell-attached patches appeared to contain only a single active channel. In these, the open probability varied from 0.02 to 0.06. Dividing the average steady-state, uncoupled current observed during whole-cell recording (−18 ± 9 pA at −50 mV, 12 cells) by the average single channel amplitude and an average open probability of 0.04 yields ≈500 active channels per cell. Since the average membrane capacitance was 57 pF (7), a cell had a surface area of ≈5700 μm^2 and an average of 1 channel per patch of 10 μm^2. Both estimates indicate that patches contained only a few active channels. Nonetheless, we are reluctant to believe that any of the patches contained only a single GAT-1 protein molecule. The following whole-cell

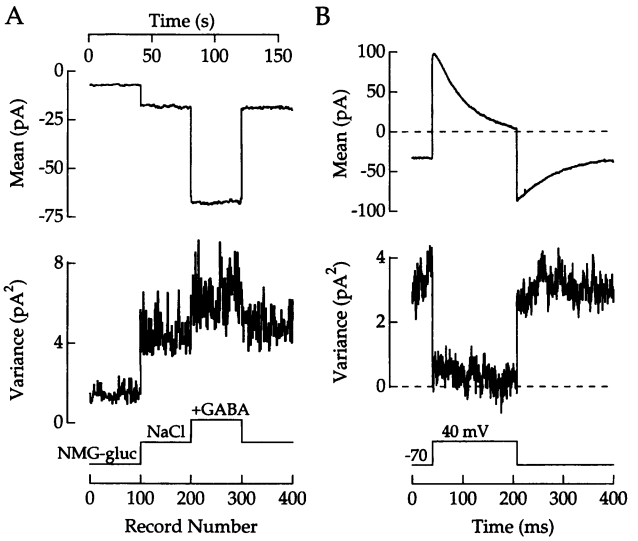

FIG. 3. (*A*) Extracellular GABA increases whole-cell current noise. The patch pipette contained cesium gluconate; the holding potential was −60 mV. The cell was superfused first with NMG gluconate, then NaCl, and finally NaCl plus 200 μM GABA as indicated in the timing trace. The mean and variance were −7.12 pA and 1.33 pA^2 during superfusion with NMG gluconate, −18.6 pA and 4.88 pA^2 during superfusion with NaCl, and −66.8 pA and 6.37 pA^2 during superfusion with NaCl plus GABA. Recording was low-pass filtered at 1 kHz. Consequently, the high-frequency noise identified in Fig. 4 is not expected to contribute to the variance. Part of the variance increase during exposure to NaCl is produced by Cl^- channels unrelated to the GAT-1 transporter. (*B*) Nonstationary fluctuation analysis of whole-cell leakage currents. First a cell was superfused with NMG gluconate. A voltage step from −70 to +40 mV was repeated 100 times and an ensemble of records was collected. Next, the cell was superfused with sodium gluconate and a second ensemble of 100 records was collected. The mean and variance at time points within each ensemble were calculated. Finally, the change in the mean (*Upper*) and variance (*Lower*) induced by extracellular Na^+ was calculated. Recordings were low-pass filtered at 1 kHz. The recording pipette contained cesium gluconate. Consequently, the steady-state current–voltage curve was rectified (see ref. 7 and Table 1) and the transient was observed in isolation at +40 mV.

experiments provide an estimate for the density of transporter molecules.

The uncoupled current has transient and steady-state components (7, 12). A nonstationary fluctuation analysis, illustrated in Fig. 3*B* (the procedure is described in the figure legend; see also ref. 13), indicates that these two components are produced by different mechanisms and may be produced by different populations of transporter molecules. First, we consider the steady-state current observed at −70 mV. The variance, 3.29 pA^2, divided by the mean, −32.9 pA, is −0.1 pA. This value should be equal to the average single channel amplitude multiplied by the fraction of time a channel was open during a burst (see ref. 14). The average single channel amplitude was −0.9 pA (weighting the amplitudes of small and large channels according to their frequency). Inspection of cell-attached records, like that in Fig. 1*A*, reveals that the fraction of open time during a burst was between 0.1 and 0.4. Thus, the numbers are in rough agreement and indicate that the steady state is produced by relatively infrequent, large events—e.g., channels opening in bursts. In contrast, a similar analysis of the transient indicates that it is produced by numerous, small events. After a depolarizing step, the mean reached a maximum of 97 pA and the variance immediately assumed a low, constant value (<0.34 pA^2). Dividing the variance by the mean indicates that events responsible for the transient are smaller than 3.5 fA. Similar results were observed in seven cells.

The transient component may be produced by the movement of ions to allosteric sites (7). If only a few ions bind each transporter, then the size of the transient allows us to estimate transporter density. If the average duration of each event is 60 ms (the time constant of the mean) then the size of an elementary event is equivalent to the movement of <85 ions. A lower limit might be the movement of 1 ion. Integrating the transient yields a charge movement of 0.1 pC/pF. If the total charge movement is divided by 1 to 85 ions, then the transporter density is 6000 to 70 molecules per μm^2. Thus, a patch of ≈10 μm^2 contains >700 and perhaps as many as 60,000 transporter molecules. A patch may contain a few active channels but a large number of transporters. The large discrepancy indicates that only rarely does a transport protein act as a channel.

The vast majority of transporters, electrically silent when GABA was absent, produced a new electrical signal when GABA was present. Although GABA produces in-gated and ex-gated transport currents, we did not expect to detect these currents in patches. During a whole-cell recording the GABA-gated currents at ±50 mV are equal to a current density of ≈0.01 pA/μm^2 (see ref. 7). Because membrane patches contain approximately 10 μm^2, the current in a patch would be only 0.1 pA, too small to be reliably measured. Nonetheless, the addition of 1 mM GABA to the intracellular solution had an electrical effect. Inspection of the traces in Fig. 2*A* indicates that the noise in the center set of traces (when GABA was present) was greater than the noise observed between channel openings in the left set of traces (when GABA was absent). This impression is confirmed by the variance (Fig. 2*B*). Furthermore, when the patch was superfused with GABA plus 1 μM SKF89976A the variance decreased to a level comparable to the minimum observed when GABA was absent (three patches).

Inhibition by SKF89976A was slowly reversible and recovery was rarely possible. Another and more convenient procedure was to superfuse the cytoplasmic face first with NMG gluconate and then a solution containing Na^+ and GABA. Although channel openings were not observed while patches were superfused with these solutions, the variance increased when NMG^+ was replaced with Na^+ plus GABA in all 21 patches excised from transfected cells but was unchanged in 8 patches pulled from untransfected cells. Because (*a*) the high-frequency noise required Na^+ plus GABA, (*b*) was blocked by a specific pharmacological agent, and (*c*) was seen only in patches excised from transfected cells, we conclude that it was due to the expression of the GAT-1 protein.

The high-frequency noise required Na^+ plus GABA, was voltage-dependent (Fig. 4*A*), and, like the in-gated current observed during whole-cell recording (7), required a group Ia cation in the extracellular solution (Table 2). We measured the spectral density of the noise induced by cytoplasmic GABA. The spectrum lacked amplitude at low frequencies and rose proportional to f^2 (Fig. 4*B*). The absence of spectral density at low frequencies indicates that the noise is not associated with a net movement of charge across the membrane; i.e., there is no measurable dc component.

A similar noise was also induced by extracellular GABA (Fig. 4 *C* and *D*). We filled pipettes with a solution containing NaCl plus 200 μM GABA and selected patches without channel openings. The cytoplasmic face was superfused with a series of solutions (Table 2). Fluctuations produced by extracellular GABA shared properties with the ex-gated current observed during whole-cell recording (7): both required either a group Ia cation or Cl^- in the intracellular solution.

How can a spectrum that rises proportional to f^2 be interpreted? The noise could be produced by charge movement within an incomplete transport cycle. Similar noise is produced by mobile carriers in a lipid bilayer (15). Another possibility is that the noise is produced by ions that hop in and out of an

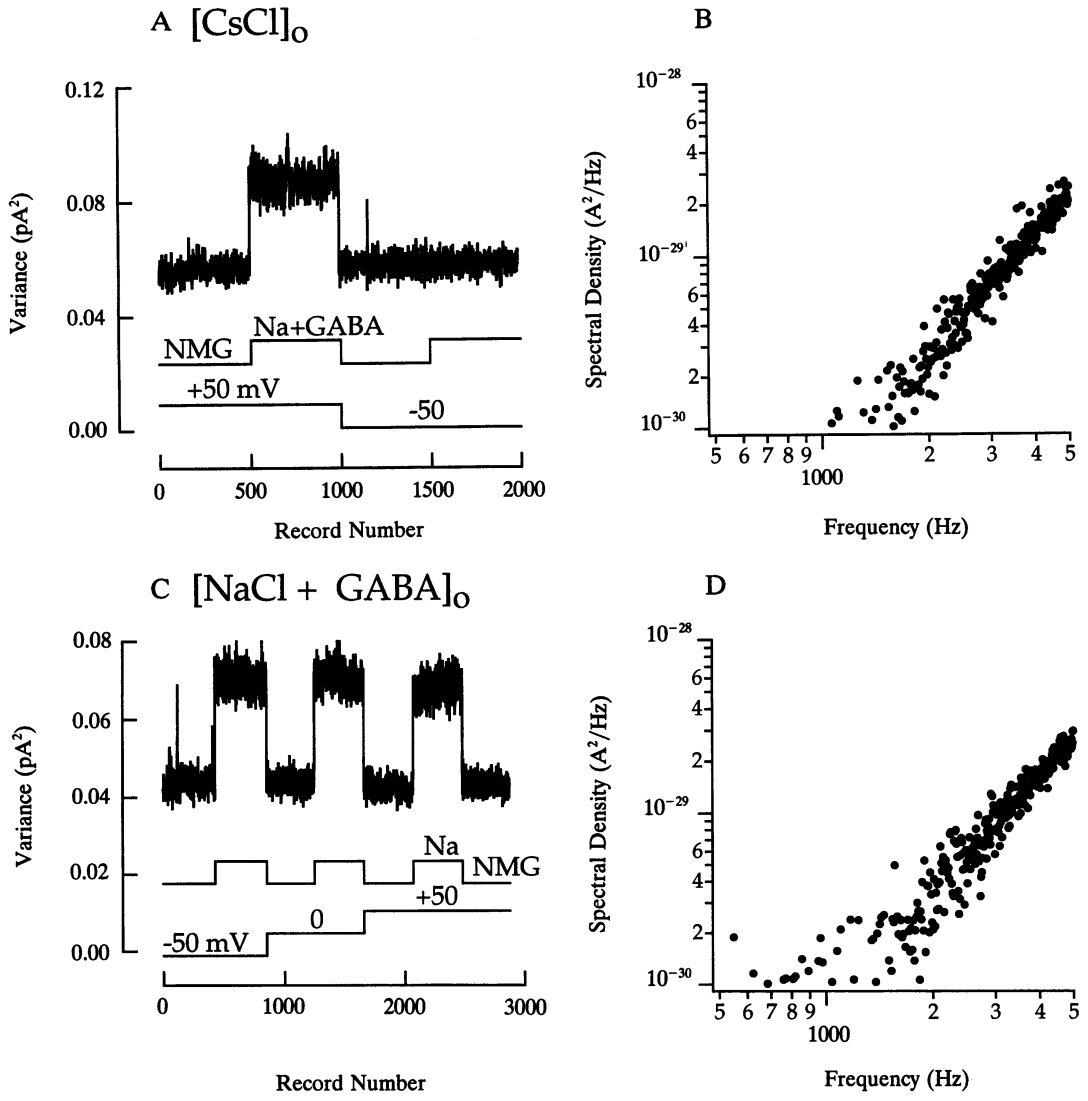

FIG. 4. Spectral density of noise induced by GABA. (*A*) Intracellular GABA. The patch pipette contained CsCl. The cytoplasmic face was superfused with NMG gluconate or NaCl plus GABA (1 mM) as indicated by the upper timing trace. Membrane voltage was maintained at +50 or −50 mV as indicated by the lower timing trace. (*B*) Spectral density of noise produced by intracellular GABA when the voltage was maintained at +50 mV. Same patch as in *A*. (*C*) Extracellular GABA. The patch pipette contained NaCl plus 200 μM GABA. The cytoplasmic face was superfused with NMG gluconate or NaCl as indicated by the upper timing trace. Membrane voltage was maintained at −50, 0, or +50 mV as indicated by the lower timing trace. (*D*) Spectral density of noise produced by intracellular NaCl (and extracellular Na^+ plus GABA) when the voltage was maintained at 0 mV. Same patch as in *C*. The same result was obtained when identical concentrations of Na^+ plus GABA were on both sides of the membrane (two patches). Individual records in *A* and *C* contained 1024 points and spanned 81.92 ms.

occluded pore. The movement of ions in an occluded pore will behave like a voltage-dependent capacitance. A resistor, R, and capacitor, C, in series produce a current noise with a spectrum

$$S(f) = \frac{4kT(2\pi fRC)^2}{R[1 + (2\pi fRC)^2]},$$

where k is Boltzmann's constant and T is absolute temperature. The spectrum is zero at dc, rises proportional to f^2, and reaches a high-frequency limit, $S(\infty) = 4kT/R$. If R is equal to the resistance of the patch pipette (3–4 MΩ), then an amplitude of 2×10^{-30} A^2/Hz at 2 kHz requires a capacitance of 1.3 pF. If a 10-μm^2 patch contains between 700 and 60,000 transporters (see above), then the noise spectrum is equivalent to a capacitance of between 10^{-15} and 10^{-17} F per transporter.

A transmembrane pore provides a simple explanation for both channel openings and high-frequency noise. Three observations provide hints for how a transporter built around a pore might work: (*i*) The binding of GABA at one membrane surface allowed Na^+ to hop into the pore from either surface (Table 2). (*ii*) The same complement of trans ions were required for the opening of channels (Table 1) and the entry of Na^+ into an occluded pore (Table 2). (*iii*) High-frequency fluctuations were observed during conditions that do not sustain a whole-cell current; although GABA-gated currents require both Na^+ and Cl^- (7), fluctuations were observed in the total absence of Cl^- (Table 2). These observations suggest that transport may be a two-step process. First, trans ions may allow Na^+ and GABA to move into a membrane pore. In the second step, Cl^- may allow Na^+ and GABA to exit across the membrane.

We conclude that the GAT-1 transporter forms both channels and translocaters: a small fraction of GAT-1 molecules function as channels; the vast majority operate as translocators that move GABA and co-ions across the membrane. The large inequality between the numbers of channels and translocators indicates that channels and translocaters function indepen-

Table 2. Ions required to observe high-frequency noise

Extracellular solution	Intracellular solution	n	Voltage (−50, +50 mV)
NaCl	NaCl, GABA	3	+ and −
Na gluconate	Na gluconate, GABA	2	+ and −
Na^+	**Na^+, GABA**		**+ and −**
CsCl	NaCl, GABA	3	+ only
Cs gluconate	NaCl, GABA	2	+ only
LiCl	NaCl, GABA	2	+ only
M^+	**Na^+, GABA**		**+ only**
NaCl, GABA	NaCl	2	+ and −
NaCl, GABA	Na gluconate	2	+ and −
Na gluconate, GABA	Na gluconate	3	+ and −
Na^+, GABA	**Na^+**		**+ and −**
NaCl, GABA	Cs gluconate	4	− only
NaCl, GABA	NMG-Cl	4	− only
Na^+, GABA	**M^+ or Cl^-**		**− only**

Every patch was superfused first with NMG gluconate and then a solution listed in the table. Every patch was tested at −50 and +50 mV. High-frequency noise was observed when a patch was exposed to a test solution and polarized to the potential indicated. The ions required for the noise increase are summarized in bold type. **M^+** indicates any group Ia cation; n is the number of patches.

dently. Once formed, channels were relatively stable during the lifetime of a patch. We do not know what change converts a translocater into a channel. The difference may be a post-translational modification or the state of aggregation. We have not explored whether the efficiency of forming channels can be influenced by second messengers or cytoplasmic factors.

Channels formed by other transporters may produce large whole-cell currents. A cloned serotonin transporter also produces an uncoupled transient current following a voltage step; however, the amplitude of the current is too large to be easily reconciled with the density of transporters in the membrane (6). The suspicion is that the current is produced by voltage- and time-dependent channels. Photoreceptors have a glutamate-gated Cl^- current that has been attributed to both channels (16) and a transporter (17). A cloned human glutamate transporter also produces a Cl^- conductance (18). The glutamate transporter in photoreceptors may form both (*i*) a translocator able to mediate the uptake and release of glutamate and (*ii*) channels able to produce a large, glutamate-gated current. Transporters that are able to form channels may produce large electrical signals and have cellular functions beyond the accumulation of substrate.

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