The Anticonvulsant Valproate Increases the Turnover Rate of γ-Aminobutyric Acid Transporters*

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Valproate is an important anticonvulsant currently in clinical use for the treatment of seizures. We used electrophysiological and tracer uptake methods to examine the effect of valproate on a γ-aminobutyric acid (GABA) transporter (mouse GAT3) expressed in Xenopus laevis oocytes. In the absence of GABA, valproate (up to 50 mM) had no noticeable effect on the steady-state electrogenic properties of mGAT3. In the presence of GABA, however, valproate enhanced the GABA-evoked steady-state inward current in a dose-dependent manner with a half-maximal concentration of 4.6 ± 0.5 mM. Maximal enhancement of the GABA-evoked current was 275 ± 10%. Qualitatively similar observations were obtained for human GAT1 and mouse GAT4. The valproate enhancement did not alter the Na⁺ or Cl⁻ dependence of the steady-state GABA-evoked currents. Uptake experiments under voltage clamp suggested that the valproate enhancement of the GABA-evoked current was matched by an enhancement in GABA uptake. Thus, despite the increase in GABA-evoked current, ion/GABA co-transport remained tightly coupled. Uptake experiments indicated that valproate is not transported by mouse GAT3 in the absence or presence of GABA. Valproate also enhanced the rate of the partial steps involved in transporter presteady-state charge movements. We propose that valproate increases the turnover rate of GABA transporters by an allosteric mechanism. The data suggest that at its therapeutic concentration, valproate may enhance the activity of neuronal and glial GABA transporters by up to 10%.

γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the central nervous system. Transport of GABA into cells is accomplished by Na⁺-dependent and Cl⁻-facilitated GABA transporters (GATs) found in the plasma membrane of neurons and glia (1–10). Thus, the GABA transporters regulate synaptic and extra-synaptic concentrations of GABA and, in this capacity, are partly responsible for the regulation of inhibitory neurotransmission in the nervous system. Because of the inhibitory role of GABA, potentiation of GABAergic neurotransmission via inhibition or reversal of the GABA transporters is believed to have therapeutic value in treating epileptic seizures and stroke (11–13). Indeed, inhibitors of the GABA transporters are known to increase GABA levels in the brain (14–16). These agents exhibit anticonvulsant activity in animal models, and one (tiagabine) that preferentially targets the most abundant GABA transporter isoform in the brain (GAT1) has been in clinical use since 1997 (16–22). Several other clinically used antiepileptic drugs are reported to act, at least in part, via potentiating GABA-mediated inhibition in the brain (11, 23); however, little is known regarding the potential effect of these drugs on the GABA transporters (24).

Valproate (2-propylpentanoate) has been in clinical use since 1967 and is effective against many types of epileptic seizures (both partial and generalized seizures). Although the exact mechanism of valproate action is not clear, its effectiveness as a broad-spectrum anticonvulsant is usually attributed to a combination of actions at multiple molecular targets (11, 25). A preponderance of evidence suggests that valproate potentiates GABAergic neurotransmission by increasing GABA levels in the brain (11, 26–32). The effect may be attributed both to enhanced GABA synthesis (stimulation of glutamic acid decarboxylase) and decreased GABA degradation (inhibition of GABA transaminase and succinic semialdehyde dehydrogenase) (11, 33–35). Correspondingly, many studies have examined valproate-induced GABA release from neurons and glia, although vesicular versus non-vesicular (i.e. transporter-mediated) release mechanisms have not been definitively addressed. These studies as well as those examining the effect of valproate on GABA uptake by neurons and/or glial cells have not provided entirely consistent results; however, the available evidence favors valproate-induced GABA release from nerve terminals in selected brain regions (e.g. 31, 32, 36–47; for review see Refs. 11 and 25). In light of this evidence, it is of interest to characterize the effect of valproate on the GABA transporters expressed in an expression system where any putative interaction may be examined by using sensitive biophysical tools.

Here, we have combined electrophysiological and tracer uptake methods to examine the effect of valproate on the GABA transporters expressed in Xenopus laevis oocytes. Our results suggest that valproate enhances the turnover rate of these transport proteins via an allosteric mechanism. The interaction of valproate with GATs opens new experimental avenues for probing the mechanism of Na⁺/Cl⁻/GABA co-transport.
Enhancement of GAT Turnover Rate by Valproate

EXPERIMENTAL PROCEDURES

Expression in Xenopus Oocytes—Stage V-VI X. laevis oocytes were injected with 50 nM cRNA for human GAT4 (48), mouse GAT4 (49), or rat Na+/iodide symporter (50). Oocytes were maintained in Barth’s medium (in mM: 88 NaCl, 1 KCl, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 0.82 MgSO₄, 2.4 NaHCO₃, 10 HEPES, pH 7.4, 50 µg/ml gentamicin, 100 µg/ml streptomycin, and 100 units/ml penicillin) at 18°C for 2–21 days until used in experiments. All of the experiments were performed at 21 ± 1°C.

Experimental Solutions—Unless otherwise indicated, experiments were performed in a NaCl buffer containing (in mM): 100 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.4. In Na⁺-free solutions, NaCl was isosmotically replaced with choline-Cl. In Cl⁻-free solutions, NaCl, KCl, CaCl₂, and MgCl₂ were isosmotically replaced with corresponding glycerol, and chloride and glycerol concentrations did not interact with the other transporters (5, 9, 10). GABA, sodium-valproate, and/or valproic acid were added to the above solutions as indicated, and the necessary pH adjustments were made in solutions containing valproate. In solutions containing sodium-valproate, the total cation and anion concentrations were maintained by isosmotic substitution of NaCl with sodium-valproate and/or sodium-glucurate. For the experiment involving the Na+/iodide symporter, ClO₃⁻ was used as a model substrate instead of iodide (for justification see Ref. 51). Unless otherwise indicated, all of the reagents were purchased from Sigma.

Tracer Uptake—Control and mGAT3-expressing oocytes were incubated for 30 min in solutions containing 100 µM GABA and various concentrations of valproate in addition to 22 nM [³H]GABA (Amersham Biosciences) or 0.65 µM [³H]valproate (American Radiolabeled Chemicals, St. Louis, MO). Oocytes were washed and solubilized in 10% SDS containing sodium dodecyl sulfate, and [³H]GABA or [³H]valproate content was determined in a liquid scintillation counter (Beckman LS 5000CE). For uptake under voltage clamp (see Fig. 2A), the membrane potential was held at −80 mV and the oocytes were initially incubated in the NaCl buffer until base line was established. GABA (100 µM) or GABA (100 µM) and valproate (5 mM) were added to the perfusion solution for 5–10 min, and the inward current was recorded. At the end of the incubation period, GABA, valproate, and the isotope were removed from the perfusion solution until the holding current returned to the base line. The oocytes were removed from the recording chamber, washed in ice-cold choline-Cl buffer, and solubilized in 10% SDS containing sodium dodecyl sulfate. The net positive charge trans-located into the cell was obtained from the time integral of the GABA-evoked inward current and correlated with GABA influx in the same cell (10, 51).

Electrophysiological Measurements and Data Analysis—The two-microelectrode voltage clamp technique was used for the recording of whole-cell transporter-mediated currents. Oocytes were voltage clamped by using the Warner Oocyte Clamp (OC-725C, Warner Instrument Corporation, Hamden, CT). In the recording experimental chamber, oocytes were initially stabilized in the NaCl buffer and the composition of the bath was changed as indicated. In all of the experiments, the reference electrodes were connected to the experimental oocyte chamber via agar bridges (3% agar in 3M KCl). Continuous holding currents were maintained at −80 mV. Currents were low pass-filtered at 1 kHz and sampled at 10 kHz. Following subtraction of the steady-state currents, the whole-cell capacitance was determined from the integral of the oocyte-capacitive transients (Cm = qVt, where q is the membrane capacitance, and Vt is the test voltage amplitude). The net positive charge translocated during the pulse protocol consisted of voltage jumps (300–400 ms) from the holding voltage (−50 mV) to test voltages ranging from −80 to −150 mV in 10- or 15-mV steps. Unless otherwise indicated, voltage pulses were separated by an interval of at least 4 s to allow for complete relaxation of mGAT3 OFF transients (10). Currents were low pass-filtered at 1 kHz and sampled at 12.5 kHz without averaging. To obtain the transporter presteady-state currents, at each Vm, the total current, I(t), was fitted to Equation 2,

\[
I(t) = I_{	ext{e}^{-at}} + I_{	ext{e}^{at}} + I_{ss}
\]

where I is the test time; I_{e^{-at}} is the oocyte-capacitive transient current with initial value I₀ and time constant t₀; I_{e^{at}} is the transporter transient current with initial value I and time constant t₂, and I_{ss} is the steady-state current (52). At each Vm, the transporter-mediated charge (Q) was obtained by time integration of the transporter transient currents. The charge-voltage (Q-V) relations obtained were then fitted to a single Boltzmann function as shown in Equation 3,

\[
Q = \frac{Q_{max} - Q_{min}}{1 + e^{(Vt - V_{1/2})/s}}
\]

where Q_{max} = Q_{dep} - Q_{free}, Q_{dep} and Q_{free} are Q at depolarizing and hyperpolarizing limits, respectively; z is the apparent valence of move-able charge; s is the fraction of the membrane electric field traversed by the charge; V_{0.5} is the potential for 50% charge movement; F is Faraday’s constant; R is the gas constant, and T is the absolute temperature.

To determine the whole-cell membrane capacitance (see Fig. 3) from a holding potential of −60 mV, the membrane was prepulsed to −100 mV for 500 ms and a 5-mV hyperpolarizing pulse (10 ms) was applied. Currents were low pass-filtered at 5 kHz and sampled at 10 kHz. After subtraction of the steady-state currents, the whole-cell capacitance was determined from the integral of the oocyte-capacitive transients (Cm = qVt, where q is the capacitance obtained from the integral of the capacitive transient current, and Vt is the test voltage amplitude). The charge-voltage (Q-V) relationship was based on the mGAT3 Q-V relationship, because at this voltage, no carrier-mediated charge movement is present (see Fig. 7C) (10).

PRESTEADY-STATE AND STEADY-STATE CURVE FITTING—By using either SigmaPlot (SPSS Science; Chicago, IL) or software developed in this laboratory (using a Marquardt-Levenberg algorithm). Unless otherwise indicated, each curve was repeated at least three times from different donor frogs. Where sample sizes are indicated (N), they refer to the number of oocytes in which the experiments were repeated. Unless otherwise indicated, reported errors represent the mean obtained from data from several oocytes.

RESULTS

Valproate Enhances mGAT3 Turnover Rate without Altering Ion/GABA Coupling.—In voltage-clamped (−60 mV) oocytes expressing mGAT3, application of valproate (5 mM) to the bathing medium had no effect on the holding current (Fig. 1A, left panel). Valproate concentrations as high as 50 mM did not elicit a mGAT3-mediated response (data not shown). In the presence of GABA (100 µM), however, the addition of valproate (5 mM) enhanced the GABA-evoked inward current by ∼100% (Fig. 1A, right panel). The effect of valproate was reversible, because after its washout from the bath, the GABA-evoked current returned to its initial level. Moreover, the effect of valproate did not diminish on repeated applications. Valproate led to an enhancement of the GABA-evoked current at all of the voltages tested (−148 to +80 mV) (Fig. 1B). After valproate washout, the GABA-evoked I-V relation was similar to that obtained before exposure to valproate (Fig. 1B). Valproate enhancement of the GABA-evoked inward current was saturable with a half-maximal concentration of 4.6 ± 0.5 mM (n = 3) (Fig. 1C). Maximum enhancement was 275 ± 10%. Correspondingly, in non-voltage-clamped oocytes, valproate enhanced GABA uptake in a concentration-dependent manner (Fig. 1D). Valproate alone (up to 50 mM) had no effect on the steady-state electrical properties of the membrane.
enhancement of mGAT3 GABA transport by valproate. A, current traces from a mGAT3-expressing oocyte maintained at a holding potential of ~60 mV. GABA and/or valproate were added as indicated by the bars. In the absence of GABA, valproate alone (5 mM) had no noticeable effect on the holding current of mGAT3-expressing cells (left panel). A similar observation was made at valproate concentrations of up to 50 mM. In the presence of GABA (100 μM), valproate (5 mM) enhanced the GABA-evoked response by ~100% (right panel). The effect of valproate was reversible with no apparent sensitization or desensitization on repeated applications. Previous applications of valproate had no effect on subsequent GABA responses. Thus, the valproate effect was seen only when GABA was present. B, current-voltage (I-V) relations of the mGAT3 GABA-evoked (100 μM) current were obtained before exposure to valproate (filled circles), in the presence of 10 mM valproate (filled squares), and after exposure to valproate (open circles). Similar I-V curves were obtained in six mGAT3-expressing cells. C, valproate enhancement of the GABA-evoked inward current (I_{GABA}) was saturable with a half-saturation concentration of 4.6 ± 0.5 mM and maximal enhancement of 275 ± 10% (n = 3) (V_m = ~60 mV). Inset shows the valproate effect within its therapeutic concentration range in the plasma and cerebrospinal fluid. D, valproate also enhanced GABA uptake into mGAT3-expressing oocytes in a concentration-dependent manner (N = 15 cells at each valproate concentration). The incubation medium contained 100 μM GABA, 22 nM [3H]GABA, and valproate at the indicated concentration. Values were normalized with respect to that in the absence of valproate (6.42 ± 0.38 pmol/min/oocyte). In control oocytes from the same batch, GABA uptake was not altered by valproate (0.29 ± 0.01 pmol/min/oocyte; N ≥ 10; data not shown). Similar results were obtained in four batches of cells.

Valproate enhancement of the GABA-evoked mGAT3 current raises the possibility that the observed effect may be attributed to reversible trafficking of mGAT3-containing vesicles to and from the plasma membrane. Thus, in cells expressing mGAT3, the GABA-evoked inward current and the valproate enhancement of the inward current were monitored (Fig. 3A) and concurrently the whole-cell membrane capacitance was measured (Fig. 3B) (see “Experimental Procedures”). In the cell shown in Fig. 3, base-line membrane capacitance was 260 nanofarads and no change was observed in the absence or presence of 5 mM valproate. A similar observation was made in four additional mGAT3-expressing cells. Thus, valproate does not lead to a significant change in the total surface area of the plasma membrane.

We tested the possibility that valproate may be recognized as a substrate by mGAT3 and, as such, transported across the plasma membrane. [3H]Valproate uptake was the same in control as well as mGAT3-expressing oocytes (N ≥ 20) (Fig. 4). Moreover, [3H]valproate uptake was not altered in the presence of GABA (100 μM) (N ≥ 20). Thus, valproate is not a transported substrate of mGAT3.

Valproate Decreases the Apparent Affinity for GABA—We examined the effect of valproate on the steady-state apparent affinity of mGAT3 for Na^+ and Cl^−, and GABA (Fig. 5). In these experiments, substrate (Na^+, Cl^−, or GABA) kinetics was ex-

However, in a small fraction of cells (~1%), high valproate concentrations (>10 mM) led to a small outward current of up to ~5 nA. These cells were not used for further investigation.

The large variability in the mGAT3 expression level between oocytes does not allow a reliable quantitative measure of the enhancement of GABA uptake relative to the GABA-evoked current. Thus, to obtain a quantitative comparison between the valproate enhancement of the GABA-evoked current and GABA uptake, we performed GABA uptake under voltage clamp in the absence and presence of 5 mM valproate. Thus, the valproate effect was seen only when GABA was present. A similar observation was made at valproate concentrations of up to 50 mM. In the presence of GABA (100 μM), valproate (5 mM) enhanced the GABA-evoked response by ~100% (Fig. 2B). The effect of valproate was reversible with no apparent sensitization or desensitization on repeated applications. Previous applications of valproate had no effect on subsequent GABA responses. Thus, the valproate effect was seen only when GABA was present.
Valproate does not alter vesicle trafficking to the plasma membrane. Panel A shows a current trace from an oocyte expressing mGAT3 and maintained at −60 mV. Base line was established in the NaCl buffer, and at the time indicated by the bar, GABA (100 μM) and [3H]GABA were perfused into the chamber. After washout of GABA and the isotope and return to the base line, the oocyte was washed in ice-cold choline-Cl buffer and counted in a liquid scintillation counter. The time integral of the GABA-evoked current trace yielded the total net-positive charge transported into the cell during the recording period. In an additional group of cells, valproate (5 mM) was also added to the perfusion solution. In control cells, GABA did not induce an inward current (data not shown).

A

B

FIG. 3. Valproate does not alter vesicle trafficking to the plasma membrane. Panel A shows a current trace from an oocyte expressing mGAT3 and maintained at −60 mV. Base line was established in the NaCl buffer, and at the time indicated by the bar, GABA (100 μM) and valproate (5 mM) were added to the perfusion chamber and the inward current (A) and the membrane capacitance (B) were monitored concurrently. To measure the whole-cell capacitance, every 5 s, the membrane was prepulsed to −100 mV for 500 ms, and a 5-mV hyperpolarizing pulse (10 ms) was applied. Whole-cell capacitance was obtained as the average of values determined for the ON and OFF capacitive transients (see “Experimental Procedures”). This protocol led to periodic gaps (~1 s in duration) in the current trace of panel A, which are too short in duration to be noticed at the time scale shown. Despite a large enhancement of the GABA-evoked inward current, the whole-cell capacitance (260 nanofarads) remained unchanged, suggesting unaltered vesicle trafficking to and/or from the plasma membrane (see “Discussion”). A similar observation was obtained in five mGAT3-expressing cells.

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A

B

FIG. 2. Tight ion/GABA coupling is maintained despite valproate-enhanced GABA transport. A, current trace from an oocyte expressing mGAT3 and maintained at −60 mV. Base line was established in the NaCl buffer, and at the time indicated by the bar, GABA (100 μM) and [3H]GABA were perfused into the chamber. After washout of GABA and the isotope and return to the base line, the oocyte was washed in ice-cold choline-Cl buffer and counted in a liquid scintillation counter. The time integral of the GABA-evoked current trace yielded the total net-positive charge transported into the cell during the recording period. In an additional group of cells, valproate (5 mM) was also added to the perfusion solution. In control cells, GABA did not induce an inward current (data not shown). B, the ratio of net inward charge (obtained from the integral of the inward current) to GABA uptake (obtained from [3H]GABA influx) determined in the same cells was 2.2 ± 0.1 charges/GABA in the absence (open circles; n = 7) and presence (filled circles; n = 8) of valproate. This experiment was repeated in two other batches of oocytes, and the results were identical to those shown here.

A

B

FIG. 4. Valproate is not a transported substrate of mGAT3. Uptake of [3H]valproate (0.65 μM; total valproate concentration, 2 mM) was examined in control and mGAT3-expressing oocytes in the absence and presence of 100 μM GABA. The experiments were performed in the NaCl buffer. Neither mGAT3 expression nor GABA (100 μM) had an effect on valproate uptake by control or mGAT3-expressing cells. Data represent the mean ± S.D. (N ≥ 20). In the same batch of oocytes, GABA uptake was 0.45 ± 0.02 pmol/min/oocyte (n = 10) in control cells and 12.23 ± 3.10 pmol/min/oocyte (n = 17) in mGAT3-expressing cells (data not shown). This experiment was repeated with oocytes from four different batches with similar results. Therefore, valproate is not transported by mGAT3. The relatively high uptake rate of valproate in control cells as well as in mGAT3-expressing cells is most probably contributed by mGAT3. The relatively high uptake rate of valproate in control cells as well as in mGAT3-expressing cells is most probably carried out at pH 7.4, it is unlikely that the observed transport rates would result from the activity of the endogenous H + -driven monocarboxylate transporter.

A

B

FIG. 5. Enhancement of GAT Turnover Rate by Valproate

Panel A shows a current trace from an oocyte expressing mGAT3 and maintained at −60 mV. Base line was established in the NaCl buffer, and at the time indicated by the bar, GABA (100 μM) and [3H]GABA were perfused into the chamber. After washout of GABA and the isotope and return to the base line, the oocyte was washed in ice-cold choline-Cl buffer and counted in a liquid scintillation counter. The time integral of the GABA-evoked current trace yielded the total net-positive charge transported into the cell during the recording period. In an additional group of cells, valproate (5 mM) was also added to the perfusion solution. In control cells, GABA did not induce an inward current (data not shown). B, the ratio of net inward charge (obtained from the integral of the inward current) to GABA uptake (obtained from [3H]GABA influx) determined in the same cells was 2.2 ± 0.1 charges/GABA in the absence (open circles; n = 7) and presence (filled circles; n = 8) of valproate. This experiment was repeated in two other batches of oocytes, and the results were identical to those shown here.

A

B

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mm valproate (100 mM Na\(^+\) and 100 \(\mu\)M GABA) \(n = 3\) (Fig. 5B). The relative enhancement induced by valproate was not dependent on [Cl\(^-\)]\(_a\) (\(\approx 150\%\) at all Cl\(^-\) concentrations) (Fig. 5B). The half-maximal concentration for GABA activation of the currents \(K_{0.5}\) was higher in the presence of 10 mM valproate (20 \(\pm\) 1 \(\mu\)M) than in its absence (8 \(\pm\) 1 \(\mu\)M) (100 mM Na\(^+\) and 96 mM Cl\(^-\)) \(n = 3\) (Fig. 5C).

**Valproate Enhancement of the Turnover Rate Is Specific to the GABA Transporters**—The effect of valproate is common to other GABA transporter isoforms (Fig. 6). Valproate enhanced the GABA-evoked inward current mediated by human GAT1 (hGAT1) and mGAT4 in a similar manner as it did for mGAT3. In general, the effects on the steady-state as well as the presteady-state properties were qualitatively similar to those shown for mouse GAT3. As valproate has significant membrane permeability (see Fig. 4), we were concerned that the effect may reflect a nonspecific membrane or cytoplasmic action of this agent. If that were true, it would be expected that another Na\(^+\)-coupled transporter with mechanistic features similar to those of GABA transporters (9, 51) would be affected in a similar fashion by valproate. When tested against the rat Na\(^+\)/iodide symporter, no valproate enhancement of the substrate-evoked current was observed (Fig. 6).

**Valproate Increases the Rate of Presteady-state Current Relaxations**—In response to step changes in the membrane voltage, GABA transporters exhibit presteady-state current transients (Fig. 7) (3, 9, 10, 53–55). These transient currents are thought to represent conformational changes associated with partial reactions of the transport cycle (see Fig. 11) and are composed of fast and slow components (10, 54). In this study, we focus only on the slow transients of mGAT3 that comprise the major fraction of the voltage-induced charge movements (10). Because of the Cl\(^-\) dependence of the presteady-state currents (3, 9, 10, 53), in these studies, valproate isoosmotically replaced gluconate while the Cl\(^-\) concentration was maintained at the values indicated. The most pronounced effect of valproate was an increase in the rate of relaxation of the transients (Fig. 7). In the GABA transporters, the relaxation of the ON transients is voltage-dependent. The time constant of the ON relaxation plotted as a function of the test voltage follows a bell-shaped distribution (see Fig. 8E for hGAT1 data) (10, 53, 54). For the OFF response, the relaxation time constants are only weakly voltage-dependent (see Fig. 8F for hGAT1 data). For the ON transients of mGAT3, the peak relaxation time constant was 36 \(\pm\) 4 ms (at \(-5\) mV) in the absence of valproate and 25 \(\pm\) 3 ms (at \(-25\) mV) in the presence of valproate \(n = 6\). The effect of valproate was more pronounced on the mGAT3 OFF transients (Fig. 7A and B, compare OFF transients; see also Fig. 8). We have shown previously that mGAT3 OFF transients relax slowly to a steady state with a time constant of \(\approx 1\) s (10). As evident in Fig. 7, panel B, the OFF transients reached steady state more rapidly in the presence of valproate \(t_{\text{off}} = 21 \pm 5\) ms; \(n = 6\) (Fig. 7B) than in its absence (Fig. 7A) (see below for further details).

Valproate led to a shift of the charge-voltage \((Q-V)\) relationship to more negative membrane potentials (Fig. 7C). \(V_{0.5}\) of the \(Q-V\) curve shifted by \(-21 \pm 3\) mV \(n = 6\) in the presence of saturating concentrations of valproate (50 mM). The valproate concentration for half-maximal shift in the \(V_{0.5}\) was 5.9 \(\pm\) 0.5 mM \(n = 6\) (Fig. 7D). Valproate had no effect on the maximum charge \(Q_{\text{max}}\) moved in response to the voltage pulses (Fig. 7, C and E). Finally, the apparent valence of the moveable charge \(z\) was not altered by valproate (Fig. 7F).

Because of the slow return of the charge (OFF transients) in mGAT3 following a voltage pulse (see Fig. 7A and Ref. 10), we elected to use hGAT1 to more clearly demonstrate the effect of valproate on the rates of the ON and OFF transients in response to voltage pulses (Fig. 8). Valproate (50 mM) led to a dramatic reduction in the time constants for the relaxation of both the ON and OFF transients (Fig. 8, A–C, E and F). The maximal charge \(Q_{\text{max}}\) was not altered at reduced [Cl\(^-\)]\(_a\) (3, 9), and in addition, similar to the observation made with mGAT3, valproate (up to 50 mM) had no effect on \(Q_{\text{max}}\) (Fig. 8D). In contrast to the observed response of mGAT3, valproate led to a right shift of hGAT1 Q-V relationship. The maximum shift in \(V_{0.5}\) was 18 \(\pm\) 2 mV \((n = 4)\). The mid-point of the Q-V curve \(V_{0.5}\) shifted to the left as [Cl\(^-\)]\(_a\) was reduced from 106 to 56 mM (gluconate replacement). \(V_{0.5}\) shifted from \(-29\) \(\pm\) 4 to \(-52 \pm 4\) mV \((n = 4)\). However, the addition of 50 mM valproate (equimolar replacement of gluconate) shifted the Q-V relationship toward positive membrane potentials. \(V_{0.5}\) shifted from \(-52 \pm 4\) to \(-33 \pm 4\) mV \((n = 4)\). The apparent valence of the moveable charge \(z\) was not significantly altered by the reduction in [Cl\(^-\)], or by addition of valproate \((1.0 \pm 0.1\) versus...
1.2 ± 0.1). Similar to the Q-V relationship, the $\tau_{ON}$-V curve shifted to the left as $[Cl^-]$ was reduced and again shifted to the right as valproate was added. Valproate led to a ~2.5-fold reduction in the ON relaxation time constants. At its maximum value (at -52 mV) $\tau_{ON}$ was 104 ± 4 ms without valproate and 42 ± 3 ms (at -33 mV) with valproate ($n = 4$). Valproate also led to a ~2.5-fold reduction in the OFF relaxation time constants (101 ± 3 ms versus 37 ± 2 ms ($n = 4$)).

Valproate Enhances the Presteady-state Charge Movements at Reduced Na⁺ and Cl⁻ Concentrations—The Na⁺, Cl⁻, and GABA dependence of mGAT3 presteady-state charge movements were examined in the absence and presence of valproate in the same cells (Figs. 9 and 10). We have shown previously that the charge movements of mGAT3 strictly depend on external Na⁺, because at zero [Na⁺]o no mGAT3-mediated charge movement is detected (10). In the absence of external Na⁺, no charge movement was detected in the absence or presence of valproate (Fig. 10A). However, significantly more charge was moved at lower [Na⁺]o in the presence of valproate than in its absence (Figs. 9, A and B, and 10A). At 10 mM [Na⁺]o, $Q_{max}$ was 8 nanocoulombs in the absence (Fig. 9A) and 15 nC in the presence of 10 mM valproate (Fig. 9B).
crease in \( Q_{\text{max}} \) at low \([\text{Na}^+]_o\) occurred despite the fact that at each concentration, the Q-V relationship was shifted toward more negative potentials (by \(-17\) mV) in the presence of valproate (see Fig. 7D). At saturating \([\text{Na}^+]_o\), \( Q_{\text{max}} \) was the same with or without valproate (Fig. 10A). Valproate led to an apparent decrease in the half-maximal concentration for \( \text{Na}^+ \) activation of the presteady-state charge movements (Fig. 10A). \( K_{\text{Na}}^{0.5} \) was \( 43 \pm 2\) mM \((n = 7)\) in the absence and \( 22 \pm 4\) mM \((n = 7)\) in the presence of 10 mM valproate. Valproate also appeared to reduce the Hill coefficient for \( \text{Na}^+ \) activation of the charge movements \((2.1 \pm 0.2 \text{ versus } 1.1 \pm 0.2 \text{ (n = 7)})\).

At zero \([\text{Cl}^-]_o\), the magnitude of mGAT3 charge movement is reduced by \( \sim 80\% \) (Fig. 9, compare panels C and E; see also Fig. 10B) \((10)\). At low \([\text{Cl}^-]_o\), more charge was preserved in the presence of valproate than in its absence (Fig. 10B). At nominal zero \([\text{Cl}^-]_o\), valproate (10 mM) increased \( Q_{\text{max}} \) from 7 to 17 nC (Fig. 9, compare panels C and D). At nominal zero \([\text{Cl}^-]_o\), \( Q_{\text{max}} \) was \( 19 \pm 1\% \) of that at 106 mM \([\text{Cl}^-]_o\) in the absence of valproate and \( 51 \pm 4\% \) in the presence of valproate \((n = 3)\) (Fig. 10B). The half-maximal concentration for \( \text{Cl}^- \) enhancement of the charge movements, however, was not altered by valproate \((16 \pm 2\text{ mM versus } 15 \pm 3\text{ mM; n = 3})\). At saturating \([\text{Cl}^-]_o\), \( Q_{\text{max}} \) was the same with or without valproate (Fig. 10B).

As GABA leads to a concentration-dependent reduction in \( Q_{\text{max}} \) \((10)\), no charge movements are observed in the presence of saturating concentrations \((1\text{ mM})\) of GABA (Fig. 9, compare panels E and F). In the absence of valproate, the GABA concentration for 50% reduction in \( Q_{\text{max}} \) was \( 6 \pm 1\mu\text{M} \((n = 4)\) and that in the presence of valproate \((10\text{ mM})\) was \( 25 \pm 1\mu\text{M} \((n = 3)\) (Fig. 10C).

**DISCUSSION**

We propose that valproate leads to an increase in the turnover rate of GABA transporters and that the effect involves an important rate-limiting step in the transport cycle. Our data do not allow us to identify the partial step in the transport cycle that is altered by valproate; however, we have shown that valproate increases the turnover rate for the forward mode of the transporter (Fig. 11A) as well as increases the rates of conformational changes of the empty carrier (presteady-state relaxations) (Fig. 11B, shaded steps). Valproate interaction with the transporter appears to be allosteric involving a site other than the GABA binding site, because valproate is not a transported substrate of GATs and valproate interaction does not compete with GABA. Indeed, valproate enhances GABA translocation across the plasma membrane in the forward transport mode. Despite enhancement of GABA transport across the plasma membrane, the ion/GABA coupling ratio remains the same, suggesting that the transport cycle remains tightly coupled. Valproate interaction with the GABA trans-
porters is specific as other short chain fatty acids such as butanoic acid and pentanoic acid were without effect (data not shown). Furthermore, valproate did not enhance the rate of transport for the Na\(^{+}\)/H\(^{+}\)/iodide symporter, a Na\(^{+}\)/H\(^{+}\)-coupled transporter with mechanistic features similar to those of the GABA transporters. Examination of transporter presteady-state kinetics suggests that valproate increases the apparent affinity of the empty transporter for Na\(^{+}\). The presteady-state charge movements further suggest that valproate can interact with the transporter in the absence of GABA and Cl\(^{-}\). However, it cannot be determined whether valproate can interact with the transporter in the absence of Na\(^{+}\), because no charge movements are induced by valproate in the absence of external Na\(^{+}\). Finally, valproate-induced enhancement is rapid and limited only by the speed of the perfusion system, and moreover, the enhancement is fully reversible.

At least three observations suggest that valproate interaction with mGAT3 is not at the GABA binding site. (i) Valproate alone neither evokes an inward current nor is it itself transported across the plasma membrane alone or in the presence of GABA. (ii) Increasing concentrations of valproate enhance both the GABA-evoked current and GABA uptake, whereas interaction at the GABA binding site would be expected to lead to competitive inhibition of GABA uptake. (iii) Valproate had no effect on the maximal charge moved in response to voltage pulses \((Q_{\text{max}})\). \(Q_{\text{max}}\) is a measure of the total number of functional transporters available to bind substrate (56). As transported substrates lead to a concentration-dependent reduction in \(Q_{\text{max}}\) (Fig. 10C) (10, 51), the results suggest that interaction of valproate with the GABA transporters occurs in an entirely different fashion from that of a transported substrate. Because of the moderate membrane permeability of valproate, it is not possible to know whether this drug acts at the extracellular, intracellular, or membrane-spanning region of the transporter.

The effect of valproate cannot be attributed to an increase in the total number of transporters caused by vesicle trafficking to the plasma membrane. Four observations support this view. (i) The total number of transporters in the plasma membrane (as determined from \(Q_{\text{max}}\)) was not changed by valproate. (ii) The effect was fully reversible and repeatable in the same cell, persisting for as long as the experiment was continued. It seems unlikely that such mGAT3-containing vesicle insertion could be balanced rapidly and precisely by recruitment of mGAT3 in retrieved vesicles. (iii) The effect was specific to the

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**Fig. 9.** Valproate increases the presteady-state charge movements at reduced Na\(^{+}\) and Cl\(^{-}\) concentrations. Current traces were recorded from a mGAT3-expressing oocyte in response to voltage pulses (200 ms) ranging from +80 to −120 mV in 10-mV steps. A and B, at 10 mM [Na\(^{+}\)], significantly more charge was seen in the presence of 10 mM valproate (B) than in its absence (A). \(Q_{\text{max}}\) was 8 nC without and 15 nC with valproate. Na\(^{+}\) was isosmotically replaced with choline. C and D, in the nominal absence of external Cl\(^{-}\), more charge was observed in the presence of 10 mM valproate (D) than in its absence (C). \(Q_{\text{max}}\) was 7 nC without and 17 nC with valproate. E and F, saturating concentrations of GABA lead to the elimination of charge movements. The relevant composition of the bathing medium is indicated (in mM) for each panel. Records from all six panels were obtained from the same cell. The scale bars shown apply to all panels.
Similar to those indicated for Fig. 9. Charge movements occur in response to voltage perturbations and represent another. The external or internal membrane surfaces as well as the translocation series of partial reactions involving binding/dissociation of ligands at GABA transporters. The transport cycle may be schematized by a panel were obtained from the same oocyte.

FIG. 10. Effect of valproate on Na\(^+\), Cl\(^-\), and GABA dependence of the presteady-state charge movements. Na\(^+\), Cl\(^-\), and GABA dependence of mGAT3 presteade-state charge movements were examined in the absence and presence of 10 mM valproate. Voltage pulses were similar to those indicated for Fig. 9. A, at nominal zero [Na\(^+\)]., \(Q_{\text{max}}\) is below the resolution of the measurements (≤2 nC). In the absence of external Na\(^+\), valproate (10 mM) does not induce presteade-state charge movements. The half-maximal concentration for Na\(^+\) activation of the change movements was 43 ± 2 mM (\(n = 7\)) in the absence of valproate (open circles) and 22 ± 4 mM (\(n = 7\)) in the presence of 10 mM valproate (filled circles). The Hill coefficient was 2.1 ± 0.2 in the absence and 1.1 ± 0.2 in the presence of valproate (\(n = 7\)). For the experiment shown, \(Q_{\text{max}}\) was 18 nC. B, valproate (10 mM) had no effect on the half-maximal concentration for Cl\(^-\) enhancement of the charge movements (16 ± 2 mM versus 15 ± 3 mM; \(n = 3\)). For the experiment shown, \(Q_{\text{max}}\) was 35 nC. C, saturating GABA concentrations lead to the elimination of presteade-state charge movements (see Figs. 9, E and F). The GABA concentration for 50% reduction in \(Q_{\text{max}}\) was 6 ± 1 μM (\(n = 4\)) in the absence of valproate (open circles) and 25 ± 1 μM (\(n = 3\)) in the presence of 10 mM valproate (filled circles). For the experiment shown, \(Q_{\text{max}}\) was 28 nC. The results shown in each panel were obtained from the same oocyte.

FIG. 11. Proposed scheme of the effect of valproate on the GABA transporters. The transport cycle may be schematized by a series of partial reactions involving binding/dissociation of ligands at the external or internal membrane surfaces as well as the translocation of the ligand-bound or empty binding sites from one membrane surface to another. C, carrier; Na\(^+\), Na\(^{\text{trans}}\); Cl\(^-\), Cl\(^{\text{trans}}\); and GABA. The subscripts o and i refer to the outward and inward, respectively, facing carrier binding sites. A, valproate enhances the overall rate for the forward transport cycle (clockwise transitions; i.e. Na\(^+\)/Cl\(^-\)/GABA co-transport into the cell). See Figs. 1, 2, 5, and 6 for supporting evidence. B, the charge movements occur in response to voltage perturbations and represent Na\(^+\) and Cl\(^-\) binding/dissociation as well as conformational changes of the empty transporter. The shaded steps correspond to those thought to be responsible for the presteade-state charge movements. Valproate decreases the time constants for the ON and OFF relaxation of the presteade-state charge movements. Thus, the rates for both the ON transitions (counterclockwise) and the OFF transitions (clockwise) are increased by valproate. See Figs. 7 and 8 for supporting evidence.

GABA transporters as the transport rate for the Na\(^+\)/iodide symporter was not altered. (iv) There was no change in the whole-cell capacitance in the presence of valproate. This is shown clearly in Fig. 3, A and B. We have shown previously that heterologous membrane proteins expressed in oocytes are targeted to the plasma membrane in 100-nm diameter vesicles containing 5–40 copies of the expressed protein (57). In the oocyte of Fig. 3A, the GABA-evoked current was enhanced by ~50 nA. If it is assumed that the enhancement was because of insertion of new mGAT3 copies in the plasma membrane, it can be estimated that a total of ~7 × 10\(^{10}\) transporters were newly inserted into the membrane (\(I = NRze\), where \(I\) is current, \(N\) is the number of transporters, \(R\) is the turnover rate (2 s\(^{-1}\) at ~60 mV; see Ref. 10), \(z\) is the net charge trans-located across the membrane per transport cycle (2.2; see legend to Fig. 2B), and \(e\) is the elementary charge). Assuming that there were 5–40 copies of mGAT3 per vesicle, 2 × 10\(^{9}\) to 14 × 10\(^{9}\) vesicles would have been expected to fuse with the plasma membrane, leading to a 2–16-fold increase in the total surface area of the oocyte. Clearly, with a resolution of ~10 nanofarads, such an increase in capacitance (260 nanofarads to at least 520 nanofarads) would have been detected (see legend to Fig. 5B).

A prominent effect of valproate was an increase in the rate of the presteade-state ON and OFF relaxations. Upon application of depolarizing voltage pulses, the ON transients represent the release of Na\(^+\) and Cl\(^-\) followed by reorientation of the empty carrier (Fig. 11B, shaded steps). Upon return from the test voltage to the holding voltage, the OFF transients represent the return of the binding sites to the external medium, Na\(^+\) and Cl\(^-\) entry into the membrane electric field, binding, and subsequent ligand-induced conformational changes (Fig. 11B) (3, 52–55). As presteade-state charge movements are obtained in the absence of GABA, the data suggest that valproate also increases the rates associated with voltage-induced conformational changes of the empty carrier.

Valproate also led to a small reduction in the apparent affinity for GABA. Both the half-maximal concentration for steady-state GABA-evoked inward current and the GABA concentration for 50% reduction in \(Q_{\text{max}}\) were increased by valproate. The values increased from ~6 μM in the absence of valproate to ~20 μM in the presence of 10 mM valproate. Apparently, valproate reversibly transforms mGAT3 into a lower affinity, higher capacity transporter. Interestingly, valproate was also reported to decrease the GABA affinity of the astroglial GABA uptake system (44), although maximal transport rates were not altered.

At saturating Na\(^+\) and Cl\(^-\) concentrations, valproate did not alter the maximal charge moved in response to voltage pulses.
Enhancement of GAT Turnover Rate by Valproate

(see Figs. 7, 8, and 10), suggesting that valproate entry/exit into/out of the membrane electric field does not contribute to presteady-state charge movements. Valproate did not alter the apparent valence of the moveable charge (ε), suggesting that the same number of charges moved the same distance within the membrane electric field in the absence or presence of valproate. Valproate led to a decrease in the half-maximal concentration for Na⁺ activation (43 versus 22 mM) of the charge movements but had no effect on the half-maximal concentrations for Cl⁻ enhancement (15 mM) of the charge movements. Therefore, the results suggest that valproate increases the apparent affinity of the empty transporter for Na⁺. Thus, by increasing the affinity of the empty carrier for Na⁺, significantly more charge can be moved at lower Na⁺/H⁺ concentrations for Cl⁻/H⁺/Na⁺ increasing the affinity of the empty carrier for Na⁺. Therefore, the results suggest that valproate increases the activity of neuronal and glial GABA transporters by up to 10%. Valproate has been shown to lead to a 15% increase in the turnover rate induced by valproate. We were unable to observe such effects under our experimental conditions. Indeed, we have provided direct evidence against different types of seizures. The wide spectrum of anticonvulsant activity against different types of seizures. The wide spectrum of anticonvulsant activity most certainly arises from diverse molecular actions. Here, we have presented data for an additional role of valproate in the central nervous system, the enhancement of the turnover rate of the GABA transporters. We predict that at therapeutic concentrations, valproate may enhance the activity of neuronal and glial GABA transporters by up to 10%.

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REFERENCES


CONCLUSION

Since its fortuitous discovery as an anticonvulsant in 1962, valproate has become one of the most widely used drugs of its class, perhaps because of its wide spectrum of anticonvulsant activity against different types of seizures. The wide spectrum of activity most certainly arises from diverse molecular actions. Here, we have presented data for an additional role of valproate in the central nervous system, the enhancement of the turnover rate of the GABA transporters. We predict that at therapeutic concentrations, valproate may enhance the activity of neuronal and glial GABA transporters by up to 10%.
Enhancement of GAT Turnover Rate by Valproate