GABA reverse transport by the neuronal cotransporter GAT1: influence of internal chloride depletion

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Bertram S, Cherubino F, Bossi E, Castagna M, Peres A. GABA reverse transport by the neuronal cotransporter GAT1: influence of internal chloride depletion. Am J Physiol Cell Physiol 301: C1064–C1073, 2011. First published July 20, 2011; doi:10.1152/ajpcell.00120.2011.—The role of intracellular ions on the reverse GABA transport by the neuronal transporter GAT1 was studied using voltage-clamp and [3H]GABA efflux determinations in Xenopus oocytes transfected with heterologous mRNA. Reverse transport was induced by intracellular GABA injections and measured in terms of the net outward current generated by the transporter. Changes in various intracellular ionic conditions affected the reverse current: higher concentrations of Na+ enhanced the ratio of outward over inward transport current, while a considerable decrease of the outward current and a parallel reduction of the transporter-mediated GABA efflux were observed after treatments causing a diminution of the intracellular Cl− concentration. Particularly interesting was the impairment of the reverse transport observed after depletion of internal Cl− generated by the activity of a coexpressed K+-Cl− exporter KCC2. This finding suggests that reverse GABA transport may be physiologically regulated during early neuronal development, similarly to the functional alterations seen in GABA receptors caused by KCC2 activity.

γ-aminobutyric acid; KCC2; Xenopus oocytes

NEUROTRANSMITTER TRANSPORTERS (NTTs), located on presynaptic terminals and surrounding glia cells in the central nervous system (CNS), are necessary for the reuptake of neurotransmitter molecules from the synaptic space. Their involvement in the termination of synaptic transmission, the prevention of neurotransmitter spread to neighboring synapses, and the maintenance of the neurotransmitter concentration below neurotoxic levels make them essential for an efficient signal transduction in the brain. Beside the uptake mode, NTTs can also work in reverse and they can release neurotransmitter in a nonvesicular, calcium-independent manner, as demonstrated, for example, for the γ-aminobutyric acid (GABA) transporter GAT1 (7, 22, 23). GAT1 is thought to play an important role in physiologically regulating the level of tonic inhibition in the CNS, thereby contributing to the control of brain excitability. Failure of this function may result in pathological conditions such as epileptic seizures, possibly caused by GABA spillover through GABA transporters (31, 32).

Under normal conditions, GAT1, a member of the SLC6 family, couples GABA uptake to that of two Na+ ions and one Cl− ion. Recently, on the basis of the atomic structure of the bacterial homologue LeuT (43), the putative external Cl−-binding site of GAT1 was identified (4, 12, 47): mutations of an uncharged serine residue in position 331 to a negatively charged amino acid converted GABA transport into a Cl−-independent process. Furthermore, it has been shown that a higher external Cl− concentration facilitates the binding of external Na+ ions necessary for the GAT1 transport cycle (13, 20, 24). These observations suggest that a negative charge is required for GABA translocation and that, in GAT1, which lacks an intrinsic negative charge, this is provided by Cl−-binding. Indeed, Cl−-independent transporters of the same family have a negative amino acid residue in the corresponding position (E290 in LeuT), and weakly Cl−-dependent transporters, such as KAAT1 and CAATCH1, have an uncharged serine residue in the corresponding position, but a negatively charged aspartate four positions before (5).

Investigating the relevance of intracellular Cl− in the reverse transport mode of GAT1 is a difficult task because of the need for controlling the cytoplasmic milieu. Anyhow, important insights were gained by few previous studies (7, 22, 23): according to these authors, intracellular Cl− and extracellular Na+ bind to the transporter in a mutually exclusive fashion. Consequently, high intracellular Cl− concentrations will sequester the transporter in an “inward facing” conformation, reducing the inward rate of charge movement underlying the pre-steady-state currents in absence of substrate, and hindering the rate of substrate uptake. Conversely, high cytoplasmic levels of Cl− should favor the reverse transport of GABA, since Cl−-binding precedes the binding of internal substrate in the reverse transport cycle (7, 22, 23).

Changes in the internal chloride concentration occur in neurons during development, because of the differential activity of the chloride-extruding cotransporters of the cation-chloride families (2, 33). The delayed expression of the potassium-chloride cotransporter KCC2 causes a progressive decrease in internal chloride concentration that is sufficient to revert from depolarizing to hyperpolarizing the GABA-induced postsynaptic potentials observed in different kinds of central neurons (3, 9, 21, 45). Indeed, hyperexcitability and epileptic seizures were reported in mice deprived of the KCC2 symporter (40), and an altered Na+-K+-2Cl− cotransporter (NKCC2)/KCC2 balance appears to decrease GABAergic inhibition in the human peritumoral epileptic cortex (11).

However, whether physiologically regulated changes in the internal Cl− concentration also influence the reverse transport mode of GAT1 and to what extent this might contribute to the regulation of neuronal activity are unknown.

On the basis of these considerations we have therefore undertaken experiments aimed to investigate the role of internal chloride on the efficiency of reverse neurotransmitter trans-
port by the neuronal transporter GAT1. Taking advantage of the possibility of coexpressing other transporters in Xenopus oocytes, we also explored the option that physiologically regulated internal chloride changes might influence the reverse transport process.

MATERIALS AND METHODS

Oocyte preparation and heterologous expression. The detailed experimental procedure was previously described (6). To prepare the mRNA for oocyte injection, the cDNA encoding the rat GABA cotransporter GAT1, cloned into the pAMV-PA vector, was linearized with NotI, while the cDNA encoding the human KCC2 cotransporter (kind gift of Prof. G. Gamba, Mexico City, Mexico), cloned into the pGEMHE vector, was linearized with NheI. Subsequently, the cRNAs were synthesized in vitro in the presence of Cap Analog and 200 units of T7 RNA polymerase. All enzymes were supplied by Promega (Milan, Italy).

Portions of ovary were removed through a small incision on the abdomen from Xenopus laevis frogs anesthetized in MS222 (tricaine methanesulfonate; SIGMA) 0.10% wt/vol solution in tap water. Animals were obtained from the Animal Experimentation Center of the University of Rome (Milan, Italy).

External solutions for recording. An ideal bathing medium consisted of NaCl, KCl, CaCl2, MgCl2, and HEPES (DI water pH 7.4). Solutions were equilibrated with 95% O2 and 5% CO2 at 20°C. The intracellular pipette solution was composed of (in mM): 96 NaCl, 2 KCl, 1 MgCl2, 5 HEPES, 1.8 CaCl2, and 2.5 pyruvate for at least 1 h at 18°C. Healthy looking stage V and stage VI oocytes were collected.

The oocytes were injected with 50 nl of water containing 12.5 ng of cRNA coding for rat GAT1 (rGAT1) or, in some experiments, also 12.5 ng of mRNA coding for the potassium-chloride symporter KCC2, using a manual microinjection system (Drummond). The oocytes were incubated at 18°C for 3–4 days in modified Barth’s saline solution [MBS, in mM: 88 NaCl, 1 KCl, 0.33 Ca(NO3)2, 0.41 CaCl2, 0.82 MgSO4, 2.4 NaHCO3, 5 HEPES] before the electrophysiological studies.

Intracellular loading of substrates. Oocytes were injected with a 50-nl drop containing variable amounts of Na+, Cl−, and GABA, as specified in the text, and buffered with HEPES at pH 7.6. With an oocyte diameter of 1.2 mm, a spherical volume of ~1 μl is obtained; therefore a 50-nl injected drop will undergo a 20× dilution. However, not all the theoretical volume may be available for free diffusion of molecules and ions (46), so that a 10× dilution factor may be more appropriate. The diffusion coefficients of ions and small organic molecules in the oocyte cytoplasm are reported to be in the order of 10−5 cm2/s (35). The expected equilibration time constant in the oocyte cytoplasm is then in the order of a few hundred seconds, and this is confirmed by experimental observations as well (8, 18).

Electrophysiology. The currents generated by the transport activity of GAT1 were investigated by classical two-electrode voltage-clamp experiments. Intracellular glass microelectrodes were filled with 3 M KCl or with saturating K2SO4 and had tip resistances between 0.5 and 2 MΩ. Agar bridges (3% agar in 3 M KCl) connected the bath electrodes to the experimental chamber. The holding potential (Vh) was kept at −40 mV, unless otherwise indicated. The standard protocols consisted of 200- or 500-ms pulses to test potentials from −120 to +60 mV in 20-mV increments. In some experiments, voltage ramps spanning the voltage from −140 to +60 mV in 1.5 s were used. To estimate the amount of intramembrane charge movement, the currents recorded in the absence of substrate were subtracted from those in its presence. The resulting traces were then integrated to calculate the amount of displaced charge, after zeroing any residual steady-state transport current.

Data analysis was performed using Clampfit 8.2 (Molecular Devices), and figures were prepared with Origin 5.0 (Microcal Software, Northampton, MA).

Uptake experiments. GABA uptake was measured as described previously (25). Briefly, groups of 8–10 oocytes were incubated for 60 min in 100 μl of solution containing (in mM) 100 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM MgSO4, 10 mM HEPES (Tris, pH 7.5) with 100 μM [3H]GABA (444 Bq/ml; 3.33 TBq/mmol), rinsed in ice-cold wash solution (100 mM choline chloride, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES/Tris, pH 7.5) and dissolved in 250 μl of 10% SDS for liquid scintillation counting.

Effect of radioactive GABA. Groups of 20–25 oocytes in triplicates were injected with 50 nl of a solution having the following composition: 98 mM NaCl (or Na-glutamate in the Cl−-depletion experiments), 1 mM MgCl2, 5 mM HEPES pH 7.6 with 100 mM [3H]GABA (10 mM final concentration in oocytes, 18.5 GBq/ml, 3.33 TBq/mmol). After 10 min of recovery in ice-cold ND96, oocytes were incubated for 60 min in 500 μl of an efflux solution having the following composition: 98 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES pH 7.6, at room temperature. The efflux solution was collected, and radioactivity was measured by liquid scintillation counting. GABA release is expressed as percentage of the total GABA injected in oocytes.

Intracellular chloride depletion. For the depletion of intracellular chloride, three methods were used. First, oocytes were incubated overnight in a hypotonic Cl−-free solution containing (in mM) 70 Na-glutamate, 2 K-glutamate, 10 Ca-glutamate, 1 Mg-glutamate, and 5 HEPES; pH 7.6. The Ca2+ concentration was elevated to 10 mM to compensate for the Ca2+-chelating action of glutamate (10). Second, oocytes were coexpressed with a mRNA encoding the potassium and chloride cotransporter KCC2 and then incubated in the hypotonic Cl−-free solution described above. Third, oocytes coexpressing GAT1 and KCC2 were incubated in isotonic solution with normal Cl−. These treatments were previously used to effectively reduce the intracellular Cl− concentration (27, 28). In these experiments the intracellular glass microelectrodes were filled with saturated K2SO4 thereby avoiding leakage of highly concentrated chloride into the cell. For the same reason, the injection drop in these experiments contained Na-glutamate instead of NaCl.

External solutions for recording. External solutions during recording consisted of (in mM) 1 M MgCl2, 1.8 CaCl2, 5 HEPES, and 98 NaCl or 98 TMACl, pH 7.6. To induce transport-associated currents, 300 μM GABA was added to the bath solution. To block transport activity, 30 μM of the specific rGAT1 blocker SKF89976A was added.

Statistical analysis. Averaged data are given as means ± SE. Significance levels are estimated by Student’s t-test, or ANOVA and Bonferroni’s test, as specified in each case in the text.

RESULTS

Detection of the outward transport current. To identify the outward transport current generated by the reverse operation of GAT1, voltage-clamp pulses were applied in different conditions, before and after the injection of GABA in the cytoplasm. Figure 1 offers a synopsis of the currents recorded in a representative oocyte in the various conditions. The traces in the top row were taken before GABA injection. Confirming previous results, the conspicuous pre-steady-state currents seen in the presence of sodium and in the absence of external GABA (GABAout = 0, left) are abolished when a saturating amount of neurotransmitter (GABAout = 300 μM) is added, a condition in which large inward steady currents are observed (middle). When external Na+ is replaced by the larger cation TMA+ (GABAoutabsent), the pre-steady-state currents are abolished (right). The dashed horizontal line shows that the steady
Fig. 1. Synopsis of the current elicited in a GABA transporter 1 (GAT1)-expressing oocyte by voltage steps from −40 mV holding potential and ranging between −120 and +40 mV (20-mV steps) in the indicated conditions. Traces in the center and bottom rows were obtained in succession starting 10 min after the injection of a 50-nl drop of concentrated GABA and NaCl solution (both at 100 mM). Dotted lines indicate the zero current level. Dashed lines in the first two rows are drawn for reference at the level of the most positive steady current in presence of external Na⁺ (Na_{out}). TMA⁺, tetramethylammonium.

The subtracted steady-state current-voltage (I–V) relationships, averaged over several oocytes, are shown in Fig. 2, together with the inward transport current elicited by the application of 300 μM GABA_{out} before and after the intracellular injection. The net inward transport current before injection (solid squares in Fig. 2) was obtained as the difference between GABA_{out} present and GABA_{out} absent, without SKF89976A, because of the very slow washout time of the blocker (37).

Assuming that in the presence of 30 μM SKF89976A the reverse current is completely blocked, the results after injection of GABA may be interpreted as follows: when no GABA is present extracellularly, the transporter is operating only in reverse mode, i.e., it is generating an outward current both in the presence and in the absence of extracellular Na⁺ (up and down triangles in Fig. 2A). Indeed, when external Na⁺ is replaced by TMA⁺ (down triangles), no inward current is observed over the entire voltage range. At the most positive voltages the outward transport currents in Na⁺ and in TMA⁺ are identical, while a small inward current is generated at negative potentials in the presence of Na⁺, possibly suggesting some leak of this ion through the transporter (24, 26). When 300 μM extracellular GABA is present, inward transport currents are generated. The I–V relationship obtained by subtracting the currents in Na⁺/SKF89976A (i.e., when the transporter is blocked) shows the usual inward-rectifying curve. This finding suggests that with 300 μM GABA outside the inward mode of operation predominates even in the presence of −10 mM GABA inside. However, when GABA is present intracellularly, the inward transport current is significantly reduced.

The observations illustrated in Fig. 2A were consistently seen in all oocytes loaded with GABA. Intracellular GABA is absolutely necessary for the production of the outward current.
as demonstrated in Fig. 2B, in which the results from a group of oocytes injected with droplets containing only NaCl but not GABA are plotted. In this case the $I-V$ curve generated by external GABA after the injection of NaCl (open circles) is identical to that obtained before the injection (solid squares), and, furthermore, no outward current can be observed as difference following SKF89976A block of the transporter in the presence of either Na$^+$ (up triangles) or TMA$^+$ (down triangles).

**Efflux of radioactive GABA.** To confirm that the outward currents measured in the oocytes loaded with GABA were truly accompanied by a GABA efflux, the electrophysiological experiments described above were complemented with the measurement of radioactive GABA fluxes. The results are shown in Fig. 3. These experiments were performed in the presence of a high external concentration of K$^+$ (98 mM KCl) to depolarize the membrane, favoring in this way the reverse operation mode, and in the absence of extracellular GABA. Replacement of external Na$^+$ with K$^+$ instead of TMA$^+$ gave identical results from the electrophysiological point of view: Fig. 3A shows indeed that the outward current observed in GABA-injected oocytes in the presence of high external potassium (up triangles) is very similar to that observed in high external TMA$^+$ (Fig. 2A, down triangles). GABA efflux from GAT1-transfected oocytes was 2.4-fold higher than that from nontransfected oocytes after the injection of the same concentration of radioactive GABA (Fig. 3B). The expression of GAT1 in the transfected oocytes was
verified by measuring the uptake of 0.1 mM GABA (Fig. 3C). These results confirm therefore that, in the same conditions used to detect the outward currents, a significant GABA efflux is observed.

**Ratio of outward to inward currents in different conditions.**

To obtain an estimate of the reverse GABA transport independent of the different expression level of the individual oocyte, we calculated for each cell the ratio between the outward current (defined as the difference between the current in Na\(^+/\)H\(^+\) minus Na\(^+/\)SKF89976A, up triangles in Fig. 2A) at +60 mV, and the absolute value of the inward transport current (defined as the difference between the current in GABA\(_{\text{out}}\) minus Na\(^+/\)SKF89976A, circles in Fig. 2A) at −120 mV. As it is shown in Fig. 4, this ratio \(I_{\text{out}}(+60)/I_{\text{in}}(-120)\) amounts to −0.38 in oocytes injected with 100 mM GABA and NaCl and tested after 10 min. The other results in Fig. 4 show that this ratio does not change significantly if the currents are measured 1 h after the injection, while lower ratios are obtained if smaller quantities of GABA are injected. GABA is absolutely necessary for the development of an outward current and cannot be replaced by proline, glycine, or histidine. Significant changes in the ratio are also observed when lower or higher quantities of NaCl are injected.

**Role of chloride ions.** The relevance of intracellular Na\(^+\) and Cl\(^-\) in the activity of GAT1 was previously reported (14, 22, 23) from experiments based on the excised giant-patch methodology. Both ions appear to be necessary for reverse transport of the neurotransmitter, and the data of Fig. 4 (column 6) confirm this finding by showing that, when GABA is injected alone, without additional NaCl, the \(I_{\text{out}}/I_{\text{in}}\) ratio is reduced.

The oocyte’s intracellular concentrations of Na\(^+\) and Cl\(^-\) have been found to be respectively around 20 and 60 mM (19). The results obtained with giant-patch experiments (23) indicate that a 60 mM chloride concentration should be almost saturating, so that a further increase of this ion is not expected to produce noticeable effects. Conversely, an increase in intracellular sodium concentration ([Na\(^+]_i\)] above 20 mM is expected to significantly enhance the outward transport current. On the basis of these considerations, the results of Fig. 4 should be ascribed to changes in Na\(^+\) rather than in Cl\(^-\).

Fig. 4. Ratio of reverse to forward transport currents in different conditions. The ratio of the outward (defined as the Na minus Na/SKF89976A at +60 mV) to inward current (defined as the GABA minus Na/SKF89976A at −120 mV) is not increased by extending the waiting time after injection from 10 min to 1 h (columns 1 and 2). Reducing the injected amounts of GABA (columns 3 and 4) or of NaCl (column 6) significantly decreases the ratio. Conversely, higher amounts of NaCl increase the ratio (column 7). No outward current is seen when oocytes are injected with only NaCl (column 4) or with other amino acids (e.g., proline, column 5). *Statistically significant conditions with respect to column 1 (one-way ANOVA with Bonferroni’s correction at \(P < 0.05\) level).

Fig. 5. Effectiveness of the chloride-depletion treatments. Representative recordings are shown of the chloride current induced by application of the calcium ionophore ionomycin (~1 μM) in a nontreated oocyte (A) and the other three oocytes treated as indicated (B–D). The holding potential \(V_h\) was −25 mV in all cases. The inward current in the control oocyte implies that the reversal potential \(E_{\text{rev}}\) of the current is more positive than −25 mV. The outward currents in the treated oocytes indicate that \(E_{\text{rev}}\) is more negative than −25 mV. Note the large outward current in the potassium-chloride cotransporter (KCC2)-expressing oocyte incubated in hypotonic, Cl\(^-\) free solution (hypo sol), suggesting that this is the most effective chloride-depleting treatment. E: representative I–V curves obtained by applying voltage ramps to the other four oocytes in the four conditions (indicated by corresponding letters), used to detect the \(E_{\text{rev}}\) of the chloride current. The vertical dotted line indicates the −25 mV holding potential. Iso, isotonic.
To examine the role of intracellular chloride, its concentration must then be reduced. To reach this goal, we used the procedures explained in MATERIALS AND METHODS: overnight exposure to hypotonic Cl⁻-free extracellular solution, and/or coexpression of the potassium and chloride cotransporter KCC2 (27, 28).

The effectiveness of these treatments was checked by measuring the reversal potential \( (E_{\text{rev}}) \) of the \( \text{Ca}^{2+} \)-induced Cl⁻ current endogenously present in Xenopus oocytes (1).

Figure 5 shows recordings of the current elicited by application of the \( \text{Ca}^{2+} \) ionophore ionomycin (~1 \( \mu \)M) while keeping the membrane potential at \( V_{\text{h}} = -25 \text{ mV} \). As previously reported (44), the control (not Cl⁻ depleted) oocytes exhibit a biphase inward current in response to the ionophore, implying that the reversal of the current occurs at more positive potentials (Fig. 5A); on the contrary the oocytes treated for chloride depletion show an outward current at the same \( V_{\text{h}} \), indicating a more negative \( E_{\text{rev}} \) (Fig. 5, B–D). The values of \( E_{\text{rev}} \) in the control and in the treated oocytes were determined applying fast voltage ramps before and during ionomycin addition (Fig. 5E), and resulted to be \(-21.0 \pm 2.0 \text{ mV} \) (SE, \( n = 18 \)) in the untreated oocytes, \(-38.3 \pm 1.9 \text{ mV} \) (\( n = 12 \)) in the oocytes incubated overnight in the hypotonic Cl⁻ free solution, \(-40.5 \pm 1.2 \text{ mV} \) (\( n = 13 \)) in the oocytes expressing KCC2, and \(-75.7 \pm 2.5 \text{ mV} \) (\( n = 14 \)) in the oocytes expressing KCC2 and incubated overnight in the hypotonic Cl⁻ free solution. These differences are statistically significant at the \( P < 0.01 \) level (paired Student’s \( t \)-test), and with extracellular Cl⁻ concentration ([Cl⁻]₀) = 104 mM, these \( E_{\text{rev}} \) values correspond respectively to [Cl⁻] = 45, 22, 21, and 5 mM, confirming that all of these treatments are effective in reducing the intracellular Cl⁻ levels.

Effects of Cl⁻ depletion on the inward transport. According to the results of Lu and Hilgeman (23), a reduction in intracellular chloride should favor the inward transport current. However, during the overnight incubation in hypotonic saline necessary to produce the intracellular chloride depletion, a change in the number of GAT1 proteins expressed on each oocyte membrane is expected, because of an unbalance between new synthesis and degradation. Therefore we performed experiments in which the amplitude of the inward transport current was measured in the same oocyte before and after overnight incubation in three groups of oocytes. The first and the second groups were transfected only with GAT1, while the third group was cotransfected with GAT1 and KCC2. Oocytes of the first group were incubated in control isotonic solution, containing a normal chloride concentration, while those belonging to the second and third groups were incubated in the hypotonic Cl⁻ free solution. Figure 6A shows the \( I-V \) relationships of the transport currents normalized for each oocyte to the current amplitude measured at \(-120 \text{ mV} \) before incubation. In the oocytes incubated in the normal isotonic solution, an average increase of the current of \(-76\% \) was observed. This increase is attributable to a rise in the number of expressed transporters on the basis of the analogous 54% increase in the maximal intramembrane charge movement measured in the same oocytes by integrating the pre-steady-state currents before and after incubation (Fig. 6B) (29, 30). The current increase was somehow larger in the two groups incubated in the hypotonic solution, amounting to approximately 90–100%, in qualitative agreement with the expected effect of low intracellular chloride, although the difference between these two groups and the one incubated in the isotonic solution is not statistically significant.

Effects of Cl⁻ depletion on the reverse transport. The effect of lowering the internal chloride concentration on the reverse transport current was investigated in oocytes expressing only GAT1, or coexpressing GAT1 and KCC2 and incubated overnight in normal extracellular solution, or in hypotonic Cl⁻ free solution. Figure 7 illustrates the results of these experiments by plotting the currents elicited by external GABA after intracellular substrate injection (defined as GABA_out minus Na/SKF89976A, squares) together with the reverse transport current (defined as Na minus Na/SKF89976A, circles). Comparing the data with those obtained in non-Cl⁻-depleted oocytes (Fig. 7A), a considerable decrease in the relative amount of outward vs. inward current is noticeable [\( I_{\text{out}} (+60)/I_{\text{in}} (-120) = 0.38 \pm 0.04 \) in Fig. 7A, 0.24 ± 0.03 in Fig. 7B, 0.11 ± 0.02 in Fig. 7C, and 0.07 ± 0.02 in Fig. 7D]. The value of the ratio in the whole of the Cl⁻-depleted oocytes is significantly different from...
the controls \((P < 10^{-7}, \text{one-way ANOVA})\), while the individual differences between the controls and the various groups (estimated with Bonferroni’s test) are significant at \(P < 0.01\) (in the oocytes coexpressing KCC2 and incubated isotonically) or better.

The effects of chloride depletion on the efflux of \(^{3}H\)GABA were also investigated. Similarly to the experiments shown in Fig. 3, these tests were conducted in high external KCl solution to depolarize the oocyte membrane. The results are illustrated in Fig. 8 and show a significant reduction in the amount of extruded GABA in all the three conditions used to lower the intracellular chloride concentration \((P < 0.01, \text{one-way ANOVA for the control condition compared with the whole of the chloride-depleted oocytes})\).

Both the electrophysiological and radiotracer experiments indicate therefore that internal chloride concentration affects

**Fig. 7.** Effects of chloride-depletion treatments on the outward transport current. \(I-V\) curves after intracellular GABA plus Na gluconate injection. Squares represent the difference GABA\(_{\text{out}}\) minus Na/SKF89976A, and circles represent the difference Na minus Na/SKF89976A. Current values were normalized before being averaged at the \(-120\) mV value of the GABA\(_{\text{out}}\) minus Na/SKF89976A condition. Data are means \(\pm\) SE \((A, n = 15; B, n = 15; C, n = 12; D, n = 9\); from two to four batches).
the balance between inward and outward cycling transport mode of GAT1.

**DISCUSSION**

The studies reported in the literature on ionic requirements for reverse mode of GABA transport by GAT1 are scarce, because of the intrinsic difficulties of controlling the cytoplasmic milieu, and have generally been performed using the patch-clamp technique either in whole cell conditions (7) or in excised patches (22, 23). In these papers it was reported that the presence of millimolar amounts of GABA on the inner side of the membrane generated an outward transport current and that intracellular sodium and chloride played a relevant role in this mechanism.

As already mentioned in the Introduction, neurotransmitter leak through the reverse operation of neurotransmitter transporters has been suggested to be involved in many pathological or abnormal conditions (17, 34, 36), including epileptic seizures possibly caused by GABA spillover through GABA transporters (31, 32). Changes in Na⁺ gradient have been shown to alter the equilibrium between forward and reverse transport of the GABA transporters, leading to an increase of extracellular GABA level and, as a consequence, to possible pathological conditions (42). In addition, reverse operation of GAT1 has also been proposed to play a physiological role in maintaining both tonic and phasic inhibition in central synapses (41).

Another emerging issue regarding the regulation of neuronal activity is the role of intracellular chloride. Fluctuations of the internal concentration of this ion appear to affect various physiological processes in neurons. Night to day variations in internal chloride are observed in the suprachiasmatic neurons (38, 39) and are believed to be involved in the regulation of the circadian cycle, again via effects on GABA receptors. Intracellular chloride concentration has also been demonstrated to affect the activity of electroneutral ion transporters, such as NCC (27, 28).

Functionally important changes in cytoplasmic chloride concentration occur in developing neurons (2, 33). In particular, in the neonatal mammalian neurons, the cytoplasmic chloride concentration is abnormally high and, as consequence, the activation of the GABA receptors causes an anomalous excitatory action. However, due to the increasing expression of the potassium-chloride exporter KCC2 during development, the internal chloride concentration in these cells is progressively lowered, converting the GABA action from excitatory to inhibitory, as commonly found in adult neurons (3, 9, 21, 45).

We considered the approach of heterologous expression in Xenopus oocytes useful to confirm the roles of internal substrates on the activity of GAT1 and, more importantly, to explore the possibility of a regulatory role of internal chloride induced by physiological means, i.e., producing chloride changes through the activity of a coexpressed potassium-chloride symporter KCC2.

**GAT1 reverse operation.** Our experiments using intact Xenopus oocytes under voltage clamp confirm that, when GABA is present in the cytoplasm at millimolar levels, an outward transport current can be detected. As expected, this current increases at positive voltages and is affected by the intracellular concentrations of Na⁺ and Cl⁻. Using the same conditions that induce the outward transport current, an efflux of radioactive GABA can be measured, confirming the reverse cotransport of ions and neurotransmitter.

To explore the effects of intracellular Na⁺ and Cl⁻ on the reverse operation of GAT1, it must be noted that, in frog oocytes, the intracellular concentrations of these ions should be in the range of 10 to 20 mM for Na⁺, and 50 to 60 mM for Cl⁻ (19). Compared with neuronal cells where GAT1 is normally expressed, the oocytes have then a significantly higher internal Cl⁻ concentration, while the Na⁺ and proton concentrations are more similar (2). As already mentioned, the results of Lu and Hilgemann (22, 23) showed that an intracellular chloride concentration of 50 to 60 mM should already have an almost saturating effect on the reverse operation, so that the additional increase of this ion caused by the injection should have only small effects. Since our results show that injections of variable amounts of NaCl (together with constant GABA) affect the ratio of the outward to inward transport currents (Fig. 4), we believe that this effect is probably due mainly to the increase of intracellular Na⁺.

**Chloride depletion.** To investigate the role of intracellular chloride, we took advantage of procedures previously shown to be able to reduce the cytoplasmic concentration of this ion (16, 27, 28). Both the incubation in hypotonic Cl⁻-free solution and the expression of potassium-chloride cotransporter KCC2 were capable of significantly lowering [Cl⁻], as evaluated from the reversal potential of the endogenous Ca²⁺-activated Cl⁻ current (Fig. 5) elicited by ionomycin application. Furthermore, the two procedures caused additive effects when used together, reducing [Cl⁻] to <10 mM.

Both forward and reverse transport currents were estimated in the Cl⁻-depleted oocytes: as shown in Fig. 6, no statistically significant changes were observed in the amplitude of the inward GABA transport current compared with the control oocytes, indicating that when GABA is not present internally, no effect of chloride on the GAT1 activity can be detected. However, a significant decrease in the ratio of the outward to inward current was observed in the chloride-depleted oocytes after GABA injection (Fig. 7).

This effect of internal chloride is confirmed by the measurements of radioactive GABA efflux that, as shown in Fig. 8, is strongly decreased after treatments causing chloride depletion.

The coexpression of KCC2 should be associated not only with a reduction of intracellular chloride but also with a loss of internal potassium, since this transporter extrudes a 1:1 ratio of potassium to chloride. However, in relative terms, the loss of potassium will be much smaller. Assuming that the decrease of chloride is ~40 mM, as observed in RESULTS, a corresponding loss for K⁺ will bring the concentration of this ion from an estimated 120 mM (19) to ~80 mM, a value that represents a
33% decrease compared with a 90% decrease for Cl\(^-\). Furthermore, it can be expected that the activity of the Na\(^+\)-K\(^+\)-ATPase will tend to efficiently stabilize the internal potassium concentration (27), while in the case of chloride, it is known that its internal concentration may be more easily altered (15). For all of these reasons, we believe that the reduction in outward transport current observed in the oocytes coexpressing KCC2 is a true effect of the reduced internal Cl\(^-\) concentration.

On the whole, our results on the action of intracellular ions on GAT1 operation are consistent with the scheme previously proposed (Fig. 9) (22), in which the balance between extracellular Na\(^+\) and intracellular Cl\(^-\) may alter the direction of the transporter cycle when sufficient internal GABA is present.

In conclusion, our experiments confirm that, when a high concentration of GABA is present in the cytoplasm, GAT1 can operate in reverse mode. The reverse transport is affected by the internal concentrations of the two ions, Na\(^+\) and Cl\(^-\), known to be involved in the forward transport cycle. The results of the chloride-depletion experiments, and particularly those in which the coexpression of KCC2 was used, may confer an interesting physiological relevance to the role of intracellular chloride. In analogy to the developmental changes in neuronal maturation, the activity of the GABA cotransporter rGAT1 preserve the mutual relation of chloride with the GABA transporter rGAT-1.

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