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Research Article

Cl^- affects the function of the GABA cotransporter rGAT1 but preserves the mutal relationship between transient and transport currents

S. Giovannardi [a], R. Fesce [a,b], E. Bossi [a], F. Binda [a] and A. Peres [a,*]

[a] Laboratory of Cellular and Molecular Physiology, Department of Structural and Functional Biology, University of Insubria, Via Dunant 3, 21100 Varese (Italy), Fax + 39 0332 421300, e-mail: antonio.peres@uninsubria.it
[b] Department of Neurosciences, S. Raffaele Institute, Via Olgettina 58, 20132 Milan (Italy)

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Abstract. The effects of reducing external Cl^- on the electrophysiological properties of the Na^+/Cl^--dependent GABA transporter rGAT1 expressed in *Xenopus* oocytes were investigated. In agreement with a recently proposed kinetic scheme, the effects of Cl^- are complex but preserve the mutual relationship that links the transport-associated current, I_{tr}, measured in saturating GABA concentration, and the transient current, I_{pre}, recorded in the absence of GABA following a voltage step from the holding potential V_h to V. In particular, $I_{tr}(V) - I_{tr}(V_h) = r \int I_{pre}(V)\, dt$, where r is the relaxation rate of I_{pre} at the same membrane potential and Cl^- concentration. The model also predicts a relationship between charge relaxation rate and apparent affinity for GABA, which is also verified in the presence of lowered Na^+ or Cl^- concentrations. In these conditions, the binding rate of GABA to the transporter is increased. All these effects are consistent with the hypothesis that interaction of the organic substrate with rGAT1 induces a conversion from a capacitive to a conductive mode of operation without strongly altering either the amount or the rate of charge movement.

Key words. Cotransporter; GABA; Cl^-; charge movement.

Several ion-coupled transporters of small organic substrates have been shown to display two (and in some cases three) characteristic kinds of current, which are thought to represent specific operating modes of the molecule. In the absence of organic substrate, a transient current is typically induced by voltage or ion concentration changes [1–3]. This kind of current, named the 'presteady-state' current (I_{pre}), has the hallmarks of a capacitive current, arising from the confined movement of charge within the membrane electrical field [1, 3]. A second kind of current that may be recorded in the absence of organic substrate is the 'leak' current, a transmembrane flux of charges present to a variable extent in several transporters [4–6]. Finally, in electrogenic transporters, a 'transport' current (I_{tr}) accompanies the translocation of the organic substrate.

In transporters belonging to the Na^+/Cl^--dependent family, these currents are affected, as the name implies, by the external and internal concentrations of these two ion species. These effects have been studied in particular with the neuronal GABA transporter rGAT1 [1, 7, 8], and several interesting observations have been made. Rather surprisingly, given the opposite charge of the two ions, reductions in either external Na^+ or external Cl^- produce qualitatively (but not quantitatively) similar effects on the presteady-state currents. Namely, negative shifts of the Q–V and τ–V curves are observed, Q being the charge underlying I_{pre} (obtained from integration of the transients)

* Corresponding author.

and τ being its relaxation time constant. Conversely, the effects of Na^+ and Cl^- reductions on the transport-associated current are somehow different: while decreasing $[Na^+]_o$ merely shifts the I_{tr}–V relationship, similar to the Q–V curve [1], lowering $[Cl^-]_o$ results in a more complex change in the I_{tr}–V curve: a reduction of the current at intermediate potentials with a recovery at more negative potentials [1].

We have recently shown that a quite simple relationship links I_{pre} and I_{tr} in the GABA transporter rGAT1, suggesting interconversion between the two kinds of current, driven by the amount of substrate present. In this paper, we successfully extend this interpretation by showing that the peculiar effects of Cl^- on the transport-associated current can be predicted from the actions of Cl^- on the presteady-state currents. The relationship between I_{pre} and I_{tr} also leads to a simple kinetic explanation of the apparent affinity for GABA. The effects of altering Na^+ and Cl^- concentrations on this parameter were therefore investigated.

Using this approach, we show that the actions of Na^+ and Cl^- on the activity of rGAT1 are due to combined effects on the rates of charge transfer and on the GABA-binding rate.

Materials and methods

cRNA preparation and *Xenopus laevis* oocyte expression

The usual procedures [9] for the isolation and injection of oocytes were employed. Briefly, the cDNA encoding the rat GAT1 cotransporter was cloned into the pAMV-PA vector. After linearization with *NotI*, cRNA was synthesized in vitro in the presence of Cap Analog and 200 units of T7 RNA polymerase. All enzymes were supplied by Promega Italia (Milan, Italy).

X. laevis frogs were anaesthetized using MS222 (tricaine methansulfonate) 0.10% (w/v) solution in water; after removal of portions of the ovary through a small incision on the abdomen, the sutured animal was returned to water. Each frog was used no more than twice; suitable postoperative care was given to the animals and the interval between operations was longer than 4 months. The experiments were carried out according to institutional and national ethical guidelines.

The oocytes were treated with collagenase (Sigma type IA) 1 mg/ml in calcium-free ND96, for at least 1 h at 18 °C. Healthy-looking stage V and VI oocytes were collected and injected with 12.5 ng of cRNA in 50 nl of water, using a manual microinjection system (Drummond). The oocytes were incubated at 18 °C for 3–4 days in NDE solution (containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes at pH 7.6, supplemented with 50 µg/ml gentamicin and 2.5 mM Na pyruvate), before electrophysiological studies.

Electrophysiology

A two-electrode voltage-clamp system was used to perform the experiments (Oocyte Clamp: Warner Instruments, Hamden, Conn.; or Geneclamp: Axon Instruments, Union City, Calif.). The holding potential was kept at –40 mV and the typical protocol consisted of 500-ms voltage pulses spanning the range –120 to +40 mV in 20-mV steps. Four pulses were averaged at each potential; signals were filtered at 1 KHz and sampled at 2 KHz.

The reference electrode was connected to the bath through an agar bridge (3% agar in 3 M KCl) to minimise Cl^- effects on junction potential. Data analysis was performed using Clampfit 8.0 (Axon Instruments) only on oocytes injected with the cRNA encoding rGAT1. Presteady-state currents were isolated by subtraction of corresponding traces in the presence of 30 µM of the specific blocker SKF89976A (Tocris; http://www.tocris.com); subtracted traces were corrected for any remaining leakage and integrated. Charge data in the text and figures always represent the average of 'on' and 'off' integrals, which never differed by more than 10% from each other. We used the convention, introduced in Fesce et al. [10], of setting the zero-charge level to the saturation value at positive potential. We call this charge Q_{in}, as it represents the amount of charge in the inner transporter position. Normalization and offset of the Q/V curves was individually done for each oocyte using Q_{max}, obtained by fitting a Boltzmann function to the Q *versus* V data obtained in control solution (98 mM $[Na^+]_o$ and 104 mM $[Cl^-]_o$), before averaging among different oocytes.

Solutions

The external control solution had the following composition (in mM): NaCl 98, $MgCl_2$ 1, $CaCl_2$ 1.8, Hepes free acid 5, at pH 7.6. When $[Na^+]_o$ was reduced, it was replaced by corresponding amounts of TMA^+ (tetramethylammonium); when the $[Cl^-]_o$ was reduced, acetate salts were substituted. The pH was adjusted with HCl, acetic acid, NaOH or TMAOH. Solutions were superfused by gravity onto the oocyte by a pipette tip placed very close (1–2 mm) to the cell. All experiments were performed at room temperature (22–25 °C).

Results

Na^+ and Cl^- effects on the transport-associated currents

Changes in external Na^+ and Cl^- concentrations produced diverse effects on the voltage-dependence of the transport-associated current of rGAT1-expressing oocytes. Figure 1 illustrates these differences: reductions in $[Na^+]_o$ diminished the current at all potentials, producing a mere shift of the I/V curves towards negative potentials (fig. 1A); on the other hand, reducing $[Cl^-]_o$ caused an in-

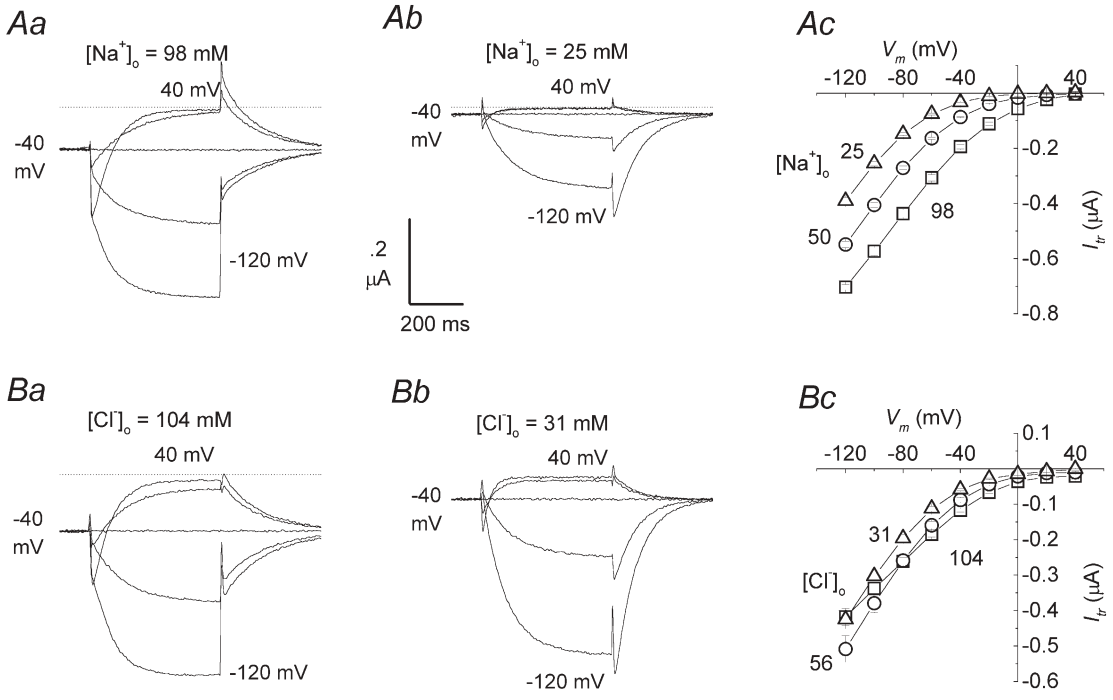

Figure 1. Effects of $[Na^+]_o$ and $[Cl^-]_o$ on the transport-associated currents. Top row: sample subtracted traces in response to voltage pulses from $V_h = -40$ mV to test potentials between –120 and +40 mV in control solution (*A a*) and in $[Na^+]_o = 25$ mM (*A b*) in the same oocyte; *A c*: *I-V* relationships in the presence of the indicated Na^+ concentrations (squares 98 mM, circles 50 mM, triangles 25 mM). Bottom row: effects of reducing $[Cl^-]_o$ from control solution (*B a*) to 31 mM (*B b*); *B c*: *I/V* relationships in the presence of the indicated Cl^- concentrations (squares 104 mM, circles 56 mM, triangles 31 mM). Data in *A c* and *B c* are averages ± SE from two batches of six oocytes each. Dotted lines indicate the zero-current level. The records were obtained by subtracting the traces in the absence of GABA from those in its presence.

creased curvature of the *I/V* relationship, resulting in a decrease in the current at moderate potentials but an increase at very negative potentials (fig. 1B). Both these actions have been reported previously [1, 9].

We recently showed [10] that, in the presence of a saturating GABA concentration, a simple relationship links the transport-associated current (I_{tr}) with the amount of inwardly displaced charge (Q_{in}):

$$I_{tr} = Q_{in}\, r = [\int I_{pre}\,(V)\, dt - \int I_{pre}\,(V \gg 0)\, dt]\, r \qquad (1)$$

where r is the charge relaxation rate, i.e. the reciprocal of the decay time constant of the presteady-state currents. Equation 1 may be derived from the three-state kinetic scheme shown in figure 2, the essential features of which are explained in the figure legend.

In previous work [10], we have shown that this equation also holds in reduced Na^+ conditions, and we tried to verify whether this same equation might help to explain the actions of Cl^- on I_{tr}. The effects of $[Na^+]_o$ and $[Cl^-]_o$ reductions on the presteady-state currents exhibited by rGAT1 in the absence of GABA were previously reported [1, 8], and are illustrated in figure 3, in terms of dependence of the amount of charge in the inner transporter position ($Q_{in} = Q(V) - Q(V \gg 0)$) and of the rate of charge relaxation (r) on membrane potential, for the same oocytes as in figure 1. Reductions in the external concentrations of either Na^+ or Cl^- shifted both Q_{in} and r towards more negative potentials; however, the shifts induced by Na^+ were larger than those caused by similar fractional reductions of Cl^-. The maximal amount of moveable charge was not altered when either ion was reduced (fig. 3Aa, Ba), while the minimal relaxation rate increased, confirming previous results (fig. 3Ab, Bb) [8].

The simple leftward shift produced by reduced $[Na^+]_o$ in the voltage dependence of the charge transfer rate caused the curves obtained in different $[Na^+]_o$ to cross each other at negative potentials. In reduced $[Cl^-]_o$, instead, the values of the charge relaxation rate at negative potentials tended to the same values as in control $[Cl^-]_o$, and even appeared to be slightly larger, although the differences were not statistically significant (fig. 3Ab, Bb).

Following equation 1, the products of the curves in figure 3Ba (amounts of charge) and figure 3Bb (rates) resulted in the *I/V* curves shown in figure 3Bc, where the experimental results of figure 1 were substantially reproduced. For comparison, figure 3Ac shows that the same holds true for the products of the corresponding curves in figure 3Aa and figure 3Ab, which reproduce well the effects of Na^+ on the transport-associated current [10].

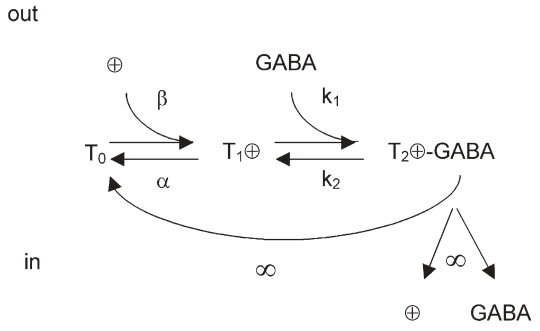

Figure 2. Simple three-state kinetic scheme that accounts for rGAT1 operation [from ref. 10]. The empty transporter (T_0) cannot bind GABA; binding and translocation of charges are tightly coupled and lead to a transporter state (T_1), with charge on the inner side of the membrane electrical field, to which GABA may bind with rate k_1; GABA binding leads to an evanescent state (T_2) that immediately dissociates charges and GABA to the cytosol, while the transporter returns to the empty state T_0, making the unbinding rate k_2 irrelevant. The charge relaxation rate (r) is equal to the sum of the unidirectional rate constants, i.e. $r = \alpha + \beta$. The ⊕ symbol refers to all the charges binding to the transporter prior to GABA binding.

Kinetic interpretation of the apparent affinity for GABA

As a corollary to equation (1), we showed [10] that in control conditions a linear relationship exists between the apparent affinity for GABA – $K_{1/2}(V)$, defined as the GABA concentration eliciting half-maximal current at potential V – and the rate of charge relaxation in the absence of GABA (r) at the same values of membrane potential. To assess whether this correlation had a more general validity, we determined the apparent affinity for GABA in conditions of reduced Na^+ or Cl^- and related it to the charge relaxation rates of figure 3. The graphs in figure 4 illustrate the *I/V* curves of the transport-associated current at different GABA concentrations in control conditions (fig. 4A), in reduced Na^+ (fig. 4B) and in reduced Cl^- (fig. 4C). Although saturation appeared to be reached at 100 µM GABA in all conditions, the GABA concentration causing half-maximal current at each potential was smaller in conditions of lowered Na^+ or Cl^-. These values ($K_{1/2}$) were estimated by fitting a sigmoidal function to semilogarithmic plots of I_{tr} versus [GABA] at

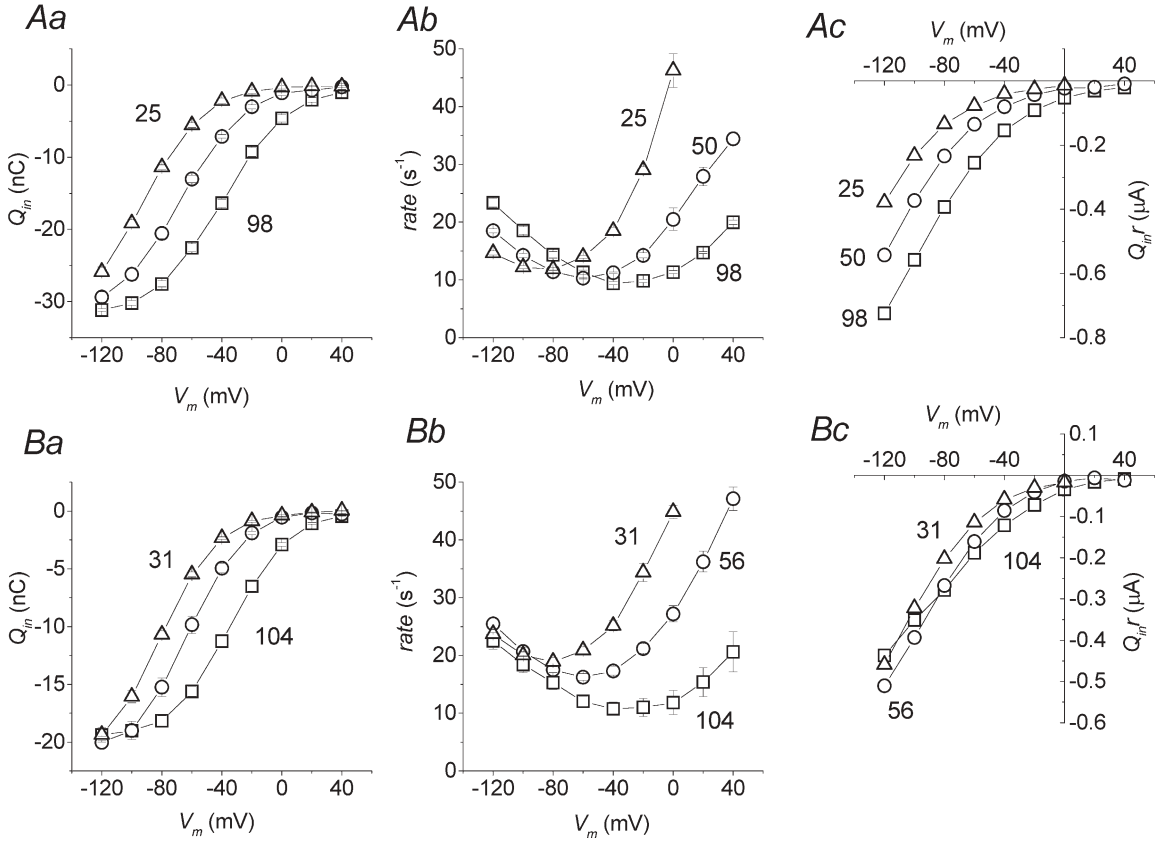

Figure 3. Effects of $[Na^+]_o$ and $[Cl^-]_o$ on the presteady-state currents. *Aa*: Q_{in}/V relationships from integration of presteady-state currents at the indicated Na^+ concentrations; the zero value of Q_{in} is set at positive potentials [10]; *Ab*: rates of decay of the presteady-state currents from single exponential fitting; *Ac*: reconstruction of the $[Na^+]_o$ effects on the transport-associated current from equation 1, using the data of panels *Aa* and *Ab*. *Ba*: Q_{in}/V relationships obtained by integrating presteady-state currents at the indicated Cl^- concentrations; *Bb*: rates of decay from single exponential fitting of presteady-state currents. *Bc*: reconstruction of the $[Cl^-]_o$ effects on the transport-associated current obtained by applying equation 1 to the data of panels *Ba* and *Bb*. Data are averages ± SE from six oocytes in *A* and from seven oocytes in *B* from two different batches.

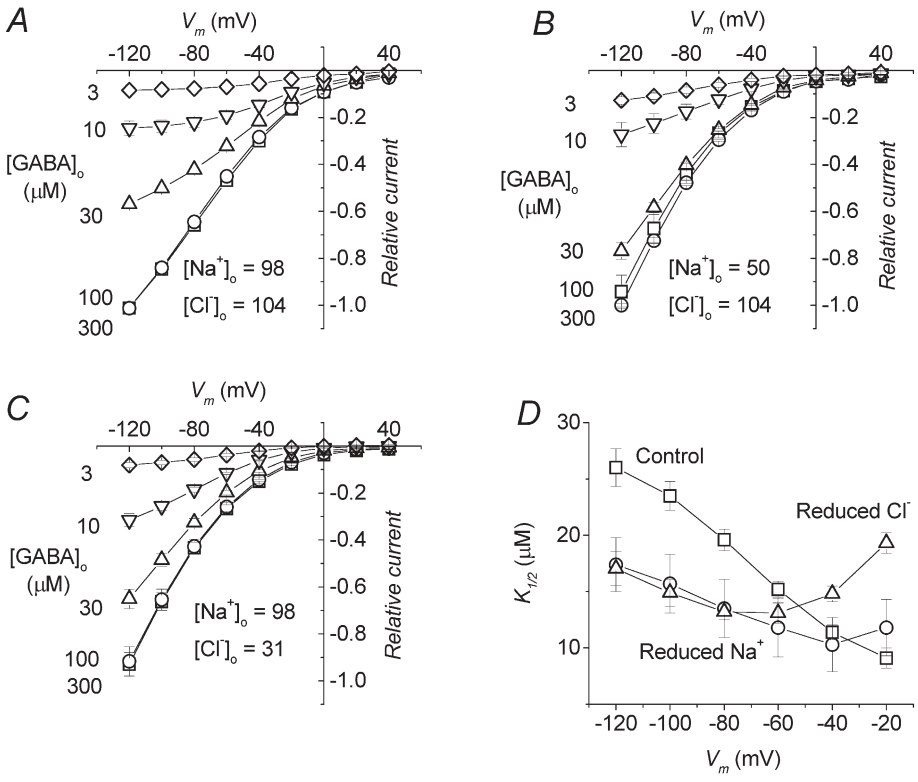

Figure 4. Apparent affinity in different $[Na^+]_o$ and $[Cl^-]_o$. *A*: control condition; *B*: reduced $[Na^+]_o$ at control $[Cl^-]_o$; *C*: reduced $[Cl^-]_o$ at control $[Na^+]_o$; *D*: apparent affinity calculated as the GABA concentration causing half-maximal I_{tr} at each potential. Squares represent the control condition; circles are data in $[Na^+]_o$ = 50 mM at control $[Cl^-]_o$; triangles are data in $[Cl^-]_o$ = 31 mM at control $[Na^+]_o$. Data are means ± SE from seven oocytes in *A* and six oocytes in *B* and *C*, from distinct batches.

each potential, and the results are shown in figure 4D for the various experimental conditions. Besides confirming its voltage-dependence, this last graph shows that at negative potentials $K_{1/2}$ was decreased, in conditions of reduced Na^+ or reduced Cl^-, compared to the control solution.

In addition to its capacity to explain the relationship between transport-associated and presteady-state currents, the kinetic scheme of figure 2 can also account for the voltage-dependence of $K_{1/2}$, giving the relation,

$$K_{1/2} = r/k_1 \qquad (2)$$

k_1 being the binding rate of GABA to the transporter.

Assuming a constant value for k_1, equation 2 predicts that plots of r versus $K_{1/2}$ should be straight lines through the origin. We plotted (fig. 5Aa, Ab, Ac) r versus $K_{1/2}$for the three ionic conditions of figure 4. While for control and reduced Na^+ conditions there was an acceptable linear relationship between these two parameters, and the fitting lines crossed the origin, for the reduced Cl^- condition the fitting line had a definitely positive intercept. The slope of the lines represents k_1 and increases from 0.84 μM^{-1} s^{-1} to 1.0 and 2.1 μM^{-1} s^{-1} in reduced Na^+ and reduced Cl^-, respectively.

Discussion

The predictions of the three-state kinetic model of figure 2 [10] have been tested in the present work in conditions of reduced Cl^- concentrations. The validity of the simple relationship between the kinetic parameters determined in the absence of GABA and the transport-associated current in its presence at saturating concentrations, i.e. the equation $I_{tr} = Q_{in} r$, was confirmed. The results show that the peculiar effects of Cl^- on the transport-associated current are reflected in the charge movement characteristics in such a way that the above relationship is verified. This finding reinforces the notion [10] that the rGAT1 transporter basically operates with the same mechanism, irrespective of the presence or absence of GABA.

Curiously enough, reducing the Cl^- concentration produces effects similar to reducing Na^+. The main difference is that low Na^+ reduces the charge relaxation rate (and the inward migration rate constant, β, in particular) at negative potential, whereas low Cl^- does not. If charge migration reflects a diffusive flow of Na^+ within the intramembrane electric field [10], then this finding is not particularly surprising. However, this phenomenon appears to have remarkable consequences for the overall shapes of the I_{tr} versus V curves. In fact, I_{tr} is still correctly

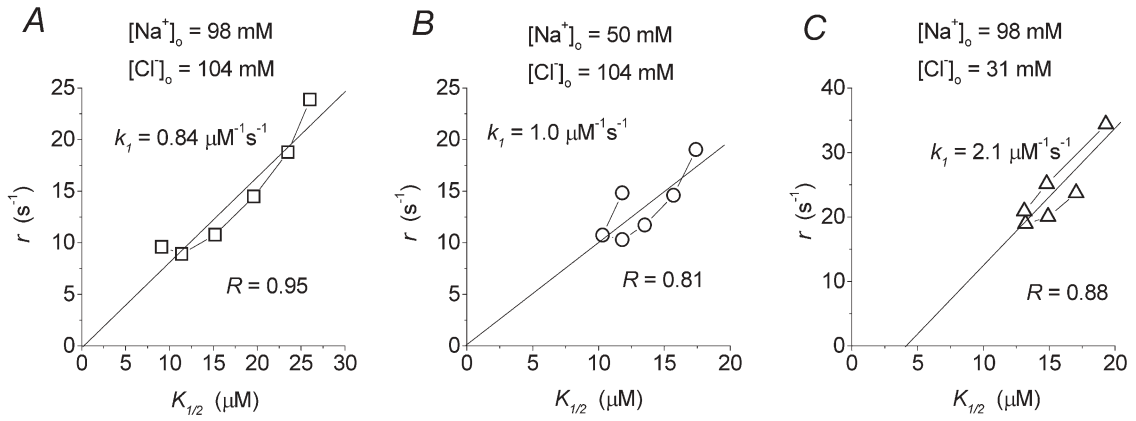

Figure 5. Plots of rate versus $K_{1/2}$ in control and reduced $[Na^+]_o$ or $[Cl^-]_o$, as indicated; data points are obtained from the experiments of figure 3 and 4, as explained in the text. Straight lines are linear regressions to the points, with values of slopes (k_1) and correlation coefficients (R) indicated. The values of the y intercepts obtained from the fitting are –0.22 ± 2.53 (A), 0.13 ± 4.88 (B) and –8.17 ± 8.69 (C) s^{-1}. The corresponding values of k_1 are 0.84 ± 0.14 (A), 1.00 ± 0.36 (B) and 2.1 ± 0.56 (C) $s^{-1} M^{-1}$.

predicted by the product $Q_{in} \times r$, so that the leftward shifts of the Q_{in} versus V curves in reduced Cl^-, combined with the unaltered values of r, produce increased transport currents at negative potential and markedly greater curvatures of the I_{tr} versus V curves (fig. 3Bc).

Our results are not sufficient to define a detailed mechanism for the action of external Cl^- on the transporter kinetic, however, the effects observed in presteady-state and transport-associated currents may be partially explained with a Donnan-like system, in which Na^+ and Cl^- equilibrate between bulk external solution and a transporter vestibule [8]. A diffusive movement of charges from the exterior into a transporter cavity is also suggested by the relatively low temperature coefficient of the inward rate of charge movement [9].

The three-state model presented here and in our previous work [10] may be considered a simplified version of the kinetic model proposed by Hilgemann and Lu [11]. Indeed, there is a strict analogy between their states E_{in} and E_{out} and our states T_0 and T_1, and, furthermore, our evanescent state T_2 corresponds to the transitional state $^*E_{in}$ in Hilgemann and Lu's model. In addition, the Hilgemann and Lu model includes stoichiometric relationships and the effects of cytoplasmic substrates, especially Cl^-, which has been shown to effectively modulate the transporter activity [12, 13].

The determination of $K_{1/2}$ for GABA in reduced Na^+ or Cl^- conditions shows that at negative potentials, this parameter is in both cases lower than in the control concentration (fig. 4). This observation is only in partial agreement with the idea that $K_{1/2}$ for GABA is directly related to the relaxation rate of charge movement, as can be derived from the three-state model [10], and consequently with a kinetic interpretation of its voltage dependence; namely, that the probability that GABA may interact with the transporter, and be subsequently transferred across the membrane, is affected by the lifetime of that particular transporter state that allows GABA loading. This lifetime is linked to the rate constants of charge movement, which in turn depend on ion concentrations and membrane voltage. Indeed, reduced concentrations of Na^+ produce a decrease in the charge relaxation rate at negative potentials (fig. 3) [14], allowing more time for GABA to board the transporter, and explaining the lower values of $K_{1/2}$. However, at negative potentials, $K_{1/2}$ is also decreased in reduced Cl^- (fig. 4D), a condition in which the charge relaxation rate is actually slightly increased (fig. 3Bb). If the GABA-binding rate, k_1, did not change, this should lead to higher values of $K_{1/2}$ in our simple kinetic model. The analysis of figure 5 shows that in low Cl^-, k_1 is significantly increased, suggesting that the decrease in $K_{1/2}$ may be due to an additional positive effect of low Cl^- on this parameter, that prevails over the accelerated charge relaxation rate. How the reduced Cl^- concentration may affect the GABA-binding rate remains a matter of speculation, although a decreased ionic strength in the proximity of a transporter vestibule may facilitate GABA access.

In reduced Cl^-, $K_{1/2}$ for GABA shows a significant increase at depolarized potentials, while this is not so for reduced Na^+ (fig. 4D). This behaviour is in agreement with equation 2, which predicts that $K_{1/2}$ should be proportional to the relaxation rate r. Indeed fig. 3Ab and 3Bb show that at a depolarized potential, r is much larger in reduced Cl^- compared to reduced Na^+. Therefore, using the above interpretation, at depolarized potentials and in reduced Cl^-, the lifetime of the transporter state to which GABA can bind will be shorter compared to the reduced Na^+ condition, and, consequently, $K_{1/2}$ will be larger.

In conclusion, the simple three-state kinetic scheme, in which GABA binds the transporter after charge movement has occurred, inducing prompt release of the sub-

strates to the cell interior, and leaving the transporter ready for a new cycle [10], is able to explain the peculiar effects of Cl^- on the transport-associated current of rGAT1. The present results therefore reinforce the notion that, in this GABA transporter, substrate binding converts an intramembrane movement of charges into the transmembrane current that accompanies GABA uptake, without strongly affecting either the amount of charge moved or the migration rates. The consequent definition of the apparent affinity for GABA allows an estimation of the binding rate, a parameter that may be useful for characterizing mutations and possibly the interaction of drugs with the transporter.

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