Differential contribution of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter NKCC1 to chloride handling in rat embryonic dorsal root ganglion neurons and motor neurons

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ABSTRACT Plasma membrane chloride (Cl^-) pathways play an important role in neuronal physiology. Here, we investigated the role of NKCC1 cotransporters (a secondary active Cl^- uptake mechanism) in Cl^- handling in cultured rat dorsal root ganglion neurons (DRGNs) and motor neurons (MNs) derived from fetal stage embryonic day 14. Gramicidin-perforated patch-clamp recordings revealed that DRGNs accumulate intracellular Cl^- through a bumetanide- and Na^+ -sensitive mechanism, indicative of the functional expression of NKCC1. Western blotting confirmed the expression of NKCC1 in both DRGNs and MNs, but immunocytochemistry experiments showed a restricted expression in dendrites of MNs, which contrasts with a homogeneous expression in DRGNs. Both MNs and DRGNs could be readily loaded with or depleted of Cl^- during GABA_A receptor activation at depolarizing or hyperpolarizing membrane potentials. After loading, the rate of recovery to the resting Cl^- concentration (i.e., $[\text{Cl}^-]_i$ decrease) was similar in both cell types and was unaffected by lowering the extracellular Na^+ concentration. In contrast, the recovery on depletion (i.e., $[\text{Cl}^-]_i$ increase) was significantly faster in DRGNs in control conditions but not in low extracellular Na^+ . The experimental observations could be reproduced by a mathematical model for intracellular Cl^- kinetics, in which DRGNs show higher NKCC1 activity and smaller Cl^- -handling volume than MNs. On the basis of these results, we conclude that embryonic DRGNs show a higher somatic functional expression of NKCC1 than embryonic MNs. The high NKCC1 activity in DRGNs is important for maintaining high $[\text{Cl}^-]_i$, whereas lower NKCC1 activity in MNs allows large $[\text{Cl}^-]_i$ variations during neuronal activity.—Chabwine, J. N., Talavera, K., Verbert, L., Eggermont, J., Vanderwinden, J.-M., De Smedt, H., Van Den Bosch, L., Robberecht, W., Callewaert, G. Differential contribution of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter NKCC1 to chloride handling in rat embryonic dorsal root ganglion neurons and motor neurons. *FASEB J.* 23, 1168–1176 (2009)

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GABA IS A MAJOR INHIBITORY NEUROTRANSMITTER in the nervous system. It hyperpolarizes and therefore stabilizes most adult neurons by increasing Cl^- conductance *via* activation of GABA_A receptors. Attention to neuronal Cl^- homeostasis has increased considerably, given the recently elucidated significance of Cl^- pathways in several physiological processes and pathological conditions (1–8). An important class of these pathways is the cation- Cl^- cotransporters (CCCs), which are electroneutral ion transporters coupling the movement of Cl^- to Na^+ and/or K^+ fluxes across the plasma membrane (9–11). They are referred to as a secondary active transport mechanism, as they use gradients for Na^+ or K^+ created by the $\text{Na}^+\text{-K}^+\text{-ATPase}$ pump.

There are three main families of CCCs: the $\text{Na}^+\text{-Cl}^-$ (NCC) and $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ (NKCC) families, which accumulate Cl^- in the cell; and the $\text{K}^+\text{-Cl}^-$ (KCC) family, which extrudes Cl^- outside the cell. KCC cotransporters (KCC1–4) are ubiquitously expressed, except the neuron-specific KCC2. They convey Cl^- extracellularly, jointly with K^+ and following the concentration gradient of the latter. The NKCC1 cotransporter is among the most active members of the CCC family (7, 10–12) and the most widely distributed isoform of the NKCC family. This transport protein accumulates Cl^- inside the cell (9–11) and is activated by phosphorylation, which occurs with a half-time of ~10 min (13). NKCC1 mediates electroneutral uptake of Na^+ and K^+ together with Cl^- , with a stoichiometry of 1:1:2. Several compounds can modulate NKCC1 activity (11, 14). The

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loop diuretic bumetanide is the most specific inhibitor of the cotransporter (15–17), the reason that NKCC1 is also named bumetanide-sensitive cotransporter 2 (BSC2). Bumetanide binding to the cotransporter requires the simultaneous presence of Na^+ , K^+ , and Cl^- , and the inhibition of the cotransporter is concentration dependent (13), with maximal inhibition obtained after ~10 min of incubation (14). Notably, the activity of NKCC1 is enhanced by the decrease in $[\text{Cl}^-]_i$ (11, 12, 15). Moreover, NKCC1-mediated Cl^- accumulation has been recently suggested to be under the control of a feedback mechanism that involves phosphorylation pathways (14, 18). In addition, extracellular $[\text{Na}^+]$ and $[\text{K}^+]$ can also modulate NKCC1 activity (13).

Because NKCC1 is able to modify $[\text{Cl}^-]_i$, it can substantially modulate GABAergic activity toward excitation or inhibition (16, 19–22). For instance, GABA is depolarizing in dorsal root ganglion neurons (DRGNs) and immature neurons mainly through high NKCC1 expression levels, whereas it has a hyperpolarizing effect in most adult neurons (7–9, 21–24). This GABA-induced excitation is an important feature of neurogenesis (25). On the other hand, this unusual excitation renders the immature brain hyperexcitable and hence more susceptible to epileptic seizures (5). Pharmacological inhibition of NKCC1 by bumetanide has even been proposed as a potential mechanism to be used in the treatment of neonatal seizures (26, 27). In addition, NKCC1 has been suggested to play a role in many other pathological processes such as neurogenic inflammation (28) and brain ischemia (29).

We have previously shown that rat cultured motor neurons (MNs) at embryonic day 14 (E14) display substantial and long-lasting $[\text{Cl}^-]_i$ changes (loading or depletion) when challenged with strong GABA stimulation at hyperpolarizing or depolarizing membrane potentials (30). Recovery was faster after $[\text{Cl}^-]_i$ depletion than after $[\text{Cl}^-]_i$ loading, and NKCC1 cotransporters were suggested to contribute to the accelerated recovery, although their expression was assumed to be low. KCCs were probably not involved in the recovery process for several reasons. First, KCC1, KCC3, and KCC4 have limited expression in the nervous system (31) and are active under hypotonic conditions (32). In addition, even if KCC2 has considerable activity under isosmotic conditions, its presence in DRGNs is controversial (6, 10, 33), and the addition of Cs^+ in the internal solutions prevents Cl^- extrusion *via* KCC2 (34).

In the present work, we studied intracellular chloride handling in E14 DRGNs and MNs in relation to the functional expression of NKCC1. We compared expression and cellular distribution of NKCC1 and studied its role in relation to $[\text{Cl}^-]_i$ under steady-state conditions and during experimentally induced $[\text{Cl}^-]_i$ depletion or loading. Gramicidin-perforated patch recordings demonstrate that $[\text{Cl}^-]_i$ in embryonic DRGNs is significantly higher than in MNs, due mainly to high NKCC1 activity. High NKCC1 activity also accelerated the

$[\text{Cl}^-]_i$ increase after depletion in DRGNs. The experimental observations could be reproduced by a model for intracellular Cl^- kinetics, which features higher activity of NKCC1 and smaller intracellular Cl^- -handling volume in DRGNs than in MNs.

MATERIALS AND METHODS

Cell culture

Both MNs and DRGNs were derived from E14 embryos of C57 black rats. MNs were prepared as described previously (35, 36). In brief, the ventral spinal cord was dissected, cut in small pieces, trypsinized [0.05% of trypsin in Hanks' balanced salt solution (HBSS)] for 15 min at 37°C, and triturated. The MN population was purified by OptiPrep (6.2%) gradient centrifugation and seeded on a preestablished glial feeder layer. The culture medium (Life Technologies, Inc., Carlsbad, CA, USA) consisted of L15 medium supplemented with glucose (3.6 mg/ml), progesterone (20 nM), insulin (5 µg/ml), putrescine (0.1 mM), conalbumin (0.1 mg/ml), sodium selenite (30 nM), penicillin (100 U/ml), streptomycin (100 µg/ml), horse serum (2%), and chick embryo extract (5%). DRGN cells were prepared as described previously (37). Briefly, DRGNs were dissected lateral to the dorsothoracic spinal cord, trypsinized (0.05% of trypsin in HBSS) for 10 min at 37°C, and triturated. They were purified by centrifugation at a relative centrifugal force of 75 for 20 min at 20°C using a 4% bovine serum albumin cushion and were seeded on poly-L-ornithine- and laminin-coated glass coverslips at a density of 90,000 cells/ml. The DRGN culture medium was composed of Dulbecco's modified Eagle's medium (F12) supplemented with fetal calf serum (10%), penicillin (50 U/ml), streptomycin (50 µg/ml), bicarbonate (0.045%), and nerve growth factor 2.5S (5–10 ng/ml). MNs and DRGNs were kept in a humidified incubator (7% CO_2) at 37°C and used between 3 to 12 days after plating.

Electrophysiology

GABA-induced currents (I_{GABA}) were recorded under voltage-clamp conditions using the gramicidin-perforated patch-clamp technique (30). The pipette solution contained 125 mM CsCl, 1.2 mM MgCl_2 , 10 mM HEPES, 2 mM Na_2ATP , and 1 mM EGTA, and the pH was adjusted to 7.3 with CsOH. Gramicidin (Fluka AG, Buchs, Switzerland) was added to the pipette solution at concentrations of 87.5 and 75 µg/ml for MNs and DRGNs, respectively. Patch pipettes with a resistance of ~3–5 MΩ were briefly dipped in gramicidin-free pipette solution and backfilled with internal solution. After seal formation, access resistance was monitored until it reached a stable value of ≤30 MΩ. GABA was locally applied by using a fast perfusion system. The external solution, a modified Krebs' solution, contained 150 mM NaCl, 6 mM KCl, 1 mM MgCl_2 , 3 mM CaCl_2 , 10 mM HEPES, and 10 mM glucose, with pH adjusted to 7.3 with NaOH. Total external $[\text{Cl}^-]$ amounted to 164 mM. In some experiments, we reduced the activity of NKCC1 using a low external Na^+ solution containing 65 mM NaCl, 100 mM *N*-methyl-D-glucamine, 6 mM KCl, 1 mM MgCl_2 , 3 mM CaCl_2 , 10 mM HEPES, and 10 mM glucose, and the pH was adjusted to 7.3 with HCl (75 mM), yielding a total external $[\text{Cl}^-]$ of 154 mM. To block voltage-gated Na^+ and Ca^{2+} channels, external solutions were supplemented with 500 nM tetrodotoxin (Alomone Laboratories, Jerusalem, Israel) and 100 µM Cd^{2+} . The

reversal potential for I_{GABA} [$E_{\text{Cl(GABA)}}$] was determined with short voltage ramps applied during GABA pulses and used to estimate $[\text{Cl}^-]_i$ based on the Nernst equation for Cl^- .

Statistics and model simulations

All data are shown as means $\pm$ SE. Statistically significant differences were evaluated with an unpaired Student's *t* test ($P < 0.05$). We used MATLAB version R14 Service Pack 3 (The MathWorks, Inc., Natick, MA, USA) to solve the differential equation describing the changes in $[\text{Cl}^-]_i$ (see Eq. 1 in the Discussion section). For this we used a variant of the built-in function "ode43" with a constant integration step of 1 s.

RESULTS

GABA-induced current responses were measured and analyzed in cultured E14 DRGNs ($n=100$) and MNs ($n=70$). DRGNs had a capacitance of 23 ± 1 pF ($n=80$), which implied that the DRGN population was almost exclusively composed of small DRGNs (38–40) of ~ 20 μm in diameter (Fig. 2C) (39). MNs had a higher capacitance of 41 ± 2 pF ($n=44$), reflecting the extensive outgrowth of neurites in MNs, whereas DRGNs showed a small number of neurites (Fig. 2C) (22).

First, we determined whether DRGNs and MNs displayed differences in GABA responsiveness and/or intracellular Cl^- content. **Figure 1A** shows typical examples of GABA-induced currents at holding potentials in the range of -120 to $+50$ mV. These currents were characterized by determining the maximum slope conductance, and reversal potentials were determined from $I_{\text{GABA}}-V$ relations (Fig. 1B). Maximum slope conductance was significantly smaller in DRGNs (0.21 ± 0.02 nS/pF; $n=6$) than in MNs (0.29 nS/pF ± 0.03 ; $n=6$). In addition, $E_{\text{Cl(GABA)}}$ values from DRGNs (-34 ± 2 mV; $n=58$) were significantly more positive than those for MNs (-56 ± 1 mV; $n=43$). These $E_{\text{Cl(GABA)}}$ values correspond to intracellular Cl^- levels of 44 ± 2 mM in DRGNs compared with 18 ± 1 mM in MNs (**Fig. 2A**). To test whether the affinity of GABA_A receptors for GABA was different between DRGNs and MNs, we determined GABA

concentration-current response relationships (Fig. 1D). Fitting of the experimental data with a Hill equation yields rather similar estimates for the effective concentration and Hill coefficient in DRGNs ($\text{EC}_{50} = 74 \pm 8$ μM ; $n_{\text{H}} = 0.71 \pm 0.04$; $n=6$) and MNs ($\text{EC}_{50} = 40 \pm 12$ μM ; $n_{\text{H}} = 0.63 \pm 0.07$; $n=6$).

It has been shown that the high intracellular Cl^- level found in mature DRGNs is related to a high expression level of NKCC1 cotransporters in these cells (7, 16, 23). In accordance with this finding, we observed that application of 10 μM bumetanide, a selective NKCC1 blocker (9, 11, 16, 17) significantly reduced $[\text{Cl}^-]_i$ in E14 DRGNs from 44 to 19 mM (57%, $n=13$) whereas $[\text{Cl}^-]_i$ in MNs only decreased from 18 to 14 mM (22%, $n=7$) (Fig. 2A). In addition, when neurons were preincubated for 30 min in low external Na^+ medium, which inhibits Cl^- entry *via* NKCC1 (41), the resting $[\text{Cl}^-]_i$ dropped significantly in DRGNs (36%, $n=6$), whereas the decrease in MNs was not statistically significant (12%, $n=5$) (Fig. 2A).

The expression of NKCC1 in DRGNs and MNs was confirmed by Western blot using the monoclonal anti-NKCC1 (T4) antibody. No clear difference in expression levels was observed between the two cell types (data not shown). In addition, immunocytochemistry experiments with the same antibody confirmed a previous report (41) showing that DRGNs showed high NKCC1 expression levels all over the cell, whereas in MNs NKCC1 was localized mainly in the neuronal expansions (data not shown).

We have shown previously that prolonged application of GABA associated with membrane hyperpolarization or depolarization induces long-lasting changes in intracellular Cl^- levels in motor neurons (30). It is known that NKCC1 is mainly a Cl^- uptake mechanism, as it only supports Cl^- efflux at very high intracellular concentrations (~ 100 mM) of this ion (11). Therefore, we hypothesized that this cotransporter should not have a strong influence in the recovery of $[\text{Cl}^-]_i$ after loading. On the other hand, a higher functional expression of NKCC1 in DRGNs should result in faster recovery of $[\text{Cl}^-]_i$ after depletion when compared with that in MNs.

Figure 1. Characteristics of GABA-activated current in DRGNs and MNs. A) Typical current traces obtained during 5-s applications of 200 μM GABA to DRGNs (left) and MNs (right). Numbers close to traces indicate corresponding membrane potential (mV). B) Examples of $I-V$ curves obtained during application of voltage ramps from -100 to $+50$ mV at peak of the GABA-activated current in DRGNs (500 μM GABA) and MNs (50 μM GABA). Normalized chord conductance and GABA equilibrium potential were 0.24 nS/pF and -32 mV and 0.39 nS/pF and -51 mV for DRGNs and MNs, respectively. C) GABA dose-response curves for DRGNs and MNs.

Figure 2. Comparison of functional expression of NKCC1 in DRGNs and MNs. Average resting $[\text{Cl}^-]_i$ [derived from measured $E_{\text{Cl}(\text{GABA})}$] in DRGNs and MNs at E14 in control conditions, after incubation with 10 μM bumetanide (10 min), and in low extracellular Na^+ (10 min). ** $P < 0.01$ vs. control treatment.

To assess the role of NKCC1 in relation to intracellular chloride dynamics, we used the following experimental procedure. In control conditions, I_{GABA} was elicited by applying a standard stimulation protocol in which DRGNs were held at -40 mV and MNs at -60 mV, and GABA was applied for 1 s every 30 s at the concentrations of 500 μM in DRGNs and 50 μM in MNs. For each cell type, the holding potentials were chosen to be close to their equilibrium potential for chloride. After three stimuli, cells were loaded with Cl^- by applying a strong depolarization in the presence of GABA at high concentration. For this, DRGNs were depolarized to $+70$ mV and MNs to $+50$ mV for 10 s while being perfused

during 5 s with GABA at 1.5 mM and 200 μM , respectively. To monitor the recovery of I_{GABA} (i.e., $[\text{Cl}^-]_i$ decrease) after loading, stimulation was resumed using the standard protocol. The effect of the loading stimulus on $[\text{Cl}^-]_i$ was tested in some cells by applying voltage ramps during the depolarizing step.

The observed shift of the equilibrium potential revealed increases in intracellular Cl^- of 13 ± 1 mM ($26 \pm 3\%$; $n=8$) and 24 ± 3 mM ($160 \pm 31\%$; $n=7$) in DRGNs and MNs, respectively. In all cases, the recovery after loading ($[\text{Cl}^-]_i$ decrease) was characterized by a single-exponential time course. As shown in Fig. 3A, B, E, the amplitude of I_{GABA} (and thus $[\text{Cl}^-]_i$) recovered from loading at similar rates in DRGNs ($\tau=2.6 \pm 0.1$ min; $n=5$) and MNs ($\tau=2.8 \pm 0.3$ min; $n=6$). Notably, the recovery was severely hindered by decreasing the frequency of stimulation of GABA receptors, indicating that the changes in $[\text{Cl}^-]_i$ after loading occurred mainly *via* Cl^- efflux through I_{GABA} . In addition, the recovery was not altered by preincubation of the cells in a medium with a low Na^+ concentration (Fig. 3C–E), a maneuver that is known to decrease the activity of NKCC1 (41). Altogether, these observations indicate that after loading NKCC1 is not involved in the decrease of $[\text{Cl}^-]_i$ to the resting level.

To test whether NKCC1 is involved in the recovery ($[\text{Cl}^-]_i$ increase) after depletion we used a protocol similar to that used for testing the effects of loading. To induce intracellular chloride depletion DRGNs were hyperpolarized to -100 mV and MNs to -120 mV for 10 s while being perfused during 5 s with GABA at 1.5 mM and 200 μM , respectively. As result, $[\text{Cl}^-]_i$ decreased by 8 ± 1 mM ($17 \pm 2\%$; $n=5$) and 5 ± 1 mM ($24 \pm 2\%$; $n=6$) in DRGNs and MNs, respectively. Figure 4 shows that the $[\text{Cl}^-]_i$ increase after depletion was significantly faster in DRGNs ($\tau=0.9 \pm 0.1$ min; $n=5$)

Figure 3. Recovery of $[\text{Cl}^-]_i$ after loading in DRGNs and MNs. A–D) Responses of GABA-induced current to Cl^- load in DRGNs and MNs, in control conditions (A, B) and in low extracellular Na^+ (C, D). Current amplitudes were normalized to values during loading pulse. Color-coded points correspond to current traces shown in insets. Open squares represent amplitudes during $[\text{Cl}^-]_i$ decrease after Cl^- loading in absence of frequent applications of GABA. I_{GABA} remained virtually unchanged for 10 min as GABA pulses were delivered only every 5 min, but recovery occurred when frequent GABA pulses resumed. Black solid lines show fit of data after depolarization, using a single-exponential function. E) Average time constant of recovery of I_{GABA} in control and in low extracellular Na^+ medium.

Figure 4. Recovery of $[\text{Cl}^-]_i$ after depletion in DRGNs and MNs. *A–D*) Responses of GABA-induced current to Cl^- depletion in DRGNs and MNs in control conditions (*A, B*) and in low extracellular Na^+ (*C, D*). Current amplitudes were normalized to values during depleting pulse. Color-coded points correspond to current traces shown in insets. Black solid lines show fit of data after depolarization, using a single-exponential function. *E*) Average time constant of the recovery of I_{GABA} in control and in low extracellular Na^+ medium.

than in MNs ($\tau = 1.6 \pm 0.1$ min; $n = 5$) and that preincubation in a medium with low Na^+ significantly slowed the changes in $[\text{Cl}^-]_i$ in DRGNs ($\tau = 1.9 \pm 0.4$ min; $n = 4$) but not in MNs ($\tau = 1.6 \pm 0.1$ min; $n = 4$). Thus, reducing the activity of NKCC1 transporter significantly slowed down the $[\text{Cl}^-]_i$ increase after depletion in DRGNs but not in MNs. These results confirm the hypothesis of higher somatic expression and/or activity of NKCC1 in DRGNs than in MNs.

DISCUSSION

The action of GABA critically depends on the Cl^- gradient across the plasma membrane and hence on the $[\text{Cl}^-]_i$. In neurons, $[\text{Cl}^-]_i$ depends on the expression level of electroneutral cation-chloride cotransporters belonging to the NKCC and KCC families (10). Using the gradient for Na^+ generated by the Na^+/K^+ -ATPase pump, NKCC1 cotransporter protein conveys Na^+ , K^+ , and Cl^- into cells under physiological conditions (9–11). Thus, NKCC1 makes an important contribution to determining the reversal potential of GABA_A-mediated currents $[E_{\text{Cl}(\text{GABA})}]$ (16, 19–22). To elucidate how NKCC1 contributes to neuronal $[\text{Cl}^-]_i$ homeostasis, we studied the expression and localization of this cotransporter in cultured rat E14 DRGNs and MNs in relation to the kinetics of changes in $[\text{Cl}^-]_i$ after intracellular Cl^- loading and depletion.

Gramicidin-perforated patch-clamp measurements revealed a significantly higher resting $[\text{Cl}^-]_i$ in DRGNs (44 mM) than in MNs (18 mM). The loop diuretic bumetanide, a strong inhibitor of NKCC1, decreased resting $[\text{Cl}^-]_i$ to final concentrations of 19 and 14 mM in DRGNs and MNs, respectively. Because passive distribution predicts a $[\text{Cl}^-]_i$ of ~ 14 mM (42), this finding suggests that NKCC1 constitutes the main mechanism for maintaining high $[\text{Cl}^-]_i$ in

DRGNs. In contrast, MNs showed low intracellular Cl^- accumulation in basal conditions, a fact that most likely reflects low NKCC1 activity in these cells (2, 30). In accordance with the effects of bumetanide, preincubation of the cells in a low Na^+ solution medium, which reduces the activity of NKCC1 (41), caused a significant decrease in the resting levels of $[\text{Cl}^-]_i$ in DRGNs but not in MNs. Notably, we found in Western blot experiments that the expression levels of NKCC1 are higher in DRGNs than in MNs. Moreover, immunohistochemistry experiments revealed that NKCC1 is expressed throughout the body of DRGNs, whereas in MNs it is expressed mainly in the dendrites.

To test further the hypothesis of higher functional expression of NKCC1 in DRGNs with respect to MNs, we conducted experiments designed to approach the kinetics of intracellular Cl^- handling. For this we monitored the time course of the amplitude of GABA-induced currents after intracellular Cl^- loading and depletion in DRGNs and MNs.

Notably, DRGNs and MNs recovered from loading $[\text{Cl}^-]_i$ with a similar time course, which mainly depended on the frequency of GABA application and was not affected by lowering the extracellular Na^+ . These findings clearly indicate that 1) the decrease in $[\text{Cl}^-]_i$ after loading represents passive Cl^- efflux *via* GABA_A receptors, 2) the recovery does not involve active Cl^- extrusion or passive Cl^- efflux *via* background Cl^- channels, and 3) consistent with previous findings (11), NKCC1 does not play an important role in extruding Cl^- . In contrast to loading, depletion was able to reveal the role of NKCC1 in the kinetics of intracellular chloride handling. Indeed, the $[\text{Cl}^-]_i$ increase after depletion was significantly faster in DRGNs than in MNs, a difference that was absent when NKCC1 was inhibited by preincubation of the cells in a low Na^+ solution.

Overall, these observations constitute strong evi-

dence for a higher functional expression of NKCC1 in DRGNs with respect to MNs. Still, it could be argued that the disparity in depletion conditions between DRGNs and MNs could somehow account for the differences in recovery between these cells. It seems clear that the recovery ($[Cl^-]_i$ increase) after depletion in DRGNs can only be attributed to secondary active Cl^- uptake, most probably through NKCC1, because during the recovery phase I_{GABA} was always inward (corresponding to passive Cl^- loss). However, understanding the recovery from depletion in MNs seems to be harder, as during the early phase of the recovery I_{GABA} was directed outward and contributed to the passive Cl^- accumulation. In contrast, in later recovery this current was directed inward and therefore contributed to Cl^- depletion.

To provide a clearer interpretation of the experimental results, we developed a mathematical model of the kinetics of intracellular Cl^- in DRGNs and MNs. For this, we simulated the $[Cl^-]_i$ kinetics by including the contributions of the I_{GABA} , the NKCC1 cotransporter, and a background Cl^- pathway. $[Cl^-]_i$ was determined by solving Eq. 1:

$$\frac{d[Cl^-]_i}{dt} = \frac{(I_{GABA}(t) + I_{BCL})/F + J_{NKCC1}}{V_{Cl}} \quad (1)$$

where $I_{GABA}(t)$ is the GABA-induced current, I_{BCL} is the current through a linear background Cl^- pathway, F is the Faraday constant, and J_{NKCC1} is the number of moles of chloride moved across the membrane per second by the NKCC1 cotransporter. As first approximation, this model assumes that intracellular Cl^- is distributed in a single compartment

of volume V_{Cl} (intracellular Cl^- -handling volume). This volume is anticipated to be smaller than the actual cell volume, given that Cl^- is not expected to diffuse into all intracellular compartments. The expressions used to calculate the terms $I_{GABA}(t)$, I_{BCL} , and J_{NKCC1} are given in the Appendix. To reproduce the experimental data, only three parameters were varied: the chloride handling volume, the conductance for chloride background current, and the maximal rate of transport through NKCC1 (see Appendix).

As shown in **Fig. 5A, B**, the model closely reproduces the time course of the $[Cl^-]_i$ increase after depletion for both DRGNs and MNs (compare with Fig. 4A, B). The time constants of recovery obtained with the model were equal to 1 and 1.57 min for DRGNs and MNs, respectively. These values are very close to those obtained experimentally ($\tau = 0.9 \pm 0.1$ and 1.6 ± 0.1 min for DRGNs and MNs, respectively). Notably, to simulate the experimental data it was necessary to consider that the maximal rate of transport of NKCC1 was 37.5-fold higher in DRGNs than in MNs (see Fig. 5C–F): in DRGNs $J_{max} = 150$ pmol/s and in MNs $J_{max} = 4$ pmol/s. The model is also useful for analyzing the role of the distinct Cl^- pathways in the kinetics of $[Cl^-]_i$. For example, Fig. 5C, D shows the contribution of NKCC1 to the change in $[Cl^-]_i$ in comparison with that of the background Cl^- current I_{BCL} . Note that the contributions of these pathways should balance each other in steady-state conditions ($d[Cl^-]_i/dt = 0$ implies that $J_{NKCC1} = I_{BCL}/F$). The model allows estimation of how both I_{GABA} and the background Cl^- current contribute to the changes in

Figure 5. Model simulations of the kinetics of $[Cl^-]_i$ in DRGNs and MNs. **A, B** Simulation of total membrane current (black line: $I_{GABA} + I_{BCL}$) during the application of the depletion protocol in DRGNs (**A**) and MNs (**B**) (compare with Fig. 4). Thick red lines represent single-exponential fit of peak current amplitudes. Insets: superposition of individual current events during GABA application in recovery phase. **C, D** Simulated changes in $[Cl^-]_i$ produced by activity of NKCC1 and background Cl^- current I_{BCL} in DRGNs (**C**) and MNs (**D**). **E, F** Cumulative contributions of NKCC1, I_{GABA} , and I_{BCL} to $[Cl^-]_i$ in DRGNs (**E**) and MNs (**F**). Note that I_{GABA} and I_{BCL} always cause Cl^- depletion in DRGNs. In contrast, they contribute to Cl^- loading during early phase of recovery in MNs. This corresponds to period in which these currents are directed outward; see bars marked with asterisk (**B, D, F**). **G, H** Simulated changes in $[Cl^-]_i$ and equilibrium potential of Cl^- during the application of the depletion protocol in DRGNs (**G**) and MNs (**H**).

$[\text{Cl}^-]_i$. In DRGNs, these pathways always cause Cl^- depletion (Fig. 5A, C, E). However, in MNs they cause loading during the early phase of the $[\text{Cl}^-]_i$ increase after depletion ($d[\text{Cl}^-]_i/dt > 0$) (Fig. 5B, D), which is manifested as a “bump” in the corresponding curves of cumulative change of $[\text{Cl}^-]_i$ (Fig. 5F).

Altogether, the model is in full agreement and gives further support to the experimental data, indicating that there is a higher functional expression of NKCC1 in DRGNs with respect to MNs. Yet, when one considers that NKCC1 is also expressed in MNs (Fig. 2B, C), it remains unclear why the experiments and the model simulations indicate that activity of NKCC1 is much higher in DRGNs than in MNs. When this point is analyzed, it is important to take into account the fact that cells were patched at the soma, and, therefore, the kinetics of intracellular Cl^- changes are expected to reflect mainly the activity of chloride membrane pathways in this region. Events occurring in the dendritic extensions were probably misrepresented in the experimental observations because of restricted diffusion and electrical access. Hence, the contrast between the rather homogeneous expression of NKCC1 in DRGNs and the almost exclusive location of this cotransporter in the dendritic regions of MNs fits with a larger contribution of NKCC1 to the intracellular (somatic) Cl^- handling in DRGNs. However, different somatic expressions of NKCC1 in DRGNs and MNs might not fully explain the remarkable discrepancy noticed in NKCC1 activity. Differences in NKCC1 activation levels could also account for it. Although we did not systematically monitor cell volume changes during our experiments, NKCC1 activation by cell shrinkage (32, 43) could also be involved. In this perspective, the crucial role of the cytoskeleton *via* F-actin, myosin II, and myosin light-chain kinase was shown in several studies (44–47).

Figure 5C, D shows that the model also complies with the changes in $[\text{Cl}^-]_i$ and Cl^- equilibrium potential observed in the depletion experiments. This was accomplished by adjusting the value of the intracellular Cl^- -handling volume V_{Cl} . In DRGNs, $V_{\text{Cl}} = 1.3$ pL, which is lower than the values obtained for MNs ($V_{\text{Cl}} = 3.6$ pL). This difference is in agreement with the higher membrane capacitance we have observed in MNs with respect to DRGNs.

It has been reported that NKCC1 is down-regulated in the perinatal period in the central nervous system (8, 48) and the spinal cord (49), which results from the action of maternal oxytocin during birth (50). Interestingly, we have observed that $[\text{Cl}^-]_i$ in DRGNs at E14 equaled those observed after maturation. Considering the results we have obtained with bumetanide and low extracellular Na^+ to inhibit NKCC1 experimentally, we postulate that the reduction of the expression of this cotransporter during the later embryogenesis results in alterations of intracellular Cl^- dynamics in DRGNs.

CONCLUSIONS

Our results indicate that the relatively low activity of NKCC1 in MNs contributes to the fact that these neurons can be subjected to substantial $[\text{Cl}^-]_i$ changes. Of note, significant Cl^- depletion may occur during spontaneous discharges caused by GABAergic pathways, which, in turn, determine important cellular processes during neurogenesis (41, 51). In contrast, high activity of NKCC1 allows DRGNs to maintain high $[\text{Cl}^-]_i$, which results in excitatory effects of GABA in resting conditions. Furthermore, the activity of NKCC1 in DRGNs allowed $[\text{Cl}^-]_i$ to recover promptly after Cl^- loss. On the other hand, $[\text{Cl}^-]_i$ recovery after an increase in $[\text{Cl}^-]_i$ was a slow process and not substantially different from that observed in MNs. Thus, DRGNs are able to sustain high $[\text{Cl}^-]_i$ and depolarizing GABA_A receptor currents during ongoing GABAergic synaptic inputs. This property is probably related to pre-synaptic inhibition occurring *via* GABA-dependent primary afferent depolarization by spinal chord interneurons, which is among the important mechanisms allowing DRGNs to perform their sensory function (1, 3, 7). Indeed, disturbances in $[\text{Cl}^-]_i$ subsequent to modification of NKCC1 activity (4) give rise to serious impairments in sensory perception (1, 6). We should, of course, be cautious in extrapolating data from embryonic cultured DRGNs to adult neurons *in vivo*. However, resting $[\text{Cl}^-]_i$ in cultured E14 DRGNs is not different from that found in mature cells (7, 16, 22, 23). [E]

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Appendix

$I_{\text{GABA}}(t)$ was expressed as

$$I_{\text{GABA}}(t) = G_{\text{GABA}}(t) \cdot (V - E_{\text{Cl}}) = G_{\text{GABA}}(t) \cdot \left(V - \frac{RT}{F} \ln \frac{[\text{Cl}]_i}{[\text{Cl}]_o} \right)$$

where V is the membrane potential, E_{Cl} is the chloride equilibrium potential, R is the gas constant, and T is the absolute temperature. $G_{\text{GABA}}(t)$ is the time-dependent whole-cell GABA conductance, which is described by an 8000-s-long train of pulses spaced every 30 s. Except the third, these pulses were expressed as

$$G_{\text{GABA}}(t) = A(0.17e^{-(t-0.33)^2/0.058} + 4.6(1 - 0.5(1 - \tanh(23(t - 0.097)))) \cdot e^{1.82(t-0.85+(t-0.85))})$$

which reproduces the amplitude and the complex shape of the GABA-induced currents recorded experimentally (Fig. 4A, B). A was equal to 18 and 40 nS for DRGNs and MNs, respectively. The third pulse, which was used in the experiments to deplete the intracellular chloride, was simulated by the equation

$$G_{\text{GABA}}(t) = B(C(1 - e^{-t/200}) \cdot e^{-t/2000} + 0.05)$$

where B was equal to 15.75 and 16.5 nS and C was equal to 0.41 and 1.3 for DRGNs and MNs, respectively.

It should be noted that the equations used to describe $I_{\text{GABA}}(t)$ are phenomenological expressions fitted to original current traces in the corresponding conditions (data not shown). Except for the parameters A and B , which have units of conductance, no attempt was made to describe in detail the nature of the parameters involved.

The background chloride current, I_{BCl} , is given by

$$I_{\text{BCl}} = G_{\text{BCl}} \left(V - \frac{RT}{F} \ln \frac{[\text{Cl}]_i}{[\text{Cl}]_o} \right)$$

where G_{BCl} is the background conductance for chloride equal to 0.69 and 0.74 nS for DRGNs and MNs, respectively.

NKCC1 activity was defined by the number of picomoles of Cl^- moved across the membrane per second, J_{NKCC1} , and was calculated as

$$J_{\text{NKCC1}} = J_{\text{max}} \frac{K_m^H}{[\text{Cl}]_i^H + K_m^H}$$

where J_{max} is the maximal rate of transport. The saturation constant K_m and the Hill coefficient H were determined by fitting the data of Gillen and Forbush (Fig. 7 in ref. 15) and were equal to 25 mM and 6, respectively.

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