INTRODUCTION

A great deal of interest has recently been focused on the observation that neurons are depolarized by γ-aminobutyric acid (GABA) in early development in several different parts of the nervous system (Ben-Ari et al. 2007). In this developmental period, the reversal potential for GABA_A receptor currents (referred to as \(E_{\text{GABA}}\)) is more depolarized than the resting membrane potential because chloride, the main carrier of the GABA_A current, is accumulated in immature neurons. The depolarizing nature of GABA is important in driving the spontaneous network activity (SNA) that is observed in virtually all developing circuits (Ben-Ari et al. 2007; O’Donovan 1999). In the spinal cord, episodes of SNA recruit the majority of neurons, drive embryonic movements, and are important in muscle, joint, and motoneuron development (Ben-Ari et al. 2007; Casavant et al. 2004; Gonzalez-Islas and Wenner 2006; Hanson and Landmesser 2004, 2006; O’Donovan 1999).

The modulation of intracellular chloride appears to be critical for the normal expression of SNA (Chub and O’Donovan 1998, 2001; Chub et al. 2006; Fedirchuk et al. 1999; Marchetti et al. 2005). During an episode of SNA, intracellular chloride is depleted as it passes out of the cell through activated GABA_A receptor channels, thus weakening the driving force for GABAergic synapses, leaving the cord relatively less excitable. Then in the quiescent interepisode interval, chloride is reaccumulated by transporters that are thought to be critical for strengthening excitatory GABAergic synapses and generating the next episode (Marchetti et al. 2005).

Several studies demonstrate that the Na^+ -K^+ -2Cl^- cotransporter, NKCC1, accumulates chloride in developing neurons (Achilles et al. 2007; Chub et al. 2006; Delpy et al. 2008; Kakazu et al. 1999; Rocha-Gonzalez et al. 2008; Rohrbough and Spitzer 1996; Sipila et al. 2006; Yamada et al. 2004). However, most of these studies block NKCC1 and still observe some chloride accumulation and it appears that in some developing neurons NKCC1 is not involved at all (Balakrishnan et al. 2003; Zhang et al. 2007). In a recent study significant chloride accumulation was observed in embryonic spinal motoneurons after blocking both NKCC1 and the chloride extruder KCC2 with furosemide (Delpy et al. 2008). The molecule(s) responsible for NKCC1-independent chloride accumulation remains unknown. In the current study we test the role of NKCC1 and other transporters in accumulating chloride in motoneurons in the chick embryo. We assess chloride levels at GABAergic synapses, which are likely to exist out in the dendrites. This allows us to determine the chloride transporters that are most relevant to setting the driving force for GABAergic inputs.

In the present report we assess \(E_{\text{GABA}}\) at synaptic sites using miniature postsynaptic currents (mPSCs) in chick embryo motoneurons. Whole cell recordings of GABAergic mPSCs were obtained at different voltage steps and current–voltage (I–V) plots were constructed, providing conductance measurements, as well as \(E_{\text{GABA}}\) for the synaptic currents. Chloride accumulators appear to define the synaptic chloride concentrations out in the dendrites. We show that although NKCC1 contributes to chloride influx in embryonic motoneurons, chloride accumulation still occurs after NKCC1 blockade and is likely mediated by the anion exchanger, AE3.

METHODS

Dissection

White Leghorn chicken eggs were incubated in a circulated air incubator (GQF Manufacturing) at 38°C. Electrophysiological experi-
ments were performed on isolated spinal cords of embryonic day 10–11 (E10–E11), stages 36–37 (Hamburger and Hamilton 1951) chick embryos. SNA has been extensively studied at these stages. The lumbar-sacral spinal cord region, with attached femorotibialis (Fem) or adductor (Add) nerves, was dissected under cooled (15°C) oxygenated Tyrode’s solution containing (in mM): NaCl, 139; KCl, 5; NaHCO3, 17; CaCl2, 3; MgCl2, 1; d-glucose, 12; tetrodotoxin (TTX), 0.001; pH was adjusted to 7.3. After dissection, the cord was allowed to recover overnight in Tyrode’s at 18°C. The cord was then transferred to a recording chamber, continuously perfused with oxygenated Tyrode’s solution that was slowly heated to recording temperature (28°C). The cord was allowed to establish stable SNA (constant frequency of bursts) before starting the experiment.

Electrophysiology: mPSCs

Patch-clamp recordings were used to acquire mPSCs in the whole cell mode. Miniature postsynaptic currents were recorded from motoneurons antidromically identified as Fem or Add motoneurons by the stimulation of their particular muscle nerves via tight-fitting suction electrodes connected to high-gain differential amplifiers (A-M Systems). Tight seals (>2 GΩ before breaking into whole cell mode) were obtained using patch electrodes pulled from thin-walled glass in stages 2–7 using a P-87 Flaming/Brown micropipette puller (Sutter Instruments) and having resistances between 5 and 10 MΩ. Series resistance during recording varied from 10 to 20 MΩ among different motoneurons and was not compensated. Recordings were terminated whenever significant increases in input resistance (>20%) occurred. Extracellular recording solution for mPSCs, unless otherwise declared, contained the following (in mM): NaCl, 139; KCl, 5; NaHCO3, 17; CaCl2, 3; MgCl2, 1; d-glucose, 12; tetrodotoxin (TTX), 20; CsCl, 5; tetrodotoxin (TTX), 0.001; pH was adjusted to 7.3 with NaOH. 6-Cyano-7-nitroquinolinoxaline-2,3-dione (CNQX, 10 μM) and 2-amino-5-phosphonopentanoic acid (AP-5, 50 μM) were usually added to the bath to isolate GABAergic mPSCs (Gonzalez-Islas and Wenner 2006). It was not necessary to block glycineergic mPSCs because they are not observed at these stages in embryonic chick motoneurons (Gonzalez-Islas and Wenner 2006). Different extracellular solutions (low Na+: 17 mM; low Cl−: 53 mM; 0 HCO3−) were obtained by substituting appropriate concentrations of choline-Cl, choline-HCO3, and Na-glucuronate. Solutions were constantly bubbled with a mixture of 95% O2−5% CO2 to maintain a pH around 7.3, except for the 0 mM HCO3− solution, which contained 10 mM HEPES and was bubbled throughout the experiment with 100% O2.

To record mPSCs, throughout the study we used five different pipette solution concentrations of chloride that are referred to as 17, 32, 52, 77, and 157 mM Cl− patch solutions. Chloride concentration was varied by substituting NaCl and Na-glucuronate, and KCl and K-glucuronate, and are shown in Table 1. pH in all cases was adjusted to 7.2 with KOH. The junction potentials were determined for each solution (Table 1) and were corrected on-line.

To block calcium-, sodium-, and potassium-voltage gated currents we added verapamil (0.1 mM), N-(2,6-dimethylphenyl carbamoyl-methyl)triethylammonium bromide (QX-314, 10 mM), TEA (10 mM), and CsCl (5 mM) to the normal pipette solution, respectively. Motoneurons were antidromically identified before QX-314 completely blocked action potentials. Once a stable motoneuron recording was obtained, we added TTX to prevent episodes of SNA from occurring and therefore also eliminate the episode-induced modulation of GABAergic mPSC amplitude. After 10–20 min when mPSC amplitudes become stable, a voltage ramp was performed to establish that voltage-activated currents were blocked (Fig. 1A). Voltage steps were then given in 10 mV increments, usually from −80 to +50 mV, although in some cases we used 20 mV steps and/or skipped voltages around the reversal potential (see following text). Each step was held for 2 min while mPSCs were acquired; however, in certain cases mPSC frequency was high and it was not necessary to record the full 2 min to collect sufficient numbers of mPSCs. Average amplitude of GABAergic mPSCs at each step was then plotted against voltage (VmPSC−Vplot). In some cases amplitudes near the reversal potential were slightly larger than predicted by our fitted line (average mPSCs were likely biased toward larger values because smaller mPSCs fall into the noise; average mPSC amplitudes of <6 pA were typically off the fitted line and were excluded). Generating mPSC I−V plots using whole cell recording should provide an accurate assessment of E\textsubscript{GABA}. Whole cell electrodes have low access resistance and recordings of embryonic motoneurons at this stage appear to provide a very good space clamp. As shown previously, mPSC amplitude and decay are not correlated as would be expected if we had a poor space clamp (Gonzalez-Islas and Wenner 2006); furthermore, we never saw inward and outward currents in the same voltage step, consistent with the idea that the entire length of the dendrite is clamped at the same voltage. Finally, at moderate and high intracellular patch chloride concentrations, the measured E\textsubscript{GABA} matched that predicted by the Nernst equation.

Whole cell currents were acquired using an EPC 8 amplifier (HEKA Elektronik), controlled by PatchMaster 2.03 software (HEKA Elektronik) via an LIH 1600 interface (HEKA Elektronik). Currents were filtered on-line at 5 kHz, digitized at 10 kHz, and analyzed using MiniAnalysis software (Synaptosoft). Average mPSC amplitudes were fitted to a linear function with Kaleidagraph plotting software (Synergy Software) to obtain E\textsubscript{GABA} and conductance. Theoretical E\textsubscript{GABA} was calculated using the Nernst equation for Cl−.

Verapamil was purchased from Calbiochem; TTX, AP-5, and CNQX, were purchased from Tocris Cookson; CsCl was purchased from Fisher Scientific; 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) was purchased from Fluka; HEPES from Acros Organics. All other chemicals and drugs were purchased from Sigma–Aldrich (St. Louis, MO). Drugs were dissolved in saline unless otherwise mentioned.

Isoguvacine puffs

Experiments measuring the evoked ventral root potentials following local application of a GABA\textsubscript{A} agonist (isoguvacine) involved six different bath solutions (Table 2). For local application, isoguvacine (100 μM) was dissolved in DPBS (Dulbecco’s phosphate-buffered saline with Ca2+ and Mg2+) and pressure applied (26–32 psi, 20- to 30-s duration) through glass micropipettes (tip diameters 2.5–5 μm) by means of Pneumatic Pico Pump (PV 800; WPI) using nitrogen gas. For repetitive (every 90 s) isoguvacine application, the duration of pressure pulses was reduced to 0.5–1 s. Micropipettes were positioned close to the Fem motor pool and the amplitude of the isoguvacine-evoked motoneuron responses were recorded (DAM 50, differential amplifier, low-pass filter; WPI) from the Fem nerve as electrotonic potentials. Signals were amplified with Cyber Amp 380, digitized with Digidata 1322A, stored in the computer, and analyzed with pClamp 9 software (Molecular Devices). To measure cellular Cl− accumulation over time, intracellular Cl− was first depleted for 50–60 min of bath application in...
the “low Cl” solution (Table 2), which completely collapsed the isoguvacine-evoked responses in motoneurons. Following this, the “low Cl” solution was changed back to normal bath solution (Table 2) and the amplitude of the nerve potentials to repetitive application of isoguvacine were measured during the following 60 min. To evaluate the transporters involved in cellular Cl accumulation we repeated the process in the presence of transporter blockers.

Statistics

Data are expressed as means ± SE. Most statistical analysis was performed using two-tailed Student’s t-tests (paired and unpaired) unless mentioned otherwise. GraphPad Instat software was used for statistical analysis.

RESULTS

Determining the reversal potential at GABAergic synapses

To assess GABAergic synaptic reversal potentials, we used whole cell recordings of GABA_A mPSCs to generate I–V plots (Jarolimek et al. 1999). One concern was that our patch solution would dialyze the cell and set the intracellular chloride concentration and therefore E_GABA. To test this we isolated spinal cords and obtained whole cell recordings of antidromically identified spinal motoneurons (Fem and Add) from E10 chicks. Voltage-gated channels and glutamatergic transmission were blocked to isolate GABAergic mPSCs (Gonzalez-Islas and Wenner 2006), which were typically recorded in voltage steps of 10 mV (Fig. 1B). The average peak amplitude of GABAergic mPSCs at each step was then plotted against the step voltage (I_mPSC–V plot). The average E_GABA and conductance were, respectively, -29.5 mV and 247 pS (Table 3, 52 mM chloride patch solution). To directly test the influence of the patch solution on intracellular chloride concentrations for GABAergic mPSCs we used patch electrodes with several different chloride concentrations in the pipette solution (17, 32, 52, 77, and 157 mM; Table 1, Fig. 2). If patch chloride concentration had set intracellular chloride, then Nernstian predictions for E_GABA would be ±10 mV from the recorded average.

TABLE 2. Composition of bath solutions (in mM)

<table>
<thead>
<tr>
<th>Solution</th>
<th>NaCl</th>
<th>Na-Prropionate</th>
<th>NaHCO_3</th>
<th>Tris-Cl</th>
<th>Tris-OH</th>
<th>Choline-HCO_3</th>
<th>HEPES</th>
<th>NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% O_25% CO_2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>“Normal”</td>
<td>134</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>“Low Cl”</td>
<td>0</td>
<td>134</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>“0 Na”</td>
<td>0</td>
<td>0</td>
<td>129</td>
<td>21</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>“0 HCO_3”</td>
<td>134</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>“0 Na” and 0 HCO_3</td>
<td>0</td>
<td>0</td>
<td>134</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>“Low Cl” and 0 HCO_3</td>
<td>0</td>
<td>134</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

All solutions also included (in mM): KCl (5), Na_2HPO_4 (0.58), MgCl_2 (1), CaCl_2 (3), d-glucose (12), and TTX (0.0025–0.005). Osmolarity was adjusted with sucrose to 345 mOsm when needed.
Chloride is the main carrier of GABAergic currents

Measurements of $E_{\text{GABA}}$ with 17 mM chloride patch electrodes suggested that either intracellular chloride was accumulated beyond 17 mM or that another ion could be involved. Since GABA$_A$ receptor channels are slightly permeable to bicarbonate ions (Ben-Ari et al. 2007; Farrant and Kaila 2007), it was possible that HCO$_3^-$ played a significant role in determining $E_{\text{GABA}}$ when we used 17 mM chloride patch electrodes, and therefore HCO$_3^-$ maintained the reversal potential at about $-30$ mV. The Goldman–Hodgkin–Katz equation predicts that bicarbonate ions do not influence $E_{\text{GABA}}$ when intracellular chloride is 17 mM (or higher), assuming the HCO$_3^-$ to Cl$^-$ permeability ratio for GABA$_A$ receptor channels at 0.2–0.4 (Farrant and Kaila 2007; Kaila 1994). However, it remained possible that $E_{\text{GABA}}$ was maintained at $-30$ mV because GABA$_A$ receptor channels in chick embryo spinal motoneurons were more permeable to HCO$_3^-$ than in other systems. Therefore we tested this possibility by depleting extracellular HCO$_3^-$, GABAergic mPSCs were acquired as described earlier to make mPSC $I$–$V$ plots and derive $E_{\text{GABA}}$ in identified motoneurons. First, $E_{\text{GABA}}$ was determined in extracellular solution containing 17 mM HCO$_3^-$ and then again after replacing the extracellular solution with a 0 mM HCO$_3^-$ solution while bubbling with 100% oxygen (17 mM chloride in the pipette). Although $E_{\text{GABA}}$ did become slightly hyperpolarized 15–45 min after depleting HCO$_3^-$ (control $-30.3 \pm 2.2$ mV vs. 0 HCO$_3^-$ $-33.9 \pm 2.2$ mV, $P < 0.05$ paired $t$-test, $n = 4$), it was far from the $-59$-mV reversal potential predicted by the 17 mM chloride patch solution. Figure 3 shows the motoneuron that exhibited the most significant change in $E_{\text{GABA}}$ following perfusion of the 0 HCO$_3^-$ solution. In the absence of HCO$_3^-$ the measured reversal potential suggested that intracellular Cl$^-$ was about 45 mM. Further, in some cases $E_{\text{GABA}}$ appeared to recover if the cells were left in the 0 HCO$_3^-$ solution for $>60$ min (not shown). Consistent with other developing systems, the results suggest that chloride is the predominant carrier of the GABAergic current in E10 chick motoneurons.

Taken together, these results suggest that at low patch chloride concentrations, chloride accumulators maintain higher chloride levels and resist the dialysis of the patch chloride. This is consistent with the possibility that the vast majority of GABAergic synaptic inputs, from which the mPSCs arise, are located out in the dendrites, further away from the influence of the patch electrode.

Chloride accumulation is partly mediated by NKCC1

Current studies suggest that in many developing neurons NKCC1 contributes to the chloride accumulation that gives rise to the depolarizing GABAergic currents. To assess NKCC1’s

TABLE 3. $E_{\text{GABA}}$ and GABA-mPSC conductance

<table>
<thead>
<tr>
<th>Recording Condition</th>
<th>$E_{\text{GABA}}$, mV</th>
<th>Conductance, pS</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 mM Cl$^-$ patch (16)</td>
<td>$-30.7 \pm 1.1$</td>
<td>336 ± 36</td>
</tr>
<tr>
<td>17 mM Cl$^-$ patch/0 mM</td>
<td>$-33.9 \pm 2.2$</td>
<td>302 ± 42</td>
</tr>
<tr>
<td>17 mM Cl$^-$ patch/10 M bumetanide bath (8)</td>
<td>$-39.1 \pm 1.0$</td>
<td>254 ± 20</td>
</tr>
<tr>
<td>17 mM Cl$^-$ patch/17 mM Na$^+$ bath (7)</td>
<td>$-38.5 \pm 1.5$</td>
<td>224 ± 17</td>
</tr>
<tr>
<td>32 mM Cl$^-$ patch (5)</td>
<td>$-24.3 \pm 2.7$</td>
<td>270 ± 14</td>
</tr>
<tr>
<td>52 mM Cl$^-$ patch (7)</td>
<td>$-29.5 \pm 2.4$</td>
<td>247 ± 20</td>
</tr>
<tr>
<td>77 mM Cl$^-$ patch (3)</td>
<td>$-25.3 \pm 1.9$</td>
<td>309 ± 25</td>
</tr>
<tr>
<td>157 mM Cl$^-$ patch (3)</td>
<td>$-0.6 \pm 3.3$</td>
<td>316 ± 64</td>
</tr>
<tr>
<td>52 mM Cl$^-$ patch/53 mM Cl$^-$ bath (3)</td>
<td>$-1.2 \pm 5.6$</td>
<td>349 ± 48</td>
</tr>
</tbody>
</table>

Number of motoneurons recorded in a given condition is in parentheses.

...
role in chloride accumulation in E10 chick motoneurons we used several techniques, each offering slightly different information and, together, providing greater confidence than that provided by any single technique. We first tested chloride accumulation in a cell-wide fashion. Muscle nerve recordings were made while a GABA agonist, isoguvacine, was locally applied to motoneurons (Fig. 4A, schematic). The isoguvacine-evoked nerve potential was then assessed as a monitor of the chloride-mediated response (TTX, a voltage-gated Na\(^+\)/H\(^+\) channel blocker was added to bath to isolate the GABA-mediated responses). We then bath applied a “0 Na\(^+\)” solution (Table 2) to completely block any Na\(^+\)/H\(^+\)-dependent transporter (e.g., NKCC1) and observed that this reduced the isoguvacine-evoked response to 44.7 ± 2.8% of the control (n = 3, Fig. 4). By 30 min after the reintroduction of normal Na\(^+\) solution the amplitude recovered to 80.6 ± 6.9% of the control. These findings suggested that there was a significant chloride accumulation that is mediated by a Na-dependent process.

To assess the progressive accumulation of chloride we first depleted intracellular chloride by bath perfusion of a low chloride extracellular solution (13 mM, Table 2) for about 50 min, followed by bath perfusion of a normal chloride solution (158 mM, Table 2) while locally applying a GABA\(_A\) agonist (100 μM isoguvacine, 0.5 s, 15-psi picospritzer) to the motor column and again recording muscle nerve potentials (Fig. 5A). This provided a measure of cell-wide chloride accumulation in the motoneuron population because these GABAergic currents grow stronger with the accumulation of chloride. Isoguvacine puffs were delivered every 90 s (Fig. 5A, lines below traces) and the isoguvacine-induced potentials increased and stabilized to predepletion levels (288 ± 50 μV, n = 4) in about 35–40 min. Chloride accumulation in the order of minutes has been described previously (Achilles et al. 2007), although small perturbations in intracellular chloride can recover more quickly in other systems (Brumback and Staley 2008). The procedure was then repeated while blocking NKCC1 with bumetanide (10 μM, added to both “low Cl” and subsequent normal chloride solutions). Isoguvacine-induced nerve potentials did begin to recover, but stabilized at a value of 34.9 ± 1.4% of that in the absence of bumetanide (25–30 min, Fig. 5B). Figure 5 shows that the initial phase of reaccumulation (phase 1) was less dependent on NKCC1 than later stages of the accumulation (phase 2). These findings suggested that NKCC1 moved chloride into the cell beyond a passive distribution of the ion, but suggested that another accumulator must exist, which might be important at lower intracellular chloride concentrations (phase 1).

We next tested chloride accumulation specifically at synaptic sites, rather than in a cell-wide manner, as before. In the chick embryo, episodes of SNA lead to significant GABAergic transmission, which depletes intracellular chloride. Although...
this weakens GABAergic currents, the currents recover as chloride is reaccumulated in the interepisode interval (Chub et al. 2006; Fedirchuk et al. 1999; Marchetti et al. 2005). We can monitor this process of chloride regulation by stimulating motoneurons projecting in one ventral root and making extracellular recordings in the adjacent ventral root. Ventral root stimulation activates R-interneurons, which then make direct synaptic projections back to motoneurons, and these GABAergic responses can be recorded in the adjacent ventral root (Wenner and O’Donovan 1999). Ventral root responses are weakened after an episode of SNA and then become progressively stronger in the interval between episodes (Fig. 6). The ventral root responses were then reduced after adding bumetanide to block NKCC1. The response was eliminated when a GABA<sub>A</sub> antagonist was applied, demonstrating the response was GABAergic (Fig. 6A). When responses were binned in consecutive 2-min intervals after an episode, we saw that the average response of the fourth bin (6–8th min) was slightly, but significantly, reduced in bumetanide (Fig. 6B). Although the ventral root response was reduced, it continued to modulate, suggesting chloride was still accumulated in the absence of NKCC1 function. These findings are consistent with the idea that NKCC1 is important in chloride accumulation at synaptic sites, but that NKCC1 was not solely responsible for the accumulation.

To more directly test chloride accumulation at synaptic sites we next used whole cell recordings and made mPSC I–V plots to determine the effect of blocking NKCC1 on \( E_{\text{GABA}} \), a measure of intracellular chloride accumulation. If NKCC1 were solely responsible for the accumulation of high chloride at GABAergic synaptic sites (\( \gtrsim 50 \text{mM} \)) then we reasoned that blocking this transporter would shift \( E_{\text{GABA}} \) toward the value predicted for the 17 mM patch chloride (\( \sim 59 \text{mV} \)) because the patch solution dialyzed the cell (Jarolimek et al. 1996, 1999). We found that inhibiting NKCC1 with 10 \( \mu \text{M} \) bumetanide hyperpolarized \( E_{\text{GABA}} \) by only 8.4 mV to \( -39.1 \text{mV} \) (Fig. 7, A and B) and this change occurred within 10 min. We also noticed that conductance was slightly reduced in the presence of the blocker (Fig. 7, A and C, Table 3). This finding suggested the possibility that bumetanide had a slight effect on GABA<sub>A</sub> receptor channel conductance in the chick embryo. We also inhibited sodium-
dependent chloride transporters by effectively collapsing the Na\(^+\) gradient (low Na\(^+\) solution, 17 mM). This maneuver hyperpolarized \(E_{\text{GABA}}\) by \(-7.8\ \text{mV} (P < 0.001, n = 7\text{ cells, Table 3})\) in the first 2 h after perfusing with the low Na\(^+\) solution (Fig. 7D). We noticed that \(E_{\text{GABA}}\) appeared to recover to control values if longer incubations were used (not shown). Therefore using a different means of inhibiting inward Cl\(^-\) movement through NKCC1, we again determined that this cotransporter was involved in chloride accumulation at synaptic sites. Because the hyperpolarization of \(E_{\text{GABA}}\) never reached \(-59\ \text{mV} predicted by the Nernst equation (given a 17 mM chloride patch solution),\) there must be another chloride accumulator. Furthermore, the apparent recovery of \(E_{\text{GABA}}\) is consistent with the idea that a Na\(^+\)-independent accumulator could compensate for the loss of NKCC1 function.

**NKCC1-independent chloride accumulation**

The anion exchanger AE3 moves chloride into the cell as it expels bicarbonate. AE3 is up-regulated in the embryonic mouse spinal cord during the period SNA is expressed (Hentschke et al. 2006). We therefore tested the possibility that chloride was accumulated by AE3 in developing chick motoneurons. We repeated the experiments described earlier, but instead of NKCC1, we tested the anion exchanger by blocking it pharmacologically with 4,4′-disothiocyanatostilbene-2,2′-disulfonic acid (DIDS). We used the technique of locally applying a GABA\(_\chi\) agonist while recording the potential produced in the muscle nerve and saw that application of DIDS reduced the response to 53.6 ± 4.7% (Fig. 8, A and B). The remaining response was largely abolished when we then added bumetanide (8.1 ± 2.9%) and this response could be partly recovered on washout to 43.0 ± 4.9% (60 min, \(n = 5\)). Because we were concerned that DIDS could have nonspecific effects (Cabantchik and Greger 1992), we repeated these experiments, blocking the exchanger in a different way, with a 0 HCO\(_3^-\) extracellular solution. Solutions were bubbled with 100% oxygen and HEPES was added to maintain the pH at about 7.3. Very similar responses to that of DIDS were produced when blocking with “0 HCO\(_3^-\)” solution (Fig. 8, C and D). The “0 HCO\(_3^-\)” solution significantly reduced the isoguvacine response to 67.9 ± 3.1% and this was further decreased to 4.4 ± 1.5% in a “0 HCO\(_3^-\) and 0 Na\(^+\)” bath solution. The response recovered to 88.5 ± 4.7% after a 45-min washout period \((n = 5\)).

In the next set of experiments we depleted and reintroduced chloride and monitored chloride reaccumulation in the presence of DIDS. We saw that the reaccumulation was inhibited compared with control values, 51.7 ± 1.1% (Fig. 9A, 50 min). When NKCC1 was also blocked with bumetanide, chloride accumulation was nearly prevented (7.1 ± 0.3% in 50 min). In a separate set of experiments, we used a “0 HCO\(_3^-\)” solution to block the anion exchanger and we observed the recovery was reduced to 65.3 ± 5.5% of the control (Fig. 9B, 50 min). Blocking AE3 with either method demonstrated that chloride accumulation was reduced. Together these findings suggest that NKCC1 significantly contributes to chloride accumulation in embryonic chick motoneurons, but that additional accumulation likely occurs through the bicarbonate-dependent anion exchanger AE3.

**DISCUSSION**

In this study we examined chloride accumulation in embryonic motoneurons using several different approaches. We determined the reversal potential and conductance of GABA\(_\chi\) synaptic currents, using whole cell recordings of GABAergic
mPSCs. Synaptic chloride accumulates to about 50 mM and this is mediated in part by the chloride cotransporter NKCC1. We also show evidence that NKCC1 is involved in cell-wide chloride accumulation, as shown by muscle nerve responses to local application of a GABAA agonist. Although reduced, synaptic and cell-wide chloride accumulation continued after NKCC1 was blocked, suggesting an additional accumulator must exist. We show evidence that this residual chloride accumulation is dependent on HCO3⁻ and is sensitive to DIDS, suggesting that, at least in embryonic motoneurons, the additional accumulator is likely to be AE3.

**Measuring synaptic chloride through mPSC I–V plots**

During development, GABAergic synapses are depolarizing and can be excitatory, leading to the expression of spontaneous network activity. Later in development GABAergic synapses become hyperpolarizing and SNA...
abates. To understand how the driving force for chloride changes and influences GABAergic synaptic strength during this period, it may be important to assess GABAergic reversal potential where the synapses are located. GABA<sub>A</sub> reversal potential is often determined by local application of a GABA<sub>A</sub> agonist, while recording from a cell using the perforated-patch technique. This technique has the advantage that intracellular contents are minimally influenced by the pipette. On the other hand, the tested reversal potential will likely assess somal or cell-wide intracellular chloride (wherever syn-

**FIG. 8.** NKCC1-independent chloride accumulation is sensitive to 4,4'-disothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and dependent on HCO<sub>3</sub>-.

Isoguvacine was puffed (100 μM, 20-s duration, every 15 min) onto the motor column while the evoked potentials were recorded in the Fem nerve. A: the Cl<sup>-</sup>/HCO<sub>3</sub>- exchanger inhibitor, DIDS (50 μM), was added to the bath (60 min), which significantly reduced the evoked potential. This response was then nearly abolished on subsequent addition of 10 μM bumetanide (45 min). The response partially recovered after drug washout (45 min). B: averages are shown in the bar chart on the right (n = 5, P < 0.001). C: a “0 HCO<sub>3</sub>-” extracellular solution (45 min) also reduced the evoked potential, which is then nearly abolished with the perfusion of “0 Na and 0 HCO<sub>3</sub>-” extracellular solution (45 min). The response recovers after normal solution is restored. D: averages are shown in the bar chart on the right (n = 3, P < 0.001). Error bars represent SE.

**FIG. 9.** Chloride accumulation following chloride depletion demonstrate a DIDS-sensitive, bicarbonate-dependent process. A: plot shows that isoguvacine-evoked potentials (100 μM, 0.5-s duration, every 90 s) increase over time in control solution following chloride depletion, but less so during reaccumulation with DIDS (50 μM); reaccumulation was nearly abolished with application of DIDS and bumetanide (10 μM, n = 4). B: plot shows that isoguvacine-evoked potentials during reaccumulation were similarly inhibited on the perfusion of a “0 HCO<sub>3</sub>-” extracellular solution (100% O<sub>2</sub>, n = 4). Error bars represent SE.
aptic and extrasynaptic GABA<sub>A</sub> receptor activations occur). Because extra- and synaptic GABA<sub>A</sub> receptors could have distinct domains of expression, it is possible that \( E_{\text{GABA}} \) measured by mPSCs versus a cell-wide application of a GABA agonist will be different because chloride concentrations can vary within an individual neuron from soma to dendrites (Chub et al. 2006; Duebel et al. 2006; Gavrikov et al. 2006; Hara et al. 1992; Jarolimek et al. 1999; Li et al. 2008; Miller and Dacheux 1983; Vardi et al. 2000; Varela et al. 2005). We have used whole cell recordings of GABAergic mPSCs to assess synaptic \( E_{\text{GABA}} \), which may provide a more specific measure of synaptic chloride. We find that intracellular chloride is about 50 mM at GABAergic synaptic locations, which are likely to be dendritic. This is considerably higher than estimates in the same cells using perforated patch recordings while locally applying a GABA<sub>A</sub> agonist (−30 mM, unpublished observations). These findings are consistent with the possibility that motoneuron dendrites have higher chloride concentrations than the soma.

Measured \( E_{\text{GABA}} \) for mPSCs was more depolarized than would be predicted by the Nernst equation when chloride in the patch solution was <50 mM. This suggests that a mechanism exists to maintain synaptic chloride at about 50 mM when patch chloride is below this. It is likely that chloride transporters actively resist dialysis out in the dendrites. This is further supported by the observation that \( E_{\text{GABA}} \) moved toward values predicted by the patch solution when we blocked chloride transporters. On the other hand, when patch chloride is high, the transporters did not effectively extrude cellular chloride. This may be due to the observation that high intracellular chloride can inhibit transporter function (Lytle and Forbush 1996). Together, the findings suggested that these embryonic spinal neurons tightly regulate synaptic chloride. Similar findings have been reported previously, where whole cell recordings show that \( E_{\text{GABA}} \) is different in the soma and dendrites of relatively mature neurons and that chloride in the patch solution did not define dendritic chloride unless the KCC2 transporter was blocked (Jarolimek et al. 1996, 1999). Making \( I-V \) plots directly from the synaptic currents themselves could prove to be a useful technique in identifying mechanisms underlying changes in mPSC amplitude because it simultaneously provides measurements of both conductance and reversal potential.

**NKCC1 contributes to chloride accumulation in embryonic spinal motoneurons**

In the E10 chick embryo spinal cord the transporter that has been thought to be largely responsible for restoring high intracellular chloride in the interepisodic interval is the NKCC1 cotransporter (Chub and O’Donovan 2001; Chub et al. 2006). NKCC1 has been shown to be important in maintaining high intracellular chloride in mature DRG cells and in many different classes of developing neuron, leading to depolarizing GABAergic currents (Alvarez-Leefmans et al. 1988; Ben-Ari et al. 2007; Payne et al. 2003; Yamada et al. 2004). In the chick spinal cord GABA appears to switch from depolarizing to hyperpolarizing shortly after E15 (Xu et al. 2005). This switch, during early development in spinal motoneurons, is thought to be mediated by a reduction in NKCC1 function and a concurrent up-regulation of KCC2 (Delpy et al. 2008; Hübner et al. 2001; Jean-Xavier et al. 2006; Li et al. 2002; Stein et al. 2004).

We have several results that confirm and extend the above-cited findings. We have used muscle nerve responses to local application of GABA<sub>A</sub> agonists to monitor chloride accumulation. We can eliminate these nerve responses by bath application of low Cl solutions. This is consistent with the idea that chloride is accumulated by secondarily active transporters dependent on a chloride gradient, such as NKCC1 and AE3. Further, these chloride-dependent nerve potentials were inhibited by reducing NKCC1 function with bumetanide, or a “0 Na<sup>+</sup>” solution. We were also able to demonstrate that NKCC1 contributes to the chloride accumulation at GABAergic synaptic sites. First, we showed that currents from GABAergic interneuronal inputs to motoneurons were reduced following bumetanide bath application. Second, using GABAergic mPSC \( I-V \) plots we determined that \( E_{\text{GABA}} \) was hyperpolarized by about 8 mV (\( E_{\text{GABA}} \) reduced to −39 mM) following NKCC1 inhibition by either bumetanide or “low Na” extracellular solutions. These results confirm earlier reports of an NKCC1-dependent chloride accumulation in embryonic spinal motoneurons. Further, they extend previous findings by showing that other Na<sup>+</sup>-dependent transporters, like the Na<sup>+</sup>–Cl<sup>−</sup> transporter (NCC), are unlikely to contribute to chloride accumulation since low and 0 mM Na<sup>+</sup> solutions had effects similar to those of bumetanide (similar reductions in \( E_{\text{GABA}} \) and nerve potentials). In addition, the results show that NKCC1 is important in chloride accumulation at GABAergic synaptic sites, likely in the motoneuron dendrites.

**Anion exchanger also appears to contribute to chloride accumulation in embryonic chick motoneurons**

Although a contributor, NKCC1 did not appear to be solely responsible for chloride accumulation in embryonic chick motoneurons. This is also consistent with previous work in several different tissues (Delpy et al. 2008; Kakazu et al. 1999; Rocha-Gonzalez et al. 2008; Rohrbough and Spitzer 1996; Yamada et al. 2004). Blockade of chloride influx through NKCC1 (bumetanide, low/0 Na<sub>out</sub>) did not prevent chloride accumulation in any of the experiments we performed (GABA<sub>A</sub> agonist-induced nerve potentials, ventral root-evoked nerve response, \( I_{\text{mPSC}}-V \)). This suggests that another transporter exists that, like NKCC1, is dependent on the chloride gradient to drive chloride into the cell. As discussed earlier it is unlikely to be the Na<sup>+</sup>–Cl<sup>−</sup> transporter NCC, which is not thought to be expressed in the nervous system (Gamba et al. 1994). The potassium chloride cotransporter KCC2 typically extrudes chloride, but under some circumstances can accumulate chloride because KCC2 can be near its thermodynamic equilibrium in mature neurons (Jarolimek et al. 1999; Payne 1997). However, it is highly unlikely that KCC2 could load chloride in embryonic motoneurons where intracellular chloride is high (~50 mM) and therefore the potassium gradient will strongly favor chloride extrusion.

Alternatively, the anion exchanger AE3 has been suggested as one potential candidate for chloride accumulation (Balakrishnan et al. 2003; Rocha-Gonzalez et al. 2008). AE3 is expressed in the developing spinal cord at the stage that SNA is exhibited (Hentschke et al. 2006) and can accumulate chloride as it extrudes HCO<sub>3</sub><sup>−</sup>, in a Na<sup>+</sup>-independent manner. We
now show evidence supporting a role in chloride accumulation for AE3. The perfusion of 0 mM HCO₃⁻ solution did lead to a slight hyperpolarization of E_GABA and this developed progressively over time (Fig. 3), which might be expected as chloride slowly leaks out of the cell. The 0 mM HCO₃⁻ solution reduces intracellular HCO₃⁻, which is necessary for AE3 function, and therefore could potentially reduce accumulated chloride. Further, DIDS and 0 mM HCO₃⁻ solutions reduced isoguvacine-evoked nerve potentials (Fig. 8) and reduced the reaccumulation of chloride in the chloride-depletion experiments (Fig. 9). A very similar finding had been reported previously, where GABA_A currents, indirectly measured by calcium imaging, were weakened by bumetanide and a 0 mM HCO₃⁻ extracellular solution in embryonic motoneurons (Kulik et al. 2000). In addition, blocking AE3 function with DIDS produced a result similar to that of the 0 mM HCO₃⁻ extracellular solution. We cannot rule out the possibility that our methods of blocking the anion exchanger alter pH, which could then change some other aspect of chloride regulation. However, we do not favor this possibility because DIDS and 0 mM HCO₃⁻ both reduce chloride accumulation, but would likely influence pH differently.

NKCC1 and AE3 appear to have distinct functions in the recovery of chloride levels following chloride depletion in embryonic motoneurons. NKCC1 seems to be responsible for approximately two thirds of the steady-state chloride accumulation, whereas AE3 is responsible for the remaining third. Although both accumulators contribute to intracellular chloride, they appear to function differently at different stages of the reaccumulation following intracellular chloride depletion. AE3 may be important at early stages of the reaccumulation when intracellular chloride is relatively low. This can be observed in Fig. 5B, where in the first 10 min (phase 1), the recovery of the evoked potential does not change significantly when NKCC1 is blocked. On the other hand, NKCC1 appears to be important in the continued recovery of the evoked potentials past the first 10 min (phase 2, Fig. 5B). Together, our results suggest that chloride accumulation throughout the cell and at GABAergic synaptic sites occurs through NKCC1, and likely through the anion exchanger as well.

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