Cross Talk Between the GABA<sub>A</sub> Receptor and the Na-K-Cl Cotransporter Is Mediated by Intracellular Cl<sup>-</sup>

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INTRODUCTION

GABA and glycine are the main inhibitory neurotransmitters in the adult mammalian CNS. Activation of GABA receptors results in the influx of Cl<sup>-</sup> and hyperpolarization of the plasma membrane (Kaila 1994). However, during fetal and postnatal development, the equilibrium potential for Cl<sup>-</sup> is more positive than the resting potential and activation of GABA receptors results in an excitatory response and a decrease in [Cl<sup>-</sup>]. (Alvarez-Leefmans et al. 1988; Cherubini et al. 1991; Misgeld et al. 1986). The excitatory action of GABA in the immature CNS is important for the development of the nervous system (Ben Ari et al. 1997; LoTurco et al. 1995). The trophic actions of GABA are mediated by membrane depolarization and a rise in [Ca<sup>2+</sup>]. (Ben Ari et al. 1997; Yuste and Katz 1991). An increase in [Ca<sup>2+</sup>], following GABA-mediated depolarization is the result of Ca<sup>2+</sup> influx from voltage-dependent Ca<sup>2+</sup> channels and glutamate ionotropic N-methyl-D-aspartate (NMDA) receptors (Ben Ari et al. 1997; Fukuda et al. 1998).

The Na-K-Cl cotransporter isoform1 (NKCC1) functions as an active Cl<sup>-</sup> transport system and contributes to the active accumulation of intracellular Cl<sup>-</sup> in immature neurons (Alvarez-Leefmans 2001; Alvarez-Leefmans et al. 1988; Sung et al. 2000). To date, only two distinct isoforms of the cotransporter, NKCC1 and NKCC2, have been identified. NKCC1 has a broad tissue distribution, while the NKCC2 isoform is only found in vertebrate kidney (Russell 2000). An emerging hypothesis asserts that the balance between the inwardly directed NKCC1 and the outwardly directed neuronal K-Cl cotransporter (KCC2) may be important in the determination of GABA<sub>A</sub> receptor-mediated depolarizing responses. This view is supported by the findings that expression of the inwardly directed NKCC1 in neurons is preceded by expression of the outwardly directed KCC2 (Clayton et al. 1998; Lu et al. 1999; Rivera et al. 1999; Sun and Murali 1999). Additionally, genetic ablation of NKCC1 leads to a decrease in [Cl<sup>-</sup>]<sub>i</sub> and abolishes the GABA-mediated depolarization in mouse dorsal root ganglion cells (Sung et al. 2000). Disruption of KCC2 resulted in frequent seizures, abolishment of respiration, and early lethality in mice (Hubner et al. 2001).

However, regulation of NKCC1 in immature neurons in response to GABA has not been studied extensively. NKCC1 activity in immature cortical neurons is significantly stimulated by activation of GABA<sub>A</sub> receptors (Sun and Murali 1999). This GABA-mediated stimulation of NKCC1 could be an important positive feedback mechanism to maintain intracellular Cl<sup>-</sup> level and GABA function in immature neurons.

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mediated Cl\(^-\) efflux. Second, NKCC1 stimulation can be via indirect signal transduction messengers that are associated with a loss of intracellular Cl\(^-\), such as cell shrinkage or membrane depolarization and a rise of [Ca\(^{2+}\)]\(i\) (Ben Ari et al. 1997; Russell 2000). In turn, cell shrinkage and an increase in [Ca\(^{2+}\)]\(i\), can stimulate NKCC1 activity (Russell 2000; Schomberg et al. 2001). In this report, we demonstrate that stimulation of NKCC1 following activation of the GABA\(_A\) receptor channel is mediated by the loss of intracellular Cl\(^-\) content but not by cell shrinkage or an increase of [Ca\(^{2+}\)]\(i\).

**Methods**

**Primary culture of rat cortical neurons**

Dissociated cortical neuron cultures were prepared using the established method described in our previous studies (Schomberg et al. 2001). Fetuses were removed at embryonic day 17 from pregnant rats (Sprague–Dawley). The cortices were rapidly dissected in HBSS and minced with scissors. Cortical tissue was incubated in a trypsin solution for 25 min at 37°C (Schomberg et al. 2001). The tissue was then rinsed with HBSS and resuspended in Eagle’s modified essential medium (EMEM) supplemented with 10% fetal bovine serum and 10% horse serum. Experiments were routinely performed on 11-day-old astroglial cells. Seventy-two hours later, cultures were refed with medium (EMEM) supplemented with 10% fetal bovine serum and 10% horse serum. Experiments were routinely performed on 11-day-old cultures in vitro (DIV), unless otherwise indicated.

**K\(^+\) influx determination**

NKCC1 activity was measured as bumetanide-sensitive K\(^+\) influx, using \(^{86}\)Rb as a tracer for K\(^+\) (Sun and Murali 1999). Briefly, cultured neurons were equilibrated for 10 min at 37°C with an isotonic HEPES-buffered minimal essential medium (MEM, 312 mOsm). The concentrations of components in HEPES–MEM (mM) were described previously (Schomberg et al. 2001). Cells were then equilibrated for 5 min in HEPES–MEM containing either 0 or 10 μM bumetanide. For assay of NKCC1 activity, cells were exposed to 1 μCi/ml of \(^{86}\)Rb in HEPES–MEM for 3 min, either in the presence or absence of 10 μM bumetanide. Radioactivity of cells extracted in 1% SDS was analyzed by liquid scintillation and \(^{86}\)Rb influx rate was calculated as the slope of \(^{86}\)Rb uptake over time and expressed as nanomol \(^{86}\)Rb per mg of protein per min. \(^{86}\)Rb influx is linear over 7 min in neurons (Schomberg et al. 2001). Quadruplicate determinations were obtained in each experiment throughout the study and protein content was measured in each sample using a method described by Smith et al. (Smith et al. 1985). Statistical significance in the study was determined by analysis of variance (ANOVA; Bonferroni/Dunn) at a confidence level of 95% (P < 0.05).

**Intracellular Cl\(^-\) content measurement**

Cells on 24-well plates were preincubated at 37°C for 0–60 min in HEPES–MEM containing 5.8 mM [K\(^+\)]\(o\), and \(^{36}\)Cl (0.4 μCi/ml), as described previously (Su et al. 2002b). Cells were then incubated in HEPES–MEM containing \(^{36}\)Cl (0.4 μCi/ml) either in the presence or absence of 10 μM bumetanide for 10 min. To specifically activate \(^{36}\)Cl in HEPES–MEM was constant under all conditions. Intracellular \(^{36}\)Cl content measurement was terminated by three washes with 1 ml ice-cold washing buffer containing (in mM) 118 NaCl, 26 NaHCO\(_3\), and 1.8 CaCl\(_2\), pH 7.40. Radioactivity of the cellular extract in 1% SDS was analyzed by liquid scintillation counting (Packard 1900CA, Downers Grove, IL). In each experiment, specific activities (counts/μmol/min) of \(^{36}\)Cl were determined for each assay condition and used to calculate intracellular Cl\(^-\) content (μmol/mg protein).

**Measurement of relative cell volume changes in a single cell**

Relative cell volume changes were estimated using video-enhanced differential interference contrast (DIC) microscopy, as described in our previous study (Su et al. 2002b). Neurons cultured on poly-L-lysine-coated coverslips were placed in an open-bath imaging chamber (Warner Instruments, Hamen, CT; bath volume 40 μl) on the stage of a Nikon TE 300 inverted epifluorescence microscope. Cells were equilibrated with an isotonic HEPES–MEM (312 mOsm) for 15 min at room temperature (Su et al. 2002a,b). Cells were exposed sequentially to isotonic HEPES–MEM (5 min), HEPES–MEM plus 30 μM muscimol (10 min), and HEPES–MEM (10 min). Cells were visualized using a Nikon 60X Plan Apo oil immersion objective lens and cell images recorded every min as described previously (Su et al. 2002b). The mean cross-sectional area (CSA) was calculated after tracing the perimeter of the cell body with MetaMorph image-processing software (Universal Imaging Corp., Downingtown, PA).

This approach to cell volume measurement can be criticized because it assumes that the soma swells and shrinks in a symmetrical manner, as if it were a sphere. This assumption has been validated in a study by Churchwell et al. (1996). Neuronal cell volume changes determined directly using optical sectioning techniques are consistent with calculated values based on measurements of CSA during hypotonic shrinkage or hypotonic swelling (Churchwell et al. 1996).

The control CSA values were obtained when cells were exposed to HEPES–MEM only. Relative changes of mean cross-sectional area (CSA) were calculated as experimental CSA/control CSA. Following each experiment, relative cell volume changes in response to HEPES–MEM buffers (238, 277, and 312 mOsm) were measured. A hypotonic buffer (238 mOsm) was prepared by reducing the NaCl concentration in the HEPES–MEM buffer to 100 mM. Buffers of 277 and 312 mOsm were prepared by holding the salt concentrations constant and adding 40 and 70 mM sucrose, respectively. Percent regulation for regulatory volume increase (RVI) was calculated as (CSA\(_{\text{min}}\) - CSA\(_{\text{max}}\))/CSA\(_{\text{max}}\) × 100, where CSA\(_{\text{min}}\) is the maximum change of CSA in response to hypertonic stress and CSA\(_{\text{max}}\) is the average CSA in the last 5 min of hypertonic stress.

**Measurement of intracellular Ca\(^{2+}\)**

Cultured neurons grown on poly-L-lysine-coated coverslips were loaded in HEPES–MEM containing 10 μM fura-2 acetoxyethyl ester (AM) and 0.1% pluronic acid at 37°C for 1.5 h. The coverslips were placed in an open-bath imaging chamber containing HEPES–MEM at ambient temperature. Using a Nikon TE 300 inverted epifluorescence microscope and a 40× Super Fluor oil immersion objective lens, neurons were excited every 10 s at 340 and 380 nm and the emission fluorescence at 510 nm was recorded. Images were collected and analyzed with the MetaFluor image-processing software. Mn\(^{2+}\) (1 mM) was used at the end of each experiment to quench the cytosolic Ca\(^{2+}\)-sensitive fluorescence, as described in our previous study (Su et al. 2000). The fluorescence intensity with 1 mM Mn\(^{2+}\) was subtracted from the value measured in the absence of Mn\(^{2+}\). The 340/380 ratio of the subtracted values was then calculated (Grynkwicz et al. 1985; Su et al. 2000).

To monitor changes of [Ca\(^{2+}\)]\(i\), the fura-2-loaded cells were equilibrated with HEPES–MEM for 10 min. The 340/380 ratios were recorded and the bath chamber buffer was changed with either 75 mM [K\(^{+}\)], HEPES–MEM (5 min) or 100 μM muscimol in HEPES–MEM (5 min), followed by HEPES–MEM alone (10 min).
Immunoprecipitation, gel electrophoresis, and immunoblotting

Cortical neurons grown on culture dishes were preincubated with isotonic HEPES–MEM at 37°C for 10 min. Cells were then either incubated with isotonic HEPES–MEM, 10 mM [$\text{Cl}^-$], or 30 [$\mu$M] muscimol for 10 min at 37°C. The incubation was stopped by adding ice-cold PBS (pH 7.4). The PBS buffer contained phosphatase inhibitors (100 mM NaF, 10 mM Na$_2$P$_2$O$_7$, 2 mM NaVO$_3$, and 0.2 [$\mu$M] microcystin) and protease inhibitors as described previously (Sun and Murali 1999). Cells were lysed and centrifuged at 320g for 5 min. The supernatant fraction was collected and centrifuged at 45,000 rpm (Ti 40 rotor, Beckman, Fullerton, CA) at 4°C for 30 min. The membrane pellet was resuspended in 0.25 ml PBS–1% SDS for 30 min at room temperature and protein content of the membrane suspension was determined (Smith et al. 1985). One milligram of membrane protein from each sample was incubated in 0.875 ml of PBS–2% CHAPS at 4°C for 30 min. Samples were centrifuged at 320g for 5 min and the supernatant was collected. Fifteen microliters of a monoclonal antibody against the human colonic T84 epithelial Na–K–Cl cotransporter (T4 ascites, Departmental Studies Hybridoma Bank, Iowa City, IA) (Lytle et al. 1995) was added and samples were rotated on a shaker overnight at 4°C. For immunoprecipitation, 40 [$\mu$M] T4 ascites was added and samples were rotated on a shaker at room temperature and then incubated overnight with a primary polyclonal antibody (R5) against a dipshophopeptidate containing T$^{125}$I and T$^{129}$I of NKCC1 (NKCC-p; 1–4000) (Fliemmer et al. 2002). The blots were rinsed with PBS and incubated with horseradish peroxidase-conjugated secondary IgG for 1 h. Bound secondary antibody was visualized using the enhanced chemiluminescence assay (ECL, Amersham Pharmacia Biotech, Piscataway, NJ).

To confirm each sample contained a similar amount of nonphosphorylated NKCC1 prior to immunoprecipitation, 15 [$\mu$g] of membrane protein from each sample was used directly for immunoblotting by T4 antibody.

Materials

Bumetanide and cytosine-1-[$\beta$-arabinofuranoside were purchased from Sigma (St. Louis, MO). Eagle’s MEM and HBSS were from Mediatech Cellgro (Herndon, VA). GABA$_A$ receptor agonist muscimol and selective GABA$_A$ receptor antagonist bicuculline were purchased from Research Biochemicals International (Natick, MA). Fetal bovine and horse sera were obtained from Hyclone Laboratories (Logan, UT). $^{86}$RbCl was purchased from NEN Life Science Products (Boston, MA). Chloride-36 was purchased from Amersham Pharmacia Biotech.

RESULTS

Activation of GABA$_A$ receptors causes a loss of intracellular Cl$^-$. In immature cortical neurons

We hypothesized that activation of the GABA$_A$ receptor decreases intracellular Cl$^-$ and thus stimulates NKCC1 activity to bring the Cl$^-$ levels back to the equilibrium. First, we investigated whether we could detect a GABA-mediated loss of intracellular Cl$^-$ in immature neurons. As shown in Fig. 1A, inset, cells were preequilibrated in HEPES–MEM with $^{36}$Cl (0.4 [$\mu$Ci/ml] for 0–60 min. A steady-state level of intracellular $^{36}$Cl$^-$ was obtained by a 10-min incubation and maintained during the 60-min equilibration. Thus, in the rest of the study, a 30-min preincubation was performed. After a 30-min equilibration with $^{36}$Cl (0.4 [$\mu$Ci/ml)], changes of intracellular Cl$^-$ content were measured when cells were exposed to HEPES–MEM ($^{36}$Cl, 0.4 [$\mu$Ci/ml]) either with or without 30 [$\mu$M] muscimol for 10 min. Intracellular $^{36}$Cl$^-$ content was 0.70 ± 0.04 [$\mu$mol/mg protein in control conditions. In the presence of muscimol, a GABA$_A$ receptor agonist, intracellular $^{36}$Cl$^-$ content was reduced to 0.53 ± 0.03 [$\mu$mol/mg protein ($P < 0.05$, Fig. 1A). Exposing cells to 10 mM Cl$^-$, resulted in a further loss of intracellular $^{36}$Cl to 0.33 ± 0.03 [$\mu$mol/mg protein ($P < 0.05$, Fig. 1A).

We used bicuculline, a GABA$_A$ receptor antagonist, to determine whether the muscimol-mediated effect is indeed via the GABA$_A$ receptor. As shown in Fig. 1B, the muscimol-mediated loss of intracellular $^{36}$Cl content was eradicated by bicuculline (0.70 ± 0.02 [$\mu$mol/mg protein) compared with a basal level of 0.70 ± 0.07 [$\mu$mol/mg protein ($P > 0.05$). In another control study, the NKCC inhibitor bumetanide had no effect on the muscimol-mediated loss of intracellular $^{36}$Cl level ($P > 0.05$, Fig. 1B). To investigate whether blocking Cl$^-$ entry via NKCC1 would reduce the basal level of intracellular Cl$^-$. we measured intracellular $^{36}$Cl content in the presence of 10 [$\mu$M] bumetanide. Intracellular $^{36}$Cl content was decreased to 0.59 ± 0.04 [$\mu$mol/mg protein (Fig. 1B). However, this change was not statistically significant from the control value (0.70 ± 0.07 [$\mu$mol/mg protein, $P > 0.05$). On the other hand, depolarizing neurons under high [K$^+$], conditions resulted in an increase of intracellular Cl$^-$. Such an increase has been reported in neurons and astrocytes (Su et al. 2002b; White et al. 1992). Under high [K$^+$], intracellular $^{36}$Cl content increased to 0.93 ± 0.08 [$\mu$mol/mg protein (Fig. 1B, P < 0.05). The increase in intracellular $^{36}$Cl was blocked with 10 [$\mu$M] bumetanide ($P < 0.05$). The data suggest that NKCC1 is important for Cl$^-$ influx as [K$^+$]$_o$ is elevated. A similar finding has been observed in astrocytes (Su et al. 2002a,b). A stimulation of NKCC1 under high [K$^+$]$_o$ can be a result of a kinetic effect of extracellular K$^+$ and Ca$^{2+}$-dependent second messenger-mediated regulatory mechanisms (Schomberg et al. 2001; Su et al. 2000). In addition, an increase in intracellular Cl$^-$ level under high [K$^+$]$_o$ has been reported to be mediated by a reversal of KCC2 (DeFazio et al. 2000; Jarolimek et al. 1999; Kakazu et al. 2000; Yamada et al. 2001).

NKCC1 activity is stimulated following cell shrinkage

Activation of the GABA$_A$ receptor leads to loss of intracellular Cl$^-$, which could result in cell shrinkage and subsequent increase in NKCC1 activity. First, we established that NKCC1 activity in neurons was stimulated by cell shrinkage. We exposed the cells to nonisotonic buffers and monitored changes of bumetanide-sensitive $^{86}$Rb uptake as a measure for NKCC1 activity. As shown in Fig. 2, NKCC1 activity in isotonic HEPES–MEM was 15.6 ± 1.7 nmol/mg protein/min. When cells were exposed to a hypertonic HEPES–MEM (238 mOsm), NKCC1 activity was reduced to 9.7 ± 1.4 nmol/mg protein/min ($P < 0.05$). In contrast, hypertonic shrinkage (HEPES–MEM, 369 mOsm) stimulated NKCC1 activity to 28.5 ± 5.4

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mM muscimol, 30 mM muscimol for 10 min. In the positive control experiments, cells were exposed to 75 mM [K+]o containing 36Cl (0.4 μCi/ml) either in the presence or absence of 10 μM bumetanide. Data are plotted as means ± SE, n = 3–4 from 2 different cultures. *P < 0.001 vs. isotonic control (Mann–Whitney rank sum test).

**Activation of GABA<sub>A</sub> receptors does not cause cell shrinkage**

We determined whether activation of the GABA<sub>A</sub> receptor causes neuron shrinkage. As a positive control, we examined whether isosmotic cell shrinkage can be detected under conditions producing a loss of intracellular Cl<sup>-</sup> (10 mM [Cl<sup>-</sup>]<sub>i</sub>). Fig. 4, A and B, illustrates that 10 mM [Cl<sup>-</sup>]<sub>i</sub> caused immature neurons to shrink gradually over 10 min (0.93 ± 0.01; P < 0.05). This shrinkage is reversible and CSAr recovered to the basal level in isotonic HEPES–MEM. The same neurons pretreated with 10 μM bumetanide for 20 min and then exposed to hypertonic HEPES–MEM plus 10 μM bumetanide, RVI was greatly attenuated (Fig. 3A). After 20 min, only 7.31 ± 2.4% of RVI was observed when NKCC1 was inhibited (Fig. 3B). This indicates that NKCC1 activity is essential for RVI in immature cortical neurons.

**FIG. 1.** Muscimol-induced intracellular Cl<sup>-</sup> loss in immature neurons. Inset: Neurons were preincubated in normal HEPES–MEM at 37°C for 10 min and then equilibrated in HEPES–MEM with 36Cl (0.4 μCi/ml) for 0–60 min. A: after a 30 min-equilibration with 36Cl (0.4 μCi/ml), neurons were treated with HEPES–MEM containing 36Cl (0.4 μCi/ml) either in the presence or absence of 30 μM muscimol for 10 min. In the positive control experiment, cells were exposed to 10 mM [Cl<sup>-</sup>]<sub>i</sub>, HEPES–MEM plus 36Cl (0.4 μCi/ml) for 10 min. Data are means ± SE; n = 5–6. *P < 0.05 vs. control; #P < 0.05 vs. muscimol-treated (Bonferroni/Dunn). B: neurons were treated with HEPES–MEM containing 36Cl (0.4 μCi/ml) in the presence of 10 μM bumetanide, 30 μM muscimol, 30 μM muscimol +10 μM bicuculline, or 30 μM muscimol +10 μM bumetanide for 10 min. In the positive control experiments, cells were exposed to 75 mM [K+]o containing 36Cl (0.4 μCi/ml) either in the presence or absence of 10 μM bumetanide for 10 min. n = 4–14. *P < 0.05 vs. control; #P < 0.05 vs. 75 mM [K+]o (Bonferroni/Dunn).

**FIG. 2.** GABA<sub>A</sub> receptor-mediated stimulation of NKCC1 activity. Neurons were preincubated in either hypotonic HEPES–MEM (238 mOsm) or hypertonic HEPES–MEM (369 mOsm) for 10 min. 86Rb influx was assayed for 3 min in the same HEPES–MEM buffers containing 1 μCi 86Rb. Data are means ± SE; n = 4–8. *P < 0.05 vs. isotonic HEPES–MEM (Bonferroni/Dunn).

**FIG. 3.** NKCC1 is required for RVI in immature neurons. A: changes in CSAr from a single representative neuron during exposure to isotonic HEPES–MEM (5 min), hypertonic HEPES–MEM (369 mOsm, 20 min); and isotonic HEPES–MEM (15 min). Bumetanide treatment study: the protocol was identical to the control study except 10 μM bumetanide was present in the entire study. B: percentage regulation was calculated in single cells from either the control or bumetanide study. Data are plotted as means ± SE, n = 3–4 from 2 different cultures. *P < 0.001 vs. isotonic control (Mann–Whitney rank sum test).
In the presence of 10 mM \([\text{Cl}^-]_0\) and bumetanide, the average values of cell shrinkage did not significantly differ from those in 10 mM \([\text{Cl}^-]_0\) \((P > 0.05)\). Cell volume recovered completely when cells were returned to isotonic HEPES–MEM. In contrast, in the presence of 10 \(\mu\)M bumetanide, cells failed to restore their volume after 15 min incubation. These results suggest that the NKCC1 is essential for RVI after isosmotic cell shrinkage following 10 mM \([\text{Cl}^-]_0\) treatment. In addition, the results imply that no detectable cell shrinkage occurred in immature neurons following activation of the GABA_A receptor.

To further establish the relationship between cell volume and \([\text{Cl}^-]_0\), we determined changes in CSAr of cultured neurons after 10 min of exposure to 50, 40, 30, 20, or 10 mM \([\text{Cl}^-]_0\). As shown in Fig. 5A, cell volume decreased linearly in response to \([\text{Cl}^-]_0\) between 50 and 20 mM \((r = 0.99)\). There was no further decrease in CSAr with 10 mM \([\text{Cl}^-]_0\).

Musculin-induced stimulation of NKCC1 is independent of \([\text{Ca}^{2+}]_0\). Depolarization of immature neurons by GABA leads to a rise of \([\text{Ca}^{2+}]_0\) via voltage-dependent \(\text{Ca}^{2+}\) channels (Ben Ari et al. 1997). In our recent studies, an increase in \([\text{Ca}^{2+}]_0\) by activation of NMDA or AMPA receptors stimulated NKCC1 activity in neurons (Schomberg et al. 2001; Sun and Murali et al. 1997). In our recent studies, an increase in \([\text{Ca}^{2+}]_0\) by activation of NMDA or AMPA receptors stimulated NKCC1 activity in neurons (Schomberg et al. 2001; Sun and Murali et al. 1997).
These observations further imply that elevation of Ca\textsuperscript{2+} mediated stimulation of NKCC1 activity (Fig. 7). The changes of 340/380 ratio in response to muscimol were smaller in some other experiments. The average change of 340/380 ratio induced by 100 μM muscimol was 0.062 ± 0.006 (n = 3). Five-minute exposure of cells to 75 mM [K\textsuperscript{+}]_o led to a larger increase of the 340/380 ratio (0.16 ± 0.013; n = 3).

We then tested whether muscimol-mediated stimulation of NKCC1 activity is sensitive to Ca\textsuperscript{2+}. As shown in Fig. 7, removal of extracellular Ca\textsuperscript{2+} did not block the muscimol-induced stimulation of NKCC1 activity. Moreover, chelating intracellular Ca\textsuperscript{2+} with 10 μM BAPTA-AM did not abolish the muscimol-mediated stimulation of NKCC1 activity (Fig. 7). These observations further imply that elevation of [Ca\textsuperscript{2+}], is not a primary signal to stimulate NKCC1 activity by muscimol.

**Muscimol-mediated increase in expression of phosphorylated NKCC1 in neurons**

A loss of intracellular Cl\textsuperscript{−} stimulates NKCC1 activity by protein phosphorylation (Darman and Forbush 2002; Lytle 1997). We investigated here whether a change of phosphorylation level of NKCC1 could be detected when the intracellular Cl\textsuperscript{−} content was altered by either low [Cl\textsuperscript{−}]_o or muscimol. The R5 polyclonal antibody against a diphosphopeptide containing T\textsuperscript{184} and T\textsuperscript{189} of NKCC1 (Flemmer et al., 2002) was used to probe the phosphorylation state of NKCC1. T\textsuperscript{184}, T\textsuperscript{189}, and T\textsuperscript{202} of the N-terminus represent a conserved phospho-regulatory domain of NKCC1 (Darman and Forbush 2002).

Cultured cortical neurons were treated for 10 min at 37°C either with isotonic HEPES–MEM, HEPES–MEM containing 10 mM [Cl\textsuperscript{−}]_o, or HEPES–MEM containing 30 μM muscimol. Figure 8, A and B, shows that a basal level of phosphorylation signal of NKCC1 was detected under control conditions. Exposing cells to muscimol or 10 mM [Cl\textsuperscript{−}]_o for 10 min triggered an increase in NKCC1-p signal by 19.0 ± 8.2 and 19.5 ± 6.5%, respectively (n = 3). The similar levels of nonphosphorylated NKCC1 in cells were observed under all three conditions (Fig. 8C). The results further suggest that the stimulation of NKCC1 activity under muscimol or 10 mM [Cl\textsuperscript{−}]_o conditions is the result of the phosphorylation of NKCC1.

**FIG. 8.** Changes of the phosphorylated NKCC1 protein expression in neurons. Cells were incubated in isotonic HEPES–MEM, 10 mM [Cl\textsuperscript{−}]_o, HEPES–MEM, or isotonic HEPES–MEM plus 30 μM muscimol for 10 min at 37°C. A 1.0 mg membrane protein from each sample was used for immunoprecipitation with T4 antibody. Following immunoprecipitation, samples were separated by 6% SDS–PAGE, transferred to a nitrocellulose membrane, and probed with the anti-phosphorylated NKCC1 antibody (see METHODS) (A and B). Fifteen micrograms of membrane protein from each sample was used directly for immunoblotting with T4 antibody (C), C, control; [Cl\textsuperscript{−}]_o, 10 mM [Cl\textsuperscript{−}]_o; MUS, 30 μM muscimol. The immunoblots were visualized by the enhanced chemiluminescence. This is a representative blot from 1 of 3 experiments.
DISCUSSION

A decrease in intracellular Cl− results in stimulation of NKCC1 activity in immature neurons

In this study, significant stimulation of NKCC1 activity followed the efflux of intracellular Cl− mediated by the GABAₐ receptor. No significant cell shrinkage was detected when GABAₐ receptors were activated. In addition, neither blocking of Ca²⁺ influx nor chelating of intracellular free Ca²⁺ abolished the muscimol-mediated stimulation of NKCC1 activity. Thus we believe that a loss of intracellular Cl−, but not a subsequent increase of Ca²⁺ or cell shrinkage, leads to NKCC1 stimulation. This stimulation is likely mediated by a direct up-regulation of NKCC1 phosphorylation because phosphorylation of NKCC1 was increased in neurons following the activation of the GABAₐ receptor. Moreover, under 10 mM [Cl−]₀, both a loss of intracellular Cl− and cell shrinkage were detected in immature neurons. A significant increase in phosphorylation of NKCC1 was observed in 10 mM [Cl−]₀ conditions. It is known that NKCC1 activity is controlled by direct phosphorylation during cell shrinkage and low intracellular Cl− (Lytte 1997; Lytte and Forbush 1992; Russell 2000). Thus the increase in phosphorylation of NKCC1 detected in 10 mM [Cl−]₀ is likely a result of effects of cell shrinkage and low [Cl−]₀.

NKCC1 activity is regulated by intracellular Cl− content in many cell types (Russell 2000). Decrease of intracellular Cl− concentration by removal of extracellular Cl− significantly stimulates NKCC1 activity in the squid giant axon (Russell 2000). In epithelium of the shark rectal gland, activation of NKCC1 during secretion is a result of CAMP-induced Cl− loss through apical chloride channels (Lytte and Forbush 1992). Phosphorylation of NKCC1 at threonine 189 (Thr 189) is directly associated with a reduction of intracellular Cl− in the shark rectal gland (Darman and Forbush 2002; Lytte and Forbush 1992). However, a kinase(s) that mediates phosphorylation of NKCC1 is still unknown. Dephosphorylation appears to be through protein phosphatase 1 (PP1c, Darman et al. 2001). Taken together, our results suggest that loss of intracellular Cl− under low [Cl−]₀ or opening of GABAₐ receptors leads to stimulation of NKCC1 activity via phosphorylation.

Significance of the GABA-mediated stimulation of NKCC1 in immature neurons

NKCC1 plays a role in maintaining intracellular Cl− in neurons, astrocytes, and oligodendrocytes of the CNS (Alvarez-Leefmans 2001; Kettenmann et al. 1991). Inhibition of NKCC1 pharmacologically or genetic ablation of NKCC1 significantly reduces intracellular Cl− concentration in neurons under control conditions and astrocytes under high [K+]₀ (Su et al. 2002a,b; Sung et al. 2000). In contrast, stimulation of NKCC1 activity in astrocytes by high extracellular potassium or elevation of NKCC1 activity in HEK 293 cells by destruction of dephosphorylation increases intracellular Cl− level (Darman et al. 2001; Su et al. 2002b). GABA-mediated depolarization in immature neurons depends on a relatively high [Cl−]₀, while intracellular Cl− level decreases following activation of GABAₐ receptor. Thus, to maintain excitatory action of GABA in immature neurons, these cells must have regulatory ion transport mechanisms that can sense a decrease in [Cl−]₀ and concurrently be able to couple to activation of Cl− entry pathways to replenish [Cl−]₀. NKCC1 is a perfect candidate for such a regulatory system, with dual functions as a sensor as well as an effector. Therefore stimulation of NKCC1 activity by GABA may serve as an effective positive feedback mechanism for immature neurons to maintain a relatively high [Cl−]₀. Such a cross-talk between Cl− channels and NKCC1 has been observed in epithelium and functions in salt reabsorption and secretion (Russell 2000).

In summary, we demonstrate here that activation of GABAₐ receptors causes Cl− efflux and reduction of intracellular Cl− in immature cortical neurons. Loss of intracellular Cl− mediated by the GABAₐ receptor stimulates NKCC1 activity. Although cell shrinkage and membrane depolarization generally accompanies the Cl− efflux, it appears that a loss of intracellular Cl− is a primary messenger for the GABA-mediated NKCC1 stimulation in immature neurons. Taken together, NKCC1 is important in regulation of intracellular Cl− in immature cortical neurons. Activation of NKCC1 by GABA may serve as a positive feedback pathway to maintain a higher intracellular Cl− level in immature neurons and thereby enable GABA to mediate membrane depolarization during neuron development.

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REFERENCES


Su G, Haworth RA, Dempsey RJ, and Sun D. Regulation of Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) cotransporter in primary astrocytes by dibutyryl cAMP and high [K\(^{+}\)]. *J Neurosci* 17: C1710–C1721, 2000.


