Early Changes in KCC2 Phosphorylation in Response to Neuronal Stress Result in Functional Downregulation

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The K⁺ Cl⁻ cotransporter KCC2 plays an important role in chloride homeostasis and in neuronal responses mediated by ionotropic GABA and glycine receptors. The expression levels of KCC2 in neurons determine whether neurotransmitter responses are inhibitory or excitatory. KCC2 expression is decreased in developing neurons, as well as in response to various models of neuronal injury and epilepsy. We investigated whether there is also direct modulation of KCC2 activity by changes in phosphorylation during such neuronal stressors. We examined tyrosine phosphorylation of KCC2 in rat hippocampal neurons under different conditions of in vitro neuronal stress and the functional consequences of changes in tyrosine phosphorylation. Oxidative stress (H₂O₂) and the induction of seizure activity (BDNF) and hyperexcitability (0 Mg²⁺) resulted in a rapid dephosphorylation of KCC2 that preceded the decreases in KCC2 protein or mRNA expression. Dephosphorylation of KCC2 is correlated with a reduction of transport activity and a decrease in [Cl⁻], as well as a reduction in KCC2 surface expression. Manipulation of KCC2 tyrosine phosphorylation resulted in altered neuronal viability in response to in vitro oxidative stress. During continued neuronal stress, a second phase of functional KCC2 downregulation occurs that corresponds to decreases in KCC2 protein expression levels. We propose that neuronal stress induces a rapid loss of tyrosine phosphorylation of KCC2 that results in translocation of the protein and functional loss of transport activity. Additional understanding of the mechanisms involved may provide means for manipulating the extent of irreversible injury resulting from different neuronal stressors.

Key words: KCC2; neurons; E_Cl⁻; Cl⁻ homeostasis; oxidative stress; cell death

Introduction

The K⁺ Cl⁻ cotransporter, KCC2, plays an important role in neuronal Cl⁻ homeostasis and in determining the physiological response to the activation of anion-selective GABA and glycine receptors (for review, see Kaila, 1994; Delpire, 2000; Payne et al., 2003). Increases in the expression level of KCC2 underlie the developmental change in GABA and glycine responses from depolarization to hyperpolarization, and different neuronal expression levels correlate with differences in GABA responses and the ability to maintain Cl⁻ homeostasis during depolarization (Kakazu et al., 1999; Rivera et al., 1999; Ueno et al., 2002; Zhu et al., 2005). Conversely, KCC2 expression levels are reduced in various pathological conditions, including axotomy and nerve crush, nerve cuff-induced chronic pain, and interictal activity, with a resultant increased [Cl⁻], and a shift of GABA-mediated responses from hyperpolarizing to depolarizing (Nabekura et al., 2002; Rivera et al., 2002, 2004; Coull et al., 2003; Toyoda et al., 2003). The changes in neuronal Cl⁻ homeostasis in these pathological conditions resemble a temporary return to an immature phenotype, speculated to be important for adaptations required to reestablish appropriate neuronal connections and functions (Payne et al., 2003; Toyoda et al., 2003).

Although protein and mRNA expression levels in tissue are clearly reduced under these pathological conditions, it is not clear whether KCC2 activity is also subject to modulation via direct phosphorylation/dephosphorylation during neuronal stress (Payne et al., 2003). In peripheral tissues, different kinases and phosphatases can modify swelling or vasodilator-induced cation-chloride transport; however, the specific pathways involved depend somewhat on cellular origin and experimental conditions (for review, see Adragna et al., 2004), and there is an array of different transporters expressed in these tissues (Payne et al., 2003). In mature hippocampal neurons, activation of receptor tyrosine kinases, in response to either neurotrophins or epileptic activity, rapidly decreases KCC2 mRNA levels and surface expression producing a corresponding depolarizing shift in the

DOI:10.1523/JNEUROSCI.3104-06.2007

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Received Feb. 27, 2006; revised Jan. 4, 2007; accepted Jan. 5, 2007.

This work was supported by Grants-in-Aid for Scientific Research (5023783) from the Ministry of Education, Culture, Sports, Sciences, and Technology, Japan and by the Japan Science and Technology Agency (J.N.).

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GABA response (Rivera et al., 2004). The rapid turnover rate of KCC2 (τ ~ 20 min) suggests that changes in surface expression may be a dominant way in which KCC2 activity is regulated (Rivera et al., 2004). In immature hippocampal neurons, however, KCC2 can be present in cells without functional KCC2 Cl⁻ transport. Moreover, it may be activated by a broad spectrum kinase inhibitor, staurosporine, or by activation of tyrosine kinases (Kelsch et al., 2001; Khirug et al., 2005). Furthermore, recent studies have demonstrated that postsynaptic spiking activity was capable of causing a rapid and sustained downregulation of KCC2 activity via a Ca²⁺ and protein kinase C-dependent mechanism (Fiumelli et al., 2005). Hence it would appear that KCC2 activity can be regulated by different cellular kinases and phosphatases signaling cascades, independent of changes in cellular mRNA or protein levels, although it is unclear whether the phosphorylation state of KCC2 itself is modified.

In the present study, we have therefore investigated the hypothesis that neuronal stress can modify KCC2 tyrosine phosphorylation state and functional activity, independent of changes in expression levels.

Materials and Methods

All relevant experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the Japanese Physiological Society.

Hippocampal cultures. Embryos were removed from pregnant Wistar rats at embryonic day 19 (E19) under ether anesthesia, and their hippocampi were dissected out and incubated in Earle’s balanced salt solution containing papain (16 U/ml; Funakoshi, Tokyo, Japan) for 20 min at 32°C. Single cells were subsequently isolated by triturating with a 5 ml plastic pipette and plated onto either 0.2% polyethyleneimine-coated glass coverslips (for electrophysiological and immunocytochemistry) or into 2 ml culture flasks (for biochemical experiments and cell viability assay), also coated with polyethyleneimine, and maintained in Neurobasal medium containing 0.5 mM L-glutamine and B27 supplement (Invitrogen), also coated with polyethyleneimine, and maintained in Neurobasal medium containing 0.5 mM L-glutamine and B27 supplement (Invitrogen, Tokyo, Japan) at 37°C and 5% CO₂. Cell cultures were fed once a week by replacing one-half of the above medium with fresh medium and used for experiments between 4 and 25 days in vitro (DIV) as indicated.

Electrical measurements. Electrical measurements were performed using the gramicidin-perforated patch-clamp recording technique (Kakazu et al., 2000; Ueno et al., 2002). The resistance between the patch pipette filled with the internal solution and the reference electrode in the normal external solution was 3–5 MΩ. Ionic currents were measured with a patch-clamp amplifier (EPC-7; List Biologic, Campbell, CA), and recorded with a sampling frequency of 10 kHz after low-pass filtering at 3 kHz. Currents were recorded, and voltage protocols applied, using pClamp software (Clampex 9; Molecular Devices, Union City, CA). Reversal potentials of GABA-activated currents (E_GABA) were recorded using voltage ramps of ±300 mV in amplitude and of 1 s duration, applied to the neuron from a holding potential (E_h) of ~50 mV (Nabekura et al., 1996; Kakazu et al., 1999). E_GABA was taken as the potential at which current responses to voltage ramps, applied just before and during GABA application, intersected. Current–voltage curves, and data analysis, were done using Clampfit 9 (Molecular Devices).

Western blots. Cells cultured under the specified conditions were harvested by washing and subsequent scraping cell cultures with a solution containing the following (in mM): 137 NaCl, 2.68 KCl, 8.1 Na₂HPO₄, 1.47 KH₂PO₄. This solution was centrifuged for 10 min at 1000 rpm at 4°C. For quantification of KCC2 and β-actin, the cell pellets were suspended in 0.25 ml of buffer (containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitor; Roche, Mannheim, Germany; and phosphatase inhibitor mixture 2; Sigma) and sonicated on ice for 30 s. The cell lysates were centrifuged for 30 min at 15,000 rpm at 4°C, and the supernatant was collected. For quantification of KCC2 and phosphorylated KCC2, the cell pellets were suspended in 1 ml of buffer (containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitor; Roche; and phosphatase inhibitor mixture 2; Sigma) and sonicated on ice for 30 s. The cell lysates were centrifuged for 10 min at 15,000 rpm at 4°C, and the supernatant was collected and mixed with protein agarose A at 4°C on a rocking platform, before additional centrifugation for 1 min at 12,000 rpm. Anti-KCC2 antibody and protein agarose A were sequentially added to the supernatant before incubating overnight, all at 4°C on a rocking platform. The pellet was washed multiple times, first with buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitor (Roche) and phosphatase inhibitor mixture 2 (Sigma), and then containing 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Nonidet P-40, 0.05% sodium deoxycholate, and then with 10 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40, 0.05% sodium deoxycholate, and then these samples were heated to 100°C in SDS sample buffer for 5 min. The protein concentrations were determined with a BCA protein assay reagent kit (Pierce, Rockford, IL). The same amount of protein was applied to each lane in all Western blotting experiments, being 2 μg for detecting KCC2 and β-actin, and 25 μg for the immunoprecipitate Western blots to quantify the phosphorylated KCC2 and total KCC2. The sample was run through a 7.5% SDS-PAGE (Bio-Rad, Hercules, CA) to separate KCC2 and phosphorylated KCC2 and β-actin, which were transferred to a Immobilon P (Millipore, Bedford, MA), and incubated for 1 h with 1% bovine serum albumin (BSA) in a TBS-T solution composed of 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20. The membranes were incubated overnight with anti-KCC2 antibody (Upstate Biotechnology, Lake Placid, NY) diluted at 1:750, anti-phosphotyrosine antibody (clone 4G10) (Upstate Biotechnology) diluted at 1:500, or anti-phosphotyrosine antibody (clone PY20) (Sigma) diluted at 1:2000, or anti-β-actin antibody (Sigma) diluted at 1:10,000. All antibody dilutions and incubations were done in TBS-T solution containing 1% BSA. After washing the membranes four times for 15 min each with TBS-T, they were further incubated for 1 h with horseradish peroxidase-conjugated secondary antibody in TBS-T buffer. Protein was visualized using an ECL detection system (Amersham Biosciences, Piscataway, NJ). The size of protein was identified using a protein size marker (Invitrogen). The band of KCC2 was detected at 150 kDa. The relative band intensities were determined by densitometry using NIH Image.

Cell surface biotinylation assay. After treatment of cultured neurons for 1 h with the appropriate experimental condition (H₂O₂ with and without Na₃VO₄), cells were immediately washed twice with ice-cold PBS. Cell surface proteins were biotinylated using 0.5 mg/ml cell impermeant, noncleavable sulfo-NHS-Biotin (Pierce) in PBS for 30 min at 4°C. The reaction was stopped with quenching solution (Pierce), and cells were harvested in 10 ml of TBS by scraping. Cells were collected by centrifugation at 500 × g for 3 min and rinsed with 5 ml of TBS and collected and aliquots were centrifuged for 3 min. Cells were resuspended in 250 μl of lysis buffer (Pierce) containing protease inhibitor mixture (Sigma). Cell lysate was sonicated for 30 s, and then centrifuged at 10,000 × g for 2 min at 4°C. Supernatants was reacted with Neutravidin Gel (Pierce) for 60 min at room temperature, before triplicate washes with wash buffer (Pierce) containing the protease inhibitor mixture (Sigma). Proteins were eluted by incubating with SDS-PAGE sample buffer containing 50 mM DTT for 60 min before being subjected to Western blotting.

³²P labeling assay for KCC2 phosphorylation. Cultured hippocampal neurons (20 DIV) were incubated for 30 min in sodium phosphate-free DMEM (P-free medium; Invitrogen), before labeling with P-free medium containing [³²P]orthophosphate (150 μCi per 60 mm dish; PerkinElmer Life Sciences, Boston, MA) for 2 h. H₂O₂ (50 μM) was then added to the medium for another 60 min. The cells were then washed three times with ice-cold PBS, and lysed with a lysis buffer. The lysates obtained by centrifugation were subjected to immunoprecipitation with anti-KCC2 antibody and rabbit control IgG, followed by the addition of 20 μl of a 50% slurry of protein G-Sepharose beads. The immunoprecipitates were analyzed by SDS-PAGE, followed by autoradiography (LAS2500; Fuji Film, Tokyo, Japan).

Immunocytochemistry. Cells cultured on coverslips were fixed by replacing medium with a solution of 4% paraformaldehyde in 0.1 mM PBS, pH 7.2, and incubated for 40 min at room temperature (RT), and then washed with TBS buffer (0.3% Triton X-100 and 1% normal goat se-
rum in PBS). Subsequently, cells were permeabilized by incubation for 1 h (RT) in a blocking solution containing 2% BSA in TNBS buffer. Anti-KCC2 antibody (at 1:325 dilution) and anti-microtubule-associated protein 2 (MAP-2) antibody (Upstate Biotechnology; at 1:100 dilution) was added and incubated overnight at 4°C (Williams et al., 1999) before being rinsed with TNBS buffer and incubated with Alexa 488 594 secondary fluorescent antibodies (Invitrogen, Eugene, OR), before final rinsing and addition of Slowfade (Invitrogen). Fluorescent images were obtained using a laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

In utero electroporation. Timed-pregnant Sprague Dawley rats were anesthetized with sodium pentobarbital (0.625 mg per 10 g of body weight, i.p.), their abdominal cavities were cut open, and the uterine horns were exposed. Approximately 1–2 μl of DNA solution was injected into the lateral ventricle of embryos at E15–E17 using a glass micropipette. Each embryo within its uterus was placed between tweezer-type electrodes (CUY650-P5; NEPA Gene, Chiba, Japan). For targeting the cortical ventricular zone, the angle of inclination of the electrode paddles, with respect to the horizontal plane of the brain, was zero. Square electric pulses (50 V; 50 ms) were applied to the electrodes five times, at 75 ms intervals, using an electroporator (CUY21E; NEPA Gene). To avoid excessive temperature loss, only a few embryos were exposed at any one time, and care was taken to quickly place them back into the abdominal cavity after electroporation. Once all embryos were electroporated, the wall and skin of the abdominal cavity were sutured, and embryos were allowed to develop normally. The KCC2 vector was a EGF-P-RcKCC2 plasmid construct. Plasmid DNA was purified with an Endofree plasmid maxi kit (Qiagen, Valencia, CA), and resuspended in 10 mM Tris-HCl, pH 8.0. Before the procedure, plasmid DNA was diluted to 1 μg/μl in PBS, and Fast Green solution was added to a final concentration of 0.03% to monitor the injection.

Data analysis. Values are the mean ± SE of at least three or four independent experiments. Statistical examination was performed by one-way factorial ANOVA combined with Scheffe’s test for all comparison pairs. Differences with p < 0.05 were considered significant.

Results
The effects of neuronal stressors on KCC2 protein expression and phosphorylation in primary hippocampal neuron cultures
To investigate changes in KCC2 protein expression and phosphorylation during neuronal stress, we used an in vitro system generating primary cultures of hippocampal neurons isolated from embryonic E19 rats. A variety of neuronal traumas have been shown previously to induce disruptions in Cl⁻ homeostasis, E_{Cl\text{,BA}}, and/or downregulation of KCC2 mRNA or protein expression. We initially investigated the effects of H₂O₂ incubation, a model of oxidative stress and free radical–induced neuronal damage (Whitemore et al., 1995). Figure 1, A and B, shows that prolonged incubation with H₂O₂ (50 μM) results in a decrease in expression levels of KCC2 protein in the membrane fraction, with this decrease becoming evident with H₂O₂ incubation periods >3 h. There was little change in levels of β-actin over the same time period. In contrast, the level of tyrosine-phosphorylated KCC2 decreases more rapidly, and a significant decrease in the ratio of tyrosine phosphorylated KCC2 to total KCC2 was seen at the earliest time point examined (1 h incubation) (Fig. 1C,D). In the experiment illustrated by Figure 1C, two samples of 2 μg of protein were applied to two separate gels of equal composition, which were then stained as described in Materials and Methods. After 3 h H₂O₂ incubation, total KCC2 levels were 88 ± 4.6% of control levels (before H₂O₂ application), whereas the ratio of tyrosine-phosphorylated KCC2 to total KCC2 declined to 16.7 ± 1.2% of control levels (n = 4). A qualitatively similar rapid loss of tyrosine-phosphorylated KCC2 in response to H₂O₂ was seen using a tyrosine-phosphorylated KCC2 antibody from a different clone PY20 (Sigma), and when KCC2 phosphorylation state was identified with autoradiography with ³²P labeling (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Our preliminary experiments had indicated that inducing seizure activity by injection of kainic acid resulted in a gradual decrease in KCC2 protein levels in subsequently excised hippocampal membranes that was preceded by more rapid decreases in tyrosine phosphorylation (data not shown). Hence we also investigated models of in vitro epileptic activity using our hippocampal...
cultures. The exogenous application of BDNF, a neurotrophin whose expression and release is increased during in vivo seizure activity, has also been shown to induce a decrease in KCC2 mRNA and protein expression and an impairment of Cl⁻ extrusion in hippocampal neurons in vitro (Rivera et al., 2002). Incubation of isolated neurons or slices with 0 Mg²⁺ has been shown to elicit hyperexcitability in vitro (Rivera et al., 2004). Figure 2, A and B, shows KCC2 protein levels in hippocampal neurons are unchanged at 1 and 3 h after incubation with BDNF, but downregulated after 6 h BDNF incubation. KCC2 protein levels are also downregulated after 3 h incubation in Mg²⁺-free culture medium. Neither of these treatments produced any change in the expression levels of β-actin. These treatments also resulted in a reduced tyrosine phosphorylation of KCC2, and, for BDNF incubation, this was observed at time points before changes in KCC2 protein levels (Fig. 2C,D). The ratio of tyrosine-phosphorylated KCC2 to total KCC2 protein was reduced by ~50% 2 h after BDNF application, at a time when total KCC2 was unchanged. Although total KCC2 expression levels were decreased by ~50% after 3 h incubation in Mg²⁺-free culture medium, the ratio of tyrosine-phosphorylated KCC2 to total KCC2 protein decreased by an even greater amount (by 77 ± 4%; n = 4) after just 2 h incubation.

**Time course of KCC2 function in primary cultured hippocampal neurons exposed to H₂O₂**

Decreases in KCC2 protein expression levels in cultured hippocampal neurons result in disruption of Cl⁻ homeostasis, increases in intracellular Cl⁻, and corresponding depolarizing shifts in E_GABA (Rivera et al., 2002; Khirug et al., 2005; Zhu et al., 2005). We therefore measured the time course of changes in E_GABA in cultured neurons exposed to H₂O₂ to investigate whether changes in KCC2 tyrosine phosphorylation state had functional consequences. Experiments were conducted on neurons after 21 DIV, a time when cultured hippocampal neurons have developed adult-like pattern of KCC2 protein levels and activity (Fiúmelli et al., 2005; Khirug et al., 2005), and we used the gramicidin-perforated patch-clamp technique to preserve intracellular Cl⁻ (Kakazu et al., 1999, 2000). Voltage ramps were applied before and after bath application of GABA (30 µM), and E_GABA was measured before, and 1, 3, 6, and 9 h after application of H₂O₂ (Fig. 3). In control conditions, GABA application produced a large outward current at a V_H of ~50 mV which reversed at ~71 ± 7.8 mV (n = 5). Furosemide shifted E_GABA to ~55 ± 3 mV (n = 5). In addition, the replacement of intracellular K⁺ with Cs⁺ (Kakazu et al., 1999, 2000) also shifted E_GABA to ~47 ± 3 mV, confirming that a furosemide- and K⁺-sensitive mechanism (i.e., KCC2) plays an important role in maintaining low intracellular Cl⁻ in adult hippocampal neurons.

One hour after application of H₂O₂, the outward current response to GABA had decreased and E_GABA already showed a shift to more positive potentials (Fig. 3), indicating that loss of tyrosine phosphorylation of KCC2 results in functional downregulation of KCC2 and an increase in intracellular Cl⁻. During continued incubation with H₂O₂, E_GABA shifted to more positive potentials with the GABA response at a V_H of ~50 mV, eventually being converted into an inward current. There seemed to be two phases of the change in E_GABA (Fig. 3), an early change occurring within 1 h of H₂O₂ treatment, and a more gradual depolarizing shift in E_GABA that occurred between 6 and 9 h after application of H₂O₂, presumably corresponding to a time when total KCC2 protein levels are decreased.

**Role of protein phosphatases in the loss of KCC2 tyrosine phosphorylation**

A loss of tyrosine phosphorylation of KCC2 may result from an increased activity of tyrosine phosphatases and/or a decreased activity of tyrosine kinases. To investigate potential underlying mechanisms, we examined the effects of sodium vanadate (Na₃VO₄), a specific inhibitor of tyrosine phosphatases (Swarup et al., 1982), on the oxidative stress-induced change in KCC2 tyrosine phosphorylation ratio. Incubation for 3 h with both Na₃VO₄ (100 µM) and H₂O₂ (50 µM) did not significantly affect
were incubated for 1 or 3 h with both Na$_3$VO$_4$ (100 μM) and phosphatase activity in control conditions. When neurons were incubated with Na$_2$MoO$_4$, but not a serine–threonine phosphatase blocker, tyrosine phosphatase blockers (e.g., phenylarsine oxide and Na$_3$VO$_4$ (100 μM)), indicating a basal level of tyrosine kinase activity. The self-consistency of these results requires that H$_2$O$_2$ stimulates kinase activity, because otherwise the 10-fold stimulation in H$_2$O$_2$ + Na$_3$VO$_4$ at 3 h would be unexplained compared with the very modest stimulation in Na$_3$VO$_4$ alone and the small decrease by H$_2$O$_2$ alone as illustrated in Figure 4D. Under conditions of intracellular Ca$^{2+}$ chelation with BAPTA-AM, H$_2$O$_2$ failed to decrease (and modestly increased) the proportion of phosphorylated KCC2 (Fig. 4C,D), suggesting that the decrease in KCC2 tyrosine phosphorylation and function may be mediated by elevation of intracellular Ca$^{2+}$.

Cell surface localization of KCC2 in hippocampal primary neuronal cultures in response to neuronal stress

It has recently been reported that the protein levels of KCC2 in hippocampal slices exposed to 0 Mg$^{2+}$-free solution or BDNF (100 ng/ml) show a decrease within 1 and 2 h, respectively (Rivera et al., 2002, 2004). The half-life of surface-expressed KCC2 in hippocampal slices exposed to 0 Mg$^{2+}$ was also decreased, from a mean of ~20 to 10 min (Rivera et al., 2004). Because this suggests a rapid translocation of KCC2 between the plasma membrane and intracellular compartments, we decided to investigate the cellular distribution of KCC2 in control conditions, and after 1 h incubation with H$_2$O$_2$, a time corresponding to when tyrosine phosphorylation is markedly reduced but total KCC2 protein is unaffected. We used both confocal microscopy and quantification of biotinylated surface proteins. Typical fluorescent images are shown in Figure 5. In control neurons, KCC2 was expressed on the plasma membrane along the proximal den-
The biotinylation assay also clearly demonstrated that \( \text{H}_2\text{O}_2 \) incubation with both \( \text{H}_2\text{O}_2 \) and \( \text{Na}_3\text{VO}_4 \), however, the surface inhibitor \( \text{Na} \) vanadate (\( \text{Na}_3\text{VO}_4 \)) (100 \( \mu \text{M} \) of KCC2 appeared to have been lost (Fig. 5, \( \text{H}_2\text{O}_2 \)). After 1 h (Fig. 5), dendrites and throughout the cell soma (Fig. 5). One hour of cell surface expression of KCC2 in cultured hippocampal neurons treated with \( \text{H}_2\text{O}_2 \).

Figure 5. Immunofluorescent staining of the cellular localization of KCC2 and quantification of cell surface expression of KCC2 in cultured hippocampal neurons treated with \( \text{H}_2\text{O}_2 \). A, KCC2 immunofluorescent images (red) in two control neurons (top panels) with MAP-2 staining (green). Note the staining of KCC2 (represented by red fluorescence) observed on the cell surface throughout the soma and particularly throughout the proximal dendrites. KCC2 immunofluorescent images after 1 h \( \text{H}_2\text{O}_2 \) incubation without (middle panels) and with the phosphatase inhibitor \( \text{Na} \) vanadate (\( \text{Na}_3\text{VO}_4 \)) (100 \( \mu \text{M} \)) (bottom panels). Note the loss of surface expression of KCC2 in response to \( \text{H}_2\text{O}_2 \), which was not observed with additional \( \text{Na}_3\text{VO}_4 \) incubation. The insets in each panel show an enlargement of the area depicted by the dotted squares. Scale bars, 10 \( \mu \text{M} \) (for each panel). B, Biotinylation assay of cell surface expression of KCC2, in control conditions, and after 1 h incubation in \( \text{H}_2\text{O}_2 \) with and without \( \text{Na}_3\text{VO}_4 \) (100 \( \mu \text{M} \)). The left panel shows a sample Western blot, and the right panel shows averaged optical densities of Western blots from three separate experiments. Error bars indicate SEM. The same total protein (2 \( \mu \text{g} \)) was applied to each lane. Surface expression of KCC2 shows a significant decrease in response to \( \text{H}_2\text{O}_2 \), which is prevented by \( \text{Na}_3\text{VO}_4 \). ** \( p < 0.01 \) compared with control.

Consequences of KCC2 functional downregulation on neuronal viability

Although a number of reports, as well as the data above, have indicated functional downregulation of KCC2 in response to neuronal injury or stress (see Introduction), it is not clear what the consequences of changes in KCC2 function are in regard to neuron viability. To begin to address this, we used a coarse, but simple, measure of cell viability, the ability of healthy neurons to exclude the membrane impermeant dye trypan blue. Incubation of cultured neurons with \( \text{H}_2\text{O}_2 \) (50 \( \mu \text{M} \)) led to a loss of cell viability, with 25 ± 12% of neurons observed unable to exclude trypan blue (Fig. 6A). Our above results showed that KCC2 function is downregulated by such oxidative stress, and consequently GABA responses become depolarizing. Therefore, we next investigated whether activation or inhibition of GABA\( \alpha \) receptors during oxidative stress could affect cell viability. Coapplication of the GABA\( \alpha \) receptor agonist, muscimol (30 \( \mu \text{M} \)), during \( \text{H}_2\text{O}_2 \) incubation, resulted in an increase in the proportion of neurons that could not exclude trypan blue (Fig. 6A), indicating enhanced neuronal death. Conversely, coapplication of the GABA\( \alpha \) receptor antagonist, 2-(3-carboxypropyl)-3-amino-6-(4-methoxy-phenyl)pyridazinium (SR 95531) (30 \( \mu \text{M} \)), resulted in a decrease in the proportion of neurons that could not exclude trypan blue. Muscimol (30 \( \mu \text{M} \)) alone did not affect this proportion (n = 3). Hence endogenous and exogenous activation of GABA\( \alpha \) receptors during oxidative stress can exacerbate the loss of cell viability during oxidative stress.

We next investigated the consequences of manipulating the levels of KCC2 that become tyrosine dephosphorylated during oxidative stress. Our first approach was to overexpress KCC2, because in neurons with excess transporters, a greater absolute number will maintain tyrosine phosphorylation during oxidative stress. To overexpress KCC2, we generated a pKCC2-ires-EGFP cDNA construct and transfected rat embryos at E15–E17 with this vector using in utero electroporation. Cortical neuronal cultures were subsequently generated from these transfected embryos at E20. We choose to use in utero-transfected cultures of cortical neurons, as opposed to in vitro transfection of hippocampal neurons, because we could achieve higher transfection rates with this method and considered it to be a less stressful stimulus for the neurons, given that they could recover from the transfection procedure in vivo. To confirm successful overexpression of KCC2, we compared \( E_{\text{GABA}} \) in cultured neurons expressing green fluorescent protein (GFP) with control neurons from the same cultures that did not express the GFP construct, at 4–5 DIV, using gramicidin-perforated patch-clamp recordings (Fig. 6B). In control neurons, GABA (30 \( \mu \text{M} \)) alone did not affect this proportion (n = 3). Our above results showed that KCC2 functional downregulation occurs in neurons expressing the construct, GABA induced an outward current at a \( V \text{m} = –58.6 \pm 0.6 \text{ mV} \) (n = 6). This is consistent with previous results in primary hippocampal neuronal cultures derived from embryonic rats, which fail to show a functional KCC2 response until ~2 weeks in vitro (Kelsch et al., 2001; Fiumelli et al., 2005; Khirug et al., 2005). At 10 DIV, incubation with control neurons with \( \text{H}_2\text{O}_2 \) (50 \( \mu \text{M} \)) for up to 9 h resulted in ~50% of neurons observed unable to exclude trypan blue. For
neurons from the same cultures that overexpressed KCC2, the loss of cell viability was markedly reduced, and this was observed at both 3 and 9 h incubation times. After 9 h incubation, for example, only ~20% of neurons were unable to exclude trypan blue (Fig. 6C).

Our next approach to investigating functional consequences of KCC2 tyrosine phosphorylation during oxidative stress was to incubate neurons with both H2O2 (50 μM) and the tyrosine phosphatase inhibitor Na3VO4 (100 μM), resulting in an increased proportion of tyrosine-phosphorylated KCC2 (Fig. 4). After 3 h of Na3VO4 and H2O2 incubation, the loss of cell viability was markedly reduced (Fig. 6C). Decrease of the loss of cell viability could also be observed in an incubation with H2O2 and other possible tyrosine phosphatase blockers, phenylarsine oxide and Na2MoO4 (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). After more prolonged Na3VO4 and H2O2 incubation (9 h), however, the proportion of neurons unable to exclude trypan blue was not significantly different from that in control neurons. Both these sets of experiments suggest that increasing the relative proportion of tyrosine-phosphorylated KCC2 protects against the loss of cell viability in response to short periods of oxidative stress, whereas increasing KCC2 expression levels can sustain cell viability for prolonged periods of oxidative stress.

**Discussion**

**Do changes in KCC2 expression levels after neuronal stress alone determine cellular KCC2 activity?**

A hyperpolarizing ionotropic GABA receptor response in neurons occurs primarily because of the ability of KCC2 to use the transmembrane K⁺ gradient to extrude Cl⁻ and hence keep [Cl⁻] low. Various neuronal traumas can result in a conversion of the GABAergic response to a depolarization. In some instances, such a depolarizing GABA response has been correlated with a decrease in KCC2 expression [axotomy (Nabekura et al., 2002; Toyoda et al., 2003), seizures (Rivera et al., 2002), neuropathic pain (Coull et al., 2003)]. Given the rapid turnover of membrane-bound KCC2 (Rivera et al., 2004), and possible subsequent degradation, these data are compatible with a hypothesis in which KCC2 expression levels may be the principal determinant of changes in cellular KCC2 activity in response to injury. However, there are also some instances of mismatches apparent between altered KCC2 expression levels and functional consequences. In a model of in vivo cortical epilepsy, Jin et al. (2005) indicate an apparent downregulation of KCC2 expression without marked changes in KCC2 activity. Fiumelli et al. (2005) reported a neuronal activity and Ca²⁺ and PKC (protein kinase C)-dependent downregulation of KCC2 function that occurred too rapidly to be mediated by alterations in KCC2 expression. Furthermore, during in vitro neuronal development of hippocampal neurons, it is clear that KCC2 can be present at early stages but nonfunctional, with activity induced by a broad kinase inhibition or with tyrosine kinase activation (Kelsch et al., 2001; Zhu et al., 2005), although whether these changes in phosphorylation were to KCC2 itself or some accessory protein is not investigated. Our results demonstrate that loss of direct KCC2 tyrosine phosphorylation occurs in response to neuronal stress and that this dephosphorylation correlates to loss of functional KCC2 activity and also precedes changes in KCC2 expression levels. Hence changes in cellular KCC2 ac-
tivity can occur in response to injury independent of changes in expression levels. The resultant initial increase of [Cl\(^-\)], led to a reduced or absent GABA hyperpolarization. During more prolonged neuronal stress, a second phase of [Cl\(^-\)]\(i\) increase was apparent that was associated with a conversion of the GABA response to a depolarization and a reduced expression of KCC2 protein. Hence we would propose that injury results in two phases of reduced KCC2 activity: an initial downregulation attributable to loss of tyrosine phosphorylation and a second phase correlated with loss of KCC2 protein levels.

Although KCC2 is primarily responsible for maintaining low [Cl\(^-\)]\(i\), and hyperpolarizing GABA responses in adult neurons (Kakazu et al., 1999), the cation-chloride cotransporter NKCC1 also contributes to neuronal Cl\(^-\) homeostasis (Okabe et al., 2003). Our present study has not addressed whether the activity of this transporter also changes with neuronal stress. Pond et al. (2006) have reported that the activity of NKCC1 in hippocampal neurons increases in response to in vitro ischemia, via an increased serine/threonine phosphorylation. An increased NKCC1 activity would result in additional increases in [Cl\(^-\)]\(i\), exacerbating the consequences of KCC2 functional downregulation.

**Mechanism linking loss of tyrosine phosphorylation to reduced cellular KCC2 activity**

The correlation between dephosphorylated KCC2 and the loss of surface expression of KCC2 is consistent with the functional downregulation being attributable to alterations in membrane trafficking of the KCC2 protein, either an increase in internalization/endocytosis or a decrease in insertion/exocytosis. Liddle is known about the mechanisms underlying these processes in regards to cation-chloride transporters, despite the recent suggestions for the related NKCC2 protein, that dynamic alterations in surface expression may mediate hormone-evoked changes in kidney salt transport (Meade et al., 2003), despite the wealth of studies on acute regulation of surface expression of other membrane proteins (Kittler and Moss, 2003; Le Roy and Wrana, 2005). Our studies show that Na\(_3\)VO\(_4\) prevented the loss of surface KCC2 expression in response to oxidative stress, suggesting that tyrosine dephosphorylation precedes loss of KCC2 surface expression. Rivera et al. (2004) reported that 0 Mg\(^{2+}\)-induced epileptic activity increased the turnover rate of biotinylated surface-expressed KCC2. It is interesting to speculate that the increased internalized KCC2 was attributable to a loss of tyrosine phosphorylation, whereas a fraction (20%), seemingly more membrane stable, remained phosphorylated and functional. Additional clarification of the underlying mechanisms responsible for membrane trafficking, and whether this involves KCC2 tyrosine dephosphorylation, is an important question for future studies.

Our data suggest that stress or injury can induce an increase in the KCC2 tyrosine phosphorylation/dephosphorylation rate, with an overall reduction in the tyrosine-phosphorylated proportion of KCC2 and a resulting rapid loss of function associated with reductions in membrane surface expression. Clearly, other signaling mechanisms exist to acutely modulate KCC2 function in response to different stimuli. In regard to tyrosine phosphorylation, activation of the receptor tyrosine kinase TrkB, in response to 0 Mg\(^{2+}\) incubation, can result in functional KCC2 downregulation and reduced KCC2 cellular expression, in a process involving both phospholipase C\(\gamma\) and the src homology 2 domain containing transforming protein (Rivera et al., 2004). Conversely, exogenous activation of the cytoplasmic tyrosine kinase src, has been shown to activate KCC2 in cultured neurons (Kelsch et al., 2001). In response to sustained postsynaptic spike activity, KCC2 function was rapidly downregulated in cultured hippocampal neurons, via a Ca\(^{2+}\)-dependent signaling mechanism that did not involve tyrosine kinases or serine/threonine phosphatases, but rather involved activation of protein kinase C (Fiumelli et al., 2005). However, in none of these previous studies was the phosphorylation state of KCC2 directly examined, so it remains unclear whether the effects were attributable to changes in the phosphorylation of KCC2 itself or of some modulatory protein.

**Functional significance of KCC2 downregulation**

Seizure activity, oxidative stress, axotomy, nerve injury, and arthritis all result in decreases in KCC2 expression and function, and a conversion of GABA-mediated responses toward depolarization. Whether this process contributes to the pathological symptoms and/or neuronal damage, or is an adaptive response to promote recovery is not totally clear and may depend on the type of insult. The conversion of GABAergic responses to depolarizing has been shown to be present in a range of in vitro and in vivo seizure models and in human epileptics (Cohen et al., 2002; Khalilov et al., 2003; Rivera et al., 2004; Jin et al., 2005) (for review, see Cosset al., 2005), and KCC2 downregulation may be important in the genesis and maintenance of seizure activity. Consistently, neonatal neurons have both a reduced KCC2 expression and show increased seizure susceptibility, although the correlation does not precisely match (Khazipov et al., 2004). BDNF-induced downregulation of KCC2 can be correlated to a model in which BDNF generates seizures in response to a variety of causes (Binder et al., 2001; Rivera et al., 2002). In addition, BDNF decreases the IPSCs in amplitude in the hippocampal excitatory neurons (Mizoguchi et al., 2003), introduced by the depolarizing shift of IPSC reversal potential (Wardle and Poo, 2003) as well as by the internalization of GABA\(_\text{A}\) receptors (Kanematsu et al., 2003). However, elevations in extracellular K\(^+\) that are likely to occur with increased neuronal activity, may reverse the direction of transport for KCC2, so that it results in Cl\(^-\) influx (Payne, 1997; Kakazu et al., 2000). Furthermore, KCC and related transporters may contribute to volume changes and cell swelling that can exacerbate seizures (Schwartzkroin et al., 1998; Haglund and Hochman, 2005). In these situations, a reduction in KCC2 activity would actually reduce GABAergic depolarization and neuronal activity, and it remains conceivable that functional downregulation may be an adaptive and neuroprotective response to seizure activity.

In our simple model of in vitro oxidative stress, overexpression of KCC2, and the subsequent maintenance of [Cl\(^-\)]\(i\), homeostasis, was neuroprotective. Similarly, inhibition of phosphatase activity in these cells, which may be expected to reduce the downregulation of KCC2 tyrosine phosphorylation and function (among other things), also enhanced neuronal survival, again suggesting that functional downregulation of KCC2 may contribute to neuronal damage. Importantly, phosphatase inhibition did not affect neuronal viability during prolonged oxidative stress, corresponding to the second phase of KCC2 downregulation attributable to loss of KCC2 expression. Although oxidative stress may cause loss of neuronal viability for multiple reasons, part of this response involved, at least in our experimental conditions, activation of GABA\(_\text{A}\) receptors. Whitemore et al. (1995) reported, for cultured cortical neurons, that H\(_2\)O\(_2\) can result in elevations of intracellular Ca\(^{2+}\). Hence one simple possibility is that, by preventing KCC2 functional downregulation and GABA-
induced depolarization, there may be reduced excitotoxic Ca$^{2+}$ influx (Sattler and Tymianski, 2000).

Additional insights into the role of KCC in different types of neuronal stress require careful consideration of the time course of functional changes in KCC2, manipulation of these changes, and monitoring the effects of these manipulations on neuronal damage and/or recovery. Understanding the mechanisms underlying functional changes in KCC2 in response to different neuronal stress may result in novel strategies for reducing neuronal damage and/or promoting neuronal recovery in different pathologi-

References


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