$GABA_A$ receptor responses are also dependent on TrkB and PLC-gamma activation, but the subsequent signaling pathways seem to change depending on the direction of the modulation and the developmental stage (Mizoguchi *et al.*, 2003a; Jovanovic *et al.*, 2004). In acutely isolated immature visual cortical pyramidal neurons, the BDNF-induced potentiation of $GABA_A$ responses is similarly blocked by PKC inhibition (Mizoguchi & Nabekura, unpublished observations), as is the transient potentiation of GABA responses seen in cultured hippocampal neurons (Jovanovic *et al.*, 2004). A similar transduction pathway appeared to mediate the BDNF-induced occlusion of the inhibitory effect of baclofen observed in the current study.

As also reported here, PKC activation is known to abolish or significantly occlude the baclofen-induced presynaptic inhibition of GABA release in the hippocampus (Dutar & Nicoll, 1988; Thompson & Gähwiler, 1992; Swartz, 1993; Jarolimek & Misgeld, 1997), by phosphorylation-induced receptor endocytosis and disruption of G-protein coupling (Swartz, 1993; Zamponi *et al.*, 1997; Bettler *et al.*, 2004). PKC activation in neurons from P7 rats failed to occlude the baclofen response. In whole rat brain (Yoshida *et al.*, 1988) and in rat hippocampal synaptosomes (Shearman *et al.*, 1991), the activity of PKC, while present at P7, increases abruptly during the first two postnatal weeks. Our results suggest that PKC is crucial to mediating the BDNF occlusion of presynaptic $GABA_B$ receptor-mediated inhibition of GABA release, and that its insufficient activity at P7 might explain the developmental change in this BDNF action.

Although the exact mechanism underlying presynaptic $GABA_B$ receptor-mediated inhibition of quantal GABA release is still obscure (Nicoll, 2004), inhibition of presynaptic Ca^{2+} channels is supposed to be most important (Misgeld *et al.*, 1995; Nicoll, 2004). In the preparation we used, baclofen did not significantly decrease the frequency of mIPSCs in the Ca^{2+}-free external solution (Mizoguchi & Nabekura, unpublished observations). Thus, in the isolated hippocampal pyramidal neurons, baclofen-induced inhibitory action mainly depends on extracellular Ca^{2+}, suggesting a possible mechanism that depends on Ca^{2+} influx through the Ca^{2+} channels.

In hippocampal slices prepared from P14 rats, BDNF significantly occluded baclofen-induced suppression of eIPSCs. Furthermore, in the presence of BDNF, baclofen did not affect PPRs, suggesting that its locus of action was presynaptic. What may be the physiological function of the BDNF-induced occlusion of $GABA_B$ autoreceptors on hippocampal GABAergic terminals? In the developing nervous system, GABAergic transmission appears before excitatory transmission and can help to establish appropriate synaptic and network connections (Ben-Ari, 2002). Presynaptic $GABA_B$ autoreceptors are present and functional in the hippocampus (current study; Jarolimek & Misgeld, 1997), and are active prior to the appearance of functional postsynaptic $GABA_B$ receptors (Ben-Ari *et al.*, 1997; Nurse & Lacaille, 1999). Other regulatory presynaptic receptors that can modulate GABA release, such as the group II metabotropic glutamate receptors (Schoepp, 2001), are absent or non-functional (Shigemoto *et al.*, 1997; Morishita & Alger, 2000) in the hippocampal CA1 region of adult rats, conferring more importance on $GABA_B$ autoreceptors for control of GABA release. Certain forms of synaptic plasticity at immature and juvenile GABAergic synapses are regulated by $GABA_B$ autoreceptors. The duration of giant GABAergic depolarizing potentials, which have been postulated to play a role in the development of appropriate neuronal networks, are regulated by activation of $GABA_B$ autoreceptors (Ben-Ari, 2002). The induction of long-term depression at excitatory hippocampal synapses is also enhanced by activation of $GABA_B$ autoreceptors (Wagner & Alger, 1995). As activity-dependent forms of long-term plasticity, including at immature and juvenile hippocampal synapses, can also be modulated by BDNF (Ikegaya *et al.*, 2002; Lu, 2003; Gubellini *et al.*, 2005; Woo *et al.*, 2005), an interaction between BDNF and presynaptic $GABA_B$ autoreceptors may contribute to the induction of this plasticity. Interestingly, the occlusion of $GABA_B$ autoreceptors by BDNF was transient and reversible, in contrast to the sustained effects of acute BDNF on $GABA_A$-mediated responses (Mizoguchi *et al.*, 2003b). BDNF has been shown to have synapse-specific activity-dependent actions at excitatory synapses (Lu, 2003). The results presented here suggest that the actions of BDNF may be temporally restricted, with activity-dependent release of BDNF switching off $GABA_B$ autoreceptors.

In the adult rat hippocampus, infusion of BDNF *in vivo* results in seizures (Scharfman *et al.*, 2002), while scavenging BDNF *in vivo* prevents kindling (Binder *et al.*, 2001). BDNF rapidly enhances excitatory transmissions (Lu, 2003) and also inhibits $GABA_A$ receptor-mediated inhibitory transmissions (Tanaka *et al.*, 1997; Mizoguchi *et al.*, 2003a) in the rat hippocampus. BDNF-induced enhancement of neural excitability might contribute to the ontogeny of epilepsy. However, BDNF is also shown to delay kindling epileptogenesis in the rat hippocampus (Reibel *et al.*, 2003). These suggest that BDNF has complex roles in the epileptogenesis. In hippocampal pyramidal neurons isolated from P14 rats, BDNF reversibly occluded the presynaptic $GABA_B$ receptor-mediated inhibition of GABA release (Figs 1 and 2). By reversibly occluding the action of $GABA_B$ autoreceptors, BDNF could temporarily enhance GABA release, facilitate GABAergic inhibition and then interfere with the BDNF-induced enhancement of neural excitability in the hippocampus.

In addition, it is also important to note that $GABA_B$ receptors are implicated in the occurrence of temporal lobe epilepsies (Gambardella *et al.*, 2003; Bettler *et al.*, 2004). $GABA_BR1$ knockout mice develop normally until P9, after which they develop severe seizures and die at about P27 (Prosser *et al.*, 2001). Thus, in the hippocampus, BDNF-induced occlusion of $GABA_B$ autoreceptors function could play important roles in the pathophysiology of epilepsy.

Although a number of recent studies have reported a diversity of acute BDNF actions on $GABA_A$ receptor-mediated transmission among different CNS regions and at different developmental stages (Brünig *et al.*, 2001; Mizoguchi *et al.*, 2003a,b; Wardle & Poo, 2003; Jovanovic *et al.*, 2004), we believe this report clearly demonstrates that BDNF also has acute actions on $GABA_B$ receptor-mediated responses. This demonstrates a novel aspect of the effects of BDNF on inhibitory transmissions in the rat hippocampus, which may have some important roles in the induction of developmental plasticity and/or pathophysiology of epilepsy.

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Abbreviations

ACSF, artificial cerebrospinal fluid; APV, 2-amino-5-phosphonopentanoic acid; BDNF, brain-derived neurotrophic factor; CNQX, 6-cyano-nitroquinoxaline-2,3-dione; DAG, diacylglycerol; DIV, days *in vitro*; DMSO, dimethylsulfoxide; eIPSC, evoked inhibitory postsynaptic current; GABA, γ-aminobutyric acid; mIPSC, miniature postsynaptic current; PKC, protein kinase C; PLC, phospholipase C; PDBu, phorbol-12,13-dibutyrate; PPR, paired-pulse ratio; TEA, tetraethylammonium; TrkB, tyrosine receptor kinase B; TTX, tetrodotoxin.

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Cellular/Molecular

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 (H_2O_2) and the induction of seizure activity (BDNF) and hyperexcitability (0 Mg^{2+}) 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 $[Cl^-]_i$, 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 correlates 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 $[Cl^-]_i$ 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

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GABA response (Rivera et al., 2004). The rapid turnover rate of KCC2 ($\tau \sim 20$ 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 Ca^{2+} 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 trituration 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, Tokyo, Japan) at 37°C and 5% CO_2. 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 d *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 (V_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 Na_2HPO_4, 1.47 KH_2PO_4. 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, St. Louis, MO) 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, 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 (H_2O_2 with and without Na_3VO_4), 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 again by centrifugation at 500 × *g* for 3 min. Cells were dissolved 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.

^{32}P labeling assay for KCC2 phosphorylatyion. 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 [^{32}P]orthophosphate (150 μCi per 60 mm dish; PerkinElmer Life Sciences, Boston, MA) for 2 h. H_2O_2 (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 (BAS2500; 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 TNBS 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 EGFP-IRES-KCC2 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 Scheffé'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_{GABA}, and/or downregulation of KCC2 mRNA or protein expression. We initially investigated the effects of H_2O_2 incubation, a model of oxidative stress and free radical-induced neuronal damage (Whittemore et al., 1995). Figure 1, *A* and *B*, shows that prolonged incubation with H_2O_2 (50 μM) results in a decrease in expression levels of KCC2 protein in the membrane fraction, with this decrease becoming evident with H_2O_2 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. 1*C*,*D*). In the experiment illustrated by Figure 1*C*, 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 H_2O_2 incubation, total KCC2 levels were 88 ± 4.6% of control levels (before H_2O_2 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 H_2O_2 was seen using a tyrosine-phosphorylated

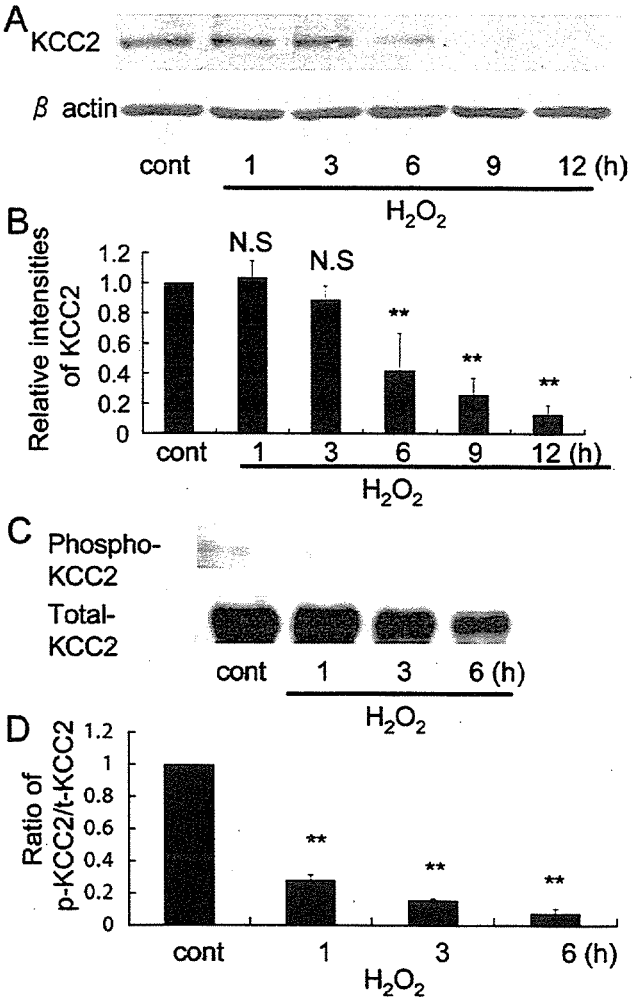

Figure 1. The time course of KCC2 protein expression and phosphorylation ratio in cultured hippocampal neurons during oxidative stress. ***A***, Western blots showing total KCC expression in membranes of hippocampal neurons, before (cont) and 1–12 h after treatment of hippocampal cultures (21–25 DIV) with H_2O_2 (50 μM). Equal amounts of protein (2 μg) were applied to each lane. The bottom panel shows expression levels of the control protein, β-actin, in parallel experiments on membranes prepared from the same cell cultures. ***B***, Averaged optical density of total membrane KCC2 expression, at 1–12 h H_2O_2 incubation, expressed relative to control levels of KCC2. Each column represents the mean and SEM of data from four experiments. ***C***, Western blots showing tyrosine-phosphorylated KCC2 (top row) and total KCC2 expression in hippocampal neurons, before (cont) and 1–6 h after treatment of hippocampal cultures with H_2O_2 (50 μM). The same amount of protein (25 μg) was added to each lane, and the same samples were used to detect both total KCC2 and phosphorylated KCC2. ***D***, Averaged ratio of optical density of tyrosine-phosphorylated KCC2 relative to total KCC2 expression (bottom row), at 1–6 h after H_2O_2 incubation. Each column represents the mean and SEM of data from four experiments. The relative amount of tyrosine-phosphorylated KCC2 is markedly decreased by 1 h after treatment with H_2O_2, before any change is observed in total KCC2 expression levels. $^{**}p < 0.01$ compared with control; N.S, not significantly different from control.

KCC2 antibody from a different clone PY20 (Sigma), and when KCC2 phosphorylation state was identified with autoradiography with ^{32}P 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

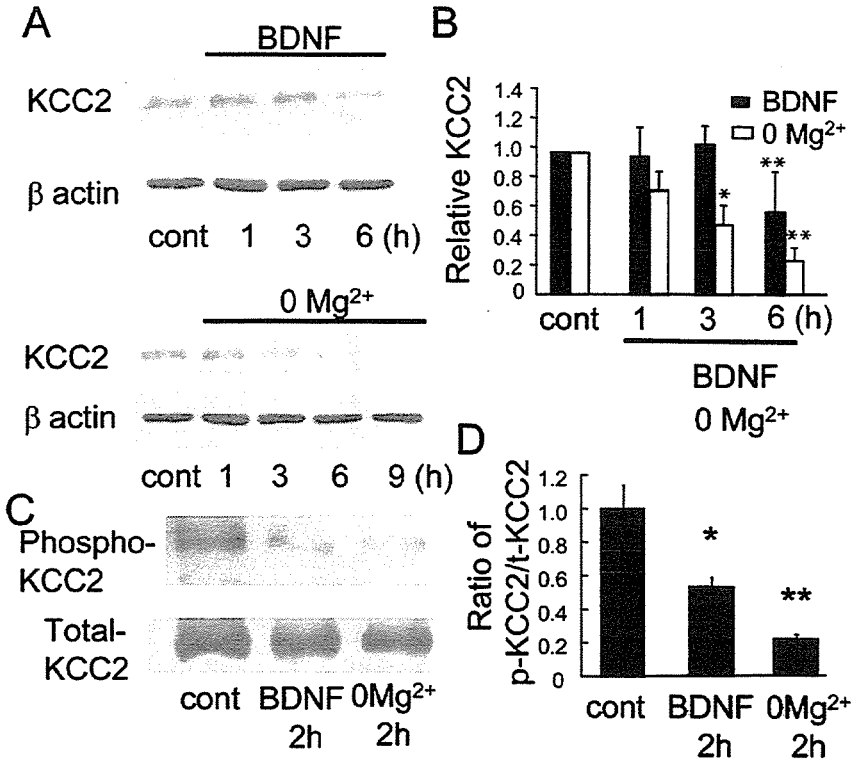

Figure 2. The time course of KCC2 protein expression and phosphorylation ratio in cultured hippocampal neurons during two different *in vitro* models of epilepsy. ***A***, Western blots showing total KCC2 expression, and control levels of β-actin expression in parallel experiments on membranes from the same cell cultures, before (cont) and 1–6 h after incubation with BDNF (20 ng/ml; top panels) or 1–9 h after incubation with 0 Mg^{2+} (bottom panels). ***B***, Averaged optical density of total membrane KCC2 expression, at 1–6 h incubation with BDNF or 0 Mg^{2+}, expressed relative to control levels of KCC2. Each column represents the mean and SEM of data from three experiments. ***C***, Western blots showing total KCC2 (bottom panel) and tyrosine-phosphorylated KCC2 (top panel) expression in control conditions (cont) and at 2 h after BDNF and 0 Mg^{2+} incubation. ***D***, Averaged ratio of optical density of tyrosine-phosphorylated KCC2 relative to total KCC2 expression, in control conditions, and at 2 h after BDNF or 0 Mg^{2+} incubation. Each column represents the mean and SEM of data from four experiments. The relative amount of tyrosine-phosphorylated KCC2 is markedly decreased before any change is observed in total KCC2 expression levels. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with control.

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 Mg^{2+} 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 Mg^{2+}-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. 2*C*,*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 Mg^{2+}-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 H_2O_2

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 H_2O_2 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 (Fiumelli 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 H_2O_2 (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 H_2O_2, 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 H_2O_2, 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 H_2O_2 treatment, and a more gradual depolarizing shift in E_{GABA} that occurred between 6 and 9 h after application of H_2O_2, 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 (Na_3VO_4), 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 Na_3VO_4 (100 μM) and H_2O_2 (50 μM) did not significantly affect

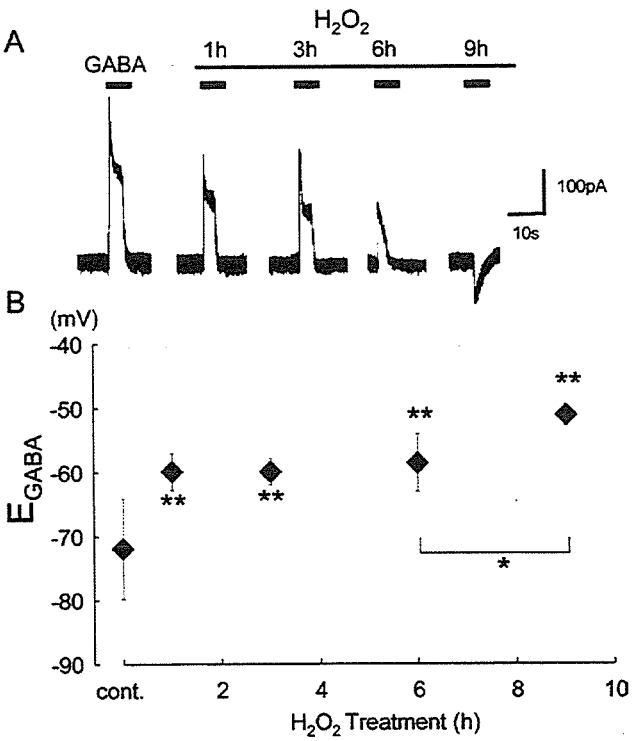

Figure 3. Changes in E_{GABA} in primary cultured hippocampal neurons during incubation with H_2O_2. ***A***, Representative whole-cell membrane currents recorded using the gramicidin-perforated patch technique, in response to application of GABA (30 μM; thick horizontal bars), recorded at a V_H of −50 mV, before and at different times during H_2O_2 (50 μM) incubation as indicated. The outward currents gradually decreased in amplitude, and eventually the response to GABA changed to an inward current response after 9 h incubation with H_2O_2. ***B***, Averaged E_{GABA} measured in neurons incubated with H_2O_2 for up to 9 h as indicated. Each data point represents the mean and SEM of E_{GABA} recorded in five neurons. H_2O_2 incubation caused E_{GABA} to rapidly (<1 h) shift to a more depolarized level resulting in a reduced outward current response at a V_H of −50 mV. Prolonged (>6 h) incubation with H_2O_2 caused an additional depolarizing shift in E_{GABA}, which could even result in an inward current at a V_H of −50 mV. $^{**}p < 0.01$ compared with the control E_{GABA}; $^{*}p < 0.05$ comparing E_{GABA} at 6 and 9 h. $[Cl^-]_o$, 159 mM.

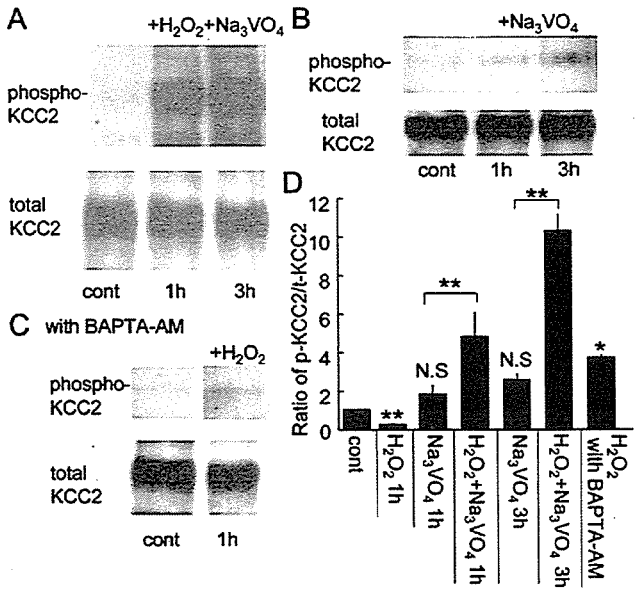

Figure 4. Effects of phosphatase inhibition and Ca^{2+} chelation on relative tyrosine phosphorylation of KCC2. ***A***, ***B***, Western blots showing expression levels of tyrosine-phosphorylated KCC2 (top rows) and total KCC2 (bottom rows) in control conditions and at 1 and 3 h after application of the phosphatase inhibitor Na_3VO_4 (100 μM) with (***A***) and without (***B***) H_2O_2. ***C***, Expression levels of tyrosine-phosphorylated KCC2 (top row) and total KCC2 (bottom row) in control conditions and at 1 h after application of H_2O_2 in the presence of BAPTA-AM (10 μM). ***D***, Averaged optical density of the ratio of tyrosine-phosphorylated KCC2 relative to total KCC2 expression levels in membrane of cultured hippocampal neurons incubated in Na_3VO_4 (100 μM) with or without H_2O_2 (1 and 3 h), or incubated with H_2O_2 and BAPTA-AM. Ratios are expressed relative to that in control conditions before incubation with any test compounds. A total of 25 μg of protein was added to each lane, and the same samples were analyzed for both total KCC2 and tyrosine-phosphorylated KCC2. Columns show mean and SEM of data from four experiments. $^{**}p < 0.01$ comparing Na_3VO_4, incubation with and without H_2O_2; $^{*}p < 0.05$ compared with control; N.S, not significantly different from control.

total KCC2 expression levels (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) (n = 4) (see also Fig. 4*A*). Incubation of cultured hippocampal neurons with Na_3VO_4 (100 μM) for 1–3 h, induced an approximate doubling of the proportion of KCC2 that was tyrosine phosphorylated (Fig. 4*B*; supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material), indicating a basal level of tyrosine kinase and phosphatase activity in control conditions. When neurons were incubated for 1 or 3 h with both Na_3VO_4 (100 μM) and H_2O_2 (50 μM), the proportion of KCC2 that was tyrosine phosphorylated increased markedly, by ~4- and 10-fold, respectively (Fig. 4*A*). In addition, an incubation with H_2O_2, and other possible tyrosine phosphatase blockers (e.g., phenylarsine oxide and Na_2MoO_4), but not a serine–threonine phosphatase blocker, okadaic acid, also increased the phosphorylated KCC2 (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). This is in stark contrast to the effects of H_2O_2 alone in causing a reduced proportion of phosphorylated KCC2 (Figs. 1, 4*D*) and suggests that oxidative stress stimulates an increased rate of both tyrosine phosphorylation and dephosphorylation (i.e., increased turnover), with dephosphorylation being dominant and the increased phosphorylation apparent only once phosphatase activity is blocked. The self-consistency of these results requires that H_2O_2 stimulates kinase activity, because otherwise the 10-fold stimulation in H_2O_2 + Na_3VO_4 at 3 h would be unexplained compared with the very modest stimulation in Na_3VO_4 alone and the small decrease by H_2O_2 alone as illustrated in Figure 4*D*. Under conditions of intracellular Ca^{2+} chelation with BAPTA-AM, H_2O_2 failed to decrease (and modestly increased) the proportion of phosphorylated KCC2 (Fig. 4*C*,*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 either 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_2O_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-

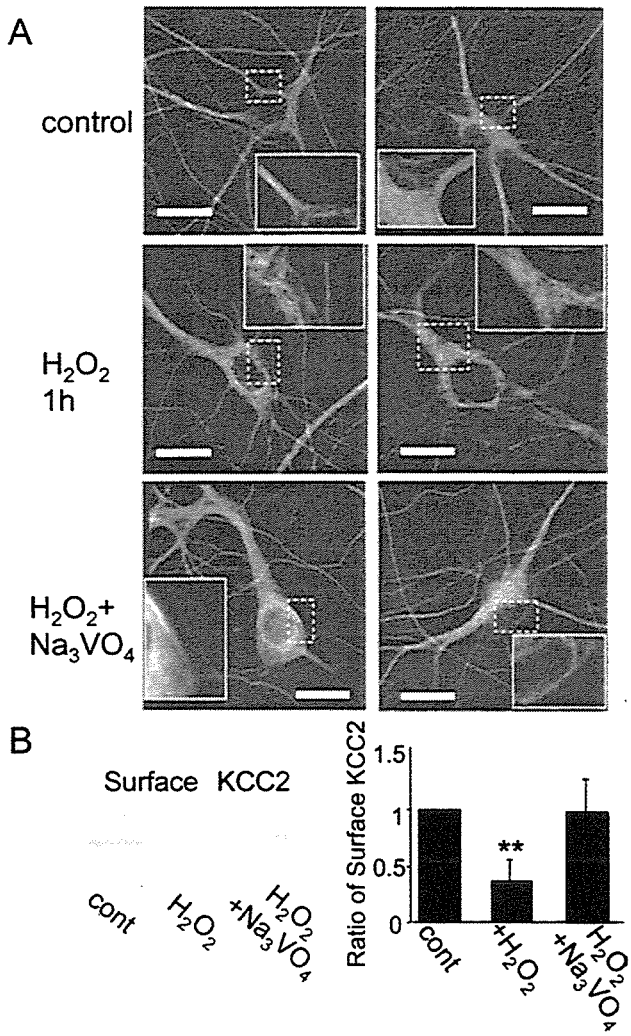

Figure 5. Immunofluorescent staining of the cellular localization of KCC2 and quantification of cell surface expression of KCC2 in cultured hippocampal neurons treated with H_2O_2. ***A***, KCC2 immunofluorescent images (red) in two control neurons (top panels) with MAP-2 staining (green). Note the staining 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 H_2O_2 incubation without (middle panels) and with the phosphatase inhibitor Na vanadate (Na_3VO_4) (100 μM) (bottom panels). Note the loss of surface expression of KCC2 in response to H_2O_2, which was not observed with additional Na_3VO_4 incubation. The insets in each panel show an enlargement of the area depicted by the dotted squares. Scale bars, 10 μm (for each panel). ***B***, Biotinylation assay of cell surface expression of KCC2, in control conditions, and after 1 h incubation in H_2O_2, with and without Na_3VO_4 (100 μM). 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 μg) was applied to each lane. Surface expression of KCC2 shows a significant decrease in response to H_2O_2, which is prevented by Na_3VO_4. $^{**}p < 0.01$ compared with control.

drites and throughout the cell soma (Fig. 5, control). One hour after incubation with H_2O_2, most of this surface immunostaining of KCC2 appeared to have been lost (Fig. 5, H_2O_2). After 1 h incubation with both H_2O_2 and Na_3VO_4, however, the surface expression of KCC2 was maintained (Fig. 5*A*, H_2O_2 + Na_3VO_4). The biotinylation assay also clearly demonstrated that H_2O_2 incubation resulted in a marked decrease in surface expression of KCC2 that could be prevented by coincubation with Na_3VO_4 (Fig. 5*B*).

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 H_2O_2 (50 μM) led to a loss of cell viability, with 25 ± 12% of neurons observed unable to exclude trypan blue (Fig. 6*A*). 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 $GABA_A$ receptors during oxidative stress could affect cell viability. Coapplication of the $GABA_A$ receptor agonist, muscimol (30 μM), during H_2O_2 incubation, resulted in an increase in the proportion of neurons that could not exclude trypan blue (Fig. 6*A*), indicating enhanced neuronal death. Conversely, coapplication of the $GABA_A$ receptor antagonist, 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)pyridazinium (SR 95531) (30 μM), resulted in a decrease in the proportion of neurons that could not exclude trypan blue. Muscimol (30 μM) alone did not affect this proportion ($n = 3$). Hence endogenous and exogenous activation of $GABA_A$ 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_{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. 6*B*). In control neurons, GABA (30 μM) evoked an inward current at a V_H of −50 mV, which reversed at −47.9 ± 5.4 mV (Fig. 6*B*, $n = 6$). 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). In contrast, in neurons expressing the construct, GABA induced an outward current at a V_H of −50 mV, which reversed at a more hyperpolarized potential of −58.6 ± 0.6 mV ($n = 6$). This indicates a reduced intracellular Cl^- resulting from overexpression of KCC2. These results also indicate that overexpression of KCC2 alone can induce functional transport in cultured neurons within the first week *in vitro*, despite other cellular processes that are typically required to mature to confer functional activity (Kelsch et al., 2001; Khirug et al., 2005). At 10 DIV, incubation with control neurons with H_2O_2 (50 μM) 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. 6*C*).

Our next approach to investigating functional consequences of KCC2 tyrosine phosphorylation during oxidative stress was to incubate neurons with both H_2O_2 (50 μM) and the tyrosine phosphatase inhibitor Na_3VO_4 (100 μM), resulting in an increased proportion of tyrosine-phosphorylated KCC2 (Fig. 4). After 3 h of Na_3VO_4 and H_2O_2 incubation, the loss of cell viability was markedly reduced (Fig. 6*C*). Decrease of the loss of cell viability could also be observed in an incubation with H_2O_2 and other possible tyrosine phosphatase blockers, phenylarsine oxide and Na_2MoO_4 (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). After more prolonged Na_3VO_4 and H_2O_2 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.

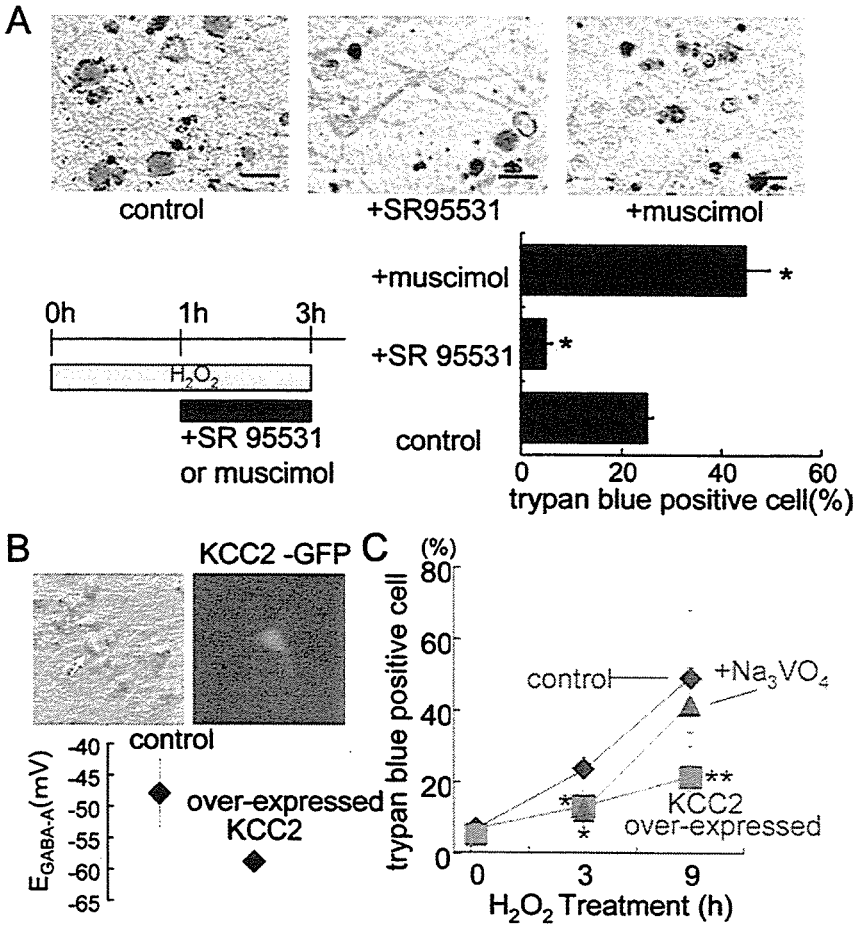

Figure 6. The role of KCC2 downregulation and $GABA_A$ receptor ligands on the survival of cultured hippocampal neurons during H_2O_2 incubation. ***A***, Top panels, Examples of cultured hippocampal neurons incubated with H_2O_2 (50 μM) for 3 h. Cell viability was assayed by trypan blue exclusion with neurons stained blue indicating cell damage and/or death. The left panel shows control neurons (H_2O_2 only). The middle panel shows neurons incubated with H_2O_2 and the $GABA_A$ receptor antagonist SR 95531 (30 μM). The right panel shows neurons incubated with H_2O_2 and the $GABA_A$ receptor agonist muscimol (30 μM). SR 95531 or muscimol were applied according to the protocol shown schematically in the bottom left panel. The right bottom panel graph shows averaged data showing the effects of H_2O_2 (50 μM) incubation for 3 h on cell viability (control) and that of additional incubation with muscimol or SR 95531. The averaged data derives from measurements of over 300 cells, in three different cell cultures. Error bars indicate SEM. $*p < 0.05$ compared with control. Scale bar, 10 μM. ***B***, Top panels, An image of culture neurons (left) and a fluorescent image of KCC2-GFP-overexpressed neurons (right). The arrow in the left image indicates the neuron expressing GFP, shown in the right image. The bottom graph shows E_{GABA} in control neurons (without GFP) and in KCC2-overexpressing neurons (with GFP; all at 4 DIV). E_{GABA} is significantly more hyperpolarized in KCC2-overexpressing neurons compared with control neurons. ***C***, The averaged time course of the loss of cultured hippocampal neuron (10 DIV) viability after 3 and 9 h H_2O_2 (50 μM) incubation (control). In neurons overexpressing KCC2 (KCC2 overexpressed; pink squares) identified with GFP, the loss of cell viability was reduced at 3 and 9 h. In neurons also incubated with the phosphatase inhibitor Na_3VO_4 (100 μM; green triangles), the loss of cell viability at 3 h was reduced but was back at control levels at 9 h. Error bars indicate SEM. $**p < 0.01$, $*p < 0.05$ compared with the same time point under control conditions.

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 $[Cl^-]_i$ 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 Ca^{2+} 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 $[Cl^-]_i$ 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. Little is known about the mechanisms underlying these processes in regards to cation-chloride transporters, despite the recent suggestion 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_3VO_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 Cγ 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 Cossart et 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 $GABA_A$ 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 $GABA_A$ receptors. Whittemore et al. (1995) reported, for cultured cortical neurons, that H_2O_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 pathological states.

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HIGH POTASSIUM-INDUCED FACILITATION OF GLYCINE RELEASE FROM PRESYNAPTIC TERMINALS ON MECHANICALLY DISSOCIATED RAT SPINAL DORSAL HORN NEURONS IN THE ABSENCE OF EXTRACELLULAR CALCIUM

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Abstract—The high potassium-induced potentiation of spontaneous glycine release in extracellular Ca^{2+}-free conditions was studied in mechanically dissociated rat spinal dorsal horn neurons using whole-cell patch-clamp technique. Elevating extracellular K^+ concentration reversibly increased the frequency of spontaneous glycinergic inhibitory postsynaptic currents (IPSCs) in the absence of extracellular Ca^{2+}. Blocking voltage-dependent Na^+ channels (tetrodotoxin) and Ca^{2+} channels (nifedipine and ω-grammotoxin-SIA) had no effect on this potassium-induced potentiation of glycine-release. The high potassium-induced increase in IPSC frequency was also observed in the absence of extracellular Na^+, although the recovery back to baseline levels of release was prolonged under these conditions. The action of high potassium solution on glycine release was prevented by BAPTA-AM, by depletion of intracellular Ca^{2+} stores by thapsigargin and by the phospholipase C inhibitor U-73122. The results suggest that the elevated extracellular K^+ concentration causes Ca^{2+} release from internal stores which is independent of extracellular Na^+- and Ca^{2+}-influx, and may reveal a novel mechanism by which the potassium-induced depolarization of presynaptic nerve terminals can regulate intracellular Ca^{2+} concentration and exocytosis. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: intracellular Ca^{2+} release, IPSC, patch-clamp, phospholipase C, depolarization.

It is well established that entry of Ca^{2+} through voltage-dependent Ca^{2+} channels plays a crucial role in the depolarization-induced release of neurotransmitters from presynaptic nerve terminals (Katz and Miledi, 1965). In addition to this depolarization-induced Ca^{2+}-influx, several studies have suggested that presynaptic depolarization per se can control the exocytotic machinery and promote neurotransmitter release even in the absence of extracellular Ca^{2+} (Parnas et al., 2000; Raiteri et al., 2002). However, the precise mechanisms by which this Ca^{2+} influx-independent release of neurotransmitters occurs, and the generality of this mechanism to central mammalian nerve terminals, have not been fully identified.

A number of different theories have been proposed for the mechanisms by which presynaptic depolarization can modulate transmitter release independent of Ca^{2+}-influx. In reviewing studies on more accessible peripheral motor nerve terminals, Parnas et al. (2000) have suggested that presynaptic depolarization causes a shift of the exocytotic machinery from an inactive to an active state. Links between presynaptic autoreceptors or presynaptic N-type Ca^{2+} channels and proteins involved with exocytosis have been suggested to mediate this effect. Other theories have proposed ionic mechanisms. Using rat brain synaptosome preparations, Storchak et al. (1998) reported that extracellular Ca^{2+}-independent release was completely abolished by removal of extracellular Na^+, suggesting that accumulation of Na^+ in the nerve terminals caused release of transmitters via a reversal of Na^+-amino acid co-transporters. Raiteri et al. (2002) also reported that elevation of extracellular K^+ increased Na^+-influx into the synaptosome, which then released Ca^{2+} from internal mitochondrial stores via the Na^+/Ca^{2+} exchanger, thereby increasing Ca^{2+}-dependent exocytosis.

A role of intracellular Ca^{2+} stores in contributing to synaptic transmission has been studied in rat cerebellum (Llano et al., 2000), rat basal ganglia (Rhee et al., 1999), frog neuromuscular junction (Narita et al., 2000) and chick retina (Warrier et al., 2005). In these reports, the release of Ca^{2+} from internal Ca^{2+} stores contributes to spontaneous and impulse-evoked vesicle exocytosis of transmitters. Furthermore, in nerve terminals isolated from magnocellular hypothalamic neurons, brief Ca^{2+}-transients caused by release from intracellular stores were directly recorded and the frequency of these events was reported to be increased by depolarization even in the absence of Ca^{2+}-influx (De Crescenzo et al., 2004). However, in this study, it was not possible to investigate the functional effects of such presynaptic depolarizations on transmitter release.

The present study was initiated to examine whether potassium-induced presynaptic depolarization affects spontaneous glycine release from spinal cord nerve ter-

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Abbreviations: IPSC, inhibitory postsynaptic current; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; TTX, tetrodotoxin; XeC, xestospongin C; 2-APB, 2-aminoethoxydiphenyl borate.

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minals in the absence of extracellular Ca^{2+}, and if so, to examine the underlying mechanisms. Using the mechanically dissociated rat spinal dorsal horn neurons, we found that, in the absence of extracellular Ca^{2+} influx, the high potassium solution does increase the frequency of spontaneous glycinergic inhibitory postsynaptic currents (IPSCs). This facilitation of glycine release involves release of Ca^{2+} from presynaptic Ca^{2-} stores.

EXPERIMENTAL PROCEDURES

Preparation

Wistar rats (12–17 days old) were decapitated under pentobarbital sodium anesthesia (80 mg/kg, i.p.), and a segment of the lumbosacral (L1–S1) spinal cord was dissected and transversely sliced at a thickness of 350 μm, using a microslicer (VT1000S; Leica, Nussloch, Germany). Slices were kept in a control incubation medium (see below) saturated with 95% O_2 and 5% CO_2 at room temperature (21–24 °C) for at least 1 h before mechanical dissociation. Slices were then transferred into a 35 mm culture dish (Primaria 3801; Becton Dickinson, Rutherford, NJ, USA) containing the standard external solution (see below), and the region of the substantia gelatinosa (lamina II) was identified under a binocular microscope (SMZ-1; Nikon, Tokyo, Japan). Details of the mechanical dissociation procedure used to isolate single neurons with adherent and functional presynaptic boutons have been given previously (Rhee et al., 1999; Akaike and Moorhouse, 2003). Briefly, mechanical dissociation was accomplished using a custom-built vibration device and a fire-polished glass pipette oscillating at about 60 Hz. The tip of the fire-polished glass pipette was lightly placed on the surface of the substantia gelatinosa and was vibrated horizontally (0.1–0.2 mm) for about 3 min. Slices were then removed and the mechanically dissociated neurons allowed to settle and adhere to the bottom of the dish for at least 15 min before recordings commenced. All experiments conformed to the guiding principles for the care and use of animals approved by the Council of the Physiological Society of Japan.

Electrical measurements

All electrical measurements were performed using the conventional whole-cell patch-clamp recording technique with a low Cl^- (12 mM) pipette solution and at a holding potential of 0 mV (EPC-9; Heka, Lambrecht, Germany). Patch pipettes were made from borosilicated capillary glass (G-1.5; Narishige, Tokyo, Japan) in two stages on a vertical pipette puller (PC-10; Narishige). The resistance of the recording pipettes filled with internal solution was 6–8 MΩ. Isolated neurons were viewed under phase contrast on an inverted microscope (DMIRB, Lica, Nussloch, Germany). Current and voltage were continuously monitored on an oscilloscope, and a pen recorder (WR3320, Graphtec, Tokyo, Japan). In most of the experiments, membrane currents were filtered at 2 kHz (FV-665, NF Electronic Instruments, Tokyo, Japan), digitized at 5 kHz, and stored on a personal computer equipped with pClamp 8.2 software (Axon Instruments, Union City, CA, USA). When rise time of spontaneous IPSCs was analyzed, the membrane currents were filtered at 8 kHz and recorded at sampling frequency of 20 kHz for a better time resolution. Experiments were performed at room temperature (21–24 °C).

Data analysis

Spontaneous IPSCs were counted and analyzed using the MiniAnalysis program (Synaptosoft, Leonia, NJ, USA), as described previously (Nakamura et al., 2003; Nabekura et al., 2004). Briefly, spontaneous events were screened automatically using an amplitude threshold of 3.5 pA and then visually accepted or rejected based upon their rise and decay times. The inter-event intervals and amplitudes of a large number of spontaneous IPSCs obtained from the same neuron were examined by constructing cumulative probability distributions, and these distributions were compared under different conditions using the Kolmogorov-Smimov test within the MiniAnalysis program. Numerical values are provided as means±S.E.M. using values normalized to the control. Differences in mean spontaneous IPSC amplitude and frequency were tested by Student's paired two-tailed test using their absolute values, rather than the normalized ones. Values of $P<0.05$ were considered significantly different.

Solutions and chemicals

The ionic composition of the incubation medium consisted of (mM): 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 24 $NaHCO_3$, 2.4 $CaCl_2$, 1.3 $MgCl_2$ and 10 glucose, saturated with 95% O_2 and 5% CO_2. The pH was 7.35–7.45. The standard external solution used for recordings from isolated neurons contained (mM): 150 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Hepes. The Ca^{2+}-free external solution contained (mM): 150 NaCl, 2.5 KCl, 3 $MgCl_2$, 2 EGTA, 10 glucose and 10 Hepes. When K^+ levels were increased, Na^+ was replaced with K^+. The Na^+-free solution was made by replacing all Na^+ of the Ca^{2+}-free solution with equimolar *N*-methyl-D-glucamine. Hypertonic solutions were made by adding 500 mM sucrose to extracellular solutions. These external solutions were adjusted to a pH of 7.4 with Tris-base. All external test solutions contained 10 μM CNQX and 10 μM AP-5 to inhibit glutamatergic postsynaptic currents. Unless indicated otherwise, the $GABA_A$ receptor antagonist SR-95531 (5 μM) was routinely added to the test solutions. The ionic composition of the internal (patch-pipette) solution for the whole-cell patch clamp recordings was (mM): 145 cesium methanesulfonate, 5 tetraethylammonium chloride, 5 CsCl, 2 EGTA, 4 ATP-Mg, 1 $MgCl_2$ and 10 Hepes, pH adjusted to 7.2 with Tris-base. The drugs and chemicals used in the present study were AP-5, ATP-Mg, BAPTA-AM, chelerythrine, CNQX, EGTA, nifedipine, ryanodine, strychnine, SR-95531, tetrodotoxin (TTX), thapsigargin, U-73343, wortmannin and xestospongin C (XeC) from Sigma (St. Louis, MO, USA), ω-grammotoxin SIA (Peptide Institute, Osaka, Japan), CGP-37157 and U-73122 from Tocris, Cookson (Avonmouth, UK) and 2-aminoethoxydiphenyl borate (2-APB) from Calbiochem (La Jolla, CA, USA). The test solutions containing drugs were applied using the Y-tube system for rapid solution change (Ishibashi and Akaike, 1995).

RESULTS

Identification of glycinergic spontaneous IPSCs and their dependence on extracellular Ca^{2+}

Fig. 1A shows the effect of strychnine on spontaneous IPSCs. The spontaneous IPSCs were fully and reversibly blocked by 1 μM strychnine, indicating that they were mediated by glycine receptors. Removal of extracellular Ca^{2+} reversibly reduced the frequency of spontaneous IPSCs to 22.9±4.4% of the control (Fig. 1B). The mean amplitude of spontaneous IPSCs was also reduced in the absence of extracellular Ca^{2+} (to 72.0±7.5% of the control). This reduced mean IPSC amplitude might be attributed to the reduced number of vesicles released or the reduction of quantal contents, as the currents evoked by exogenous application of 30 μM glycine in control and Ca^{2+}-free conditions were not significantly different ($n=6$; not shown). The results indicate that Ca^{2+} influx into these nerve terminals predominantly contributes to the genera-

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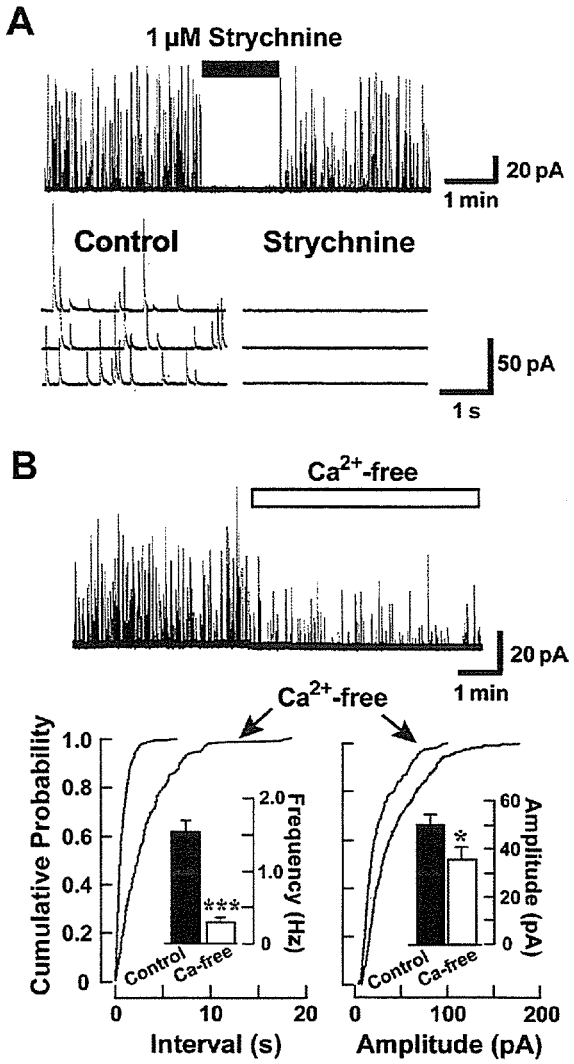

Fig. 1. Isolation of glycinergic spontaneous IPSCs in mechanically dissociated dorsal horn neurons, and the effect of Ca^{2+}-free external solution. Currents were recorded at a holding potential of 0 mV in the presence of 10 μM CNQX, 10 μM AP-5 and 5 μM SR-95531. (A) Effect of strychnine (1 μM) on the spontaneous IPSCs. Lower panels show typical current traces in the absence and presence of strychnine with an expanded time scale. The figure is representative of eight reproducible experiments showing that, under these conditions, spontaneous synaptic currents are exclusively glycinergic. (B) Effect of removal of extracellular Ca^{2+} on the characteristics of spontaneous IPSCs. Typical cumulative probability plots for inter-event intervals (lower left panel) and for IPSC amplitudes (lower right panel) for currents recorded with or without 2 mM extracellular Ca^{2+}. Insets show mean IPSC frequency (left) and amplitude (right) with each column representing mean±S.E.M. of the data from 12 neurons. * $P<0.05$, *** $P<0.001$.

tion of spontaneous IPSCs, but some residual glycine release remains in the absence of Ca^{2+} influx.

Modulation of spontaneous IPSCs by increased K^+ concentration in the absence of extracellular Ca^{2+}

In the presence of 2 mM Ca^{2+}, as shown in Fig. 2A, application of 30 mM K^+ rapidly evoked fast transient outward current. This transient outward current was fully inhibited by 3 μM strychnine ($n=4$, data not shown), suggesting that this current was caused by a massive release of glycine from presynaptic nerve terminals. In the Ca^{2+}-free solution that contained 2 mM EGTA, the initial transient outward current evoked by 30 mM K^+ was never observed. On the other hand, application of 30 mM K^+ in Ca^{2+}-free conditions slowly increased the spontaneous IPSC frequency (Fig. 2A). When we calculated the current charges carried by synaptic currents during application of 30 mM K^+ (Fig. 2B), the charge in the presence of 2 mM Ca^{2+} was increased immediately after application of 30 mM K^+. On the other hand, the integrated IPSC charge in the absence of extracellular Ca^{2+} was slowly increased after application of 30 mM K^+ (Fig. 2B).

To study the extracellular Ca^{2+}-independent increase of the IPSC, the following experiments were all performed in the absence of extracellular Ca^{2+}. Fig. 2C shows the cumulative distributions of the inter-event intervals and amplitudes of spontaneous IPSCs before, during and after application of 30 mM K^+. The distribution of inter-event intervals was significantly shifted to the left by high K^+ solution, indicating an increase in spontaneous IPSC frequency. The mean frequency of spontaneous IPSCs increased to 1250.3±266.1% during perfusion of 30 mM K^+ ($P<0.001$; $n=18$). The cumulative distribution of spontaneous IPSC amplitude was shifted to the right during 30 mM K^+ perfusion, with the mean IPSC amplitude being increased to 127.5±5.4% of control ($P<0.01$; $n=18$). However, the amplitude of outward currents induced by exogenously applied glycine (30 μM) in control and in the 50 mM K^+ solution was not significantly different (Fig. 2E), being 268±41 and 252±29 pA, respectively ($n=5$). The results suggest that high-potassium solution acts presynaptically to facilitate glycine release at these synapses in the absence of extracellular Ca^{2+}. The enhancement of mean IPSC frequency and amplitude was dependent on the concentration of extracellular K^+ (Fig. 2D), with frequency being significantly increased at all K^+ concentrations examined while mean amplitude was significantly increased at 30 and 50 mM. On the other hand, the application of high K^+ had no effect on the rise time of spontaneous IPSCs. The 10–90% rise time of all detected spontaneous IPSCs before and during application of 50 mM K^+, recorded in four neurons, was 0.99±0.02 ($n=131$) and 1.01±0.03 ms ($n=383$, $P>0.1$), respectively. To reveal whether the increase in IPSC frequency during application of the high K^+ solution is attributable to increase in the number of release-ready synaptic vesicles, we measured the readily releasable pool of synaptic vesicles. We recorded synaptic response to hypertonic sucrose (Fig. 2F) that triggers exocytosis of primed vesicles (Rosenmund and Stevens, 1996). In eight neurons examined, the responses to 0.5 M sucrose were significantly increased from control (364±56 pC) to 30 mM K^+ condition (570±89 pC, $P<0.05$). These findings suggest that the increase in spontaneous IPSC frequency might be accompanied with a significant increase in the number of primed vesicles.

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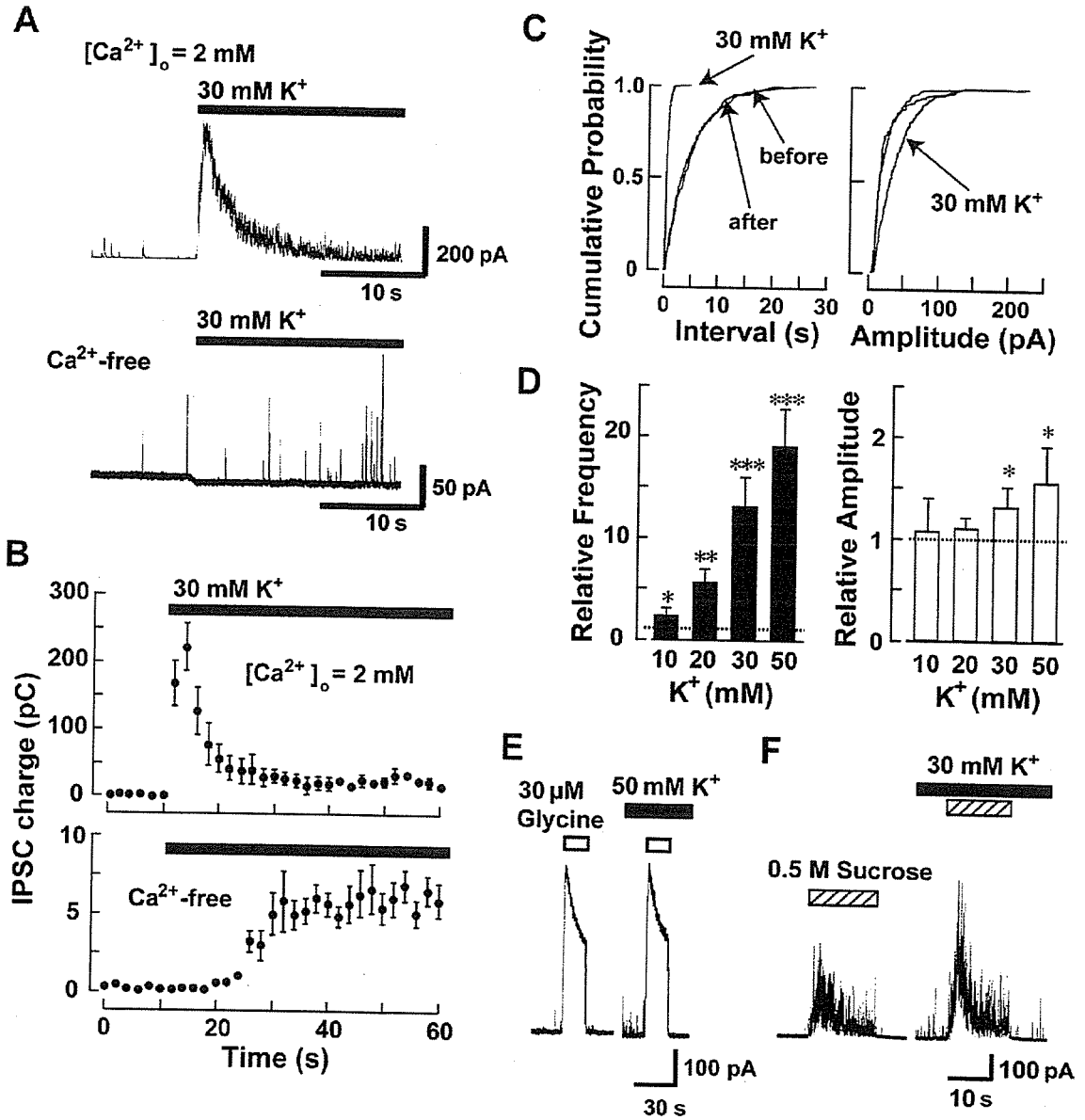

Fig. 2. High potassium-induced increase in spontaneous IPSCs in the absence of extracellular Ca^{2+}. (A) The current response induced by 30 mM K^+ (as indicated by bar) in the absence and presence (2 mM) of extracellular Ca^{2+}. The current traces in upper and lower panels were obtained from the same neuron. The figure was representative of five neurons. (B) Effect of 30 mM K^+ on the integrated IPSC charges. The integrated charge (pC) of IPSCs occurring every 2 s was pooled, and the mean values were plotted (B). Each point represents the mean±S.E.M. of the data from five neurons. (C) The effect of 30 mM K^+ on the cumulative distribution of IPSC frequency (left) and amplitude (right). (D) Concentration-dependent effects of elevated K^+ concentration on the frequency and amplitude of spontaneous IPSCs. Each column represents the mean±S.E.M. of the data from 6 to 15 neurons. Frequency and amplitude are expressed relative to values recorded during the control conditions. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. (E) Glycine (30 μM)-induced postsynaptic currents in control conditions and during perfusion with high K^+. (F) Effect of hypertonic sucrose (0.5 M) solution in control and high K^+ conditions. The figure was representative of eight reproducible experiments.

Effects of voltage-dependent Ca^{2+}- and Na^+-channel antagonists on the high potassium-induced facilitation of glycine release

Although our experimental conditions eliminated any Ca^{2+} influx, the high potassium-induced depolarization would still cause activation of presynaptic voltage-dependent Ca^{2+} channels. Activation of N-type Ca^{2+} channels has been reported to cause a Ca^{2+}-independent enhancement of transmitter release via interactions with presynaptic SNARE proteins involved in exocytosis (Mochida et al., 1998). Furthermore, dihydropyridine receptors are known to function in skeletal muscle as a voltage sensor, triggering intracellular Ca^{2+} release for excitation–contraction coupling (Iino, 1999). Thus, we investigated the effects of ω-grammotoxin SIA; which inhibits N- and P/Q-type voltage-activated Ca^{2+} channels by shifting the voltage de-

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pendence of activation to more positive values and by slowing the activation kinetics (McDonough et al., 1997), and of nifedipine, a dihydropyridine antagonist that inhibits charge movements and excitation–contraction coupling in skeletal muscle (Rios and Brum, 1987). Both ω-grammotoxin SIA (0.5 μM, n=5) and nifedipine (3 μM, n=6) had no effect on the basal spontaneous IPSC frequency (data not shown). In the presence of 0.5 μM ω-grammotoxin SIA and 3 μM nifedipine, the application of 30 mM K^+ still increased the spontaneous IPSC frequency to 1157.2± 117.9% (n=6) and 1216.0±283.8% (n=5), respectively (Fig. 3). These values were not significantly different from that obtained in control conditions. Thus, the voltage-dependent Ca^{2+} channels might not be involved in the high potassium-induced increase in spontaneous IPSC frequency.

The potassium-induced presynaptic depolarization might cause an influx of Na^+ into presynaptic nerve terminals through voltage-dependent Na^+ channels, which may itself promote release or give rise to an increased mitochondrial Na^+/Ca^{2+} exchange. Thus, we investigated the effects of the voltage-dependent Na^+ channel inhibitor TTX and mitochondrial Na^+/Ca^{2+} exchanger inhibitor CGP-37157 on the high K^+-induced increase in IPSC frequency. However, the application of 30 mM K^+ increased the spontaneous IPSC frequency to 1233.1±270.0% (n=5) and 1390.5±154.2% (n=5) in the presence of 1 μM TTX and 30 μM CGP-37157, respectively (Fig. 4A and C). These values were not significantly different from that obtained in control conditions. In addition, 30 mM K^+ increased the spontaneous IPSC frequency to 1310.8± 288.5% (n=7) in the absence of extracellular Na^+. This observation, coupled with the pharmacological experiments described above, also rules out Na^+ entry via voltage-dependent Ca^{2+} channels. However, the recovery of IPSC frequency after returning to the control K^+ concentration (2.5 mM) was prolonged in Na^+-free conditions (Fig. 4D). Extracellular Na^+ is important in homeostasis of intracellular Ca^{2+} concentration via the activity of the Na^+/Ca^{2+} exchanger of plasma membrane (Lee et al., 2002). This more sustained enhancement of high potassium-induced glycine release in the absence of external Na^+ led us to investigate any contribution of presynaptic intracellular Ca^{2+}.

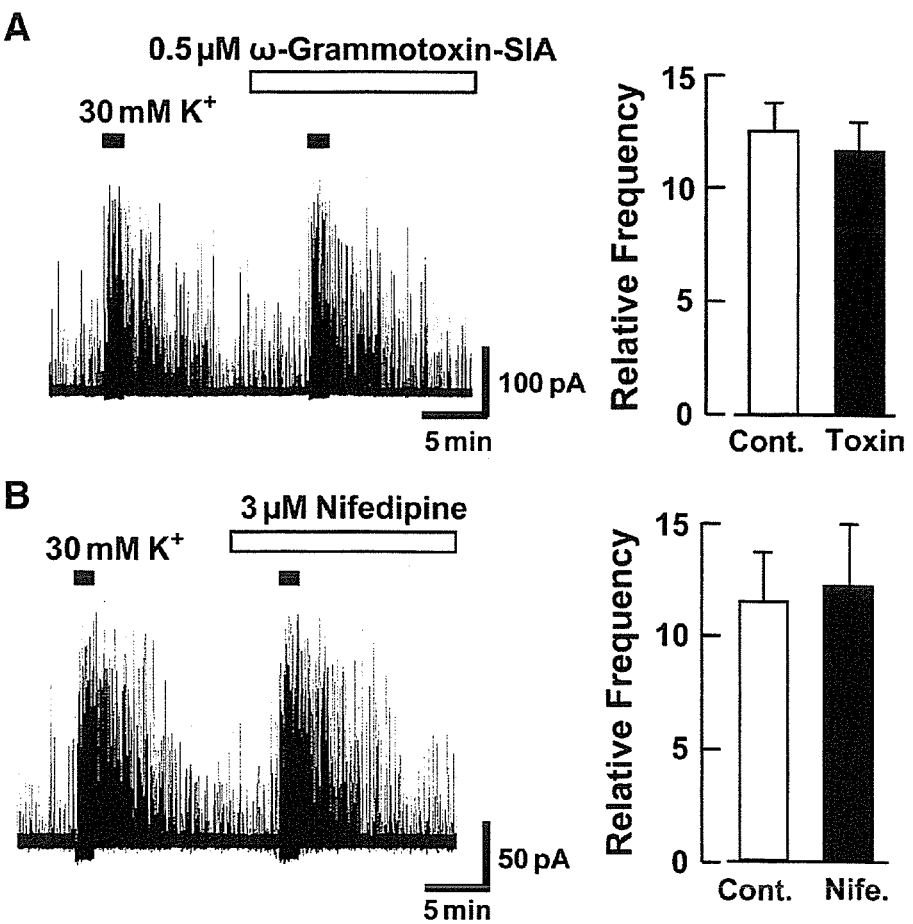

Fig. 3. Effects of Ca^{2+} channel antagonists on the high potassium-induced facilitation of glycine-release. (A) (Left panel) Current trace showing the effects of ω-grammotoxin-SIA (0.5 μM) on the high potassium-induced increase in the frequency of spontaneous IPSCs. (Right panel) Averaged effects of ω-grammotoxin-SIA (0.5 μM). Each column represents the mean±S.E.M. of the data from five neurons. (B) Typical example (left panel) and averaged data (right panel, mean±S.E.M., n=6) showing the effect of nifedipine (3 μM) on the high K^+-induced increase in spontaneous IPSCs.

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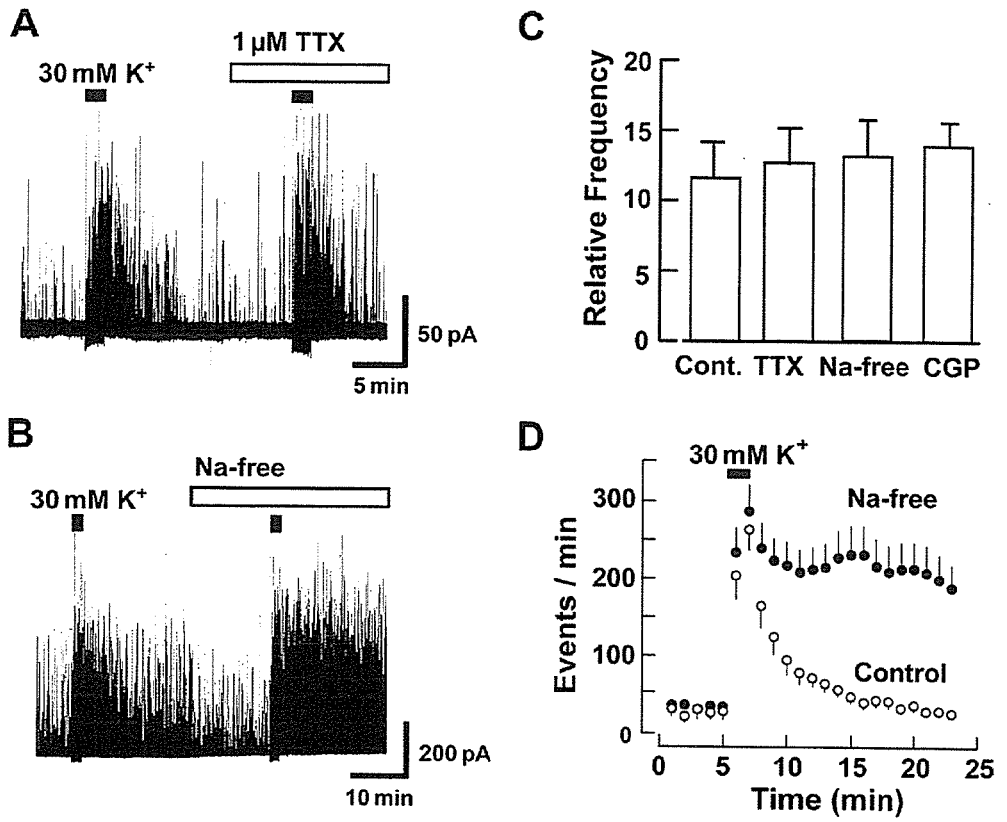

Fig. 4. The effects of voltage-dependent Na^+ channel inhibitor, mitochondrial Na^+/Ca^{2+} exchanger inhibitor and removal of extracellular Na^+ on the potassium-induced facilitation of spontaneous IPSC. (A) Sample trace showing the lack of effect of TTX (1 μM) on the high K^+-induced increase in spontaneous IPSCs. (B) Sample trace showing the more sustained effect of potassium-induced potentiation of spontaneous IPSCs in external Na^+-free conditions. For the Na^+-free solution, all external Na^+ was replaced with equimolar *N*-methyl-D-glucamine. (C) Averaged data showing the relative facilitation of spontaneous IPSC frequency in response to 30 mM K^+ under control condition (cont), in the presence of 1 μM TTX and 30 μM CGP-37157 (CGP), and in the absence of external Na^+-free. Each column represents the mean±S.E.M. of the data from five to seven neurons. (D) The time course of the K^+-induced potentiation of IPSC frequency in control and Na^+-free conditions. The number of spontaneous IPSCs occurring every 1 min was pooled and plotted. Each point represents the mean±S.E.M. of the data from seven neurons.

Facilitation is due to high potassium-induced release of Ca^{2+} from intracellular stores

To test the possibility that the enhanced release may result from an increase in presynaptic Ca^{2+}, we pretreated isolated neurons with BAPTA-AM, a membrane permeable Ca^{2+} chelator. BAPTA-AM (10 μM) itself reduced the spontaneous IPSC frequency to 71.0±9.8% of the control ($P<0.05$, $n=7$). In the presence of 10 μM BAPTA-AM, the high K^+-induced facilitation of IPSC frequency was greatly reduced to 146.8±18.9% of the BAPTA-AM condition ($n=7$, Fig. 5A). In addition, the high K^+-induced increase in the spontaneous IPSC amplitude was also inhibited in the presence of BAPTA-AM (98.4±5.5% of the BAPTA-AM condition, $n=7$). As influx of external Ca^{2+} was eliminated in our conditions we considered that Ca^{2+} must be arising from the release from intracellular stores.

Thapsigargin is known to inhibit endoplasmic reticulum (ER) Ca^{2+}-pump and therefore has been used to deplete intracellular Ca^{2+}-stores (Rhee et al., 1999; Yang et al., 2003). Application of thapsigargin (1 μM) in Ca^{2+}-free conditions increased the basal IPSC frequency. At 10 min after application, the IPSC frequency was increased from 0.247±0.04 to 1.97±0.76 Hz ($P<0.01$, $n=7$). At this point, 30 mM K^+ increased the IPSC frequency to 3.85±1.35 Hz ($n=7$). With 60 min treatment of thapsigargin, the basal IPSC frequency was reduced to 0.36±0.09 Hz, and the high K^+-induced increase in IPSC frequency was markedly reduced (169.8±20.8% of the thapsigargin condition, Fig. 5D), suggesting a contribution of intracellular Ca^{2+} release. On the other hand, ryanodine (50 μM), an inhibitor of endoplasmic reticulum ryanodine-sensitive Ca^{2+} release channels (Sutko et al., 1997), was without effect on the potassium-induced potentiation (Fig. 5D).

To investigate the signaling pathway to release of Ca^{2+} from internal stores, we firstly investigated the effects of the phospholipase C (PLC) inhibitor U-73122 and its inactive isomer U-73343. These agents had no significant effect on the basal IPSC frequency (96.2±7.5% of the control, $n=4$) and amplitude (104.2±9.3% of the control). When high K^+ was applied in the continued presence of either 1 μM U-73122 or 1 μM U-73343, we did not observe any robust potentiation of IPSC frequency ($n=4$, data not shown). When neurons were briefly (5 min) pretreated with 1 μM U-73122 before application of high K^+, U-73122

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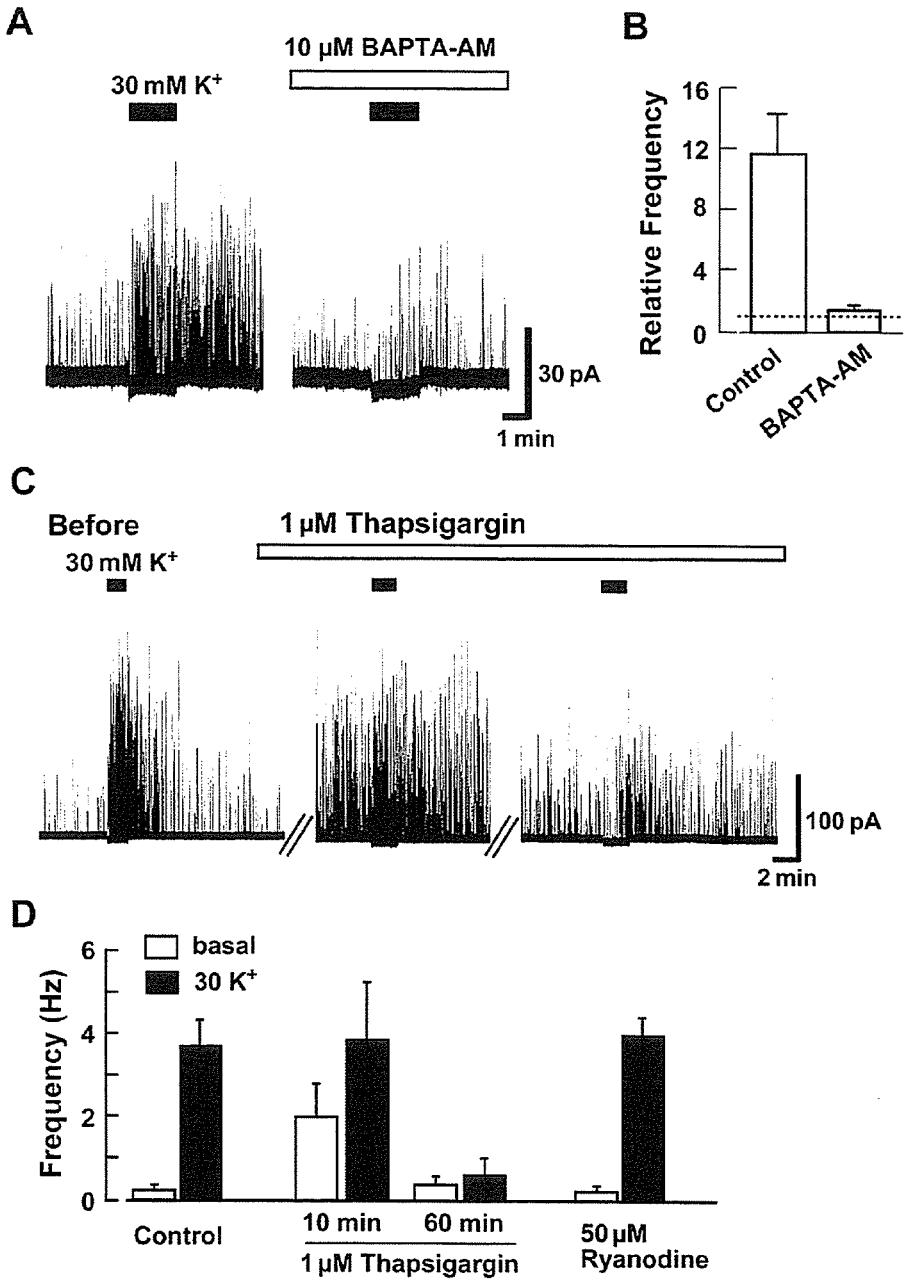

Fig. 5. Contribution of Ca^{2+} release from intracellular Ca^{2+} stores to the K^+-induced potentiation of glycine release. (A) Sample trace showing the effects of 30 mM K^+ on the frequency of spontaneous IPSCs in control conditions (left panel) and, in the same neuron, in the presence (right panel) of 10 μM BAPTA-AM. BAPTA-AM was continuously applied from 5 min before application of high-potassium. (B) Summary of the effect of BAPTA-AM on the facilitation of glycine release by high K^+. Each point represents the mean±S.E.M. of the data from seven neurons. (C) Sample trace showing the effect of depletion of intracellular Ca^{2+} stores by long-term treatment (60 min) with 1 μM thapsigargin. (D) Summary of the effect of 1 μM thapsigargin and 50 μM ryanodine on the relative enhancement of glycine release by high K^+. Each column represents the mean±S.E.M. of the data from five neurons.

selectively reduced the potentiation of IPSC frequency in response to subsequent application of 30 mM K^+ (Fig. 6A–C). In eight neurons tested, the high K^+ (30 mM)-induced increase in IPSC frequency before and after treatment of 1 μM U-73122 was 1104.8±143.5 and 245.8±60.3%, respectively. In addition, after the pretreatment of U-73122, 30 mM K^+ failed to cause a significant potentiation of the IPSC amplitude (105.8±7.4%, n=8). The irreversible inhibition of the PLC-mediated Ca^{2+} mobilization by U-73122 was reported in neuronal NG108-15 cells (Jin et al., 1994). The results suggest the contribution of PLC to the potassium-induced synaptic potentiation.

Activation of PLC cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) into two second messengers diacyl-

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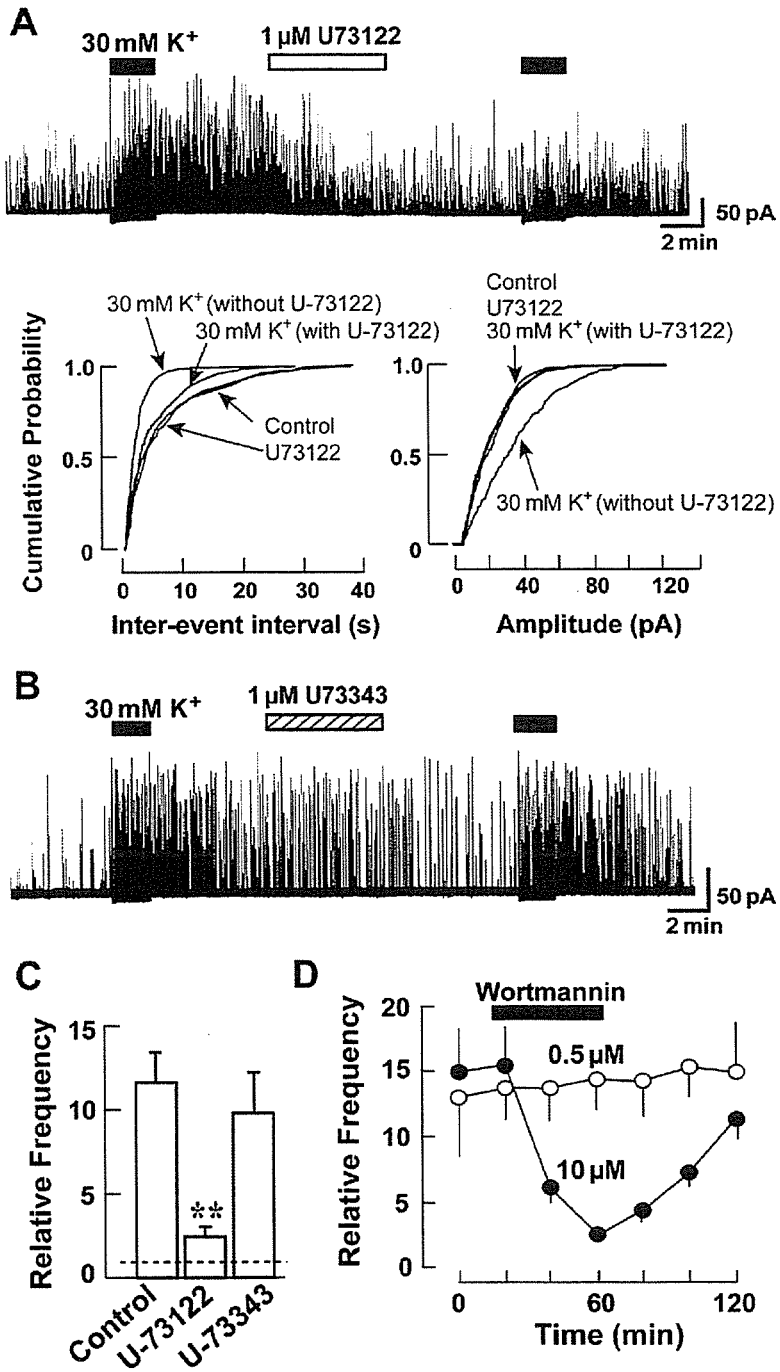

Fig. 6. Effects of U73122 and wortmannin on the K^+-induced potentiation of glycine release. (A) Sample current trace showing inhibition of the high potassium-induced potentiation of spontaneous glycine release by pretreatment with U-73122 (1 μM). Lower panels show cumulative distributions of inter-event interval (left panel) and amplitude (right panel) of spontaneous IPSCs in control conditions (control), after application of U-73122 (U-73122), and during application of 30 mM K^+ without or with U-73122 treatment. (B) Sample current trace showing lack of any effect of pretreatment with the inactive isomer U-73343 (1 μM) on the potassium-induced synaptic potentiation. (C) Averaged data showing the relative enhancement of spontaneous IPSC frequency in response to high K^+ in control conditions, and after brief pretreatment with U-73122 and U-73343. Each column represents the mean±S.E.M. of the data from five to eight neurons. ** $P<0.01$. (D) The high K^+ (30 mM)-induced potentiation of IPSC frequency before, during and after application of 0.5 μM (open circle) or 10 μM (closed circle) wortmannin. High K^+ solution was applied for 2 min every 20 min. Each point represents the mean±S.E.M. of the data from five to six neurons.

glycerol and inositol 1,4,5-trisphosphate (IP_3). Since diacylglycerol is known to activate protein kinase C (PKC), we investigated the effect of the PKC inhibitor chelerythrine on the high K^+ action. Application of 3 μM chelerythrine had

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