Chloride Influx Aggravates Ca^{2+}-Dependent AMPA Receptor-Mediated Motoneuron Death

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AMPA receptor-mediated excitotoxicity has been implicated in the pathogenesis of stroke, neurotrauma, epilepsy, and many neurodegenerative diseases such as motoneuron disease. We studied the role of Cl^- in AMPA receptor-mediated Ca^{2+}-dependent excitotoxicity in cultured rat spinal motoneurons. Using the gramicidin perforated patch-clamp technique, the intracellular Cl^- concentration could be calculated from the reversal potential of the GABA-induced current. The membrane depolarization caused by AMPA receptor stimulation resulted in Cl^- influx through 5-nitro-2(3-phenylpropyl-amino) benzoic acid- and niflumic acid-sensitive Cl^- channels. Cl^- influx during AMPA receptor stimulation aggravated excitotoxic motoneuron death by two mechanisms: an increase of AMPA receptor conductance and an elevation of the Ca^{2+} driving force through a partial repolarization. The Cl^- influx during AMPA receptor stimulation was enhanced by coadministration of GABA. This resulted in an increased Ca^{2+} influx and an enhanced cell death, suggesting that concomitant GABAergic stimulation may aggravate excitotoxic motoneuron death.

Key words: **AMPA receptor; calcium; motoneuron; chloride; excitotoxicity; amyotrophic lateral sclerosis**

Introduction

Influx of Ca^{2+} through glutamate receptors triggers neuronal death in stroke, neurotrauma, epilepsy, and many neurodegenerative diseases such as motoneuron disease (Coyle and Puttfarcken, 1993; Lipton and Rosenberg, 1994; Yamada, 1998; Doble, 1999). In motoneurons, Ca^{2+} influx through AMPA receptors is the major trigger for excitotoxic cell death (Carriedo et al., 1996; Van Den Bosch et al., 2002). A high proportion of Ca^{2+}-permeable AMPA receptors in motoneurons contributes to their selective vulnerability to excitotoxicity (Van Den Bosch et al., 2000; Van Damme et al., 2002b).

The role of movements of Cl^- ions in excitotoxicity is poorly understood. Osmotic swelling attributable to Na^+ and Cl^- influx is thought to contribute to acute excitotoxic cell death in neocortical and hippocampal neurons (Choi, 1987; Rothman et al., 1987). Membrane depolarization caused by glutamate receptor stimulation results in Cl^- influx and subsequent cell swelling in hippocampal neurons (Inglefield and Schwartz-Bloom, 1998). Segmental varicosity formation in dendrites during sustained glutamate receptor stimulation is dependent on influx of Na^+ and Cl^- ions (Hasbani et al., 1998), but these morphological changes are reversible and not associated with neuronal cell death (Hasbani et al., 1998; Al-Noori and Swann, 2000; Hasbani et al., 2001). Little is known about the role of Cl^- influx in Ca^{2+}-dependent motoneuron death.

In the present study, we studied AMPA receptor stimulation-induced changes in $[Cl^-]_i$ and its effects on Ca^{2+}-dependent excitotoxicity in cultured rat spinal motoneurons. Our findings suggest that Cl^- influx during AMPA receptor stimulation, which occurs through background Cl^- channels, or Cl^- influx mediated by $GABA_A$ receptor activation during excitotoxic stimuli, enhances Ca^{2+} influx and excitotoxic motoneuron death.

Materials and Methods

Motoneuron cultures. Motoneurons were cultured as described previously (Vandenberghe et al., 1998; Van Den Bosch et al., 2000) following procedures approved by the local ethical committee. In brief, ventral spinal cords were dissected from 14-d-old Wistar rat embryos and dissociated. A motoneuron-enriched neuronal population was purified from the ventral spinal cord by centrifugation on a 6.5% metrizamide cushion and was cultured on a pre-established glial feeder layer. The culture medium consisted of L15 supplemented with sodium bicarbonate (0.2%), glucose (3.6 mg/ml), progesterone (20 nM), insulin (5 μg/ml), putrescine (0.1 mM), conalbumin (0.1 mg/ml), sodium selenite (30 nM), penicillin (100 IU/ml), streptomycin (100 μg/ml), chick embryo extract (5%), and horse serum (2%). As described previously, >80% of the cells in culture are motoneurons as shown by immunostaining with the motoneuron marker peripherin (Van Damme et al., 2002a). Cultures were kept in a 7% CO_2 humidified incubator at 37°C. Neurons were used for experiments after 1 week in culture.

Cell death experiments. Motoneuron cultures on day 8 in culture were exposed to AMPA receptor agonists for 30 min at 37°C in a modified Krebs' solution (in mM): 122.3 NaCl, 5.9 KCl, 10 $CaCl_2$, 1.2 $MgCl_2$, 11.6 HEPES, and 11.5 glucose, pH 7.3 with NaOH. The NMDA receptor antagonist MK-801 (10 μM) was added during all exposures. The survival of motoneurons was quantified by counting motoneurons in a marked area of 1 cm^2 under phase contrast before and 24 hr after the exposure, with the observer blinded to the treatment protocol. For cell death experiments, *n* refers to the number of cultures studied.

Electrophysiology with combined Ca^{2+} measurements. For perforated patch-clamp recordings, gramicidin, which is impermeable to Cl^- and leaves the $[Cl^-]_i$ undisturbed (Abe et al., 1994; Akaike, 1994; Rhee et al., 1994; Kyrozis and Reichling, 1995), was used. Pipettes were back-filled with pipette solution containing 50–75 μg/ml gramicidin, after tip-filling with gramicidin-free solution. Gramicidin was dissolved in DMSO (50 $\mu g/\mu l$) before each experiment. Pipettes had a resistance of 2–4 MΩ when filled with intracellular solution. After seal formation, the progress

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of perforation was followed by evaluating the decrease in series resistance. Cells were accepted for study if the series resistance (R_s) dropped below 30 MΩ and remained stable during the experiment. All recordings were perforated-patch recordings, unless stated otherwise. For whole-cell recordings, R_s was ≤10 MΩ, and no gramicidin was added to the pipette solution unless the perforated patch was ruptured, as indicated. Cells were held at a membrane potential of −60 mV, and *I–V* relationships were generated using voltage ramps from −100 to +50 mV. If the extracellular Cl^- concentration ($[Cl^-]_e$) was changed, an agar bridge was used as bath electrode. Using the Nernst equation, $[Cl^-]_i$ was calculated from the reversal potential of the GABA-induced current. To avoid GABA-induced changes in $[Cl^-]_i$, brief pulses of 50 μM GABA were given, and ramps from −100 to −40 mV were applied to limit Cl^- currents during ramping.

Experiments were performed in the presence of 500 nM tetrodotoxin (TTX), 10 μM MK-801, and 100 μM Cd^{2+} to block voltage-gated Na^+ channels, NMDA receptors, and voltage-operated Ca^{2+} channels, respectively. Niflumic acid (NFA) and 5-nitro-2(3-phenylpropyl-amino) benzoic acid (NPPB) were used as Cl^- channel blockers. Kainate and (±)-AMPA hydrobromide were used as agonists of AMPA receptors. Application of 100 μM kainate induced large inward currents at negative membrane potentials. The selective AMPA receptor blocker LY300164 inhibited 95.5 ± 1.5% of the kainate current at −60 mV ($n = 5$), indicating that in this culture model, kainate stimulates almost exclusively AMPA receptors, as has been described previously (Van Den Bosch et al., 2000).

Signals were recorded using an L/M-EPC7 List-Medical amplifier, filtered at 3 kHz, sampled at 2 kHz, and analyzed off-line (Digidata 1200, pClamp8; Axon Instruments). The normal pipette solution consisted of (in mM): 125 CsCl, 1.2 $MgCl_2$, 10 HEPES, 2 Na_2ATP, and 1 EGTA, pH adjusted to 7.3 with CsOH. The standard extracellular solution contained (in mM): 99.1 NaCl, 30 TEACl, 5.9 KCl, 3.2 $CaCl_2$, 1.2 $MgCl_2$, 11.6 HEPES, 11.5 glucose, pH adjusted to 7.3 with NaOH. TEACl was added to this solution to avoid kainate-induced inhibition of voltage-gated K^+ channels (Van Damme et al., 2002a). To lower the extracellular Cl^- concentration, NaCl and TEACl were replaced by methanesulfonic acid (free acid) or isethionic acid (sodium salt). Because both anions can bind Ca^{2+}, the free Ca^{2+} concentration was measured and corrected. No changes in intracellular pH (using the pH-sensitive dye 5-and-6-carboxy SNARF-1 AM) were noted when the $[Cl^-]_e$ was lowered (data not shown). The Ca^{2+}- and Na^+-free solution was a nominal Ca^{2+}-free solution supplemented with 2 mM EDTA disodium salt, and Na^+ was replaced by equimolar amounts of *N*-methyl-D-glucamine.

For Ca^{2+} imaging combined with electrophysiology, neurons were incubated with Calcium Green-5N AM in the culture medium for 20 min at 37°C. Calcium Green-5N was dissolved in DMSO (2 μg/μl) and used at a final concentration of 5–10 μM with 0.02% pluronic acid. Neurons were illuminated at 488 nm, and emitted fluorescence was collected at wavelengths >515 nm using a photomultiplier tube. Fluorescence signals collected from regions covering the cell soma and proximal neurites were filtered at 100 Hz, sampled at 2 kHz, and analyzed off-line (Digidata 1200, pClamp 8; Axon Instruments). No Cd^{2+} was added during Ca^{2+} measurements, and verapamil (100 μM) was used as an inhibitor of voltage-gated Ca^{2+} channels, as indicated. Intracellular Ca^{2+} signals are shown as $\Delta F/F$, i.e., fluorescence increase divided by prestimulus fluorescence.

Materials and statistics. Media and additives were obtained from Invitrogen (Grand Island, NY), TTX was from Calbiochem (San Diego, CA), MK-801 was from Tocris Cookson (Bristol, UK), Calcium Green-5N AM and pluronic acid were from Molecular Probes (Eugene, OR), and gramicidin was from Fluka (Seelse, Germany). LY300164 was kindly provided by Dr. J. D. Leander from Eli Lilly (Indianapolis, IN). All other chemicals were from Sigma (St. Louis, MO). Average data are shown as mean ± SEM, and Student's *t* tests were used to calculate significance. When more than two groups were compared, a one-way ANOVA with Bonferroni comparison was used.

Results

Accumulation of intracellular Cl^- during AMPA receptor-mediated depolarization enhances excitotoxic motoneuron death

To elucidate the role of Cl^- and Cl^- channels in motoneuron excitotoxicity, we first estimated $[Cl^-]_i$ in cultured motoneurons. We recorded GABA-induced currents with the gramicidin perforated patch-clamp technique and calculated $[Cl^-]_i$ from E_{GABA}, the reversal potential of the GABA-induced current (Abe et al., 1994; Akaike, 1994; Rhee et al., 1994; Kyrozis and Reichling, 1995). Under control conditions(extracellular $[Cl^-]$ = 143.8 mM), E_{GABA} amounted to −58.5 ± 0.7 mV, which corresponded to a $[Cl^-]_i$ of 14.6 ± 0.4 mM (range 8.3–26.2 mM; $n = 73$). These values are similar to what has been reported previously (Li et al., 1998). In contrast, under "whole-cell" conditions with the standard pipette solution (127.4 mM Cl^-), the GABA-induced current reversed at −0.1 ± 1.5 mV ($n = 5$), which approximated the predicted Cl^- equilibrium potential of −3 mV. These observations indicate that the gramicidin perforated patch-clamp technique preserves $[Cl^-]_i$, thereby validating this technique to monitor changes in $[Cl^-]_i$ during motoneuron stimulation.

Using this technique, we determined $[Cl^-]_i$ in motoneurons under depolarizing or hyperpolarizing conditions. First, we applied 100 μM kainate for 30 sec under current-clamp conditions, which depolarized the membrane potential from its resting value of −57.3 ± 1.0 to −12.1 ± 0.7 mV ($n = 78$; $p < 0.001$). Concomitantly, E_{GABA} shifted to a more positive potential, which corresponded to a rise in $[Cl^-]_i$ from 14.7 ± 0.9 to 19.5 ± 1.0 mM ($n = 21$; $p < 0.001$) (Fig. 1*A*). The elevation of $[Cl^-]_i$ during AMPA receptor stimulation was abolished by Cl^- channel blockers (Fig. 1*B*): in the presence of 50 μM NPPB or 100 μM NFA, $[Cl^-]_i$ was 14.3 ± 1.1 mM before and 14.1 ± 1.2 mM after kainate application in current clamp ($n = 6$; $p = 0.24$).

We evaluated whether this Cl^- influx could be explained by the kainate-induced membrane depolarization, which generates a net electrochemical driving force for Cl^- influx. We therefore attempted to mimic the accumulation of $[Cl^-]_i$ under voltage-clamp conditions. Indeed, clamping the membrane potential for 30 sec at −20 mV shifted E_{GABA} to more positive potentials equivalent to an increase of $[Cl^-]_i$ from 16.9 ± 1.1 to 22.9 ± 1.5 mM ($n = 15$; $p = 0.002$) (Fig. 1*C*). In contrast, hyperpolarization to −90 mV resulted in a small reduction of $[Cl^-]_i$ to 14.2 ± 1.2 mM ($n = 15$; $p < 0.001$). Again, these changes in $[Cl^-]_i$ were blocked by application of NPPB (50 μM) (Fig. 1*D*) or NFA (100 μM; data not shown) which is consistent with movement of Cl^- ions through Cl^- channels.

This presumed Cl^- current in motoneurons was further characterized by studying the NPPB- and NFA-inhibited current in a Ca^{2+}- and Na^+-free external solution (Fig. 1*E*). The NPPB- and NFA-sensitive current displayed outward rectification with a current amplitude of 0.49 ± 0.05 pA/pF at 0 mV ($n = 10$) (Fig. 1*F*). After rupture of the perforated patch and establishing whole-cell conditions with the pipette solution containing 127.4 mM Cl^-, the current trace shifted to more positive potentials (Fig. 1*F*). The near absence of inward currents precluded a reliable determination of a reversal potential for the NPPB- and NFA-sensitive currents. However, the parallel movement of the current trace and of the predicted E_{Cl} after increasing intracellular Cl^- suggests that the NPPB- and NFA-sensitive current is carried by Cl^- ions. The NPPB- and NFA-sensitive current could still be elicited in the presence of 50 μM picrotoxin and 50 μM bicuculline (data not shown).

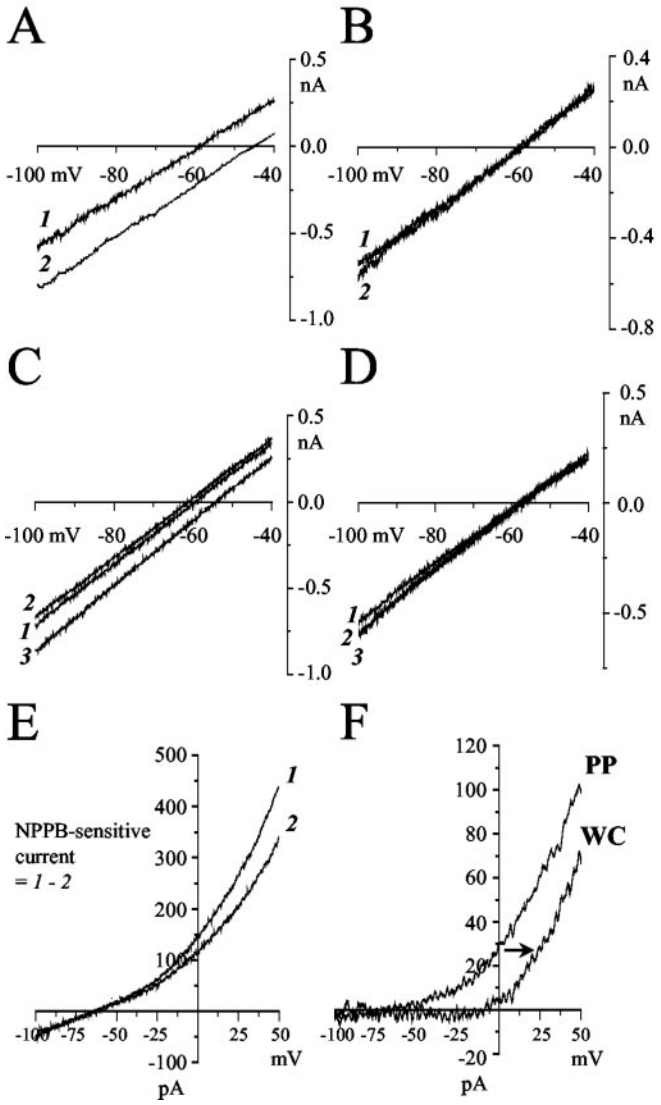

Figure 1. Influx of Cl^- during depolarization. *A*, *I–V* relation of the GABA-induced current (50 μM) obtained by subtraction of a voltage ramp from −100 to −40 mV before GABA application from a ramp during GABA application before (1) and immediately after (2) 30 sec of kainate application in current clamp. A shift of E_{GABA} toward more positive potentials can be noted, corresponding to an increase in $[Cl^-]_i$. *B*, *I–V* relation of GABA currents (50 μM) before (1) and immediately after (2) 30 sec of kainate application in current clamp in the presence of 50 μM NPPB, recorded from the same motoneuron as in *A*. The shift of E_{GABA} during kainate application is prevented. *C*, *I–V* relation of GABA currents (50 μM) from a holding potential of −60 mV (1), after 30 sec of clamping the membrane at −90 mV (2), and after 30 sec of clamping at −20 mV (3). A shift of E_{GABA} toward the clamped potential can be noted. *D*, *I–V* relation of GABA current (50 μM) from a holding potential of −60 mV (1), after 30 sec of clamping the membrane at −90 mV (2), and after 30 sec of clamping at −20 mV (3) in the presence of 50 μM NPPB, recorded from the same motoneuron as in *C*. The shifts in E_{GABA} are prevented. *E*, A voltage ramp from −100 to +50 mV was given before (1) and after (2) 30 sec application of 50 μM NPPB in a Ca^{2+}- and Na^+-free external solution. Note the reduction of the membrane conductance at positive potentials. 1–2 yields the NPPB-inhibited current. *F*, *I–V* relation of the NPPB-inhibited current in a Ca^{2+}- and Na^+-free external solution obtained in the same neuron with the perforated patch-clamp technique (PP). After rupture of the perforated patch [whole cell (WC)], the NPPB-sensitive current shifts toward the calculated E_{Cl} of −2.6 mV, confirming that it is a Cl^- current.

The finding that E_{GABA} (−58.5 ± 0.7 mV) approximates the resting membrane potential (−57.3 ± 1.0 mV) suggests that in motoneurons Cl^- is passively distributed over the plasma membrane. In addition, motoneurons possess a background outwardly rectifying Cl^- conductance sensitive to NPPB and NFA

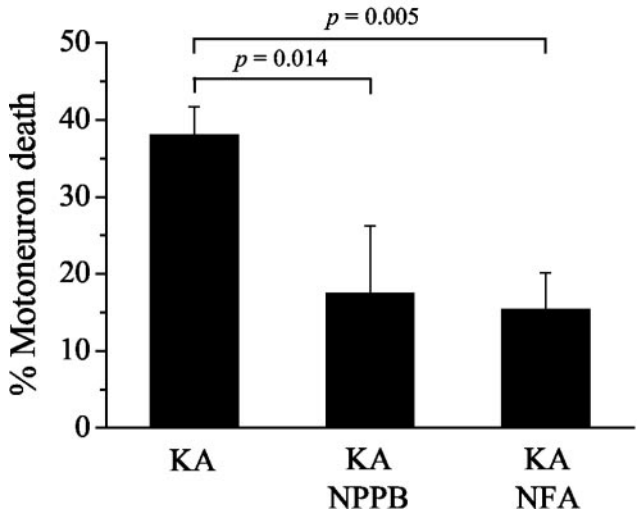

Figure 2. Effect of Cl^- channel blockers on AMPA receptor-mediated excitotoxicity. Motoneuron death induced by a 30 min exposure to 300 μM kainate (KA) in the absence and presence of the Cl^- channel blockers NPPB (50 μM; $n = 5$) and NFA (100 μM; $n = 6$).

that allows accumulation of intracellular Cl^- ions during membrane depolarization.

Subsequently, we investigated whether the intracellular accumulation of Cl^- during kainate application affected the AMPA receptor-mediated Ca^{2+}-dependent motoneuron death. As shown in Figure 2, NPPB (50 μM) and NFA (100 μM), which prevented Cl^- accumulation during kainate application as mentioned, substantially protected against kainate-induced motoneuron death. Motoneuron death was reduced by 47.4 ± 22.1% ($n = 5$; $p = 0.014$) and 62 ± 11.2% ($n = 6$; $p = 0.005$) by NPPB and NFA, respectively.

Part of this protective effect could be caused by a small but statistically significant inhibitory effect of NPPB and NFA on the AMPA receptor currents. The inhibition of the AMPA receptor current at −60 mV was 13.8 ± 1.8% ($n = 18$; $p < 0.001$) for 50 μM NPPB and 8.5 ± 1.5% for 100 μM NFA ($n = 13$; $p < 0.001$). The AMPA block by NPPB and NFA was independent of changes in $[Cl^-]_i$, because it was also observed in the whole-cell configuration with 127 mM Cl^- in the pipette solution (data not shown).

These findings suggest that Cl^- influx induced by AMPA receptor-mediated depolarization enhances the AMPA receptor-mediated Ca^{2+}-dependent motoneuron death.

The effect of Cl^- on AMPA receptor-mediated intracellular Ca^{2+} accumulation and excitotoxicity in motoneurons

To further elucidate whether changes in $[Cl^-]_i$ affected kainate-induced motoneuron death, we manipulated $[Cl^-]_i$ by incubating motoneurons in an extracellular solution with low $[Cl^-]$ ($[Cl^-]_e \cong 16$ mM: NaCl replaced by methanesulfonic acid or isethionic acid).

Incubation of motoneurons in a low $[Cl^-]_e$ solution induced a progressive decline of $[Cl^-]_i$ to 3.3 ± 0.7 mM ($n = 10$; $p < 0.001$) (Fig. 3*A*), and this occurred with a time constant of 21.8 ± 3.2 sec ($n = 5$). This intracellular depletion of Cl^- ions had a clear effect on AMPA receptor-mediated excitotoxicity, as expected. In low $[Cl^-]_e$ conditions, kainate-induced motoneuron death was reduced by 50.4 ± 9.8% ($n = 4$; $p = 0.021$) and 52.1 ± 9.9% ($n = 6$; $p = 0.002$) in the methanesulfonic acid- and the isethionic acid-containing solutions, respectively (Fig. 3*B*). These changes were not accompanied by changes in intracellular pH (see Mate-

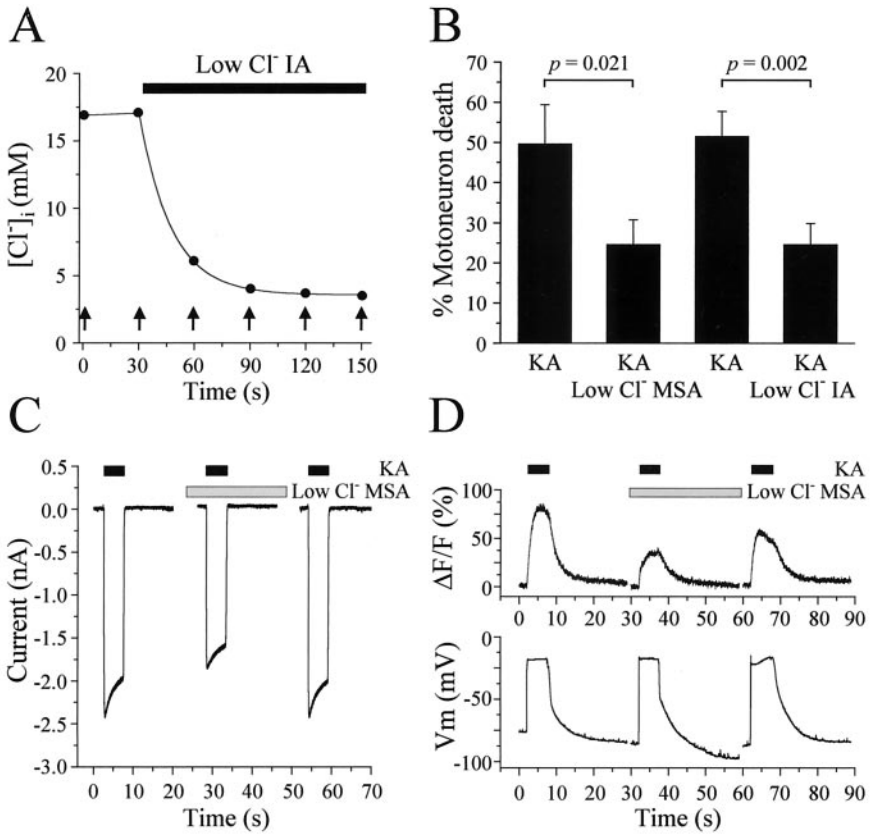

Figure 3. Lowering the extracellular Cl^- concentration reduces kainate-induced currents, Ca^{2+} transients, and motoneuron death. *A*, Time course of $[Cl^-]_i$, calculated from E_{GABA}, which was determined every 30 sec during brief pulses of 50 μM GABA (indicated by arrows) before and during a switch to a low $[Cl^-]_e$. A low $[Cl^-]_e$ (~16 mM) was obtained by replacing NaCl with isethionic acid (IA). *B*, Motoneuron death induced by a 30 min exposure to 300 μM kainate (KA) in normal or low $[Cl^-]_e$. The low $[Cl^-]_e$ solution was obtained by replacing NaCl with methanesulphonic acid (MSA) ($n = 4$) or isethionic acid (IA) ($n = 6$). C, KA-induced current at −60 mV in normal and low $[Cl^-]_e$. The low $[Cl^-]_e$ was allowed to wash in before and wash out after the measurement for 30 sec. *D*, KA-induced Ca^{2+} transients and changes in membrane potential in a normal and low $[Cl^-]_e$. Ca^{2+} signals are shown as increase in fluorescence divided by baseline fluorescence ($\Delta F/F$).

rials and Methods). To study the neuroprotective mechanism of lowering $[Cl^-]_e$ on excitotoxic motoneuron death, we studied its effect on kainate-induced AMPA receptor currents and Ca^{2+} transients. The AMPA receptor current was reduced by 20.3 ± 2.9% at −60 mV ($n = 11$; $p = 0.009$) in the methanesulfonic acid-containing buffer and by 23.0 ± 2.4% in the isethionic acid-containing buffer ($n = 10$; $p < 0.001$) (Fig. 3*C*). Concomitantly, Ca^{2+} transients induced by application of kainate were reduced by 27 ± 6% ($n = 11$; $p = 0.001$) and 25 ± 5% ($n = 9$; $p = 0.001$) in the methanesulfonic acid- and the isethionic acid-containing buffer, respectively (Fig. 3*D*, top trace). Surprisingly, the kainate-induced depolarization did not significantly change in the low $[Cl^-]_e$ solution [by 0.9 ± 2.1 mV ($n = 6$; $p = 0.68$) and 2.2 ± 2.4 mV ($n = 6$; p 0.40) in the methanesulfonic acid- and the isethionic acid-containing buffer, respectively (Fig. 3*D*, bottom trace)]. These findings suggest that under normal $[Cl^-]_e$ conditions, the influx of Cl^- during kainate application exerts a repolarizing effect, thereby limiting the AMPA receptor-mediated depolarization. Under conditions of low $[Cl^-]_e$, the Cl^- influx is reduced and consequently has almost no repolarizing effect. Thus, the simultaneous reduction by low $[Cl^-]_e$ of both depolarizing (Na^+ and Ca^{2+} influx) and repolarizing (Cl^- influx) currents results in a comparable degree of depolarization.

Several factors may contribute to the reduction of AMPA receptor currents in the presence of low $[Cl^-]_e$: (1) an inhibitory effect of low $[Cl^-]_e$ on the AMPA receptor; (2) an inhibitory effect oflow $[Cl^-]_i$ on the AMPA receptor; (3) an inhibitory effect of methanesulfonic acid and isethionic acid themselves on the AMPA receptor; and (4) a desensitization of the AMPA receptor induced by endogenous glutamate release. To distinguish among these components, we manipulated the $[Cl^-]_i$ in the presence of normal $[Cl^-]_e$ by varying the Cl^- concentration in the pipette solution ($[Cl^-]_{pip}$) in whole-cell recordings. The AMPA receptor current density induced by 20 μM kainate at −60 mV was 6.0 ± 0.8 ($n = 18$), 8.9 ± 1.0 ($n = 18$), and 10.3 ± 0.9 pA/pF ($n = 12$) for a $[Cl^-]_{pip}$ of 3, 17.4, and 127.4 mM, respectively ($p = 0.01$). This correlation between $[Cl^-]_i$ and the current amplitude suggests that the reduction of AMPA receptor currents in low $[Cl^-]_e$ is caused by an inhibitory effect of low $[Cl^-]_i$ on the AMPA receptor. Furthermore, AMPA receptor currents were also reduced by 24.4 ± 0.8% ($n = 3$) in low $[Cl^-]_e$ in the presence of 100 μM of the inhibitor of AMPA receptor desensitization, cyclothiazide, suggesting that AMPA receptor desensitization caused by endogenous glutamate release is not involved.

Taken together, these data suggest that Cl^- influx during AMPA receptor stimulation potentiates excitotoxic motoneuron death through an enhancement of AMPA receptor conductance.

Cl^- influx during AMPA receptor stimulation induces a relative hyperpolarization, which enhances the Ca^{2+} influx

The potentiating effect of $[Cl^-]_i$ on AMPA receptor currents was observed under voltage-clamp conditions (−60 mV), indicating that at least part of the amplification of the AMPA current by the Cl^- influx is independent of the membrane potential. In the next set of experiments, we tested whether Cl^- influx could also increase AMPA currents through its repolarizing effect on the membrane potential. Therefore, we studied the effect of a forced hyperpolarization step on the AMPA receptor-induced Ca^{2+} transient. This was done in the presence of 100 μM verapamil to minimize changes in intracellular Ca^{2+} concentrations caused by Ca^{2+} influx through voltage-gated Ca^{2+} channels. Application of 100 μM kainate for 30 sec in current clamp resulted in a stable elevation of the Ca^{2+} signal and the membrane potential (Fig. 4*A*). A repolarization step of 15.5 ± 1.1 mV during kainate stimulation resulted in an increase of the Ca^{2+} signal of 117 ± 23% ($n = 8$; $p = 0.002$) (Fig. 4*B*). Thus, Cl^- influx during kainate stimulation can affect AMPA currents and the subsequent Ca^{2+} transient through two mechanisms: (1) accumulation of $[Cl^-]_i$, which potentiates the AMPA receptor (as shown before), and (2) a repolarization that increases the driving force for Na^+ and Ca^{2+} influx.

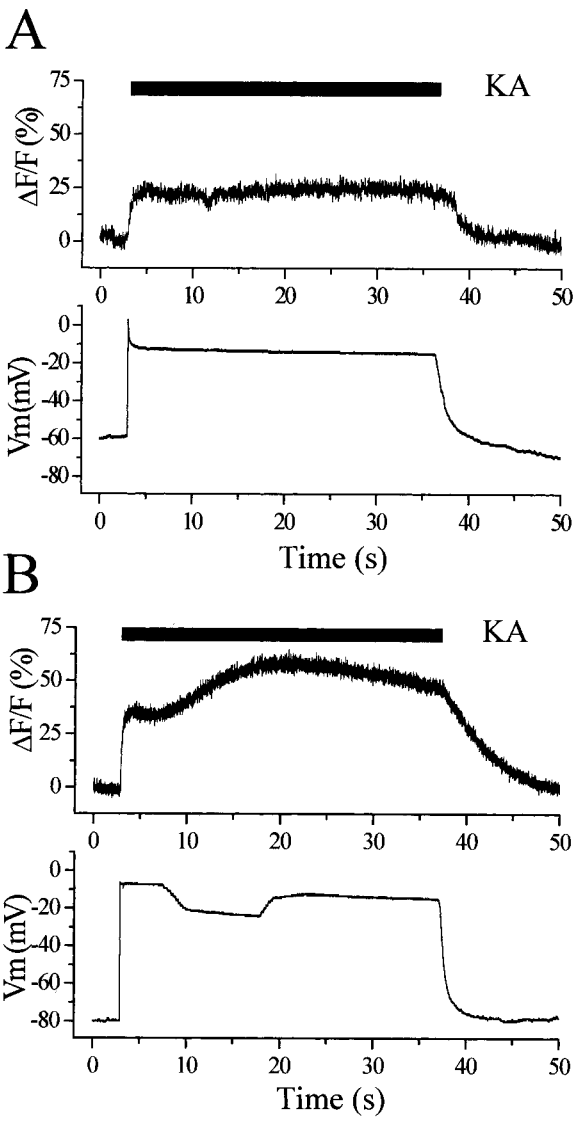

Figure 4. Repolarization during AMPA receptor stimulation enhances the Ca^{2+} influx. *A*, Kainate (KA)-induced Ca^{2+}-transient (top trace) and changes in membrane potential (bottom trace) during a longer application of 100 μM KA (±30 sec). To limit Ca^{2+} flux through voltage-gated Ca^{2+} channels during changes in membrane potential, 100 μM verapamil was added. *B*, A repolarization in current clamp during a KA application (bottom trace) induces a large increase in the Ca^{2+} signal (top trace). In this example, a 74% increase of the Ca^{2+} signal was achieved.

GABA enhances AMPA receptor-mediated motoneuron death

Motoneurons are known to have a high GABAergic and glycinergic input (Price et al., 1976; Magoul et al., 1987; Carlton and Hayes, 1990; Plaitakis, 1991), and during conditions of excitotoxicity a concomitant release of glutamate and GABA occurs. As a consequence, changes of $[Cl^-]_i$ that may affect AMPA receptor-induced cellular processes may occur during concomitant GABAergic and excitotoxic stimulation. We therefore studied the effects of GABA-induced Cl^- changes on AMPA receptor currents, AMPA receptor-mediated depolarizations, AMPA receptor-mediated Ca^{2+}-transients, and AMPA receptor-mediated excitotoxicity.

Application of 200 μM GABA at −90 mV for 30 sec induced a large inward current (= Cl^- efflux) (Fig. 5*A*), resulting within 15 sec in a large shift of E_{GABA} to more negative potentials (Fig. 5*B*, trace 2), corresponding to a decrease of $[Cl^-]_i$ to 4.9 ± 0.2 mM ($n = 5$). Conversely, application of GABA at −20 mV generated a large outward GABA current (= Cl^- influx) (Fig. 5*A*), which resulted in a large shift of E_{GABA} to more positive potentials (Fig. 5*B*, trace 3), corresponding to an elevation of $[Cl^-]_i$ to 44.0 ± 4.1 mM ($n = 5$). The AMPA receptor current amplitude was determined before and immediately after the imposed changes in $[Cl^-]_i$. GABA application at −90 mV, which resulted in a change of $[Cl^-]_i$ to 4.9 mM, reduced the AMPA receptor current by 24 ± 3% ($n = 9$; $p < 0.001$). In contrast, GABA application at −20 mV, which increased $[Cl^-]_i$ to 44 mM, enlarged the AMPA receptor current by 13 ± 5% ($n = 10$; $p = 0.048$) (Fig. 5*C*). Furthermore, when the whole-cell AMPA receptor current recordings with different $[Cl^-]_{pip}$ were normalized to the average value of the 17.4 mM-containing pipette solution (although 17.4 mM is not exactly the same as the basal $[Cl^-]_i$), the results of the whole-cell measurements and perforated-patch experiments could be plotted on the same graph (Fig. 5*D*) ($p = 0.004$), demonstrating the potentiating effect of $[Cl^-]_i$ on the AMPA receptor current.

Application of 200 μM GABA during kainate application in current clamp repolarized the membrane potential in steady-state conditions with 6.5 ± 1.4 mV ($n = 7$; $p = 0.003$) and augmented the Ca^{2+} transient by 94 ± 33% ($n = 7$; $p = 0.01$) (Fig. 5*E*). The Ca^{2+} increase during GABA application can be ascribed to the concomitant Cl^- accumulation that potentiates the AMPA receptor current (see above) and to the repolarization that provides a larger driving force for Ca^{2+}. Because kainate-induced excitotoxicity critically depends on the influx of Ca^{2+} via AMPA receptors, one would predict that inhibitory neurotransmitters such as GABA actually enhance excitotoxic motoneuron death. As shown in Figure 5*F*, application of 250 μM GABA indeed increased motoneuron death during kainate exposure by 38.1 ± 6.2% ($n = 5$; $p = 0.012$) (Fig. 5*F*). The excitotoxicity-aggravating effect of GABA was blocked by 50 μM picrotoxin (Fig. 5*F*), confirming that it is linked to Cl^- influx through the $GABA_A$ receptor. Application of 50 μM picrotoxin or 1 μM strychnine without exogenous GABA application did not affect the kainate-induced motoneuron death ($n = 4$; $p = 0.496$ and 0.379, respectively), indicating that there is no endogenous GABA release in our culture system.

AMPA receptor desensitization does not prevent Cl^- influx and aggravation of excitotoxic motoneuron death

Physiological AMPA receptor agonists rapidly induce AMPA receptor desensitization, and AMPA receptor desensitization has been shown to limit excitotoxic neuron death (Zorumski et al., 1990; Van Den Bosch and Robberecht, 2000). In previous experiments, kainate, which induces little desensitization, was used. The desensitizing agonist AMPA, which elicited steady-state currents that were only 30.6 ± 6.0% of the kainate currents ($n = 7$) (Fig. 6*A*), induced smaller amounts of excitotoxic motoneuron death, but nevertheless a considerable degree of membrane depolarization and Cl^- influx was observed. A high input resistance in motoneurons can explain these findings. Levels of membrane depolarization, Cl^- influx, and motoneuron death induced by kainate and AMPA are summarized in Table 1. Cl^- influx during AMPA receptor stimulation with AMPA also aggravated excitotoxic motoneuron death. In a low Cl^--containing external solution, AMPA-induced motoneuron death was reduced by 47.5 ± 14.1% ($n = 3$; $p = 0.016$), and GABA application aggravated AMPA-induced motoneuron death with 51.6 ± 13.1% ($n = 3$; $p = 0.015$) (Fig. 6*B*).

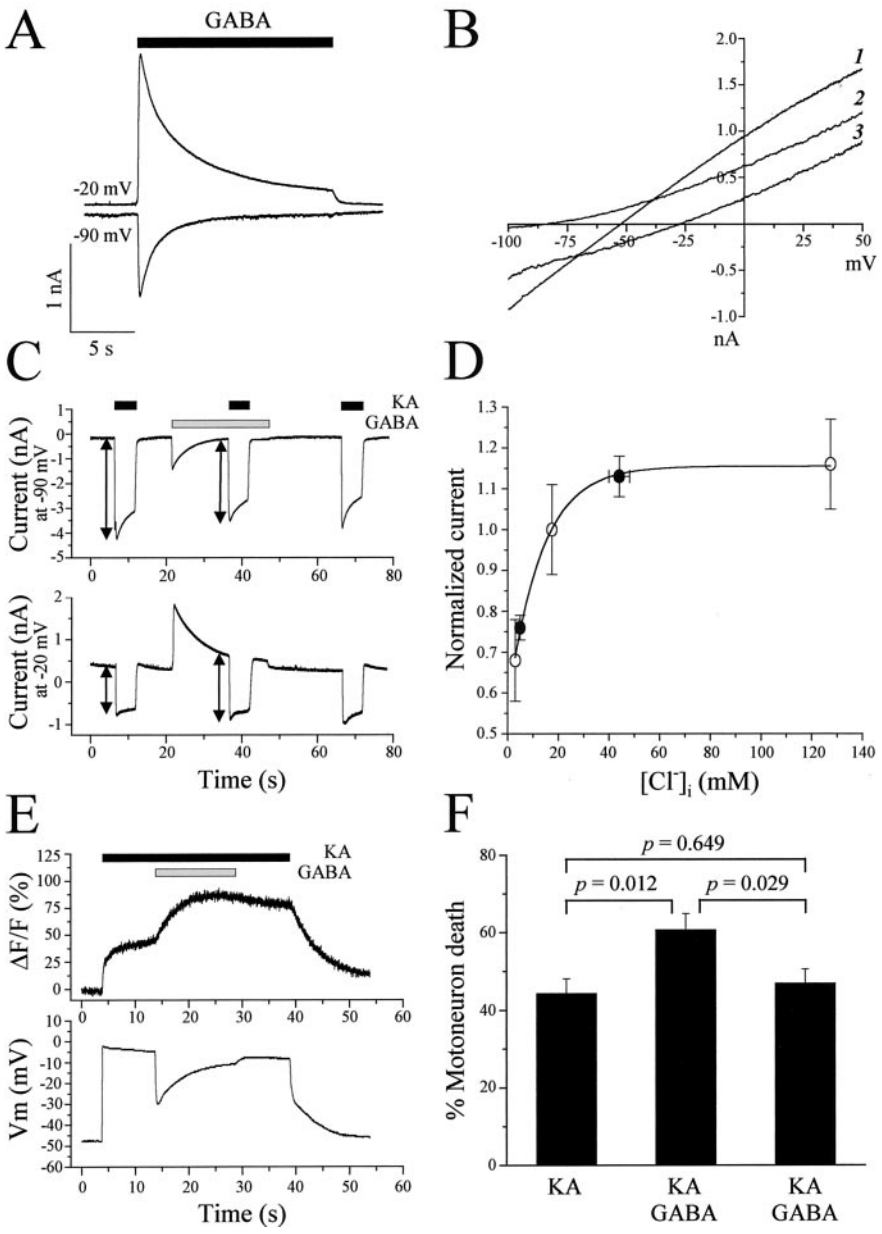

Figure 5. Effects of GABA on $[Cl^-]_i$, AMPA receptor currents, depolarization, Ca^{2+}-signals, and excitotoxic motoneuron death. *A*, GABA-induced current (200 μM) at −90 and −20 mV. *B*, *I–V* relation of GABA current (200 μM) at rest (1), after 15 sec GABA application at −90 mV (2), and after 15 sec GABA application at −20 mV (3). A large shift of E_{GABA} toward the clamped potential can be noted. *C*, Kainate (100 μM) (KA)-induced current before and after 15 sec application of GABA at −90 mV (top trace) and at −20 mV (bottom trace) using the gramicidin perforated patch-clamp technique. GABA application at −90 mV reduces $[Cl^-]_i$ to 5 mM, whereas GABA application at −20 mV heightens $[Cl^-]_i$ to 44 mM. The concomitant changes in the AMPA receptor current amplitude are indicated by arrows. *D*, Relation between AMPA receptor current amplitude and $[Cl^-]_i$. Whole-cell recordings of kainate-induced currents (20 μM) at −60 mV were performed for three different pipette solutions with 3, 17.4, and 127.4 mM Cl^-, respectively. Normalization of current densities to the average of the 17.4 mM-containing pipette solution allowed presentation of these whole-cell measurements (○) on the same plot as the perforated-patch recordings (●). Data points could be fit with a first order exponential equation. The physiological $[Cl^-]_i$ (14.6 mM) is situated on the steep part of the curve. *E*, Application of 200 μM GABA reduces the KA (100 μM)-induced depolarization (bottom trace). This is accompanied by an increased Ca^{2+} influx (top trace). To limit Ca^{2+} flux through voltage-gated Ca^{2+} channels during changes in membrane potential, 100 μM verapamil was added. *F*, Motoneuron death induced by a 30 min exposure to 300 μM KA in the absence and presence of 250 μM GABA ($n = 5$). The excitotoxicity–aggravating effect of GABA is prevented by application of 50 μM picrotoxin ($n = 5$).

Discussion

The intracellular Cl^- concentration ($[Cl^-]_i$) is an important parameter for neuronal excitability (Li et al., 1998; Singer et al., 1998; Ehrlich et al., 1999; Kakazu et al., 1999; Rivera et al., 1999). The precise value of $[Cl^-]_i$ in neurons depends on the balance between active extrusion of Cl^- from the cytosol (e.g., by K^+-Cl^- cotransporters or Cl^- pumps), active accumulation of Cl^- in the cytosol (e.g., by Na^+-K^+-2 Cl^- cotransporters), and passive movement through Cl^- channels in the plasma membrane. In this study, we show that in cultured rat spinal motoneurons, the E_{Cl} is close to the resting membrane potential, indicating a relatively weak activity of the active transporters relative to the background Cl^- conductance. Indeed, electrophysiological characterization of these motoneurons revealed an outwardly rectifying Cl^- current that was sensitive to NPPB and NFA. Furthermore, we show that this particular constellation (passive Cl^- distribution and a background Cl^- channel) significantly modulates the neuronal response to excitotoxic stimuli.

Accumulation of Cl^- ions in response to AMPA receptor stimulation

A critical finding in this study is the intracellular accumulation of Cl^- in motoneurons during stimulation of AMPA receptors in current clamp. Kainate and AMPA were used as agonists of AMPA receptors. Kainate displayed little desensitization, whereas AMPA-induced currents desensitized quickly to steady-state levels of only 30% of the kainate-induced currents. Because motoneurons have a low input resistance (Van Damme et al., 2002a), application of AMPA in current clamp still resulted in a considerable degree of depolarization and Cl^- influx. Because a voltage clamp to −20 mV resulted in a nearly identical increase in $[Cl^-]_i$, we conclude that Cl^- accumulation during kainate or AMPA application can be ascribed entirely to a change in electrochemical driving force favoring net Cl^- influx. Indeed, from the near-equilibrium distribution of Cl^- at resting membrane potential (approximately −60 mV), it follows that a depolarization, as triggered by kainate or AMPA, reduces the outwardly directed electrical gradient such that the inwardly directed concentration gradient starts to dominate and creates a net driving force for Cl^- influx. Because the Cl^- influx during depolarization was abolished in the presence of NPPB and NFA, we conclude that Cl^- ions enter motoneurons through these NPPB- and NFA-sensitive outwardly rectifying Cl^- channels. The molecular identity of this Cl^- channel remains unknown because of the lack of sufficiently specific tools to identify neuronal Cl^- channels at the molecular level. It is unlikely that this Cl^- channel is a ligand-gated channel, because Cl^- influx also occurred in the absence of any added agonist, e.g., during membrane depolarization in volt-

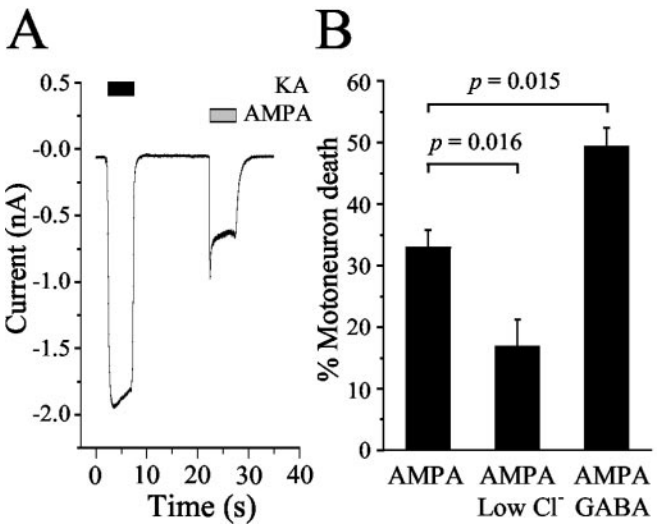

Figure 6. Cl^- influx aggravates AMPA-induced motoneuron death. *A*, Kainate (KA)- (100 μM) and AMPA (100 μM)-induced current at −60 mV recorded from the same motoneuron. *B*, Motoneuron death induced by a 30 min exposure to 1 mM AMPA in normal extracellular solution, in low $[Cl^-]_e$ solution with methanesulphonic acid, and in normal extracellular solution with 250 μM GABA ($n = 3$).

age clamp. The Cl^- current in motoneurons can also not be ascribed to any of the known plasma membrane ClC channels, because expression of ClC-1 and ClC-Ka/b is restricted to, respectively, skeletal muscle (ClC-1) and the kidney and the inner ear (ClC-Ka/b)(Jentsch et al., 2002). ClC-2 has a broader expression pattern, including the brain, but it is activated by hyperpolarization and closed at potentials more than −40 mV (Thiemann et al., 1992; Jentsch et al., 2002), which is difficult to reconcile with the Cl^- influx during depolarization of motoneurons. Moreover, voltage-gated Cl^- channels have not yet been described in motoneurons. Because the Cl^- influx was observed in the absence of protein kinase A or G stimulation, one can also exclude the cystic fibrosis transmembrane conductance regulator. Possible candidates for the motoneuron Cl^- current are background Cl^- channels or Ca^{2+}-activated Cl^- channels. An outwardly rectifying, NPPB-sensitive background Cl^- current has been described in endothelial cells and probably corresponds to volume-regulated anion channels (VRAC) that are partially activated under isotonic conditions (Voets et al., 1996). Ca^{2+}-activated Cl^- channels (CaCC) have been described in motoneurons (Owen et al., 1984, 1986), can display outward rectification (Nilius et al., 1997; Frings et al., 2000), and can be blocked by NPPB and NFA (Nilius et al., 1997). Unfortunately, VRAC and CaCC have not yet been identified at the molecular level (Jentsch et al., 2002), which leaves the hypothesis that VRAC or CaCC channels, or both, are responsible for the Cl^- influx in motoneurons difficult to test.

Cl^- influx aggravates excitotoxic motoneuron death

A second remarkable finding is that Cl^- influx during AMPA receptor stimulation significantly contributes to the excitotoxic motoneuron death. As described previously, Ca^{2+} influx through Ca^{2+}-permeable AMPA receptors is critical for AMPA receptor-mediated motoneuron death *in vitro* (Carriedo et al., 1996; Van Den Bosch et al., 2000, 2002; Van Damme et al., 2002b). It was shown previously that AMPA receptor desensitization limits excitotoxicity (Zorumski et al., 1990; Van Den Bosch and Robberecht, 2000). Likewise, AMPA resulted in smaller amounts of motoneuron death as compared with kainate. We now show that conditions that reduce Cl^- influx (e.g., NPPB, NFA, low $[Cl^-]_e$) protect motoneurons against both kainate- and AMPA-induced excitotoxicity. At least two mechanisms contribute to this protective effect. First, the AMPA receptor current amplitude is directly proportional to the $[Cl^-]_i$, with the steepest part of the curve situated around the $[Cl^-]_i$ found in resting motoneurons. Consequently, a small increase or decrease in $[Cl^-]_i$ during AMPA receptor stimulation will significantly alter the AMPA receptor current and hence the degree of cell death. At present, the mechanism of the modulation of AMPA receptors by Cl^- ions remains elusive. This modulation seems to be independent of changes in intracellular pH, because no changes in intracellular pH were observed if the $[Cl^-]_e$ was lowered. Cl^- ions may have a direct stimulatory effect on AMPA receptors, or alternatively, Cl^- may operate indirectly, e.g., by altering the phosphorylation status of the receptor. There are some indications that Cl^- ions favor the activation of G-proteins (Higashijima et al., 1987), and Cl^--sensitive protein kinases have been described (Lanius et al., 1993; Treharne et al., 1994; Muimo et al., 1998).

A second reason why reduced Cl^- influx protects against AMPA receptor-mediated cell death is that repolarization caused by Cl^- influx effectively clamps the membrane potential, thereby maintaining a large driving force for Ca^{2+} influx. Thus, the combination of inhibition of the AMPA receptor caused by intracellular Cl^- depletion on the one hand and a reduced driving force for Ca^{2+} entry on the other hand explains the neuroprotection observed in low extracellular Cl^-. In contrast, the protective effect of the Cl^- channel blockers NPPB and NFA cannot be attributed solely to reduced Cl^- influx, because these compounds also had a small but significant inhibitory effect on the AMPA receptor. NPPB and NFA are also known to inhibit NMDA receptors (Lerma and Martin del Rio, 1992). The combined activity of AMPA receptor block and Cl^- channel block constitutes a unique pharmacological profile with respect to neuroprotection against glutamate-mediated excitotoxicity.

GABA aggravates excitotoxic motoneuron death

A correlate of the neuroprotective action by reduced Cl^- influx is that increased Cl^- influx could worsen excitotoxic cell death. Indeed, simultaneous application of GABA and kainate or AMPA increased cell death as compared with the single application of AMPA receptor agonists. With respect to the mechanism, we were able to show that GABA application induces a large Cl^- influx leading to an accumulation of intracellular Cl^- on the one hand and to an efficient repolarization of the membrane potential on the other hand. Both factors enlarge the Ca^{2+} influx through the AMPA receptor, which explains the more pronounced cell death. The enhancement of excitotoxic motoneuron death by GABA is contrary to the neuroprotective actions of GABA agonists observed in other experimental models in which excitotoxicity contributes to neuronal death, such as ischemia or neurotrauma. In ischemia, not only the extracellular glutamate concentration is elevated, but also the GABA concentration (Mrsulja et al., 1978; Bosley et al., 1983; Graham et al., 1990; Hutchinson et al., 2002). This is thought to be neuroprotective, and GABAergic drugs have been shown to produce varying degrees of neuroprotection after cerebral ischemia in animal models (Schwartz-Bloom and Sah, 2001). In the spinal cord, $GABA_A$ receptor stimulation has also been shown to produce neuroprotection in models of neurotrauma (Farooque et al., 1999) and ischemia (Madden, 1994; Lapchak et al., 2000). How-

Table 1. Summary of changes in membrane potential, chloride influx, and motoneuron death induced by kainate (KA) and AMPA

	ΔVm (mV)	$\Delta[Cl^-]_i$ (mM)	% Motoneuron death
100 μM KA in 3.2 mM $[Ca^{2+}]_e$	45.2 ± 1.1 ($n = 78$)	4.7 ± 0.6 ($n = 21$)	
100 μM AMPA in 3.2 mM $[Ca^{2+}]_e$	37.8 ± 3.1 ($n = 5$)	3.6 ± 0.5 ($n = 6$)	
100 μM KA in 10 mM $[Ca^{2+}]_e$	41.4 ± 2.5 ($n = 6$)	5.7 ± 1.2 ($n = 6$)	33.6 ± 3.3 (Van Den Bosch and Robberecht, 2000)
300 μM KA in 10 mM $[Ca^{2+}]_e$	48.2 ± 2.0 ($n = 6$)	6.7 ± 1.7 ($n = 6$)	46.0 ± 2.0 (Van Damme et al., 2002a)
300 μM AMPA in 10 mM $[Ca^{2+}]_e$	31.8 ± 2.0 ($n = 10$)	4.2 ± 0.5 ($n = 10$)	21.7 ± 1.8 ($n = 4$)
1 mM AMPA in 10 mM $[Ca^{2+}]_e$	37.5 ± 1.6 ($n = 5$)	5.4 ± 1.0 ($n = 5$)	33.3 ± 2.0 ($n = 4$)

ever, our findings suggest that Cl^- influx through $GABA_A$ receptors or through Cl^- channels during sustained glutamate receptor stimulation would rather be harmful to spinal motoneurons. AMPA receptor-mediated excitotoxicity contributes to the selective motor neuron loss in transgenic mice overexpressing mutated superoxide dismutase 1 (SOD1) (Canton et al., 2001; Van Damme et al., 2003), which is a model for familial amyotrophic lateral sclerosis. In preliminary experiments we studied the effect of the $GABA_A$ agonist muscimol on mutant SOD1 mice. In agreement with our hypothesis, treatment with muscimol shortened the survival of mutant SOD1 mice.

Of particular interest with respect to motoneuron death is that spinal motoneurons have a very high inhibitory GABAergic and glycinergic innervation (Price et al., 1976; Magoul et al., 1987; Carlton and Hayes, 1990; Plaitakis, 1991) that would render them very vulnerable to excitotoxic conditions. Furthermore, this may also explain the high expression level of KCC2 in motoneurons (Hubner et al., 2001), not only in the vicinity of inhibitory synapses (Hubner et al., 2001), but also around excitatory synapses (Gulyas et al., 2001). In counteracting Cl^- influx, KCC2 could exert a neuroprotective effect.

We therefore conclude that in addition to their well documented role in setting neuronal excitability, Cl^- ions could play a vital role in pathophysiological processes such as glutamate-mediated excitotoxicity, albeit in a different relation to the excitatory neurotransmitters. Although Cl^- influx suppresses neuronal excitability and thereby counteracts the action of excitatory neurotransmitters such as glutamate, our data suggest that Cl^- influx during exposure to pathological amounts of glutamate actually amplifies the excitotoxic action of glutamate. This additive effect of Cl^- influx on cell death could have direct implications for neuroprotective strategies. Contrary to the observation that Cl^- channel openers (e.g., GABA agonists) offer neuroprotection in some models of excitotoxicity, our data indicate that pharmacological strategies to reduce glutamate-mediated excitotoxicity in motoneurons should focus on Cl^- channel blockers and on activators of Cl^- extrusion systems such as KCC2.

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