Alterations of N-Methyl-D-aspartate Receptor Properties after Chemical Ischemia[1]

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ABSTRACT

Sublethal ischemic challenges can protect neurons against a second, more severe hypoxic insult. We report here that nonlethal chemical ischemia induces a transient alteration of NMDA receptors in rat cortical neurons in culture. Cells were incubated with 3 mM KCN in a glucose-free solution for 90 min. Analysis of NMDA receptor unitary events in patches excised from KCN-treated neurons showed an increased incidence of a small conductance channel 24 h after chemical ischemia. Whole-cell recordings of NMDA-induced currents 1 day after cyanide exposure revealed a significant increase in voltage-dependent extracellular Mg^{2+} block compared with untreated neurons. The block reverted to control levels within 48 h. Both of these changes in the NMDA receptor could decrease the overall current flowing through the channel. Message levels for the NMDA receptor subunits NR1, NR2A, and NR2B were not different between the chemically challenged neurons and control cells, whereas NR2C message was barely detectable in either group. These results suggest that the alterations in NMDA receptor properties after KCN exposure may contribute to the molecular mechanisms that are activated in neurons to withstand lethal ischemic events in the brain after preconditioning.

Neuronal tissue can become tolerant to severe ischemic insult after a sublethal ischemic challenge both in vivo and in vitro (Schurr et al., 1986; Kitagawa et al., 1990; Kirino et al., 1991; Liu et al., 1992; Ying et al., 1997). This process, termed "ischemic tolerance" or "ischemic preconditioning", has been proposed to be expressed as a consequence of the induction of a number of proteins, including heat shock proteins (Kogure and Kato, 1993; Liu et al., 1993; Sakaki et al., 1995; Akins et al., 1996; Gage and Stanton, 1996; Bergeron et al., 1997; Pringle et al., 1997). However, the precise subcellular molecular mechanism responsible for the observed neuroprotection is yet to be determined. Because neuronal cell death resulting from ischemic events can be associated with abnormal activation of NMDA receptors (Sattler et al., 2000), it is possible that alterations in receptor function could be partly responsible for ischemic tolerance (Lowenstein et al., 1991; Kato et al., 1992; Marini and Paul, 1992). Ischemia, anoxia, and injury have been shown to trigger changes in the levels of phosphorylation of NMDA receptor subunits (Zhang et al., 1996; Takagi et al., 1997; Braunton et al., 1998; Pittaluga et al., 2000) or in the receptor subunit composition itself (Perez-Velazquez and Zhang, 1994; Small et al., 1997; Zhang et al., 1997), potentially producing prosurvival modifications in the function of this channel. These could include either a decrease in overall NMDA receptor activity or perhaps a dissociation between receptor gating and cell death (Sattler et al., 1999). In this study, we use a sublethal chemical ischemic stimulus (Hartnett et al., 1997b) to test whether any detectable functional changes in the NMDA receptor could account for the observed neuroprotection.

Materials and Methods

Tissue Culture. Approved institutional guidelines were followed for the care and sacrifice of the animals. Cortical cultures were prepared from embryonic day 16 Sprague-Dawley rats as previously described (Hartnett et al., 1997a). Briefly, cortices were enzymatically dissociated and the resultant cell suspension was adjusted to 600,000 cells/ml and plated onto 12-mm poly(L-lysine)-coated coverslips in a growth medium composed of a volume to volume mixture of 80% Dulbecco's modified minimum essential medium, 10% Ham's F-12 nutrients, 10% bovine calf serum (heat-inactivated, iron-supplemented; Hyclone, Logan, UT) with 25 mM HEPES, 24 U/ml penicillin, 24 μg/ml streptomycin, and 2 mM L-glutamine. Cultures were maintained at 37°C, 5% CO_2, and medium was partially replaced on a Monday-Wednesday-Friday schedule. Glial cell proliferation was inhibited after 2 weeks in culture with 1 to 2 μM cytosine arabinoside, after which the cultures were maintained in growth medium containing 2% serum and without F-12 nutrients. Cultures were used for all experiments at 25 to 29 days in vitro.

ABBREVIATIONS: KCN, potassium cyanide; NR, N-methyl-D-aspartate receptor subunit; NMDA, N-methyl-D-aspartate; PKC, protein kinase C.

Chemical Ischemia. Cortical cultures were exposed for 90 min to 3 mM potassium cyanide (KCN) prepared in sterile, glucose-free balanced salt solution (composition: 150 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, and 10 mM HEPES) for 90 min at 37°C, 5% CO_2. Cyanide treatment was terminated first by rinsing (200:1) and then replacing the treatment solution with growth medium (2% serum, no F-12). The KCN concentration and exposure time used were found to be the most extreme chemical ischemic conditions that failed to produce neuronal toxicity and induce tolerance against NMDA toxicity (Hartnett et al., 1997b). These parameters were established by testing various concentrations of KCN at 30-min incremental incubation periods. Neuronal viability was determined 18 to 20 h after KCN exposure using a lactate dehydrogenase-based in vitro toxicology assay kit (Sigma, St. Louis, MO). Samples (40 µl) of medium were assayed spectrophotometrically (490:630) according to the manufacturer's protocol, to obtain a measure of cytoplasmic lactate dehydrogenase released from dead and dying neurons (Hartnett et al., 1997a). No toxicity was observed with KCN treatments for as long as 90 min (data not shown).

Electrophysiological Measurements. Recordings were performed at 25°C. The extracellular solution contained 150 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, 10 µM glycine, 250 nM tetrodotoxin (for whole-cell measurements only) and was adjusted to a pH of 7.2 with 0.3 N NaOH. The intracellular pipette solution contained 140 mM CsF, 10 mM EGTA, 1 mM $CaCl_2$, 10 mM HEPES and was adjusted to a pH of 7.2 with CsOH. Patch electrodes (2 MΩ) were used for whole-cell recordings with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Signals were filtered at 1 kHz and digitized at 2 kHz (Digidata 1200; Axon Instruments). Extracellular application of 30 µM NMDA alone or in conjunction with 500 µM Mg^{2+} was performed using a fast perfusion multibarrel system (Warner Instruments, Wallingford, CT). Data were collected and analyzed using commercially available software (pCLAMP; Axon Instruments). The degree of extracellular Mg^{2+} block was determined by measuring the steady-state response to NMDA in the presence and absence of the cation. Single-channel measurements were performed on outside-out patches using 10 to 15 MΩ electrodes at room temperature. Signals were filtered at 2 kHz, stored on videotape, and later replayed and digitized at 10 kHz. Single-channel data were analyzed using pCLAMP software using a 50% threshold detection criterion. Extracellular application of 3 µM NMDA at various Mg^{2+} concentrations was performed by complete bath exchange (Brimecombe et al., 1997).

RNase Protection Assay. Total RNA was prepared from cortical neurons grown on poly(L-lysine)-treated six-well tissue culture plates under control conditions and 24 h after cyanide exposure. RNA was isolated and purified using a commercially available kit (Rneasy; Qiagen, Santa Clarita, CA). The integrity of the isolated RNA samples was assessed using denaturing agarose gel electrophoresis and ethidium bromide staining to visualize the 18S and 28S rRNA. Using this method, the usual yield was 30 to 50 µg of RNA for each condition. NMDA receptor-subunit and rat-specific cyclophilin (Ambion, Austin, TX) probes were labeled with [^{32}P]UTP (DuPont-NEN, Boston, MA) in a solution also containing nucleotides, unlabeled UTP, SP6, or T7 RNA polymerase, dithiothreitol, and Rnasin. DNase I was used to degrade the cDNA template. Unincorporated nucleotides were removed using phenol/chloroform extraction, and the RNA probes were precipitated with ethanol. Samples (10 µg) of total RNA were hybridized to the prelabeled probes (5 × 10^5 cpm) overnight at 50°C. Unhybridized RNA was digested with RNase A (Boehringer Mannheim, Indianapolis, IN) and RNase T1 (Sigma), which preceded the addition of 1% SDS and proteinase K (Boehringer Mannheim) to the reaction mixture. The protected RNA was extracted, precipitated, and run on a denaturing acrylamide/urea gel. Bands were visualized using a phosphorimaging instrument (Storm; Molecular Dynamics, Sunnyvale, CA). The intensity of each band was measured and normalized to cyclophilin levels to provide a quantification, in relative units, of the amount of NMDA receptor-subunit message present in each sample.

Results

KCN-Induced Changes in Single NMDA Receptor Channels. Outside-out patches were excised from control neurons and from cells that had been exposed for 90 min to 3 mM KCN a day earlier. Analysis of total amplitude histograms of events elicited by 3 µM NMDA at (60 mV, 24 h after the hypoxic challenge, revealed the appearance of an additional single-channel amplitude in many KCN-treated cells that was not usually present in control patches (Fig. 1). This second amplitude was smaller than the most commonly occurring main conductance. Only 3 of 17 (17.6%) control patches had events with amplitude histograms that were best fit by two Gaussian functions. In contrast, the smaller amplitude was readily detected in 13 of 26 (50.0%) amplitude distributions of channels in patches excised from the KCN-treated neurons. This increased incidence of small conductance events after chemical hypoxia was statistically significant ($P < .05$, Fisher's exact test for 2 × 2 tables). Nonetheless, the peaks of the two Gaussian components were not different between control (-3.2 ± 0.1 and -2.7 ± 0.3 pA) and KCN-challenged neurons (-3.2 ± 0.1 and -2.5 ± 0.1 pA). Open time distributions of the NMDA-activated events were virtually identical in both groups of neurons and very similar to those published previously for outside-out patches in this neuronal preparation (Tang and Aizenman, 1993). The mean open times calculated from these distributions were 3.9 ± 0.3 ms for control patches, and 4.2 ± 0.2 ms for the patches excised from KCN-treated neurons.

Alteration in Mg^{2+} Sensitivity after Chemical Ischemia. The presence of a smaller conductance in the patches excised from the previously hypoxic neurons suggested that NMDA receptors in these cells could have undergone a structural change, including the possibility of an alteration in their subunit composition. Such a change could include, for example, an increased contribution by NR2C to the functional pool of receptors (Perez-Velazquez and Zhang, 1994; Small et al., 1997; Zhang et al., 1997). NR2C-containing recombinant NMDA receptors are blocked to a lesser degree by extracellular Mg^{2+} compared with other subunit combinations (i.e., NR1/NR2A or NR1/NR2B; Monyer et al., 1994; Takahashi et al., 1996). To test for this possibility, whole-cell responses to 30 µM NMDA were obtained in control neurons, as well as in cells treated with 3 mM KCN for 90 min, 24 h before the recordings. Currents were obtained at various membrane voltages in the presence or absence of 0.5 mM Mg^{2+}. Surprisingly, we observed a small but statistically significant increase in the degree of block produced by Mg^{2+}, detectable at all potentials tested in the KCN-treated cells (Fig. 2, A and B). Recordings were obtained in some cells at 48 h rather than 24 h after the KCN exposure, and in this case the degree of Mg^{2+} block had reverted to control levels (Fig. 2B). Under all conditions, current amplitudes in the absence of Mg^{2+} were linear with voltage and reversed near 0 mV. Interestingly, the temporal changes in magnesium sensitivity observed here closely parallel the time course of neuroprotection observed against NMDA toxicity after the chemical preconditioning of our cultures (Hartnett et al., 1997b).
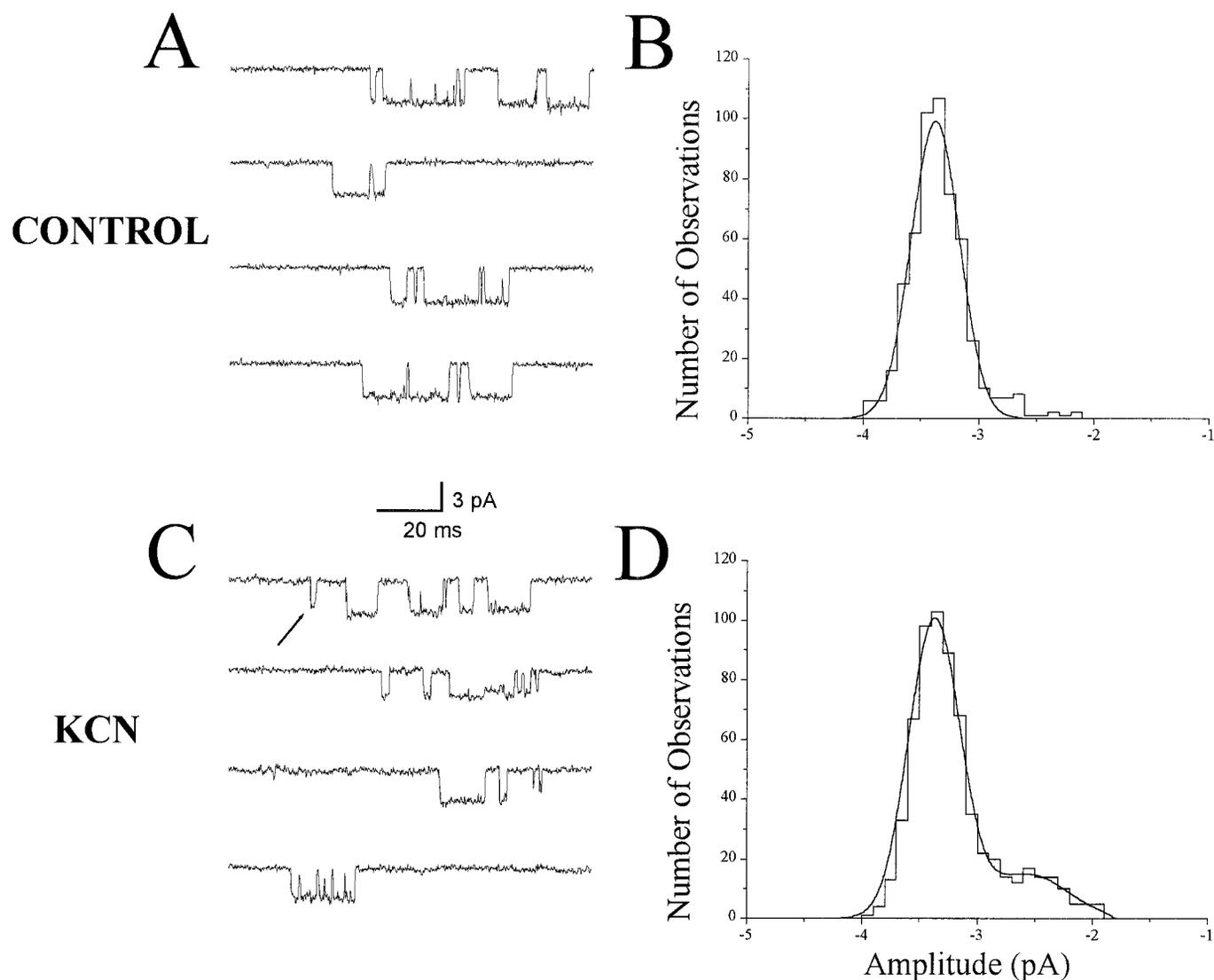
Fig. 1. Analysis of unitary events in KCN-pretreated neurons suggests an increased incidence of small conductance channels. A, representative single-channel recordings from an outside-out patch excised from a control cortical neuron during continuous exposure to 3 μM NMDA at −60 mV. B, amplitude histogram obtained from the events shown in A was fit with a single Gaussian function. The mean amplitude for this patch was −3.37 pA at −60 mV. C, representative single-channel recordings from an outside-out patch excised from a cortical neuron pretreated with 3 mM KCN (90 min) approximately 24 h before recording. Traces represent 3 μM NMDA-activated channels at a holding potential of −60 mV. Arrow points to smaller conductance channel. D, amplitude histogram obtained from the events in C reveals the presence of two conductance levels at −60 mV, one with a mean amplitude of −3.36 pA and another with a mean amplitude of −2.60 pA. Arrow points to smaller conductance channels. Similar measurements were performed in a total of 17 control and 26 KCN-treated neurons.

NMDA Receptor Expression Remains Unaltered after KCN Treatment. We used an RNase protection assay to quantify message levels for the NMDA receptor subunits expressed in control and KCN-treated neurons (Fig. 3). No significant changes in NR1, NR2A, or NR2B message could be detected in cultures 24 h after a 90-min exposure to 3 mM KCN, compared with control (Table 1). NR2C message was below quantifiable levels in both control and cyanide-pretreated cells, similar to what has been reported previously for cortical neurons in vitro (Zhong et al., 1994). These data argue that the observed changes in channel conductance or magnesium block are not due to a gross change in NMDA receptor composition in the chemically challenged neurons.

Increased protein kinase C (PKC) activity has been associated with decreased NMDA receptor Mg^{2+} block (Chen and Huang, 1992; Zhang et al., 1996; Pittaluga et al., 2000).

Although PKC has been implicated in ischemic preconditioning in both heart and brain, the precise cytoprotective roles of this enzyme are somewhat ill-defined and controversial (Reshef et al., 1997; Przyklenk and Kloner, 1998). Nonetheless, a decrease in PKC activity would be consistent with other neuroprotective paradigms (Favaron et al., 1990), and with an increase in NMDA receptor Mg^{2+} block. We thus performed a series of preliminary studies to examine whether the PKC activator phorbol-12-myristate-13-acetate (1 μM), as well as the nonselective phosphatase inhibitors *ortho*-vanadate (500 μM) and okadaic acid (30 nM), could abolish the actions of KCN in protecting neurons from a subsequent NMDA exposure. The results obtained with these substances were inconclusive because their use together with KCN resulted in widespread neurotoxicity.
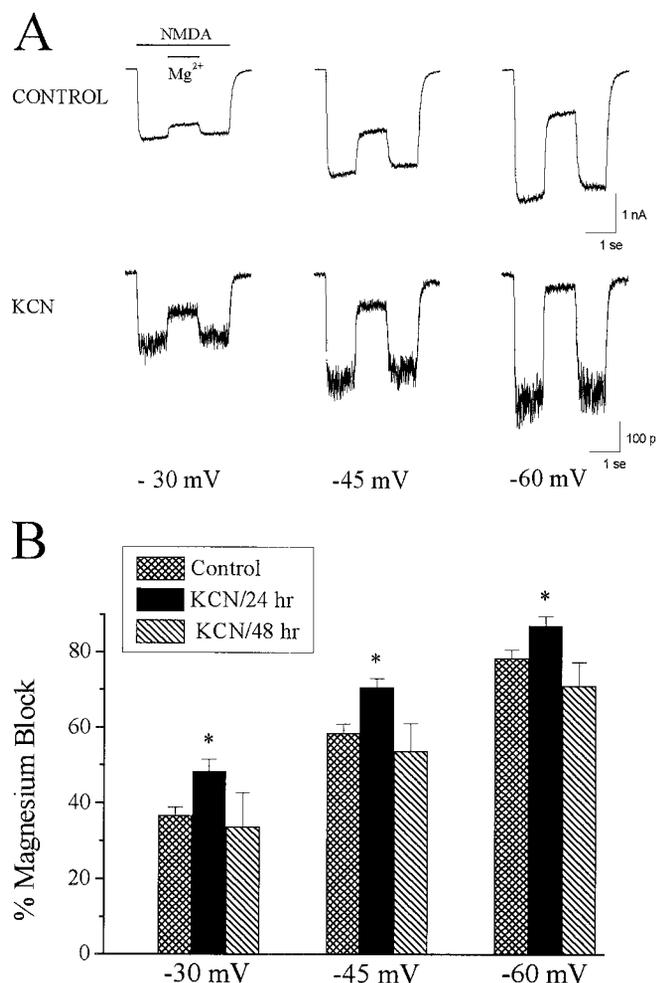
A

B

Fig. 2. Whole-cell patch-clamp recordings obtained 24 h after sublethal KCN challenge reveal an increase in voltage-dependent Mg^{2+} block. A, whole-cell recordings obtained from cortical neurons 24 h after exposure to either control (top) or KCN (bottom). The bars above the first trace denote the extracellular application of 30 μM NMDA alone or in conjunction with 500 μM Mg^{2+}. Neurons were clamped at the holding voltages indicated. B, current traces such as those shown in A were used to measure the percentage block (mean ± S.D.) by magnesium in control neurons ($n = 31$) and in cells exposed to KCN either 24 h (KCN/24 h; $n = 14$) or 48 h (KCN/48 h; $n = 5$) before the recordings. ∗ denote a significant increase in block ($P < .05$, unpaired t test).

Discussion

Cyanide inhibits mitochondrial respiration by preventing the oxidation of cytochrome a_3, thereby obstructing the electron transport chain and oxidative phosphorylation. Cytochrome a_3 together with cytochrome a form the cytochrome c oxidase complex, which is the terminal enzyme in the electron transport chain and the cellular respiratory component that uses molecular oxygen. Hence, cyanide toxicity is analogous to cellular anoxia. In neurons, cyanide treatment has been used as a model of hypoxia, and, more significantly, it has been extensively used in examining the relationship between oxygen starvation and excitotoxic processes (Novelli et al., 1988; Dubinsky and Rothman, 1991; Zeevalk and Nicklas, 1992; Pocock and Nicholls, 1998). Other investigators have reported that chemical inhibition of mitochondrial function or impairment of glucose use can condition neurons against subsequent ischemia or excitotoxic injury (Riepe et al., 1997; Lee et al., 1999; Weih et al., 1999). In the present
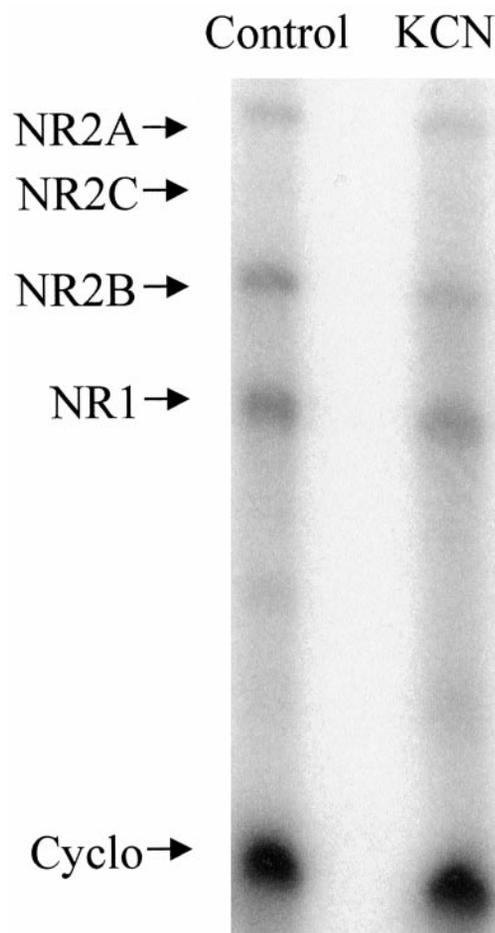

Fig. 3. Sublethal chemical hypoxia has no detectable effect on NMDA receptor-subunit message. Total RNA samples were obtained 24 h after control or cyanide challenge from cortical neuronal cultures. Samples (10 μg) were used for a RNase protection assay and run on an acrylamide/urea gel (under *Materials and Methods*). Chromatograms such as this one were visualized on a phosphorimaging device to generate the data shown in Table 1.

TABLE 1

Effects of chemical ischemia on NMDA receptor subunit message levels

Mean ± S.E. intensities of hybridized RNA bands as determined by an RNase protection assay (see Fig. 3) for the various NMDA receptor subunits expressed by cortical cultures under control and 24 h after chemical ischemia (3 mM KCN for 90 min). The numbers shown are normalized to the cyclophilin signal on the same hybridization and are given in relative units. The n values represent the number of separate hybridizations, each to independent RNA preparations, in which a given probe was used.

NMDA Receptor Subunit	Control	KCN-Treated
NR1 ($n = 3$)	0.183 ± 0.0057	0.179 ± 0.0022
NR2A ($n = 5$)	0.039 ± 0.0010	0.038 ± 0.0008
NR2B ($n = 5$)	0.049 ± 0.0017	0.052 ± 0.0014
NR2C ($n = 5$)	ND	ND

ND, not detectable.

study we treated neurons with a sublethal concentration of KCN in a glucose-free solution (chemical ischemia) to evaluate whether this type of ischemic preconditioning can produce measurable alterations in NMDA receptor function.

We observed that patches excised from KCN-treated neurons had a high incidence of a small conductance channel. This result is consistent with observations from previous studies where small conductance channels were also detected

in postischemic, albeit not preconditioned, neurons (Tsubokawa et al., 1995; Zhang et al., 1997). In addition, the increased presence of these small conductance channels would be consistent with investigations showing a rapid up-regulation of the NR2C NMDA receptor subunit during hypoxia/ischemia (Perez-Velazquez and Zhang, 1994; Small et al., 1997). Recombinant NR2C-containing receptors generally have smaller conductances than those assembled with either NR2A or NR2B (Brimecombe et al., 1997). Hence, the functional expression of NR2C-containing channels could, in theory, be used as a neuroprotective strategy by the cell (Boeckman and Aizenman, 1996). These receptors not only have smaller conductances, but also have lower Ca^{2+} permeability compared with other subunit configurations (Burnashev et al., 1995), and their functional expression is the only nonlethal combination in nonneuronal cells (Boeckman and Aizenman, 1996). Because NMDA receptors assembled with NR2C have also been shown to have lower sensitivity to extracellular Mg^{2+} block (Monyer et al., 1994; Takahashi et al., 1996), we tested whether Mg^{2+} sensitivity was altered in NMDA-induced whole-cell responses in neurons previously exposed to KCN. Surprisingly, the level of block produced by this cation increased, rather than decreased. Although this finding would be inconsistent with the aforementioned hypothesis, it is consistent with the fact that NR2C message was barely detectable under any conditions in our cultures. The significance, or mechanism, behind the increased appearance of the small conductance channel in KCN-treated neurons is unclear, as is its potential role in the neuroprotection observed.

A correlation between NMDA receptor-mediated extracellular Mg^{2+} block and excitotoxic cell death was recently established. Sakaguchi et al. (1997) evaluated NMDA receptor-channel properties, including Mg^{2+} block, in hippocampal slice preparations at differing stages in culture. Three-week-old cultures became increasingly more resistant to Mg^{2+} block (-70 mV) and were concomitantly more susceptible to NMDA receptor-mediated excitotoxicity. Younger cultures, which exhibited a greater sensitivity to Mg^{2+} block, were resistant to NMDA receptor-mediated excitotoxicity in the presence, but not the absence, of extracellular Mg^{2+}. Thus, it is conceivable that a relationship exists between the ischemic tolerance induction process and increase in Mg^{2+} block observed here. That is, it may be the result of the activation of subcellular pathways that trigger neuroprotection, including changes in NMDA receptor properties. One such pathway may include a decrease in PKC activity, which has been shown to be protective against glutamate toxicity (Favaron et al., 1990). Moreover, activation of PKC has been shown to result in a decrease of Mg^{2+} block of NMDA receptor channels (Chen and Huang, 1992; Pittaluga et al., 2000). Additional work in this area is currently underway in our laboratory with true ischemia because experiments conducted with PKC activators and phosphatase inhibitors were unsuccessful due to the widespread cytotoxicity induced by these compounds with chemical ischemia. Elucidation of the mechanisms responsible for the changes observed in the NMDA receptor after ischemia could yield novel neuroprotective strategies for disease states where overactivation of this receptor has been implicated.

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