

## Subunit-specific Interactions of Cyanide with the N-Methyl-D-aspartate Receptor\*

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Cyanide can potentiate N-methyl-D-aspartate receptor-mediated physiological responses in neurons. Here we show that this phenomenon may be attributable to a subunit-specific chemical modification of the receptor directly by the toxin. N-Methyl-D-aspartate (30  $\mu$ M)-induced whole cell responses in mature (22–29 days *in vitro*) rat cortical neurons were potentiated nearly 2-fold by a 3–5-min treatment with 2 mM potassium cyanide, as did a similar treatment with 4 mM dithiothreitol. A 1-min incubation with the thiol oxidant 5,5'-dithiobis(2-nitrobenzoic acid) (0.5 mM) readily reversed the potentiation induced by either cyanide or dithiothreitol. Cyanide did not increase further currents previously potentiated by dithiothreitol nor was it able to potentiate responses during brief co-application with the agonist. Transient expression studies in Chinese hamster ovary cells with wild-type and mutated recombinant N-methyl-D-aspartate subunits (NR) demonstrated that cyanide selectively potentiated NR1/NR2A receptors, presumably via the chemical reduction of NR2A. In contrast, currents mediated by NR1/NR2B receptors were somewhat diminished by the metabolic inhibitor. Some of the effects of cyanide on NR1/NR2B receptors may be mediated by the formation of a thiocyanate adduct with a cysteine residue located in NR1. Cyanide thus is able to distinguish chemically between two different N-methyl-D-aspartate receptor subtypes and produce diametrically opposing functional effects.

Cyanide is a mitochondrial poison that adversely affects cellular respiration by inhibiting the reoxidation of cytochrome  $a_3$  by molecular oxygen, thereby obstructing the electron transport chain and oxidative phosphorylation (1). Cytochrome  $a_3$  and cytochrome  $a$  form the cytochrome  $c$  oxidase complex, which is the terminal enzyme in the electron transport chain. Transferring electrons to oxygen, cytochrome  $c$  oxidase is the cellular respiratory component responsible for the critical need for oxygen. Thus, cyanide intoxication in cells is akin to oxygen starvation. In neuronal systems, cyanide treatment has been a widely used model of hypoxia (2–4), particularly in relation to excitotoxic processes. For example, cyanide can raise extracellular glutamate levels (5, 6), increase glutamate-triggered intracellular  $Ca^{2+}$  elevations in neurons (7, 8), and potentiate

glutamate toxicity (7, 9). Moreover, specific inhibitors of N-methyl-D-aspartate (NMDA)<sup>1</sup> receptors can inhibit cyanide-induced  $Ca^{2+}$  influx in neurons (7, 10, 11) as well as neurotoxicity (12–14).

Interestingly, a direct interaction between cyanide and the NMDA receptor has recently been demonstrated. Cyanide treatment of cultured rat hippocampal or cerebellar neurons potentiated NMDA-induced physiological responses, including single channel activity in excised outside-out membrane patches (15, 16). However, the precise site of action of cyanide at the receptor remained to be elucidated. In the present investigations we have evaluated the possible interaction of KCN on the NMDA receptor redox modulatory sites (17). Via these sites, disulfide-reducing agents such as dithiothreitol (DTT; see Refs. 17 and 18), dihydrolipoic acid (19), or tris(carboxyethyl)phosphine (20) enhance NMDA receptor-mediated physiological responses, whereas thiol-containing oxidants (21, 22), reactive quinones (23, 24), or oxygen-derived free radicals (25, 26) can reverse the effects of reductants or depress native responses. Cyanide has well established properties as a disulfide reducing agent in many preparations (27–30), and thus an effect on the NMDA thiol-sensitive sites would not be surprising. Nonetheless, the experiments described here demonstrate that cyanide can be used to distinguish between different NMDA receptor subtypes by producing either a potentiation or a depression of the physiological responses mediated by this ligand-gated ion channel.

### EXPERIMENTAL PROCEDURES

**Tissue Culture**—Tissue culture and all common reagents were purchased from Sigma, excluding the following: iron-supplemented bovine calf serum, HyClone Laboratories (Logan, UT), and minimal essential medium, Life Technologies, Inc. Chinese hamster ovary cells (CHO-K1; ATTC CCL61) were grown in Ham's F-12 nutrient medium with 10% fetal bovine serum, and 1 mM glutamine (CHO media) in 50- or 200-ml flasks at 37 °C in 5%  $CO_2$ . Cells were passaged at a 1:10 dilution at 80% confluency, approximately every 2 days, no more than 30 times. Cerebral cortices were obtained from E-16 Sprague-Dawley C-D rats and dissociated according to methods described previously (31). Briefly, cortices were incubated in minimal essential medium solution containing 0.03% trypsin for 2 h at 37 °C. Dissociated cells were plated at a density of  $3\text{--}5 \times 10^5$  cells/ml in growth medium (v/v 80% Dulbecco's modified Eagle's minimum, 10% Ham's F12 nutrient mixture, 10% heat-inactivated iron-supplemented bovine calf serum, 25 mM HEPES, 24 units/ml penicillin, 24  $\mu$ g/ml streptomycin, and 2 mM L-glutamine) into 35-mm tissue culture dishes containing five 12-mm poly-L-lysine-coated glass coverslips each, for electrophysiological recordings. Cells were maintained at 37 °C in 5%  $CO_2$ . Growth medium was changed three times per week. After 15 days in culture, non-neuronal cell proliferation was inhibited with 2  $\mu$ M cytosine arabinoside after which the growth medium contained 2% serum and no F-12. Cells were mostly

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<sup>1</sup> The abbreviations used are: NMDA, N-methyl-D-aspartate; NR, NMDA receptor subunit; KCN, potassium cyanide; CHO, Chinese hamster ovary; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine; DIV, days *in vitro*; PCR, polymerase chain reaction.

used between 22 and 29 days *in vitro* (DIV), although some later experiments utilized cells at 8 DIV.

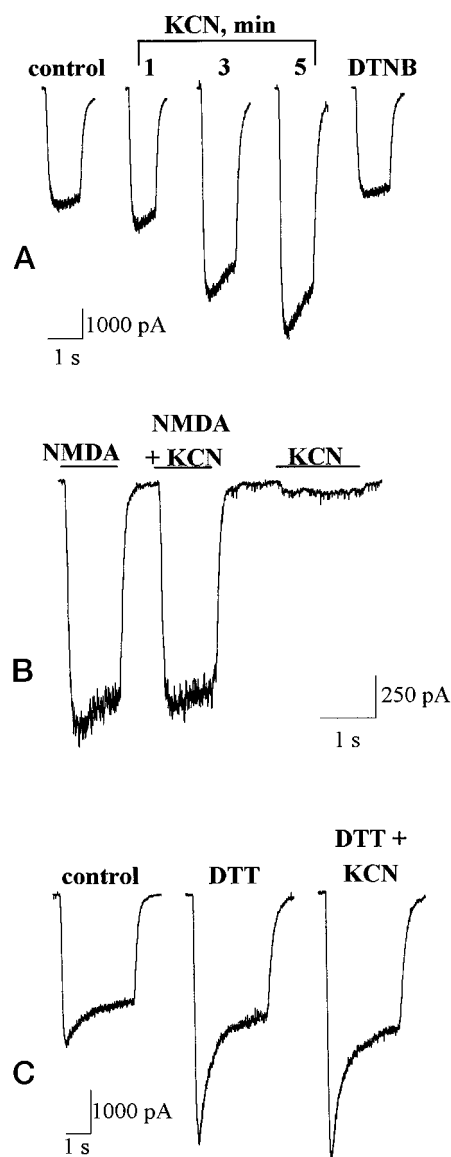
**Transfection Protocol**—The cDNAs for the NMDA subunits and the positive transfection marker green fluorescent protein (32) were previously subcloned into mammalian expression vectors (33–35). Cells were seeded at  $3 \times 10^5$  cells per well in 6-well plates (35-mm wells) approximately 24 h prior to transfection with 1.3  $\mu\text{g}$  of total DNA and 6  $\mu\text{l}$  of LipofectAMINE (Life Technologies, Inc.) in 1 ml of serum-free CHO media per well. Of the total DNA 0.3  $\mu\text{g}$  were green fluorescent protein-containing plasmid, and the remaining 1  $\mu\text{g}$  was composed of a 1:3 ratio of NR1- to NR2-containing plasmids (34). After a 5-h incubation at 37 °C in 5% CO<sub>2</sub> with the transfection solution, cells were refed with CHO medium containing 300  $\mu\text{M}$  ketamine or 1 mM 5,7-dichlorokynureate to prevent the cell death that accompanies NMDA receptor expression (34). Cells were used for recording approximately 40–50 h after the start of the transfection.

**Site-directed Mutagenesis**—A construct containing the cDNA for rat NR1 pN60 (36) was used as template for site-directed mutagenesis to produce NR1(C744S). Mutagenic primers (36-mers) were designed to mutate the codon for cysteine 744, TGC, to that of serine, TCC, utilizing a commercially available PCR-based procedure (Stratagene, La Jolla, CA). Cycling parameters were initial denaturation at 94 °C for 1.25 min; 18 cycles of 94 °C for 0.75 min, 55 °C for 1 min, 68 °C for 15 min; and a final extension at 68 °C for 10 min. To remove methylated template DNA, the PCR products were restriction-digested with *DpnI* (Stratagene). Epicurian Coli XL-1 Blue supercompetent *Escherichia coli* (Stratagene) were heat shock-transformed with the PCR products and colonies selected; plasmid DNA was isolated and digested with *HindIII* and *NotI* (Life Technologies, Inc.) to excise the mutated NR1 cDNA. This was subcloned into the equivalent restriction sites of pRc/CMV. Sequencing of the restriction fragment was performed to confirm the presence of the desired mutation and absence of spontaneous unwanted sequence alterations. The NR1(C744A,C798A) double mutant was the kind gift of S. Traynelis, Emory University, Atlanta, GA.

**Whole Cell Recordings**—Electrophysiological measurements were obtained at a membrane voltage of  $-60$  mV using the whole cell patch-clamp configuration. Methods of data acquisition and analysis have been described previously (18). The external recording solution contained 150 mM NaCl, 2.8 mM KCl, 1.0 mM CaCl<sub>2</sub>, 10 mM HEPES, and 10  $\mu\text{M}$  glycine (pH 7.2). For recordings from cortical neurons 0.25  $\mu\text{M}$  tetrodotoxin (Calbiochem) was added to this solution. Patch electrodes (2–4 M $\Omega$ ) were filled with 140 mM CsF, 10 mM EGTA (Sigma), 1 mM CaCl<sub>2</sub>, and 10 mM HEPES (pH 7.2). NMDA, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), DTT, potassium cyanide (KCN) or *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) were dissolved in extracellular solution and applied onto cells by a multi-barrel fast perfusion system (Warner Instrument Corp., Hamden, CT). All results are expressed as the mean  $\pm$  S.D. For statistical comparisons a parametric test (*t* test) was used, unless the standard deviations of the two groups analyzed were significantly different from each other, in which case a non-parametric test was utilized (Mann Whitney two sample test).

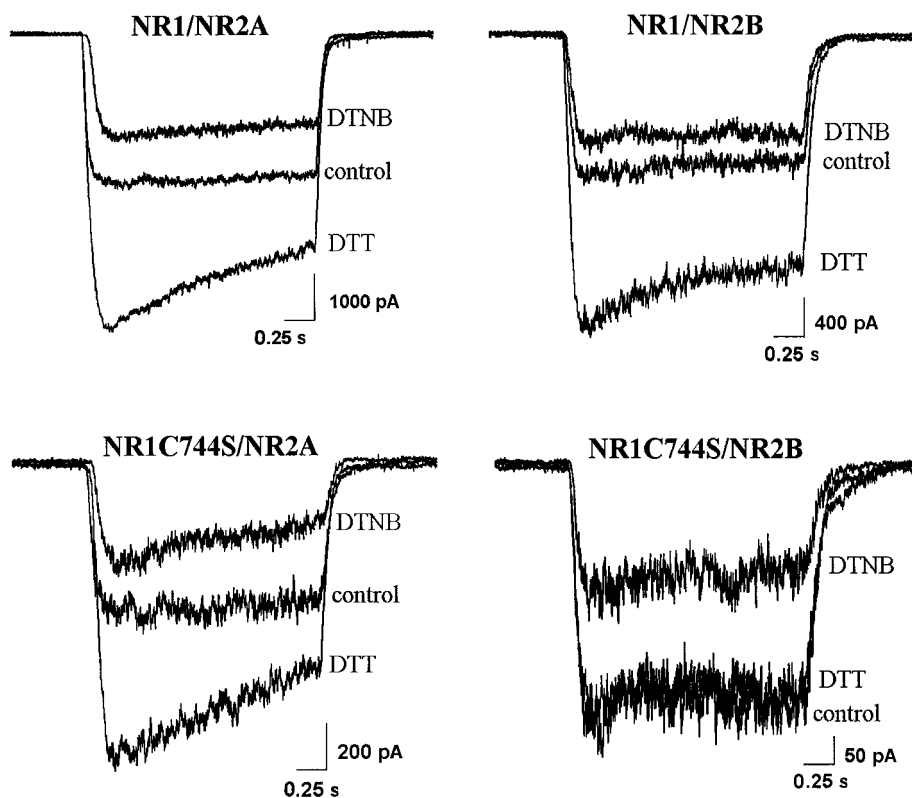
## RESULTS

**Potentiation of NMDA-induced Whole Cell Responses by KCN in Cortical Neurons**—Cyanide as a disulfide-reducing agent is used in biochemical experiments at concentrations in the range of 1–40 mM (27–29). Since the NMDA receptor reduction process is both time- and concentration-dependent (18), we opted to use a concentration roughly 2–20 times higher than the concentrations utilized by Isom and co-workers (100  $\mu\text{M}$  to 1 mM; see Refs. 15 and 16) to accelerate and maximize the reduction process but without damaging the cells. Cortical neurons were therefore incubated for several minutes in extracellular recording solution containing 2 mM KCN. Whole cell measurements of NMDA (30  $\mu\text{M}$ ) receptor-mediated currents were performed under control conditions and at 1-min intervals during the KCN treatment (Fig. 1A). During the KCN incubation itself, we noted a rapid, small, variable increase in inward leak current, which always decreased in amplitude and eventually disappeared a few seconds later, even while the toxin was still present in the bath. We observed that the NMDA-induced responses increased in amplitude in a time-dependent fashion during the cyanide exposure. After a 3-min treatment with the



**FIG. 1. Potentiation of NMDA-induced whole cell currents by KCN in mature neurons.** A, KCN (2 mM) applied via rapid perfusion produced a time-dependent enhancement of currents elicited by 30  $\mu\text{M}$  NMDA applied to a cortical neuron from a second perfusion barrel (in the absence of KCN). The oxidizing agent DTNB (0.5 mM) reversed this effect of KCN within 1 min. Currents were recorded at  $-60$  mV under the whole cell voltage clamp configuration in this and subsequent figures. For all figures, each treatment was rinsed off the cells with control solution prior to the next application of a redox substance. B, NMDA (30  $\mu\text{M}$ )-induced currents are not substantially altered when KCN (2 mM) is rapidly co-applied from a second perfusion barrel. KCN, when applied alone, produces a small, irregular increase in inward current. Bars above the traces show application intervals of the indicated drugs. C, currents elicited by 30  $\mu\text{M}$  NMDA are potentiated by a 9-min incubation with 4 mM DTT but are not further enhanced by a subsequent treatment with DTT plus 2 mM KCN together (6 min).

mitochondrial inhibitor, the NMDA-generated currents had increased  $1.6 \pm 0.8$ -fold ( $x\text{-fold} = x_2/x_1$ ;  $n = 10$ ) over control responses; by 5 min, responses were potentiated  $1.8 \pm 0.4$ -fold ( $n = 3$ ). This action of KCN in cortical neurons is similar to that reported earlier for NMDA-induced responses in cerebellar granule cells (16). The oxidizing agent DTNB (0.5 mM) quickly ( $<1$  min) and effectively reversed the actions of KCN and further depressed the NMDA-induced current amplitudes to  $81.1 \pm 17.3\%$  ( $n = 3$ ) of the initial control responses (Fig. 1A). Hence, KCN produced effects on the NMDA receptor resem-



**FIG. 2. Redox sensitivity of recombinant NMDA receptors expressed in CHO cells.** NMDA ( $30 \mu\text{M}$ )-evoked responses were recorded under control conditions, following a 3-min incubation with 4 mM DTT, and after a 1-min treatment with 0.5 mM DTNB, in CHO cells transfected with the NMDA receptor subunits indicated above the traces. Note the failure of DTT to potentiate the NMDA-induced current in cells expressing the NR1(C744S)/NR2B receptor configuration.

bling those previously reported for the sulfhydryl-reducing agent DTT (17, 18).

We also monitored whole cell responses to brief (1–1.5 s) applications of either NMDA ( $30 \mu\text{M}$ ) or KCN (2 mM) alone or both these drugs together (Fig. 1B). Currents induced by NMDA + KCN were slightly ( $6.0 \pm 5.3\%$ ;  $n = 6$ ) but significantly ( $p < 0.001$ , paired  $t$  test) smaller than those induced by NMDA. Moreover, KCN alone was able to elicit inward currents but with maximal amplitudes that were only  $8.0 \pm 4.2\%$  ( $n = 6$ ) of the NMDA-elicited responses. These KCN-induced currents are thus likely responsible for the aforementioned transient increase in current during the first few seconds of the prolonged incubations with the toxin. The potentiating actions of KCN on the NMDA-induced currents thus required relatively longer incubation periods ( $>3$  min) with the toxin, again reminiscent of the actions of DTT in this preparation (18).

To test whether DTT and KCN altered NMDA receptor-mediated currents via a similar mechanism, we obtained NMDA-induced responses during a prolonged treatment with DTT (4 mM, approximately 10 min) and following a subsequent exposure to DTT together with 2 mM KCN (6 min; Fig. 1C). Currents were potentiated during both these treatments  $2.6 \pm 1.3$ - and  $2.9 \pm 1.6$ -fold, respectively ( $n = 9$ ). The ratio of DTT + KCN to DTT potentiation for all cells tested was  $1.1 \pm 0.2$ . Therefore, KCN was unable to potentiate further NMDA responses after the DTT treatment, suggesting that both these agents act via a common site.

**NMDA Receptor Subunit-specific Effects of KCN**—To facilitate the examination of the putative actions of KCN on the redox-sensitive sites of the NMDA receptor, we first investigated the actions of DTT and DTNB on recombinant receptors expressed in CHO cells (33–35). We used site-directed mutagenesis to substitute cysteine 744 of the NR1 subunit with a serine residue (NR1(C744S)). This mutation abolishes DTT sensitivity in recombinant NMDA receptors composed of NR1 together with either the NR2B, NR2C, or NR2D subunit but not NR2A (37), as this latter subunit may contain a separate

DTT-sensitive site (38, 39). NR1 or NR1(C744S) subunits were transiently transfected into CHO cells along with either NR2A or NR2B to yield four separate groups of cells, each expressing one of the following subunit combinations: NR1/NR2A, NR1(C744S)/NR2A, NR1/NR2B, and NR1(C744S)/NR2B. NMDA ( $30 \mu\text{M}$ )-induced responses were recorded from these cells (Fig. 2) during control conditions and following treatments with DTT (4 mM, 3 min) and DTNB (0.5 mM, 1 min). NMDA-evoked currents in NR1/NR2A-, NR1(C744S)/NR2A-, and NR1/NR2B-expressing cells were similarly potentiated by DTT (Table I). As expected, NMDA-induced responses in cells transfected with NR1(C744S)/NR2B were unaltered by the DTT treatment (Table I). In contrast, DTNB depressed the currents in all subunit configurations (Table I). Interestingly, the effects of DTNB on NR2B-containing receptors, but not on NR2A, were significantly greater for the mutated form of NR1, when compared with the wild-type ( $p < 0.05$ , Mann-Whitney two sample test). In all cases, the inhibitory effects of DTNB were fully reversible by a subsequent incubation with 4 mM DTT. The depressive actions of DTNB on the NR1(C744S)/NR2B subunit configuration could thus be due to the formation of a mixed disulfide (between one-half of the DTNB molecule and a sulfhydryl group in the protein) with a second critical cysteine present on NR1. This amino acid, Cys-798, is also important for the formation of the redox-sensitive site on this subunit (37), perhaps by forming a disulfide bond with Cys-744 (40, 41). A mixed disulfide should be subject to reduction by DTT, and this appears to be the case.

A separate group of CHO cells transfected with the four aforementioned subunit configurations was also incubated with KCN (2 mM, 3–6 min). We observed that cyanide produced a  $1.4 \pm 0.1$ -fold potentiation of  $30 \mu\text{M}$  NMDA-elicited responses in cells expressing NR1/NR2A receptors ( $n = 6$ ), with DTNB (0.5 mM) rapidly ( $<1$  min) reversing this effect (to  $82.1 \pm 14.1\%$  of control,  $n = 5$ ; Fig. 3A). Moreover, KCN had a very similar effect to 4 mM DTT in CHO cells transfected with NR1(C744S)/NR2A receptors ( $1.8 \pm 0.3$ -fold increase,  $n = 4$ ; Fig. 3B). The

TABLE I  
Effects of redox agents on recombinant NMDA receptor-mediated currents

Currents elicited by a 30  $\mu$ M NMDA application to CHO cells transfected with the NMDA receptor subunits indicated were recorded under control conditions, following a 3-min incubation with 4 mM DTT and after a 1-min treatment with 0.5 mM DTNB. The values given are means  $\pm$  S.D. from 4 to 7 experiments.

NMDA receptor subunit composition	DTT	DTNB
	-fold increase of control response amplitude	% control response amplitude
NR1/NR2A	2.1 $\pm$ 0.6	73 $\pm$ 15
NR1(C744S)/NR2A	2.3 $\pm$ 1.3	55 $\pm$ 13
NR1/NR2B	1.9 $\pm$ 0.5	78 $\pm$ 23
NR1(C744S)/NR2B	1.0 $\pm$ 0.3	47 $\pm$ 86

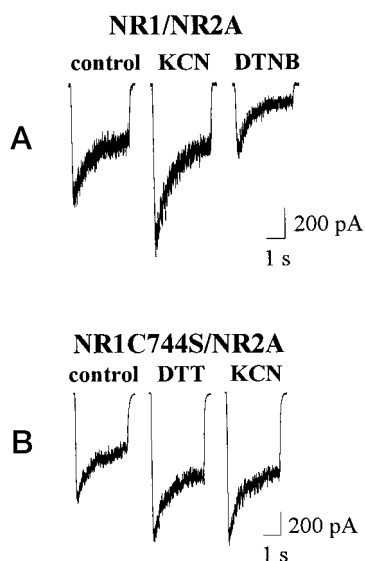


FIG. 3. Effects of KCN on NR2A-containing receptors. A, KCN (2 mM, 3 min) potentiates 30  $\mu$ M NMDA-elicited whole cell responses in CHO cells expressing NR1/NR2A. This effect of KCN was readily reversed by 0.5 mM DTNB (1 min). B, DTT (4 mM) and KCN (2 mM) treatments (3 min) independently enhanced 30  $\mu$ M NMDA-induced currents in CHO cells expressing NR1(C744S)/NR2A receptors.

potentiating action of KCN was spontaneously reversible upon washout, albeit slowly, similar to what has been observed following DTT treatment in native receptors (18). A concentration-response curve for the potentiating action of KCN on wild-type NR1/NR2A receptors after 3-min incubations was then generated (Fig. 4). The data were fitted to a logistic function; the calculated  $EC_{50}$  of KCN potentiating NMDA-induced responses in this preparation was 0.5 mM. Surprisingly, 2 mM KCN did not potentiate but, in fact, somewhat depressed NMDA-induced currents in cells transfected with NR1/NR2B (to 83.2  $\pm$  14.4% of control;  $n$  = 4), even though 4 mM DTT produced its expected response augmentation in these cells (Fig. 5A). A more pronounced current depression by KCN was observed in cells expressing the DTT-insensitive NR1(C744S)/NR2B receptor configuration (to 49.3  $\pm$  15% of control;  $n$  = 3; Fig. 5B). The depressive actions of KCN on the mutant receptor were not reversible by a subsequent treatment with 4 mM DTT. Similar to the effects of DTNB on NR2B-containing receptors, KCN produced a significantly greater depression of the NMDA-induced currents in cells expressing NR1(C744S)/NR2B subunits, when compared with NR1/NR2B ( $p$  < 0.05, Mann-Whitney two sample test).

The depressing actions of KCN on NR1/NR2B or NR1(C744A)/NR2B receptors could be due to reaction of these substances with either Cys-744 or Cys-798 in the wild-type

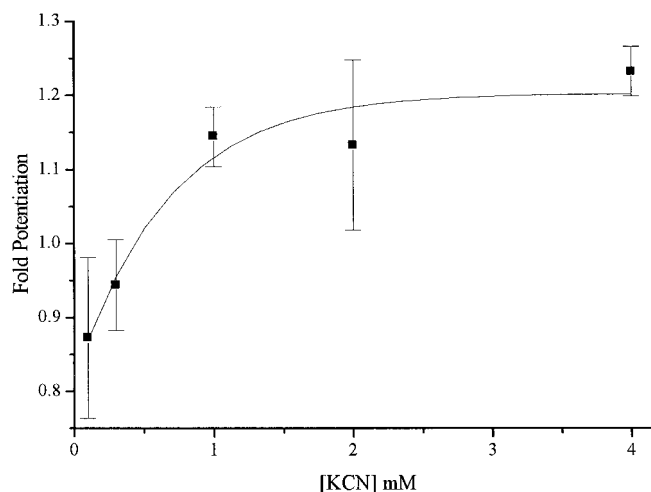


FIG. 4. Concentration-response curve for the potentiating actions of KCN on NR1/NR2A receptors. From data such as those shown in Fig. 3A, the fold increase in 30  $\mu$ M NMDA-mediated current amplitude was plotted versus the KCN concentration, after a 3-min incubation with the metabolic inhibitor. Symbols represent the mean  $\pm$  S.E. for 3–6 cells tested at each concentration of KCN. The data were fitted to a logistic function; the calculated  $EC_{50}$  was 0.5 mM.

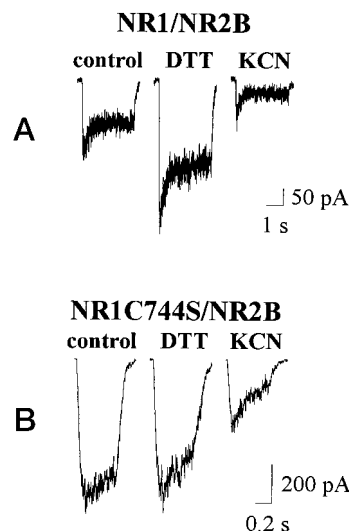


FIG. 5. Effects of KCN on NR2B-containing receptors. A, DTT (4 mM, 3 min) enhanced but KCN (2 mM, 3 min) depressed the NMDA responses in cells transfected with NR1/NR2B subunits. B, in cells expressing DTT-insensitive NR1(C744S)/NR2B receptors, KCN still depressed the NMDA-evoked currents.

subunit or with the remaining Cys-798 in the case of the mutant. These interactions would lead to the formation of a DTT-insensitive thiocyanate adduct. In order to test this hypothesis we obtained a series of recordings with a double NR1(C744A,C798A)/NR2B mutant. Interestingly, the inhibitory actions of 2 mM KCN (2 min) were significantly decreased but still present in the double mutant, when compared with the single mutant ( $p$  < 0.05, unpaired  $t$  test; Fig. 6). This effect was not reversible by 4 mM DTT. Responses obtained from NR1(C744A,C798A)/NR2B receptors after cyanide treatment were 84.2  $\pm$  19.9% ( $n$  = 6) of control currents, a value very similar to the inhibitory effect of KCN on wild-type NR1/NR2B receptors. Interestingly, 0.5 mM DTNB had a similar inhibitory effect on NR1(C744A,C798A)/NR2B receptors (84.2  $\pm$  6.9% of control,  $n$  = 5) as in the wild-type NR1/NR2B subunit configuration and significantly different from its effect on the single mutant NR1(C744S)/NR2B ( $p$  < 0.0001; unpaired  $t$  test). These results support the hypothesis that DTNB and KCN interact

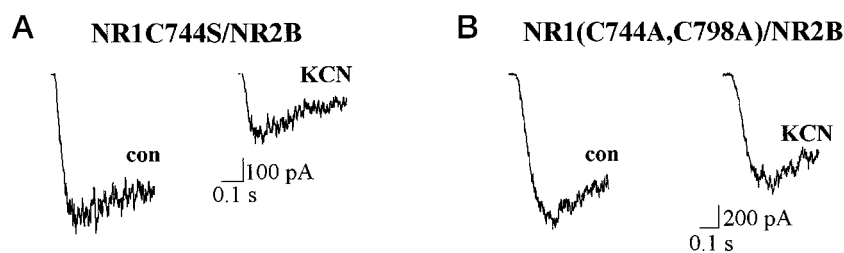


FIG. 6. Mutation affecting KCN sensitivity of NR2B-containing receptors. *A*, as noted earlier, KCN (2 mM, 3 min) depressed 30  $\mu$ M NMDA-induced currents in CHO cells expressing NR1C744S/NR2B receptors. *B*, this effect of KCN is substantially decreased in cells expressing double mutant NR1(C744A,C798A)/NR2B receptors.

with Cys-798 in the single NR1 mutant and suggest a novel effect of these substances with a different site in either the wild-type or double mutant NR1/NR2B receptor. Comparative measurements were obtained on the NR1(C744A,C798A)/NR2A subunit combination. A 3-min incubation with either 2 mM KCN or 4 mM DTT produced a  $1.7 \pm 0.5$ -fold ( $n = 4$ ) or a  $2.2 \pm 0.8$ -fold ( $n = 4$ ) potentiation, respectively, of these receptors.

**Effects of KCN on NMDA-induced Responses in Neurons at Early Developmental Stages**—Cortical neurons in culture have been shown to express primarily, but not solely, NR1 and NR2B early in development (42, 43). As neurons mature *in vitro*, expression of NR2A becomes more prominent. Hence, KCN was tested for its ability to alter NMDA (30  $\mu$ M)-induced responses in young neurons (8 DIV). The metabolic inhibitor produced a modest potentiation of these responses after a 5-min incubation (to  $1.2 \pm 0.1$ -fold of control,  $n = 5$ ). It is possible that the relatively small amounts of NR2A expression at this developmental stage are sufficient to prevent a current depression by KCN in these neurons, such as that seen for the NR1/NR2B combination. Nonetheless, the aforementioned potentiating actions of KCN observed in mature neurons were more prominent than in the younger cells ( $p < 0.05$ , Mann-Whitney two sample test). Interestingly, 2 mM KCN applied for 3–5 min to CHO cells transfected with NR1, NR2A, and NR2B produced neither a potentiation nor a depression of NMDA-elicited currents ( $n = 4$ ). One must interpret this result with caution, however, since the proportion of functional receptors that co-assemble all three subunits in this situation is not known.

**Actions of KCN on NR1/NR2A Are Not Due to Zinc Chelation**—A recent report (39) has suggested that the previously proposed redox-sensitive site on the amino-terminal of NR2A (38) may actually represent a high affinity zinc recognition site. Through this site, low background zinc levels present in extracellular solutions may constitutively inhibit NR1/NR2A channel activity. Since DTT has good metal chelating properties (44), Paoletti and co-workers (39) proposed that the rapid onset and quickly reversing component of the effects of the reducing agent on NR1/NR2A-mediated currents actually represents the removal and re-introduction of endogenous zinc block. We therefore evaluated whether the actions of KCN on NR1/NR2A may, in fact, be mediated by a similar mechanism as this substance can also chelate metals to some extent (45). We initially tested the actions of 1  $\mu$ M TPEN, a high affinity zinc chelator (46), on NMDA-elicited currents in mature (25–29 DIV) cortical neurons (Fig. 7A) and in NR1/NR2A-transfected CHO cells (Fig. 7B). TPEN did not alter the peak currents elicited by the agonist in both these preparations ( $92.3 \pm 13.5\%$  of control,  $n = 8$  for neurons;  $94.2 \pm 11.4\%$  of control,  $n = 5$  for NR1/NR2A), although a small effect on NR1/NR2A current desensitization was noted. This suggests that the levels of zinc in our external recording solution may be below the binding affinity of this metal for its site on NR2A (approximately 5 nM;

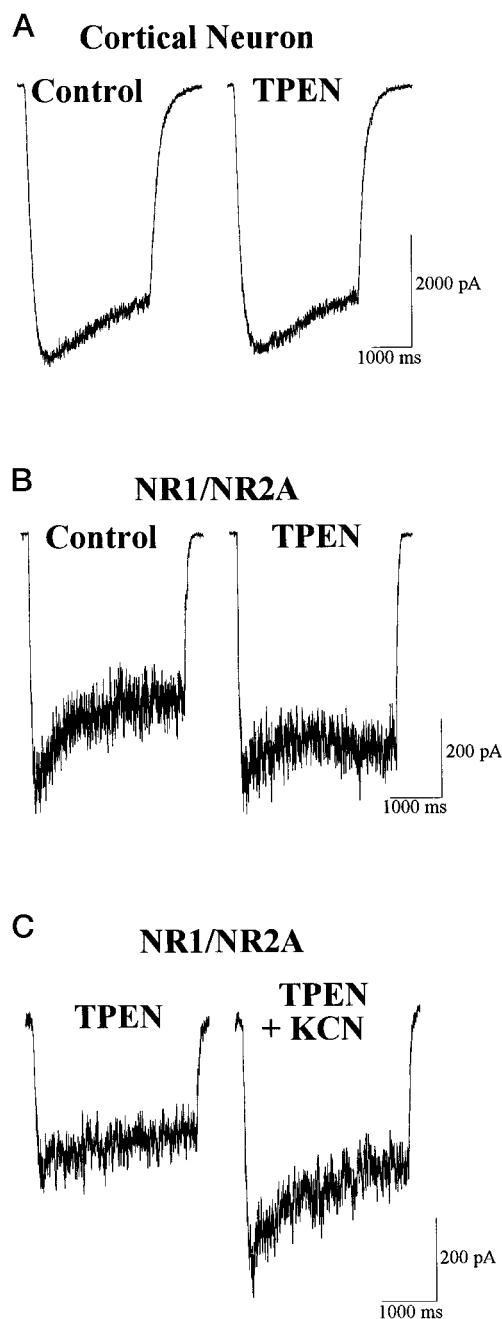
see Refs. 39 and 47). We investigated the integrity of our TPEN solution by evaluating its effect on chelating exogenously added zinc on NMDA receptor-mediated currents in cortical neurons. One  $\mu$ M TPEN significantly ( $p < 0.001$ , paired *t* test) reversed the blocking actions of 1  $\mu$ M zinc on these currents, that is the steady-state responses to 30  $\mu$ M NMDA in the presence of zinc were  $64.1 \pm 10.1\%$  of control, whereas in zinc and TPEN they were  $86.7 \pm 10.6\%$  of the currents produced by NMDA alone ( $n = 6$ ). Finally, and most importantly, the inclusion of 1  $\mu$ M TPEN in all external solutions did not affect the potentiating actions of 2 mM KCN on responses mediated by the activation of NR1/NR2A receptors ( $1.5 \pm 0.3$ -fold increase,  $n = 4$ ; Fig. 7C). This suggests that the actions of KCN on this receptor configuration are not due to removal of zinc.

#### DISCUSSION

Cyanide has been used extensively to characterize and identify critical thiols in a variety of enzymes and other proteins (28–30, 48, 49). The present study not only confirms the previously proposed direct interaction of the toxin with the NMDA receptor (15, 16) but suggests a possible effect on the functional thiol groups of the protein. Our data indicate that the modifications of these sites by KCN can produce different, subunit-dependent physiological outcomes as follows: either an enhancement or a depression of receptor channel function. Hence, mature cortical neurons in culture, which have been shown to express comparatively high levels of NR2A, in conjunction with NR1 and NR2B (42, 43), show a more pronounced sensitivity to KCN-mediated NMDA current potentiation than younger neurons, which express primarily NR1/NR2B receptors and less NR2A. This is in stark contrast to the potentiating actions of DTT on native NMDA receptors, which we have recently reported to be substantially larger in immature cultured neurons than in older cells (50).

Studies by Köhr *et al.* (38) and Sullivan *et al.* (37) suggested that the NMDA receptor contained two separate redox agent-sensitive centers. Köhr and co-workers (38), by using chimeric NR subunits, showed that the extracellular amino-terminal of NR2A contained a DTT-sensitive site(s) that influenced NMDA-induced currents. Investigations by Sullivan *et al.* (37) employed site-directed mutagenesis to demonstrate that Cys-744 and Cys-798 of NR1, located in the extracellular loop between the putative transmembrane domains III and IV, were critically important for rendering redox sensitivity to NR1/NR2B, NR1/NR2C, and NR1/NR2D receptors but, as expected, not to the NR1/NR2A subunit combination.

As mentioned earlier, a recent study has questioned the existence of a redox modulatory site on NR2A. Paoletti *et al.* (39) have suggested that the observed rapid effects of DTT on NR1/NR2A receptors represent zinc chelation and removal from solution with the consequent relief of a high affinity block by the metal. We believe that not all of the effects of DTT on NR1/NR2A receptors can be explained by zinc chelation, however. First, DTT (and KCN) produces a slow potentiation of the



**FIG. 7. Actions of KCN on NR1/NR2A are not due to zinc chelation.** *A*, whole cell currents from a mature cortical neuron in culture in response to 30  $\mu\text{M}$  NMDA under control conditions or in the continuous presence of 1  $\mu\text{M}$  TPEN. *B*, similar responses obtained from a CHO cell expressing NR1/NR2A receptors. Note that in both *A* and *B*, TPEN does not increase the peak response to the agonist, although a small effect on current desensitization can be noted in the CHO cell response. *C*, NMDA (30  $\mu\text{M}$ )-induced currents in a CHO cell expressing NR1/NR2A receptors recorded in the continuous presence of 1  $\mu\text{M}$  TPEN before and after a 3-min treatment with 2 mM KCN.

currents in our experiments, which is different from the rapid onset (<10 s) of the DTT and TPEN effects seen by Paoletti *et al.* (39) in their investigations. Second, the strong metal chelator TPEN, in our hands, had practically no effect on NMDA receptor-mediated currents in either mature neurons or in CHO cells expressing NR1/NR2A receptors, suggesting that background zinc levels in our preparation are very low. These observations may lead one to conclude that the slow effects produced by DTT or KCN on native receptors on mature neurons or on NR1/NR2A recombinant receptors may then be due

to the interactions of these substances with the redox site on NR1(Cys-744 and Cys-798; see Ref. 37). However, these substances work well in potentiating currents mediated by NR1(C744S)/NR2A or NR1(C744A,C798A)/NR2A receptors, which would suggest that a separate redox site exists on either NR1 or NR2A. The fact that KCN depresses slightly the currents mediated by NR1/NR2B receptors, which contain Cys-744 and Cys-798 on NR1, potentially as the only DTT-sensitive site(s), would seem to indicate that a KCN/DTT-sensitive site does lie within NR2A in NR1/NR2A receptors.

A recent study from our laboratory (35) demonstrated that two DTT-sensitive sites could be electrophysiologically distinguished in recombinant NMDA receptors, at least at the single channel level. Redox agents were shown to affect both open channel frequency and open time in NR1/NR2A receptors but only frequency in NR1/NR2B channels. The behavior of receptors putatively composed of NR1/NR2A/NR2B subunits was more akin to the latter configuration. The present investigations show that two putative redox-active sites can be chemically distinguished by cyanide. This toxin mimics the actions of DTT for the NR1/NR2A subunit configuration, potentiating NMDA-induced currents, but behaves more like DTNB at the NR1/NR2B receptor by depressing the responses. As NR2A is expressed in our cultures (43) and in similar preparations (42), at the time we perform most of our recordings the effects of KCN on NR2A appear to override, or somehow occlude, the actions of the toxin on any NR2B-containing receptors that are present in the neurons (NR1/NR2B or NR1/NR2A/NR2B). This complex subunit interaction is complemented by our recent single channel studies (35) that revealed a masking of a subunit-specific redox property when both NR2A and NR2B co-assembled in the same receptor with NR1. Therefore, detection of the modification of the NR1 redox site by KCN may not be possible, at least macroscopically, when NR2A is present in the final receptor and regardless of whether or not there is co-assembly with NR2B. This possibility is strengthened by the fact that the redox site on NR1 can only be alkylated with *N*-ethylmaleimide (18) when this subunit combines with NR2B but not with NR2A (38).

The inhibitory actions of KCN on NR1(C744S)/NR2B receptors could be due to the cyanolation (formation of a thiocyanate adduct) of Cys-798 of NR1. It is unlikely, however, that such a chemical modification can produce equivalent allosteric changes on the receptor as those induced by the hypothetical formation of a disulfide bond between Cys-744 and Cys-798 after DTNB-induced oxidation (40, 41). Therefore, it remains to be seen what effects cyanide has, if any, on NR1(C798S)/NR2B receptors. Experiments are currently underway to address these issues. Nonetheless, the effect of DTNB on NR1(C744S)/NR2B receptors are similar to that produced by KCN on these channels and significantly larger than its effect on NR1/NR2B channels. Since KCN also depresses NMDA-stimulated currents in cells expressing wild-type NR1/NR2B subunits, it is attractive to speculate that the preferred effect of the toxin on this receptor configuration is cyanolation of the same site where DTNB oxidizes. In addition, this site may be neither Cys-744 or Cys-798 on NR1, as the actions of both DTNB and KCN are similar in wild-type or double mutant NR1(C744A,C798A)/NR2B receptors. Although this possibility can only be definitively tested by further studies, it may pave the way for future experiments aimed at identifying additional sites susceptible to thiol modification on all NMDA receptor subunits, which could also be susceptible to cyanolation.

In summary, cyanide represents the first described molecule able to distinguish chemically between two putative redox sites on the NMDA receptor. Furthermore, the chemical reduction of

the receptor by cyanide, leading to the potentiation of NMDA-induced currents in neurons, may be sufficient to account for the enhancement in excitotoxicity produced by the metabolic inhibitor, similar to what has been seen for DTT (51). The ability of cyanide to produce different effects on NMDA receptor function, depending on the subunit composition, further supports the notion that two or more putative redox sites modify NMDA receptor function by different allosteric mechanisms (35). Finally, the use of cyanide and cyanide adducts may help to characterize biochemically the mechanisms whereby the redox modulatory sites influence NMDA receptor channel activity.

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