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Regulation of the NMDA receptor by redox phenomena: inhibitory role of ascorbate

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Redox phenomena seem to modulate activity of the *N*-methyl-D-aspartate receptor. Some reductants (ascorbate, hydroquinone) inhibit, while others (dithiothreitol, mercaptoethanol, penicillamine) potentiate NMDA receptor function. Ascorbate inhibits binding of [^{3}H]glutamate and [^{3}H]thienylcyclohexylpiperidine to the NMDA receptor complex, and impedes NMDA-gated currents in isolated neurons; dithiothreitol-like reductants enhance NMDA-induced currents. The ability of reductants to alter function of the NMDA receptor is abolished by oxidation.

Redox mechanisms regulate numerous cellular processes, including receptor function[9]. Ascorbic acid (AA) is a bio-reductant abundant in all mammalian tissues, including the central nervous system (CNS). Most mammals, excluding guinea pigs, flying mammals and primates, synthesize AA. The AA content in the rat brain is 200–300 μg/g wet tissue (1.1–1.7 mM)[24]. As the estimated extracellular levels of AA in the brain are 100–500 μM[24,29], the intracellular concentrations should be greater, resulting from rapid and saturable transport from the plasma[28]. The CNS levels of AA remain relatively constant despite variations in intake[24,28].

Because glutamate evokes secretion of AA from synaptic compartments[6,23], we were interested if AA and redox phenomena might play a role in glutamatergic neurotransmission. In this study, we explored interactions of various reductants with excitatory amino acid receptors activated by *N*-methyl-D-aspartate (NMDA). Using biochemical and electrophysiological methods, we found that redox phenomena may regulate NMDA receptor function. We postulate the existence of a novel redox-sensitive regulatory center(s) at this receptor. Preliminary data from this study have been published[17].

NMDA receptors respond to agonists by increasing cation (Na^+, Ca^{2+}, and K^+) conductance in a voltage-dependent manner, exerting excitatory actions on neurons[2,5]. NMDA receptors participate in fundamental CNS functions, such as synaptic plasticity and learning[20], but their overstimulation leads to accumulation of intracellular Ca^{2+}, resulting in neurotoxicity. Such phenomena are involved in neuronal damage due to ischemia[26] or seizures. Function of the NMDA receptor is allosterically potentiated by glycine[2,27] and is inhibited by zinc[30]. NMDA-gated cationic conductance is impeded by Mg^{2+}, in a voltage-dependent manner[2], and by dissociative anesthetics, such as ketamine and phencyclidine, which bind to an open state of the channel[1].

We characterized the pharmacological profile of sites that bind [^{3}H]glutamate in crude synaptosomal membranes from rat forebrain by comparing inhibition of [^{3}H]glutamate binding by NMDA, quisqualate, and kainate, which interact with distinct classes of glutamate receptors[11,14,18]. The IC_{50} for inhibition of 20 nM [^{3}H]glutamate binding by glutamate was 340 ± 141 nM (n = 3; Fig. 1A). NMDA inhibited 50 ± 4% of [^{3}H]glutamate binding (IC_{50} = 5.9 ± 1.7 μM; n = 3). Quisqualate inhibited [^{3}H]glutamate binding biphasically, displacing 39 ± 2% of the specific binding at 1–10 μM concentrations (IC_{50} = 340 ± 22 nM; n = 3), and producing greater inhibition at concentrations > 10 μM, suggesting a concentration-dependent interaction with two populations of sites. Kainate was inactive at concentrations up to 1 mM. Hence, 20 nM [^{3}H]glutamate labeled mainly two populations of sites in our system; about 50% of the binding seemed to involve NMDA receptors and about 40%, quisqualate receptors.

Next, we examined AA effects on [^{3}H]glutamate binding to NMDA receptors in the presence of 5 μM quisqualate. AA inhibited [^{3}H]glutamate binding to the NMDA receptors (IC_{50} = 510 ± 53 μM; n = 3; Fig. 1B), being about 1000 times less potent than glutamate (IC_{50} = 460 ± 122 nM; n = 3; Fig. 1B). The atypical

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dose–response curve of AA suggested positive cooperativity of AA action at doses > 300 μM. Up to 3 mM AA did not significantly alter the pH of the assay buffer used for [^{3}H]glutamate binding (50 mM Tris-acetate, pH 7.2).

To determine if AA alters the state of the NMDA-gated channel, we examined effects of AA, among other reductants, on [^{3}H]N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine (TCP) binding. TCP (an analog of phencyclidine) is a potent blocker of the NMDA receptor-gated channel; and [^{3}H]TCP binding serves as a biochemical probe of channel function, because it is increased by NMDA receptor agonists and decreased by antagonists[3, 15]. Several reductants inhibited [^{3}H]TCP binding (Fig. 2), with the following K_i values: AA, 436 ± 29 μM (n = 7); dehydroascorbic acid, 4.8 ± 0.6 mM (n = 3); sodium ascorbate, 5.1 ± 0.8 mM (n = 3); hydroquinone 5.0 ± 1.3 mM (n = 3); sodium sulfite, 11.1 ± 0.8 mM (n = 3). Dithiothreitol had little effect on [^{3}H]TCP binding, inhibiting less than 40% of binding at 30 mM concentration (n = 3). The effect of AA on [^{3}H]TCP binding was typified by positive cooperativity (n_H = 1.5), attributable partially to a decrease in pH of the assay buffer (5 mM Tris-HCl, original pH = 7.4). Other reductants did not alter pH. Since lowering of pH decreases [^{3}H]TCP

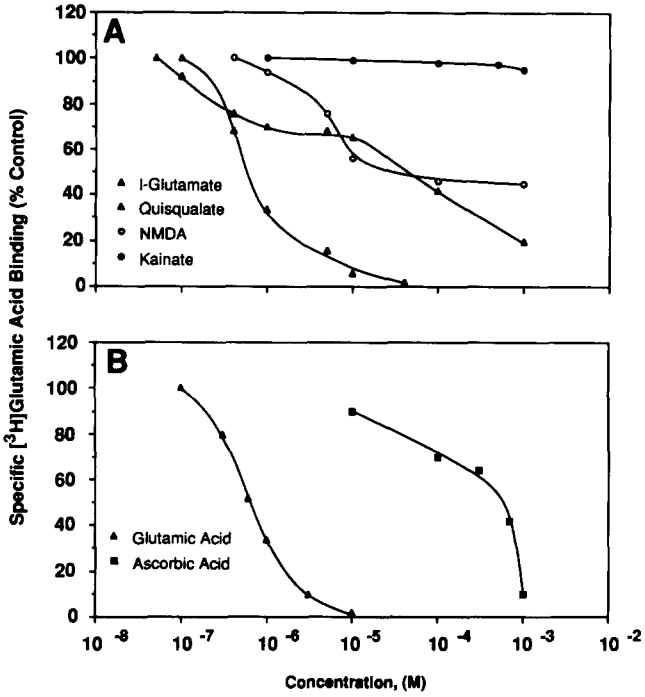

Fig. 1. [^{3}H]Glutamate binding to crude synaptosomal membranes. A: inhibition by specific ligands for excitatory aminoacid receptors. B: interaction of ascorbate with NMDA receptors. Binding was assayed in well-washed crude synaptosomal fractions from rat (male Fisher 344; 4-month-old) forebrains. Tissue was homogenized in 15 vols. of 0.32 M sucrose/50 mM Tris-HCl buffer (pH 7.4), using a glass-Teflon homogenizer. The homogenate was centrifuged at 1000 g for 10 min, and the resulting supernatant fluid was centrifuged at 40,000 g for 10 min. The pellet was resuspended by homogenization with a polytron in 30 vols. of 5 mM Tris-acetate, pH 7.2, allowed to stand for 20 min on ice and centrifuged for 10 min at 40,000 g. The resulting pellet was washed 2 times by homogenization and centrifugation, and was frozen at –70 °C. On the day of experiment the pellet was thawed, washed 5 times with 15 vols. of ice-cold buffer (50 mM Tris-acetate; pH.7.2) by homogenization and centrifugation, and was suspended in buffer. The membranes (250 μg protein) were incubated with 20 nM [^{3}H]glutamate (NEN, spec. act. 51.9 Ci/mmol) on ice for 20 min in the presence or absence of competitors in a total volume of 0.5 ml. The reactions were terminated by filtration over Whatman GF/C filters, and the filters were washed twice with 4 ml of ice-cold buffer. The radioactivity on the filters was measured by liquid scintillation spectrometry. Non-specific binding was determined in the presence of 1 mM glutamate. All experiments were done in triplicate. Data are mean values from 3 experiments.

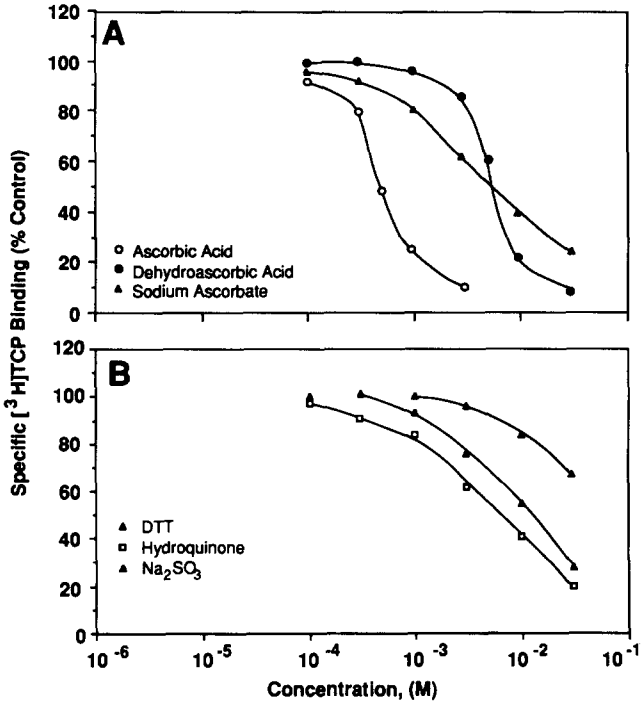

Fig. 2. [^{3}H]TCP binding. A: inhibition by ascorbic acid, dehydroascorbic acid, and sodium ascorbate. B: inhibition by hydroquinone, sodium sulfite (Na_2SO_3), and dithiotreitol (DTT). [^{3}H]TCP binding was assayed as described before[16]. Rat forebrains were homogenized in 15 vols. (g/ml) of 5 mM Tris-HCl buffer, pH 7.4 using a Polytron and homogenates were centrifuged at 40,000 g for 10 min. The pellets were rehomogenized in 15 ml of buffer, the homogenates centrifuged, and the pellets frozen at –70 °C. On the day of the experiment the pellet was thawed, washed twice with 5 mM Tris-HCl, suspended in the buffer, and used for assays. The membranes (400 μg protein) were incubated in 25 °C with 8 nM [^{3}H]TCP (NEN, Boston, MA; 60 Ci/mmol) in the presence or absence of drugs. After a 45-min incubation, the reactions were terminated by filtration under vacuum over Whatman GF/C filters, presoaked in 0.05% polyethyleneimine. The filters were rinsed 3 times with 3 ml of buffer and radioactivity on the filters was measured by liquid scintillation spectrometry. Protein was measured by Bradford method (Bio-rad, Richmond, CA) with bovine serum albumin as the standard. Non-specific binding was determined in the presence of 200 μM phencyclidine; at a ligand concentration of 8 nM it represented about 5% of the total binding. Data are mean values from 3–7 experiments.

binding (data not shown) and inhibits the function of NMDA receptors[7], it is probable that hydronium ions act in synergy with AA to close NMDA-gated channels, thus decreasing TCP binding. Scatchard analysis of the saturation binding data revealed that AA (500 μM) reduced the apparent density of [^{3}H]glutamate recognition sites (–57%; $n = 3$; $P < 0.05$, Dunnett's test) and decreased the affinity of [^{3}H]TCP binding sites ($P < 0.05$; Table I). AA also abolished both glutamate- and glycine-mediated potentiation of [^{3}H]TCP binding (results not shown).

The interaction of reductants with the NMDA receptor was also tested in electrophysiological studies on cultured cerebral cortical neurons from the rat. Perfusion of neurons with AA decreased NMDA-induced inward currents (Fig. 3A). AA, 1 mM, inhibited the current by 42 ± 11% ($n = 4$) and 3 mM inhibited by 48 ± 4% ($n = 8$). Steady-state blockade usually occurred within 1–2

TABLE I

Scatchard analysis of the effects of ascorbic acid on binding of [^{3}H]glutamate and [^{3}H]TCP to the NMDA receptor

Binding was assayed as described (Fig. 1) in the presence of 5 μM quisqualic acid in every tube. Twelve concentrations of both [^{3}H]glutamate (5–450 nM) or [^{3}H]TCP (1–200 nM) were used. AA concentration was 500 μM; the assays were performed in duplicate. The equilibrium binding data were analyzed by the LIGAND computer programs[21]. Data represent means ± S.E.M. for the number of experiments shown in parenthesis. * Denotes statistically significant changes from control (Dunnett's test, $P < 0.05$).

	K_d (nM)	B_{max} (fmol/mg protein)
[^{3}H]Glutamate		
Control ($n = 7$)	279 ± 32	2025 ± 143
Ascorbic acid ($n = 3$)	284 ± 52	886 ± 30*
[^{3}H]TCP		
Control ($n = 5$)	11.7 ± 1.3	1700 ± 89
Ascorbic acid ($n = 3$)	25.3 ± 1.8*	1503 ± 22

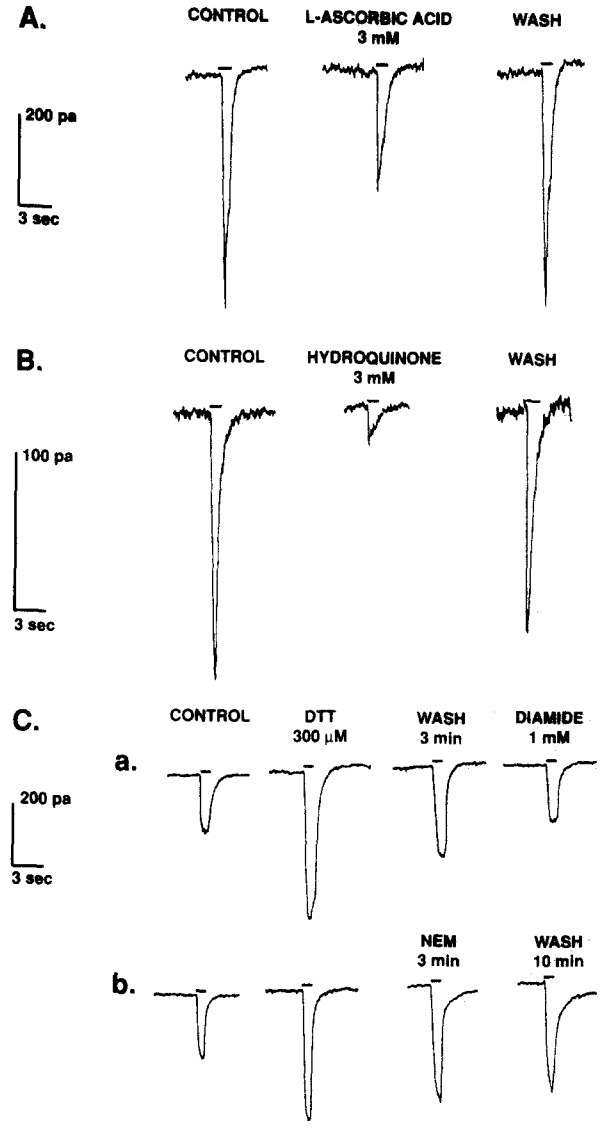

Fig. 3. Effects of various reductants on NMDA-evoked currents in cultured cortical neurons. Neurons from medial cortex (hippocampal enriched) of fetal rat brain (E 15) were prepared according to previously described methods[25]. The cultures were used in electrophysiological experiments 3 weeks after plating. For recordings, the cells were bathed in a bicarbonate-phosphate buffer of the following composition: (mM) NaCl 138.6, KCl 3.35, $NaHCO_3$ 21, $NaHPO_4$ 0.58, $CaCl_2$.5, $MgCl_2$ 0.01, glucose 12. Tetrodotoxin (0.3 μM) was present in the buffer to suppress synaptic activity. Recordings were made at room temperature on the stage of an inverted phase microscope. The cells were superfused with the recording solution at a rate of 1 ml/min. Drugs were dissolved in the recording medium, and were applied by a glass capillary tube (50 μm internal diameter), affixed to a polyethylene U-tube, positioned about 50–100 μm lateral to the cell[22]. This arrangement allowed rapid and reliable application of different drug solutions to the same cell. NMDA (500 μM) was dissolved in the buffer and was applied to the cell from a puffer pipette (3 μM tip) at 1-min intervals. The puffer pipette was positioned so that the stream from it joined the stream from the perfusion pipette. A high concentration of NMDA was used because it was diluted and diverted by the stream from the perfusion pipette. Stable responses to NMDA were obtained before any drug application, and the position of the application pipettes was never changed during recordings. Whole cell recordings were obtained using an Axoclamp II amplifier, with the single electrode voltage clamp in the continuous mode, from cells clamped at –50 mV. A: effect of Ascorbic acid (AA) on NMDA evoked responses. NMDA was applied for 1 s every 60 s. The buffer solution from the miniperfusion pipette was replaced with solution containing AA (3 mM). The response shown under AA was recorded 3 min after the beginning of AA perfusion. Recovery was obtained 2 min after resumption of perfusion of the control buffer. B: effect of hydroquinone on NMDA responses. Hydroquinone was applied in a similar manner as above, but to a different cell. Recovery ensued within 2 min after reinstatement of control solution. C: effect of dithiothreitol (DTT) on NMDA-evoked currents. a: DTT enhanced the NMDA response within 2 min after the beginning of its application. At 3 min after removal of DTT the response was still enhanced. Application of diamide (1 mM), returned the response to the control level. Replacement of diamide with the control solution had no effect on the response (not shown). b: DTT application (300 μM, 3 min) potentiated the NMDA response in a subsequent application to the above cell. Replacement of DTT with *N*-ethylmalemide (NEM; 300 μM, 5 min) impeded recovery of the response; 10 min after removal of NEM enhancement of the response persisted.

min, with rapid recovery (> 90%) of the original peak current after reperfusion of the cell with control buffer. Hydroquinone, 1 mM, reversibly inhibited the NMDA-gated current by 41 ± 17% (n = 3), and 3 mM inhibited the current by 71 ± 9% (n = 6; Fig. 3B). At 300 μM concentration, neither AA nor hydroquinone notably decreased NMDA-induced current. Neither of these compounds significantly changed the pH of the buffer or produced any current alone. Air-oxidized hydroquinone (3 mM; n = 2) was inactive, as was dehydroascorbic acid (3 mM; n = 5) (data not shown), suggesting that reducing properties are essential for the NMDA-inhibitory effects of AA and hydroquinone.

Surprisingly, dithiothreitol (DTT), which reduces cystine disulfide bonds, markedly potentiated NMDA-induced currents at micromolar to low millimolar concentrations. The EC_{50} for DTT was about 10 μM, and maximal potentiation (+75 ± 18%) was produced by 30 μM–3 mM DTT (Fig. 3C; n = 16). DTT, at concentrations which enhanced electrophysiological responses to NMDA (< 3 mM), did not alter [^{3}H]TCP binding (Fig. 2). This lack of effect of DTT on [^{3}H]TCP binding (where theoretically potentiation should be expected), could possibly result from destruction of DTT-sensitive centers on the receptor during membrane preparation procedures, which included freezing and thawing. The slight inhibition of [^{3}H]TCP binding by DTT > 3 mM appears to be not related to the action of this compound on NMDA receptor function.

Two other disulfide-reductants, mercaptoethanol (1 mM) and penicillamine (100 μM–3 mM), also potentiated NMDA-induced currents by 164 ± 19% (n = 2) and 169 ± 12 (n = 3), respectively (data not shown). None of these compounds produced any current alone. Oxidation of DTT in the test tube with diamide (1–3 mM) abolished the ability of DTT to potentiate NMDA-induced currents. The DTT effect was reversible upon perfusion of cells with the normal medium. This process was time-dependent, as return to control levels of the NMDA response took 4.8 ± 1.8 min (n = 6). However, when the cells were rapidly perfused with diamide (1–3 mM), after DTT treatment, a return to control levels or below occurred within 1 min (Fig. 3Ca). Diamide alone did not alter the NMDA-mediated response. In contrast, when neurons were perfused with the alkylating agent, *N*-ethylmalemide, following DTT treatment, the increased response to NMDA persisted (Fig. 3Cb).

Our results show that various reductants distinctively alter NMDA receptor function. Reduction mediated by DTT-like compounds that is associated with the breakage of disulfide bonds, potentiates NMDA receptor function, whereas reduction mediated by AA or hydroquinone, inhibits receptor function. AA inhibits the binding of [^{3}H]glutamate and [^{3}H]TCP to the NMDA receptor, and it reversibly blocks NMDA-gated currents in neurons. Redox phenomena are implicated in the AA/hydroquinone-mediated inhibition of NMDA receptor function because oxidized forms of these compounds did not decrease NMDA-induced currents. Also the DTT-mediated potentiation of NMDA receptor function appears redox-related, as it was rapidly reversed by treatment of the cell with the oxidant, diamide. Oxidation of DTT with diamide also abolished the ability of DTT to enhance NMDA-induced currents. In contrast, alkylation of the sulfhydryl groups after DTT treatment prolonged the facilitatory effect (Fig. 3C).

We propose that the NMDA receptor contains novel regulatory center(s) sensitive to tissue redox state. The rapid, reversible inhibition of NMDA receptor activity by reductants, such as AA or hydroquinone, may result from conformational changes produced by alteration of the electric charge of the receptor. In contrast, breakage of the protein disulfide bonds by reductants such as DTT, mercaptoethanol, or penicillamine, which potentiate NMDA receptor function, suggests the existence of regulatory sulfhydryl center(s) on the receptor.

Redox mechanisms may comprise NMDA receptor regulatory systems, which are sensitive to tissue metabolic state. As electron transport occurs across the plasma membrane[19], reduction of the redox-sensitive center may proceed either by extra- or intracellular mechanisms. Thus, it is conceivable that the reduction-mediated inhibition of the NMDA receptor is related to the intriguing resistance of neurons, containing high levels of NADPH-diaphorase, to NMDA-mediated injury[10].

The possible physiological role of enhanced NMDA receptor activity associated with breakage of disulfide bonds on the receptor is yet to be defined. However, reductive inhibition of NMDA receptors may be physiologically mediated by AA, as the millimolar levels of AA, which are active, are likely to be achieved postsynaptically during intense neuronal activity[6,23]. Theoretically, high CNS levels of AA could be neuroprotective, as inhibition of the NMDA receptor protects neurons from ischemic damage[26]. Indeed, we observed protection by AA of cerebral cortical neurons in cultures against NMDA- and glutamate-induced toxicity (Majewska and Bell, *Neuroreport,* in press). During ischemia, possible neuroprotective actions of AA could be synergistically enhanced by accompanying acidosis, which promotes positive cooperativity of channel closing, reflected by [^{3}H]TCP binding. This notion is supported by the fact that acidosis curtails NMDA receptor function[7]. Moreover, AA may play a neuroprotective role during stress, which is accompanied by elevation of plasma and brain levels of this reductant[4,12].

Under normal conditions, CSF levels of AA may be too low to impede the functioning of NMDA receptors, but during intense neuronal activity or acute stress, when AA is released from the adrenal glands and synaptic compartments, its level in the CSF may be adequate to attenuate the activity of NMDA receptors. The pattern of diurnal rhythms of AA in the brain and plasma[8] also suggests that peak levels of this compound could serve to protect neurons from overstimulation. In contrast to neuroprotective actions of AA, DTT was recently shown to exacerbate NMDA receptor-mediated neurotoxicity[13], consistent with our observation that DTT potentiates NMDA action.

In conclusion, our findings suggest that the NMDA receptor contains redox-sensitive regulatory center(s). Because the naturally occurring reductant, AA, at physiological concentrations, antagonizes function of the NMDA receptor, it is possible that it may function as a neuroprotective agent.

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