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Reduction of NMDA receptors with dithiothreitol increases [^{3}H]-MK-801 binding and NMDA-induced Ca^{2+} fluxes

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1 We have investigated the modulation of N-methyl-D-aspartate (NMDA) receptor activation by the sulphydryl redox reagents dithiothreitol (DTT) and 5,5-dithio-bis-2-nitrobenzoic acid (DTNB).

2 Increases in [^{3}H]-MK-801 binding produced by glutamate, glycine and spermidine were enhanced by DTT (2 mM) and diminished by DTNB (0.5 mM).

3 The inhibition of [^{3}H]-MK-801 binding by CGS 19755 and 7-chlorokynurenate was not altered by 2 mM DTT. However, the potency of the competitive polyamine antagonist, arcaine, was decreased by DTT.

4 NMDA-induced Ca^{2+} fluxes into primary cultures of rat forebrain neurones were enhanced by DTT in a DTNB-reversible fashion. In addition to augmenting the magnitude of NMDA-induced increase in intracellular free Ca^{2+}, 10 mM DTT also prolonged the duration of the Ca^{2+} signal. However, DTT had no effect on the increase in Ca^{2+} produced by depolarizing neurones with 50 mM KCl.

5 These studies show that the reduction of disulphide bonds on the NMDA receptor complex by DTT increases activation. The precise site of these groups remains unclear but they are unlikely to form an integral part of the glutamate, glycine or polyamine binding domains. The enhancement of the activation of the NMDA receptor by DTT is associated with increased Ca^{2+} fluxes. The possible pathophysiological consequences of receptor reduction are discussed.

Introduction

Activation of the N-methyl-D-aspartate (NMDA)-preferring subtype of glutamate receptor is subject to modulation by a variety of endogenous substances, including glycine, Mg^{2+}, Zn^{2+} (see Collingridge & Lester, 1989 for review) and polyamines (Ransom & Stec, 1988; Reynolds & Miller, 1989a; Williams *et al.*, 1989). In most cases the physiological consequence of the actions of these modulators is not entirely clear due to the difficulty in manipulating modulator levels *in vivo*. However, the action of substances that modulate the NMDA receptor have been the subject of great interest because of the likely therapeutic utility of NMDA receptor-directed drugs in neurological disorders including stroke, neurodegenerative diseases and epilepsy.

It was recently demonstrated that dithiothreitol (DTT) could enhance NMDA responses electrophysiologically (Aizenman *et al.*, 1989). As the actions of DTT could be fully reversed by 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), which oxidizes sulphydryl groups, it appeared that the actions of DTT were due to a chemical modification of one or more disulphide bonds on the NMDA receptor. Other studies have generally confirmed that the action of DTT on the NMDA receptor promotes activation (Lazarewicz *et al.*, 1989; Levy *et al.*, 1990; Sacaan & Johnson, unpublished observations). Furthermore, DTT enhances synaptic activity in areas of the brain where NMDA receptors are thought to be involved, including the hippocampus (Tolliver & Pellmar, 1987; 1988; Tauck & Ashbeck, 1990). However, the precise mechanism of action of DTT remains unclear.

In this study we have further investigated the actions of DTT on NMDA receptors. We have employed [^{3}H]-MK-801 to label the NMDA receptor-channel complex. This ligand can be used to monitor drug action at all of the known modulator sites on the NMDA receptor and, thus, it represents a powerful tool for probing the actions of DTT (Reynolds & Miller, 1988; 1989a; Ransom & Stec, 1988). In addition, we have studied the actions of DTT and DTNB on changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) produced by NMDA. This is a particularly important component to consider because the pathophysiological actions of NMDA appear to be Ca^{2+}-dependent (Choi, 1987).

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Methods

Radioreceptor binding assays

[^{3}H]-MK-801 binding to well washed membranes prepared from frozen rat brains (Pel-Freez, Rogers AR) was performed as previously described (Reynolds & Miller, 1989a). Assays consisted of 1 ml 10 mM HEPES/NaOH, pH 7.4 which contained 50–100 μg protein, 0.5 nM [^{3}H]-MK-801 and drugs as appropriate. Non-specific binding was determined by using 30 μM MK-801. Following a 2 h incubation at room temperature (21–25°C) assays were terminated by vacuum filtration over glass fibre filters (Schleicher and Schuell, Keene NH). Radioactivity was determined by liquid scintillation counting with an efficiency of approximately 50%. Basal [^{3}H]-MK-801 binding under these conditions ranged from 60–127 c.p.m. Estimates for EC_{50} values, as well as basal and maximum levels of binding for compounds which increased [^{3}H]-MK-801 binding were obtained using a simplex curve fitting routine which employed the following equation:

$$E = E_{max} \cdot D/(D + EC_{50}) + B$$

where E is the increase in binding for a given drug concentration, D, with a maximal response E_{max} and basal (unstimulated) binding of B. Spermidine, as previously noted (Reynolds & Miller, 1989a), produces a biphasic curve (see Figure 1). Only data points on the ascending phase of the dose-response curve were analysed for EC_{50} estimation. Dose-response curves performed in the presence of DTT or DTNB were compared to control curves by a repeated measures analysis of variance (ANOVA) test using both redox state and concentration as within subject factors. *Post hoc* comparison of means was performed by Tukey's HSD test with P values of less than 0.05 being considered significantly different.

Intracellular Ca^{2+} assays

$[Ca^{2+}]_i$ was monitored in primary cultures obtained from embryonic rat (E17) forebrain as previously described (Reynolds & Miller, 1989b). Cells were plated onto poly-D-lysine-coated glass cover slips in Dulbecco's modified Eagle's medium (DMEM) containing 10% v/v foetal bovine serum, 100 u ml^{-1} penicillin and 5 $\mu g\,ml^{-1}$ streptomycin. After 24 h the medium was replaced by DMEM supplemented with antibiotics and 10% v/v horse serum, and the cover slips inverted. Cells were kept in a humidified incubator with 95% air, 5% CO_2 until use, which was usually 12–21 days. At the time of use, cover slips were turned over and rinsed in HEPES-buffered salt solution (HBSS) of the following composition (mM): NaCl 137, KCl 5, $MgSO_4$ 0.9, $NaHCO_3$ 3, NaH_2PO_4 0.6, K_2HPO_4 0.4, glucose 5.6 and HEPES 20, pH adjusted to 7.4 with NaOH. Cells were then incubated in HBSS containing 5 mg ml^{-1} bovine serum albumin and 5–7 μM fura-2 AM (Molecular Probes, Eugene OR, U.S.A.) for approximately 1 h at 37°C. Cover slips were then rinsed for 10 min in HBSS before use.

Recordings of fura-2 fluorescence were made as follows. Light from a 200 W mercury-xenon lamp (Oriel Corporation, Stratford CT, U.S.A.) was filtered by 340 and 380 nm narrow band-pass filters (Omega Optical, Brattleboro VT, U.S.A.). The filters were mounted in a wheel driven by computer-controlled stepping motor (Ludl Electronic Products, Hawthorne NY, U.S.A.) that moved filters rapidly (approx. 100 ms) to alternate between illumination at 340 and 380 nm wavelengths. The recording chamber (Biophysica Inc. Baltimore MD, U.S.A.) was mounted on the stage of a Nikon Diaphot fluorescence microscope. Fluorescence from single neurones was obtained by using a rectangular diaphragm to limit the visual field of the video port of the microscope. Levels of fluorescence were detected and quantified by a Thorn EMI bialkali photomultiplier and a C10 photon counting system (Thorn EMI Electron Tubes Inc, Fairfield NJ, U.S.A.). The photon counting system was interfaced with a 30386-based IBM compatible computer by custom designed data acquisition software. Values for $[Ca^{2+}]_i$ were estimated by comparing ratios obtained from single neurones with known values on a standard curve constructed with a range of EGTA-buffered Ca^{2+} concentrations. Values for $[Ca^{2+}]_i$ were calculated from the equation:

$$[Ca^{2+}]_i = K \cdot (\text{Ratio} - \text{Rmin})/(\text{Rmax} - \text{Ratio})$$

where K is a constant for the affinity of fura-2 for Ca^{2+} (Grynkiewicz *et al.*, 1985). The values for K, Rmin and Rmax were obtained from standard curves.

To monitor the effects of DTT and DTNB on NMDA-induced changes in $[Ca^{2+}]_i$ single neurones were first tested without addition of DTT or DTNB. DTNB (0.5 mM) was then added for 60 s and subsequently washed out before the addition of agonist. This condition gave the response to the agonist with a consistently oxidized state of the receptor. For each additional response the 60 s DTNB application was followed by 120 s incubation with the appropriate concentration of DTT. This agent was also washed out before the addition of agonist. Agonists were applied by perfusion, and application continued until the $[Ca^{2+}]_i$ change had reached a clear maximum. Reaching a maximum response rarely took more than 40 s. Following each addition of agonist, levels of $[Ca^{2+}]_i$ were allowed to return to baseline before additional drug treatments were performed. Baseline levels of $[Ca^{2+}]_i$ were usually stable for the duration of the experiment, which was usually less than 90 min. In experiments with 50 mM KCl, osmolarity was adjusted by substituting an appropriate amount of NaCl with KCl.

Drugs

[^{3}H]-MK-801 (15 Ci $mmol^{-1}$) was purchased from Du Pont/NEN (Boston MA, U.S.A.). Unlabelled MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) was a generous gift of Dr Richard Ransom (Merck, Sharp and Dohme, West Point PA, U.S.A.). CGS 19755 (1-(*cis*-carboxypiperidine-4yl)methyl-1-phosphonic acid) and 7-chlorokynurenate were generous gifts from Dr Paul Ornstein (Eli Lilly, Indianapolis, IN, U.S.A.). All other drugs and chemicals were from commercial sources.

Results

Effects of DTT and DTNB on [^{3}H]-MK-801 binding

Several studies have demonstrated that [^{3}H]-MK-801 binding to well-washed rat brain membranes is sensitive to the state of activation of the NMDA receptor (Reynolds *et al.*, 1987; Foster & Wong, 1987). We employed the [^{3}H]-MK-801 binding assay to attempt to establish the mechanism of action of DTT. Preliminary experiments showed that DTT produced a small increase (10–20%) in [^{3}H]-MK-801 binding to well-washed membranes at concentrations between 20 μM and 6 mM, while concentrations of this reducing agent above 8 mM decreased binding. To investigate the possible mechanisms underlying this action of DTT, we performed dose-response curves to the positive modulators of [^{3}H]-MK-801 binding, namely glutamate, glycine and spermidine (Figure 1). We employed 2 mM DTT as the test concentration to avoid the inhibition of binding seen at higher levels. DTT increased [^{3}H]-MK-801 binding at each concentration of glutamate tested. In contrast, the oxidizing agent DTNB generally decreased [^{3}H]-MK-801 binding. A similar pattern was observed when dose-response curves to glycine were performed (Figure 1b). DTT had rather modest effects on the increase in [^{3}H]-MK-801 binding produced by spermidine (Figure 1c). The ascending phase of the curve was shifted to the left slightly, but there was no difference in the maximum response. Interestingly, higher concentrations of spermidine more effectively decreased [^{3}H]-MK-801 binding in the presence of DTT. In the presence of DTNB, spermidine increased [^{3}H]-MK-801 binding less effectively when compared to controls.

The binding parameters for the agonists shown in Figure 1 are summarized in Table 1. DTT and DTNB did not alter the EC_{50} of the agonists tested. The fold increase in binding shown in Table 1 is expressed as the agonist-induced increase in binding over basal levels in the presence of DTT and DTNB as appropriate. From these analyses it was apparent that the principle action of DTT was to raise the baseline level of binding rather than to modify the glutamate- or glycine-induced increase in [^{3}H]-MK-801 binding. In contrast, DTNB tended to decrease basal binding. However, when binding was expressed as a proportion of control (i.e. no DTT or DTNB), as is shown in Figure 1, the actions of DTT and DTNB were more apparent.

Table 1 Effects of dithiothreitol (DTT) and 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) on ligand binding to N-methyl-D-aspartate (NMDA) receptors

	EC_{50} (μM)	*Fold increase*[a]
Glutamate		
Control	0.484 ± 0.15	3.17 ± 0.2
+ DTT 2 mM	0.364 ± 0.07	2.73 ± 0.1
+ DTNB 0.5 mM	0.456 ± 0.09	2.75 ± 0.3
Glycine		
Control	0.372 ± 0.04	3.04 ± 0.6
+ DTT 2 mM	0.359 ± 0.05	3.11 ± 0.3
+ DTNB 0.5 mM	0.442 ± 0.11	2.44 ± 0.03
Spermidine		
Control	8.12 ± 0.47	13.5 ± 3.6
+ DTT 2 mM	6.78 ± 0.59	12.1 ± 3.5
+ DTNB 0.5 mM	9.47 ± 0.39	10.4 ± 2.7

Results are the mean ± s.e.mean of 4–5 curves performed in duplicate as described in Methods.
[a] Fold increase in [^{3}H]-MK-801 binding above basal levels.

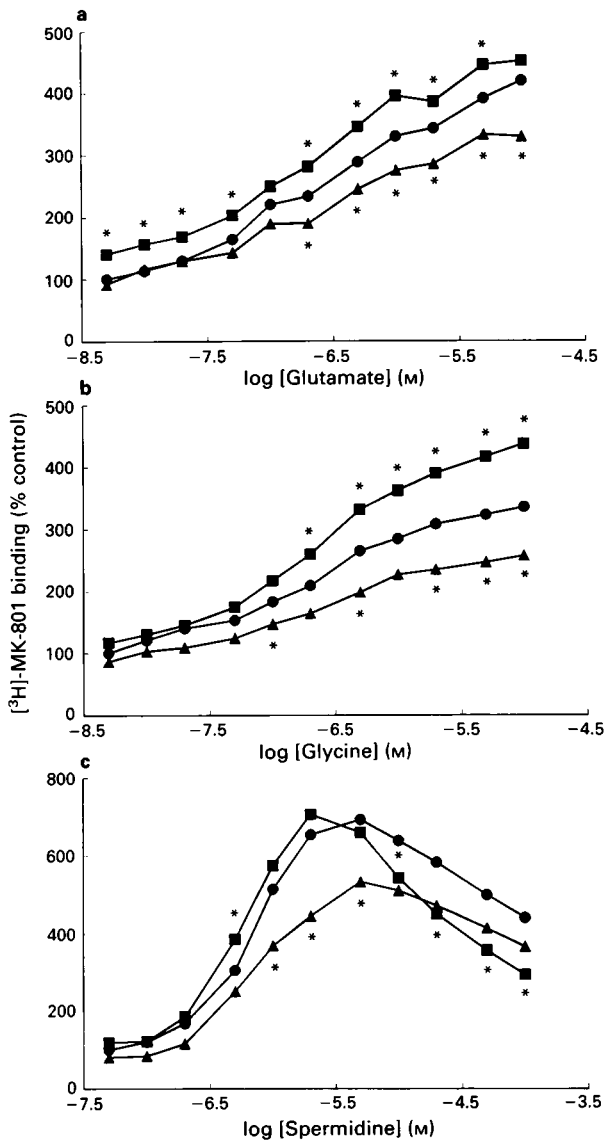

Figure 1 Effects of dithiothreitol (DTT) and 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) on the modulation of [^{3}H]-MK-801 binding by glutamate, glycine and spermidine. Concentration-response curves were performed using (a) glutamate, (b) glycine and (c) spermidine in the absence of redox reagents (●), or in the presence of 2 mM DTT (■) or 0.5 mM DTNB (▲). Curves with glutamate were obtained in the presence of 0.03 μM glycine, while curves with glycine were obtained in the presence of 0.1 μM glutamate. Curves with spermidine were obtained in the presence of 0.1 μM glutamate and 0.03 μM glycine. Standard error bars were omitted for clarity and usually represented less than 15% of the values shown. Results represent the mean of 4–5 experiments preformed in duplicate. Data points on curves performed in the presence of DTT and DTNB that are significantly different ($P < 0.05$) from corresponding values on the control curve are indicated by an asterisk.

We also examined the effects of DTT on the inhibition of [^{3}H]-MK-801 binding by competitive antagonists of glutamate, glycine and spermidine, namely CGS 19755, 7-chlorokynurenate and arcaine, respectively (Table 2). DTT had rather modest effects on the binding of these antagonists. However, a small but significant decrease in the affinity of the spermidine antagonist, arcaine, was found.

Effects of DTT and DTNB on NMDA-induced $[Ca^{2+}]_i$ changes

NMDA and glycine applied together to rat forebrain neurones in culture produced a rapid rise in $[Ca^{2+}]_i$. We tested 9 cells with up to 4 DTT concentrations per cell. Preliminary

Table 2 Effects of dithiothreitol (DTT) on antagonist binding to N-methyl-D-aspartate (NMDA) receptors

	IC_{50} (μM)
CGS 19755	
Control	4.20 ± 0.51
+ DTT 2 mM	2.99 ± 0.32
7-Chlorokynurenate	
Control	4.74 ± 0.88
+ DTT 2 mM	5.91 ± 1.30
Arcaine	
Control	0.79 ± 0.28
+ DTT 2 mM	4.58 ± 0.32**

Results are the mean ± s.e.mean of 4–6 curves performed in duplicate as described in Methods.
** Significantly different from control ($P < 0.01$, Student's t test).

experiments demonstrated that DTT (2 mM) could enhance responses to NMDA and glycine. To avoid differences in DTT effects arising from variations in the basal redox state of the neurones (Aizenman *et al.*, 1989) we used the following protocol: NMDA (30 μM) and glycine (1 μM) were applied to each cell at the beginning of the experiment, and then following a 60 s incubation with 0.5 mM DTNB, which was washed out before the agonists were re-applied. Following DTNB treatment responses to NMDA and glycine were reduced to 88.8 ± 5.1% of control. DTNB was then re-applied and washed out before each DTT treatment in order to return each cell to an oxidized state. Recordings from a typical cell are shown in Figure 2a. DTT increased NMDA and glycine-induced $[Ca^{2+}]_i$ changes between 1 and 10 mM. This effect could be completely reversed by the addition of DTNB. The concentration-dependence of the action of DTT on peak $[Ca^{2+}]_i$ responses to NMDA and glycine are shown in Figure 2b.

Interestingly, 10 mM DTT always increased the time taken to recover from the NMDA and glycine stimulus. Thus, while $[Ca^{2+}]_i$ normally returned to basal levels rapidly when the agonists were washed out, $[Ca^{2+}]_i$ remained elevated for as long as 20 min following treatment with 10 mM DTT. Although the actual recovery time varied from cell to cell a prolongation was seen in all cells tested with 10 mM DTT ($n = 8$). This phenomenon was seen less frequently with 3 mM DTT and never seen at all with 1 mM DTT. In order to determine whether this represented an action of DTT on Ca^{2+} homeostasis rather than a specific effect on NMDA receptors, we depolarized cells with 50 mM KCl to activate voltage-sensitive Ca^{2+} channels. As shown in Figure 3, this procedure produced rapid and reversible $[Ca^{2+}]_i$ changes that were similar in magnitude to those produced by NMDA with glycine. However, while the $[Ca^{2+}]_i$ signal produced by NMDA was increased by 10 mM DTT, KCl-induced increases in $[Ca^{2+}]_i$ were not altered. This demonstrated that DTT apparently did not alter Ca^{2+} homeostatic processes in the cell, but modified responses that were associated with NMDA receptor function.

Discussion

These studies have confirmed and extended the observation that activation of the NMDA receptor can be modulated by redox reagents (Aizenman *et al.*, 1989). One goal of this investigation was to establish the mechanism by which DTT produced its effects. We employed the [^{3}H]-MK-801 binding assay to monitor the effects of DTT on the actions of glutamate, glycine and spermidine. We observed a general increase in [^{3}H]-MK-801 binding which was consistent with the increase in NMDA receptor function associated with DTT. However, we did not find any specific alterations in the effects of the modulators tested. As DTT increased [^{3}H]-MK-801 binding even in the presence of maximal levels of glutamate

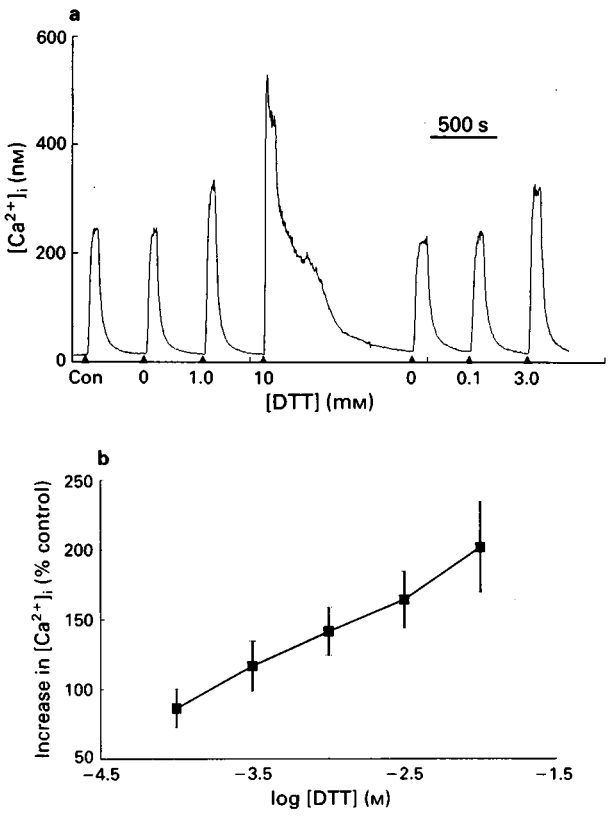

Figure 2 Effects of dithiothreitol (DTT) on N-methyl-D-aspartate (NMDA) and glycine-induced $[Ca^{2+}]_i$ changes in rat single cultured forebrain neurones. (a) $[Ca^{2+}]_i$ recording from a single neurone. NMDA (30 μM) and glycine (1 μM) were added at times indicated by triangles. Agonists were applied for 40 s and then removed by perfusion. With the exception of the first response (marked 'Con') cells were treated for 60 s with 0.5 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) followed by 120 s incubation with the concentration of DTT indicated, which was then washed out before agonist application. DTNB treatment alone reduced responses to 89 ± 5% of control levels. Where the DTT concentration is zero, cells were pretreated with DTNB only before NMDA and glycine application, thus indicating that DTNB completely reversed the actions of DTT. Following agonist application cells were perfused continuously until $[Ca^{2+}]_i$ levels returned to baseline. (b) Concentration-response relationship for effect of DTT on responses to NMDA and glycine. The data represent the peak NMDA and glycine-induced increase of $[Ca^{2+}]_i$ above baseline $[Ca^{2+}]_i$ levels in DTT-treated cells and are expressed as a percentage of control (untreated) responses. Each data point represents the mean of 4–8 cells and vertical lines show s.e.mean. Up to six different DTT concentrations were tested per cell. Responses to NMDA and glycine following DTNB but not DTT treatment near the end of each cell were 94 ± 10% of control responses, suggesting that responses were relatively stable even over the 60–90 min duration of these experiments.

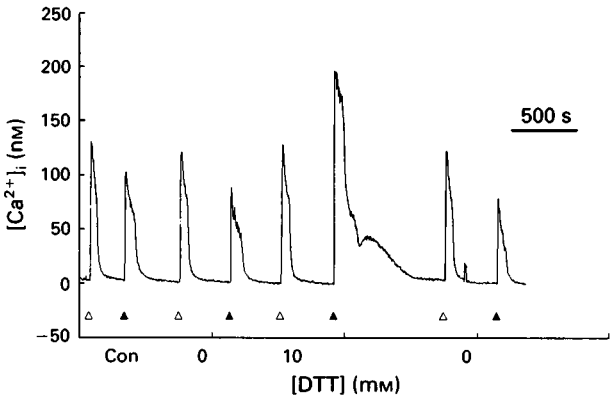

Figure 3 Effects of dithiothreitol (DTT) on $[Ca^{2+}]_i$ responses produced by N-methyl-D-aspartate (NMDA) and glycine and by depolarization with KCl in single cultured rat forebrain neurones. The trace represents a $[Ca^{2+}]_i$ recording from a single neurone. NMDA (30 μM) and glycine (1 μM) together, or KCl (50 mM) were added at times indicated by closed and open triangles, respectively. Agonists were applied for 40 s and then removed by perfusion. With the exception of the first response (marked 'Con') cells were treated for 60 s with 0.5 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) followed by 120 s incubation with the concentration of DTT indicated, which was then washed out before agonist application. Where the DTT concentration is zero, cells were pretreated with DTNB only before NMDA and glycine application, thus indicating that DTNB completely reversed the actions of DTT. Following agonist application cells were perfused continuously until $[Ca^{2+}]_i$ levels returned to baseline. This trace is representative of four separate cells tested under these conditions.

and glycine it is unlikely that DTT acts simply by increasing the affinity of these ligands. Consistent with this, we did not observe an increase in affinity of glutamate and glycine. Moreover, DTT did not increase the degree to which glutamate and glycine enhanced [^{3}H]-MK-801 binding. Finally, DTT had little effect on the actions of the competitive glutamate and glycine antagonists CGS 19755 and 7-chlorokynurenate. Together these findings suggest that the redox site that is the target of the action of DTT and DTNB is probably not located within the binding site for glutamate or glycine.

The actions of polyamines are a little more complex. It has been proposed that polyamines are positive modulators of the NMDA receptor based on their effects on [^{3}H]-MK-801 or [^{3}H]-TCP binding (Ransom & Stec, 1988; Reynolds & Miller, 1989a; Williams *et al.*, 1989). DTT did not increase the magnitude of the stimulant actions of spermidine, and the concentration-response curve was only slightly shifted to the left. However, DTT did enhance the inhibitory actions of spermidine which are observed at higher concentrations of this polyamine. We have previously found that the decrease in [^{3}H]-MK-801 binding produced by high concentrations of polyamines is more pronounced when the receptor is fully activated (Reynolds & Miller, 1989a). Thus, the interaction between spermidine and DTT is consistent with a more activated state of the receptor. Conversely, DTNB reduced the overall action of spermidine. In addition, we observed that the actions of the competitive polyamine antagonist, arcaine (Reynolds, 1990), were less potent in the presence of DTT. The significance of these findings remains obscure as a physiological effect of the polyamines has not yet been demonstrated. In the absence of a profound modification of the actions of spermidine it seems unlikely that DTT or DTNB modifies the polyamine binding site directly either. As polyamines are synthesized intracellularly and there is little evidence to suggest that they are secreted, it seems reasonable to suggest that the polyamine site may be located on the intracellular face of the receptor. The physiological actions of DTT that we have observed are probably restricted to the external face of the receptor, since this reducing agent is not lipid permeable. In addition, it is unlikely that the effects of DTT are mediated by a second messenger as effects on NMDA receptors have been observed electrophysiologically in excised outside-out patches of membrane (Wright & Nowak, 1990). Thus, it is unlikely that the actions of DTT are mediated by a modification of the polyamine site.

Perhaps the most interesting findings of this study relate to the effects of DTT on NMDA-induced Ca^{2+} influx. Many of the physiological and pathophysiological consequences of activating NMDA receptors, including neuronal cell death, are directly related to Ca^{2+} and presumably increased $[Ca^{2+}]_i$ (MacDermott *et al.*, 1986; Choi, 1987). In this study we have demonstrated that the increases in ligand binding to the NMDA receptor are accompanied by increases in NMDA-induced changes in $[Ca^{2+}]_i$. These actions of DTT may be considered specific chemical changes to NMDA receptors in that (i) DTT does not have to be present during NMDA exposure to produce an enhanced response, (ii) all the effects of DTT can be reversed by DTNB, and (iii) DTT does

not alter changes in $[Ca^{2+}]_i$ produced by KCl depolarization. In contrast to results obtained with the [^{3}H]-MK-801 binding assay, we did not observe a decrease in NMDA-induced changes in $[Ca^{2+}]_i$ at higher concentrations of DTT. However, unlike the [^{3}H]-MK-801 binding assay, DTT was washed out before the addition of agonists, whereas DTT was present continuously during the incubation of the binding assay. The inhibition observed in the [^{3}H]-MK-801 assay probably reflects a low affinity inhibitory action of DTT that is unrelated to modification of sulphydryl groups.

An intriguing action of higher concentrations of DTT is illustrated by Figure 2a. Following treatment with 10 mM DTT the duration of the NMDA-induced $[Ca^{2+}]_i$ change was drastically increased. The time taken for the recovery from the NMDA application, which lasted 40 s, was greatly in excess of the time necessary to exchange the bath volume completely with rinse solution many times. We initially suspected that the prolonged Ca^{2+} signal was a consequence of delayed extrusion of free Ca^{2+} from the cytoplasm. However, as demonstrated in Figure 3, a response to a potassium depolarization of similar magnitude to the NMDA response was not altered by DTT. This observation apparently excludes the possibility that altered Ca^{2+} homeostasis can account for the difference. It is possible that NMDA and KCl increase $[Ca^{2+}]_i$ in different compartments within the cell, which would allow differential modulation of Ca^{2+} homeostasis by DTT. It is also interesting to note that the concentration-dependence of the two actions of DTT may be different. Thus, DTT increases the peak $[Ca^{2+}]_i$ response at lower concentrations than are required to prolong the $[Ca^{2+}]_i$ signal. This may indicate that modification of two or more distinct disulphide bonds are involved in these processes. Nevertheless, the physiological consequences of this modification remain to be determined.

Several studies have shown that a certain minimum time of trauma is required in cultured neurones in order to produce delayed cell death. If the NMDA receptor can be reduced *in vivo* by physiological agents, it is possible that this would result in a sensitizing effect to marginal traumatic events. Alternatively, regional variations in constitutive levels of reduction or oxidation could underlie the selective vulnerability that has been observed in many neurotoxicity studies (Pulsinelli, 1985; Wieloch, 1985).

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