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Long-Lasting Modification of the *N*-Methyl-D-Aspartate Receptor Channel by a Voltage-Dependent Sulfhydryl Redox Process

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SUMMARY

Ionic currents through the *N*-methyl-D-aspartate (NMDA) receptor channel are modulated by sulfhydryl redox reagents. We report here a novel form of redox modulation that alters NMDA channel kinetics in a voltage-dependent manner. The effects of the thiol reductant dithiothreitol (DTT) and the oxidizing agent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) on NMDA-activated whole-cell currents were examined at various transmembrane voltages in cultured rat cortical neurons. DTT produced a similar level of potentiation of NMDA-induced currents at both −60 mV and +30 mV. However, the reversal of this potentiation by a sulfhydryl-oxidizing agent was dependent on the holding potential, because DTNB decreased the DTT-potentiated NMDA responses more effectively at negative voltages. Interestingly, the NMDA peak current-voltage relationship became substantially outwardly rectifying when sequential DTT/DTNB treatments took place at a positive holding potential, but not under any other circumstances. Single-channel recordings from outside-out patches revealed that this phenomenon was likely produced by a significant and long-lasting 2.3-fold prolongation of the mean open time of NMDA channels at a positive holding potential. Thus, a voltage-dependent chemical alteration in NMDA receptor structure modified the kinetic properties of the associated ion channel.

Thiol-reducing agents have been observed to dramatically enhance NMDA-mediated responses, whereas oxidizing agents can decrease native responses and reverse the effects of reductants (1). The primary mechanism by which sulfhydryl redox agents modulate NMDA-mediated ionic currents is by altering the frequency of single-channel openings (2, 3), although small changes in the single-channel conductance have been observed (3). In addition to being altered by sulfhydryl reagents (1, 4–8), the redox-sensitive site on the NMDA receptor is modified by oxygen-derived free radicals (9) and the essential nutrient pyrroloquinoline quinone (10). Recently, nitric oxide has also been suggested to interact with this site (11), although this has not been a consistent observation (12). In the present report we describe a novel form of redox modification of this receptor that can be produced only at positive transmembrane potentials and is apparent only at such positive potentials. This effect appears to be the result of a long-lasting chemical modification of a structural site closely associated with a voltage-sensitive gating control mechanism of the NMDA receptor.

Experimental Procedures

Tissue culture. Cortices from embryonic day 16 C-D rats were dissociated as described previously (13). The growth medium consisted of a mixture of 80% (v/v) Dulbecco's modified Eagle's medium, 10% Ham's F-12 nutrient mixture, 10% heat-inactivated iron-supplemented calf serum, 25 mM HEPES, 24 units/ml penicillin, 24 μg/ml streptomycin, and 2 mM glutamine. The dissociated cells were plated onto 35-mm plastic dishes, each containing five 12-mm collagen/poly-L-lysine-coated coverglasses. Growth medium was changed on a Monday/Wednesday/Friday schedule. Non-neuronal cell proliferation was inhibited for 72 hr with 2 μM cytosine arabinoside 2 weeks after dissociation. Cells were used for electrophysiological recordings 1 week after inhibition.

Solutions. The extracellular solution was based on Hanks' salts and contained 137 mM NaCl, 1 mM $NaHCO_3$, 0.34 mM $NaHPO_4$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 2.5 mM $CaCl_2$, 5 mM HEPES, 22.2 mM dextrose, 0.001 g/liter phenol red, 0.5 μM tetrodotoxin, 5 μM (−)-bicuculline methiodide, and 1 μM glycine; pH was adjusted to 7.2 with NaOH. The intracellular pipette solution contained (in mM): CsCl, 140; $MgCl_2$, 1; $CaCl_2$, 1; EGTA, 2.25; and HEPES, 10; pH was adjusted to 7.2 with CsOH.

Recording conditions. Electrophysiological measurements were performed at room temperature (22°) using the whole-cell and outside-out configurations of the patch-clamp technique (14). The resistances of the patch electrodes were measured to be 3–5 MΩ for whole-cell recordings and 5–10 MΩ for single-channel measurements. Current signals were amplified using an Axopatch 1-C patch-clamp amplifier (Axon Instruments), filtered with an 80-dB/decade low-pass Bessel filter, and digitized with a computer interface system (TL-1-125 DMA Labmaster; Scientific Solutions). Data were collected and analyzed using commercially available software (pClamp; Axon Instruments).

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; DTT, dithiothreitol; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Single-channel data were also stored with a videocassette recording system (Neurocorder) for later analysis. During whole-cell recordings, signals were filtered at 500 Hz (−3-dB cutoff) and digitized at a rate of 1–2.5 kHz. During single-channel measurements, the signals were filtered at 1–2 kHz (−3-dB cutoff) and digitized at 5–10 kHz. Single-channel analysis was performed with pClamp software, using a 50% threshold criterion. Because the NMDA channel currents reversed near 0 mV, single-channel conductances were obtained by fitting a Gaussian function to the amplitude distribution and dividing the peak amplitude by the holding potential. All transitions were included in the analysis of open time distributions. The reference electrode was a Ag-AgCl wire connected to the extracellular solution by a 2 M KCl/1% agarose bridge.

Drug perfusion. Cells were continuously superfused with the extracellular solution at a rate of 0.5 ml/min. The agonist, dissolved in extracellular solution, was applied to the cells under study by pressure ejection (PicoPump; World Precision Instruments) from micropipettes placed in close proximity (20–30 μm) to the cell. With this technique, it is likely that the entire cell, including its processes, did not receive a homogeneous concentration of applied drug at the same time. The thiol redox reagents DTT (Calbiochem) and DTNB (Calbiochem) were applied slowly via the superfusate for 2–4 min. The effects of DTT and DTNB on NMDA-mediated whole-cell currents were normally tested while a given cell was continuously held at a given potential. A 30-sec wash period was always interposed between the DTT and DTNB treatments. For quantitation of the results, we generally averaged two or three peak responses for a given cell under each redox and voltage condition.

Results

It was originally reported that redox reagents modify the size of NMDA receptor-mediated whole-cell currents without altering the general shape of the current-voltage (I-V) relationship (1). In those studies, redox reactions were carried out while cells were voltage-clamped at −60 mV, and all subsequent testing was performed after the reagents had been rinsed off the cells. In the present experiments, we first examined the thiol reduction and oxidation processes at the NMDA receptor at various holding potentials, to determine the effects of transmembrane voltage on the chemical modification of the receptor.

Effect of membrane potential on redox modulation. Results are expressed as the mean ± standard error. During whole-cell recordings, rat cortical neurons were treated sequentially via the superfusate with 4 mM DTT (2–4 min) and 500 μM DTNB (2–4 min) at either −60 mV or +30 mV holding potentials. Fig. 1 shows that DTT produced similar levels of potentiation of 30 μM NMDA-induced currents at both −60 mV (2.0 ± 0.1-fold increase; $n = 8$) and +30 mV (1.9 ± 0.1-fold increase; $n = 8$). However, the reversal of this potentiation by an oxidizing agent was dependent on the holding potential, inasmuch as DTNB decreased the DTT-potentiated NMDA responses by 64.3 ± 3.1% at −60 mV, whereas it diminished these responses by only 31.5 ± 4.8% at +30 mV (Fig. 1). These two values differed significantly ($p < 0.001$; paired t test). This result shows that 500 μM DTNB is a less effective oxidizing agent at positive membrane potentials than at negative voltages. In fact, further oxidation was readily obtained when the membrane potential was changed to −60 mV. For example, Fig. 2 shows representative currents elicited by repeated NMDA applications in a cell in which sequential 4 mM DTT and 500 μM DTNB treatments were performed while the cell was continuously voltage-clamped at +30 mV (Fig. 2, *third* and *fourth traces*). As can be noted, DTNB did not effectively reverse the actions of DTT at this potential (see also Fig. 1). After these

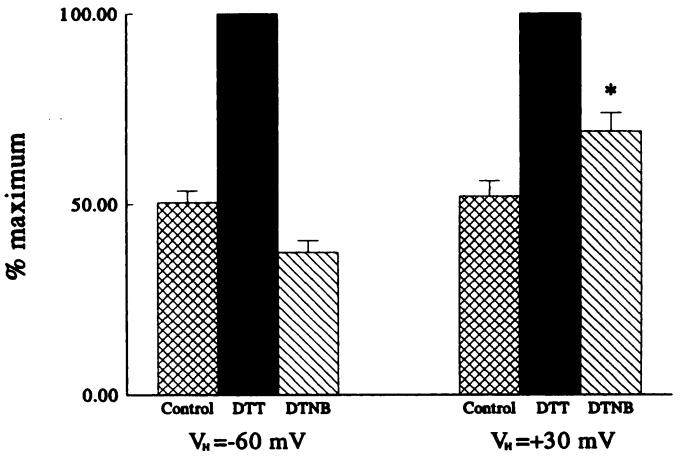

Fig. 1. Effects of DTT and DTNB on NMDA responses at different holding potentials. Mean whole-cell currents in a cortical neuron *in vitro* in response to 30 μM NMDA under control or oxidized (500 μM DTNB) conditions are expressed as a percentage of the maximum potentiated currents obtained after incubation of the cells with 4 mM DTT. Neurons were voltage-clamped continuously at either −60 or +30 mV. Whereas DTT produced a nearly 2-fold potentiation of the responses at either holding potential, DTNB was more effective in reversing the effects of DTT at −60 mV (64%) than at +30 mV (32%). Values are the mean ± standard error ($n = 8$), * Significantly different from DTNB treatment at −60 mV ($p < 0.001$; paired t test).

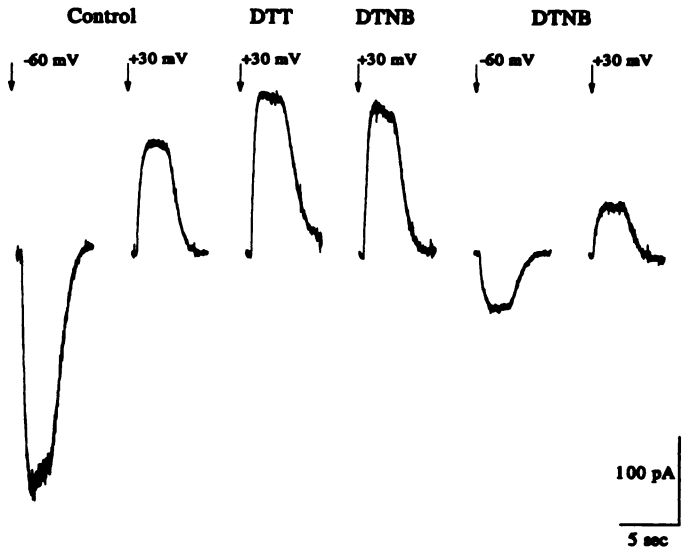

Fig. 2. Representative sequential whole-cell responses to 30 μM NMDA in a cortical neuron after exposure to 4 mM DTT or 500 μM DTNB at different holding potentials. Note that DTNB was able to oxidize the receptor more efficiently after the potential was switched from +30 to −60 mV. Similar results were obtained in five additional cells.

treatments, the holding potential of the cell was switched to −60 mV and a second DTNB treatment ensued. This was sufficient to promote further oxidation, because subsequent NMDA-mediated responses (Fig. 2, *fifth* and *sixth traces*) were diminished, relative to the control currents (Fig. 2, *first* and *second traces*). Traces shown in Fig. 2 are representative, because NMDA responses were obtained repeatedly throughout the treatments.

These results demonstrate that, although some oxidation does take place at positive potentials, the action of DTNB in reversing the effects of the DTT-induced potentiation is highly voltage dependent. Finally, it is noteworthy that treatment

with DTT at either negative (see Fig. 4 in Ref. 1) or positive (Fig. 3) potentials is sufficient to potentiate NMDA responses tested at other potentials. This suggests that the primary events that result in the redox modulation of NMDA responses at a given membrane potential, as manifested by a potentiation of the response by DTT and the reversal of this potentiation by DTNB, are likely the result of the chemical modification of a common site or sites in the receptor molecule. The primary difference noted so far, however, is the pronounced inability of DTNB to fully reverse the actions of DTT at positive, but not negative, membrane potentials.

Changes in the current-voltage relationship. During the course of the experiments described above, we observed that, after the sequential DTT/DTNB treatments at positive potentials (but not a negative potentials; see Ref. 1), the I-V relationship of peak NMDA responses became substantially outwardly rectifying (Fig. 4). We were alerted to this phenomenon by experiments such as that shown in Fig. 2. Specifically, compare the first two responses with the last two traces in Fig. 2. As can be noted, the sequential sulfhydryl redox treatments at +30 mV produced a change in the relative ratio of the responses at −60 and +30 mV, compared with pretreatment conditions. At a positive holding potential, a 2–4-min treatment with 4 mM DTT followed by a 30-sec wash period and a 2–4-min treatment with 500 μM DTNB resulted in a long-lasting change in the I-V properties of the whole-cell NMDA responses (Fig. 4). Furthermore, this rectification was not reversed after the cell was held at −60 mV for 5–10 min (Fig. 4), nor was it dependent on the direction that the cells were depolarized or hyperpolarized to establish the I-V relationship. Therefore, the voltage- and time-dependent effect on the probability of channel opening of NMDA receptors that was recently reported (2)

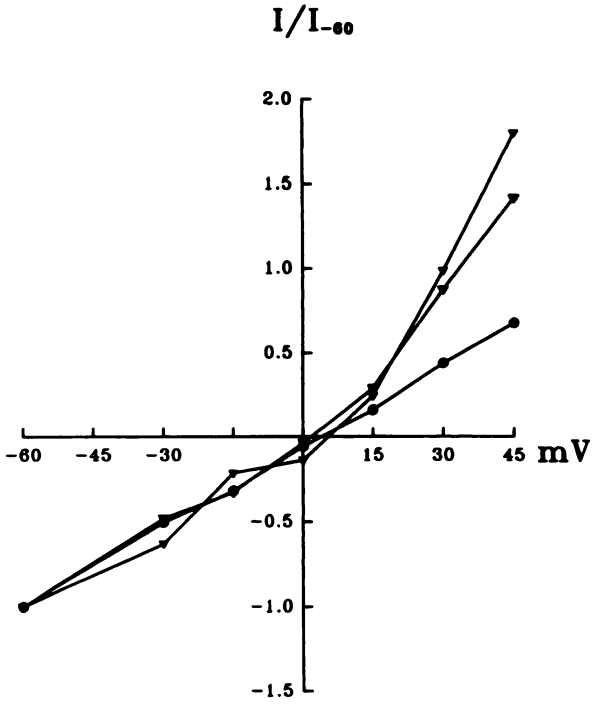

Fig. 4. Whole-cell NMDA I-V relationships become outwardly rectifying after reduction and oxidation at positive potentials. Whole-cell peak NMDA current-voltage relationships for a single cell under control conditions (●), after reduction with 4 mM DTT and oxidation with 500 μM DTNB at +30 mV (▽), and after rinsing of the cell with control solution for 5 min at −60 mV (▼) are shown. Values represent the average of three responses at each potential and have been normalized to the response measured at −60 mV under each condition, to facilitate comparison of the three relationships; standard error bars (<10%) were omitted for clarity. Similar results were obtained in a total of six cells.

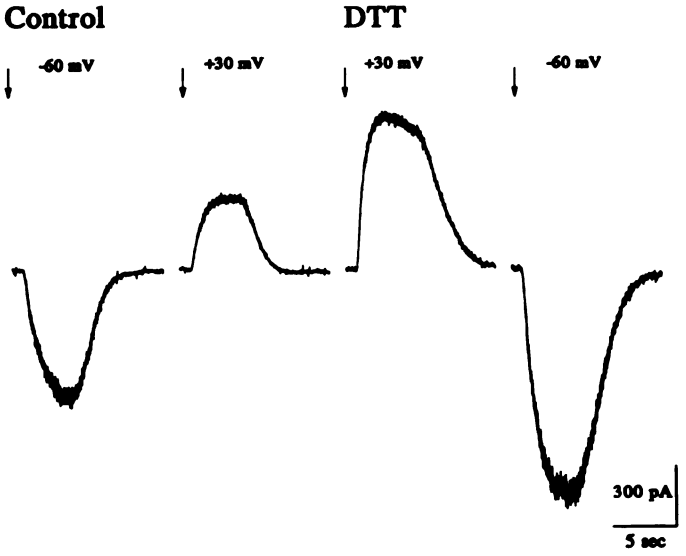

Fig. 3. Incubation with 4 mM DTT at +30 mV induces a potentiation of the NMDA-mediated response at a negative potential. Whole-cell currents elicited by 30 μM NMDA in a cortical neuron under control (pretreatment) conditions at two different holding potentials are shown (*first two traces*). While the cell was continuously voltage-clamped at +30 mV, treatment with DTT was performed and the potentiating actions of this agent on the NMDA-induced response were verified (*third trace*). Immediately after the washout of DTT, the holding potential was switched to −60 mV and a response to NMDA was elicited (*fourth trace*). Note that the potentiating actions of the reducing agent are also apparent at this potential. Similar results were obtained in five additional cells.

appeared not to be responsible for the phenomenon observed here.

Exposure to 4 mM DTT alone ($n = 4$) or exposure to 500 μM DTNB alone ($n = 3$) at +30 mV was not sufficient to produce the observed modification in the I-V relationship. This was most easily assessed by measuring the peak current amplitudes obtained at −60 mV and +30 mV before and after the redox treatment at +30 mV and calculating the absolute ratio of these two responses (Fig. 5). In addition, maintaining cells at +30 mV with no redox treatments for up to 10 min did not result in rectification ($n = 5$) (Fig. 5). In these records, however, a transient hysteresis in the I-V relationship, similar to that shown by other investigators (2), was sometimes observed (data not shown).

We then attempted to reverse the rectification by performing the following experiment ($n = 4$). After the sequential DTT/DTNB treatments at +30 mV (which resulted in the appearance of rectification), we treated the cell under study again with DTT at +30 mV, then rinsed the reducing agent off the cell, and finally switched the membrane potential to −60 mV. The presence of rectification was then tested before and after a second treatment with DTNB, this time at −60 mV. This procedure, however, was unsuccessful in reversing rectification. In addition, prolonged wash periods (>5 min) at either −60 mV or +30 mV were also ineffective. These results suggest that a long-lasting modification of the receptor via a novel, voltage-sensitive, redox site has been produced. Furthermore, this site is distinct from the redox site uncovered previously (1, 3),

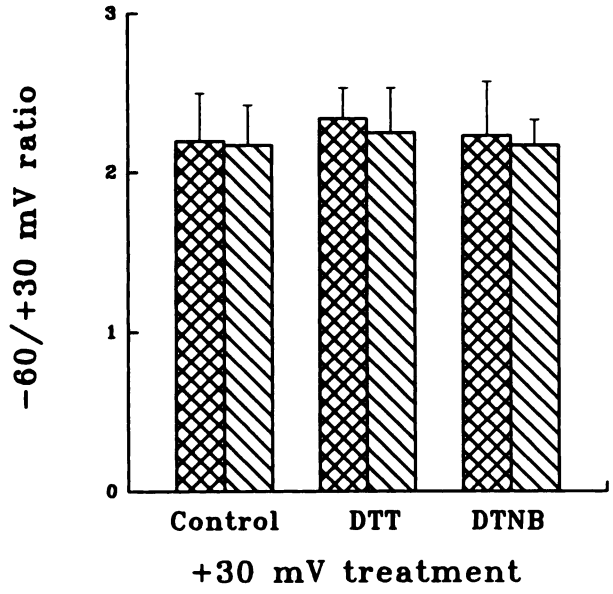

Fig. 5. Incubation with DTT or DTNB alone at +30 mV is insufficient to induce rectification of NMDA-mediated whole-cell currents. Responses to 30 μM NMDA were measured at −60 and +30 mV before (▩) and after (▩) a particular treatment, and the ratios of the absolute peak current values were determined for each condition. Any significant decrease from a ratio of 2 was taken as an indication that the outward rectification had appeared. Ratios were calculated for each cell before and after treatment with either 4 mM DTT (n = 4) or 500 μM DTNB (n = 3) at +30 mV. Control ratio values are from cells whose treatment was simply voltage-clamping at +30 mV for a prolonged period of time (10 min), without a chemical treatment (n = 5). Values are the mean ± standard error.

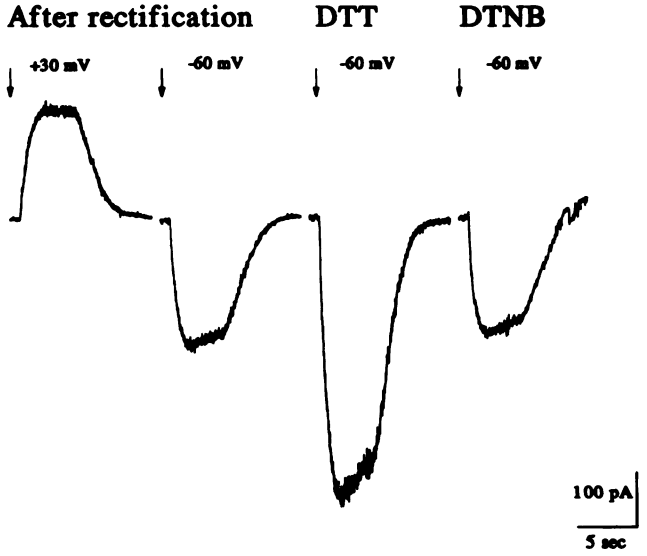

Fig. 6. Presence of 'traditional' redox modulation after the appearance of rectification. Whole-cell currents measured in response to 30 μM NMDA applications in a cortical neuron are shown. Previously, this cell had been continuously voltage-clamped at +30 mV and sequentially treated with 4 mM DTT and 500 μM DTNB to induce the rectification phenomenon. Redox reagents were rinsed off the cell and NMDA responses were elicited at +30 and −60 mV (*first two traces*). Notice that the amplitudes of these two responses are of similar magnitude, indicating that the rectification had indeed appeared. NMDA responses were then elicited after additional treatments with DTT and DTNB while the cell was continuously held at −60 mV (*last two traces*). Note that a substantial potentiation was induced by DTT and that DTNB was able to reverse this effect. Similar results were obtained in a total of four cells.

because redox reagents still modify the whole-cell responses after the effect appears. Thus, DTT and DTNB can just as effectively potentiate and depress NMDA responses at negative holding potentials after the manifestation of the rectification (Fig. 6).

The sequential DTT/DTNB treatments were performed at various holding potentials to define voltage requirements for producing the permanent change in the whole-cell I-V relationship. Once again, we used the −60/+30 mV response ratio as an index of rectification. Fig. 7 shows that the rectification effect was only produced when the redox treatments were performed at voltages more positive than 0 mV. This suggests that the molecular mechanism necessary for this outcome may require a reversal of the transmembrane charge distribution or some related event.

Study of unitary conductances. Single-channel recordings were used to further characterize the mechanism of the observed rectification. Outside-out patches were obtained from cells that had been exposed to 500 μM DTNB before the experiment. Therefore, all receptors studied under pre-redox treatment conditions were actually in the oxidized form. This was done to prevent variations in the starting redox state from patch to patch (1). Upon patch excision, 10 μM NMDA-activated unitary conductances were recorded at −60 mV and +30 mV under control conditions and after sequential reduction (500 μM DTT, 2 min) and oxidation (500 μM DTNB, 2 min) of the patch at +30 mV. Lower concentrations of the reducing agent were used here, compared with those used in the whole-cell studies, to prevent any potential damage to the patches. We previously showed that this concentration of DTT is effective in substantially modifying NMDA single-channel behavior (3). Only patches that survived the redox treatments were included in the analyses to compare the data from a single patch before and after the procedure (n = 5).

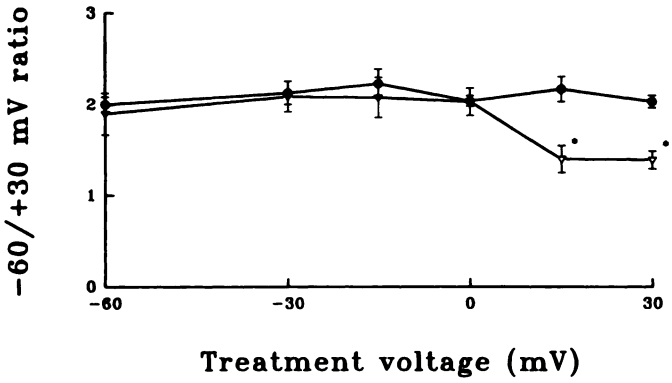

Fig. 7. Voltage dependence of the appearance of the outward rectification after redox treatment. Whole-cell responses to 30 μM NMDA were measured at −60 and +30 mV and the ratios of the absolute peak current values were determined. A decrease from a ratio of 2 was taken as an indication that the outward rectification had appeared. Ratios were calculated for each cell before (●) and after (▽) a sequential DTT/DTNB treatment at the indicated voltages. Rectification appeared only when the sequential redox treatments were performed at voltages more positive than 0 mV. Values represent the mean ± standard error for four to eight cells. *, Significantly different from the pretreatment ratio, $p < 0.001$, paired t test.

After sequential redox treatments at +30 mV, we observed a significant prolongation of the open time of NMDA-induced unitary conductances (Fig. 8) at this potential (τ_o = 5.5 ± 1.3 msec for control and τ_o = 12.9 ± 2.6 msec for treated; $p < 0.005$;

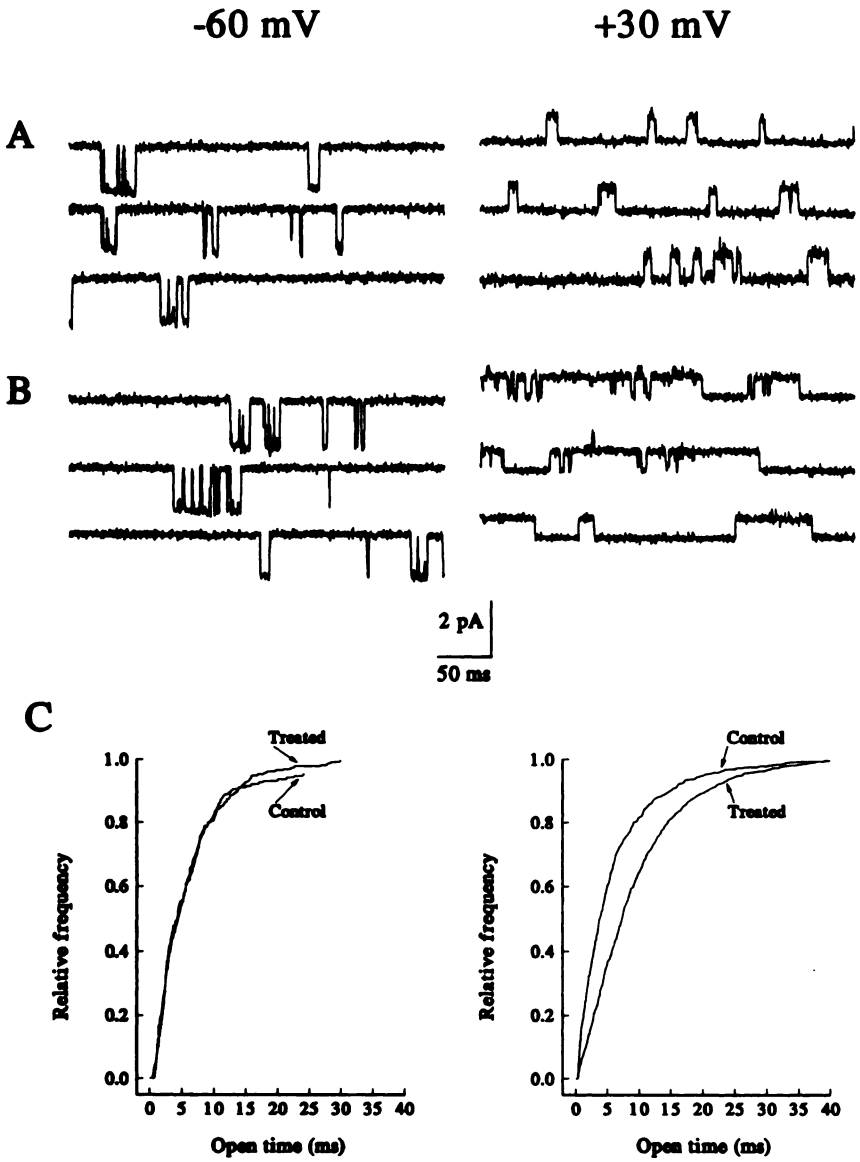

Fig. 8. NMDA single-channel behavior after redox treatments at +30 mV. A and B, NMDA (10 μM)-activated single-channel currents from an outside-out patch excised from a cortical neuron and voltage-clamped at either −60 mV (*left*) or +30 mV (*right*), before (A) and after (B) sequential 500 μM DTT/500 μM DTNB treatments at +30 mV. Data were filtered at 2 kHz and digitized at 10 kHz. No events were apparent when NMDA was excluded from the perfusion solution. Total amplitude histograms (not shown) were fitted by a Gaussian distribution, and a single main conductance was calculated at either voltage for both control (34.6 at −60 mV and 34.0 pS at +30 mV) and redox-treated (34.0 pS at both −60 and +30 mV) conditions. C, Cumulative open time histograms for NMDA-activated events obtained at −60 mV (*left*) and +30 mV (*right*) before and after redox treatments. Histograms, which are for the events from the same patch as shown above, were best fitted with a single-exponential function having the following time constants (in msec): −60 mV control, 5.1; −60 mV treated, 5.1; +30 mV control, 4.9; +30 mV treated, 9.6. For all patches tested ($n = 5$) there was a significant increase in the mean open time at +30 mV after treatment, compared with control ($p < 0.005$, paired t test; see text for values).

paired t test) but not at −60 mV ($\tau_o = 6.5 \pm 2.9$ msec for control and $\tau_o = 5.5 \pm 0.8$ msec for treated). No significant changes in single-channel conductance were observed from records obtained at either potential before or after the redox treatments (31.5 ± 0.7 pS; range, 30.2–32.9 pS; data not shown). The fact that we used 2.5 mM Ca^{2+} in the extracellular solutions and 1 mM Mg^{2+} in the internal solution is probably responsible for the single-channel conductance observed here being smaller than that reported by others (15–17). In addition, under our recording conditions we seem to resolve only one main conductance state (3).

A reversible increase in the frequency of openings was detected at +30 mV, compared with −60 mV under control conditions (3.2 ± 0.9-fold increase; range, 1.2–6.2-fold; $n = 5$), similar to that described earlier (2). This effect was not significantly altered after the sulfhydryl redox treatments at +30 mV (1.7 ± 0.7-fold increase; range, 0.8–4.3-fold; $n = 5$). Finally, all parameters remained essentially unchanged in the patches we were able to rinse for 5–7 min at −60 mV in control solution ($n = 3$). These results suggest that the increase in the single-channel open time at +30 mV after the redox treatments is likely responsible for the observed outward rectification of the whole-cell responses. This is due to the fact that both effects are produced by similar treatments and are seemingly very long-lasting.

Discussion

In this paper we report two separate voltage-dependent sulfhydryl redox-related phenomena occurring at the NMDA receptor. First, DTNB could completely reverse the potentiating actions of DTT when DTNB was applied at −60 mV but not at +30 mV. Second, the NMDA-mediated currents become outwardly rectifying as a result of the reduction treatment being followed by oxidation at positive potentials, but not under any other circumstances. Thus, our study of the actions of DTNB in reversing the effects of DTT at various transmembrane potentials led to the discovery of the long-lasting rectification. It is noteworthy that, even after the rectification appears, the redox sensitivity of the NMDA receptor, as reported in our original experiments (1), remains essentially unchanged; at negative holding potentials DTT still potentiates NMDA-induced responses, and DTNB reverses this effect.

The first phenomenon, the inability of DTNB to completely reverse the potentiating actions of DTT at positive potentials, could be the result of a membrane potential-sensitive change in the oxidation potential of the redox-sensitive site or the result of allosteric changes in the receptor structure. The fact that the action of DTT does not change with voltage argues for the latter possibility. Using higher concentrations of DTNB could have addressed the likelihood that this agent is a much weaker oxidizing agent at positive potentials. That is, increasing the concentration of this substance 10-fold would make it possible to test whether a lack of complete reversal of the effects of DTT at positive potentials is reflective of a change in the effectiveness of DTNB. Unfortunately, DTNB is at its limit of solubility at 0.5–1 mM in aqueous solutions, and thus higher concentrations could not be tested. In addition, concentrations of this oxidizing agent as low as 100 μM appear sufficient to substantially oxidize the receptor at negative holding potentials (1).

The aforementioned studies have uncovered a new sulfhydryl redox effect that modifies NMDA receptor channel properties. The new sulfhydryl redox effect is manifested as an outward rectification of the NMDA whole-cell I-V relationship and as an increase in the open time of NMDA-activated unitary conductances at positive potentials. The rectification of the NMDA-induced whole-cell responses is likely a direct result of the observed voltage-dependent changes in the single-channel open time. This change cannot be reversed by additional thiol redox treatments nor does it spontaneously reverse, at least within the limited time frame of our observations.

We can only speculate on the possible chemical events underlying this phenomenon, but it is possible that a disulfide bond in the receptor becomes strained or exposed at positive potentials so that it can be altered by the sequential thiol redox treatments. This alteration may become not easily reversible due to newly introduced steric hindrances or new covalent changes after oxidation with DTNB. These changes somehow become insensitive to further reduction by DTT. Additional work will reveal whether other reducing agents may be effective in reversing the rectification or whether other oxidizing agents mimic the actions of DTNB. Nevertheless, the results presented here provide new details on potential structural sites on the NMDA receptor that can modify its channel properties. Although it is unlikely that the phenomenon observed here has any physiological significance, uncovering these sites by mutagenetic studies may reveal some fundamental properties of the mechanism of gating modification of this and related receptor channels.

Acknowledgments

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