Single-Channel Currents from Diethylpyrocarbonate-modified NMDA Receptors in Cultured Rat Brain Cortical Neurons

JANET L. DONNELLY and BARRY S. PALLOTTA

From the Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7365

ABSTRACT The role of histidine residues in the function of N-methyl-D-aspartate (NMDA)-activated channels was tested with the histidine-modifying reagent diethylpyrocarbonate (DEP) applied to cells and membrane patches from rat brain cortical neurons in culture. Channels in excised outside-out patches that were treated with 3 mM DEP for 15–30 s (pH 6.5) showed an average 3.4-fold potentiation in steady state open probability when exposed to NMDA and glycine. Analysis of the underlying alterations in channel gating revealed no changes in the numbers of kinetic states: distributions of open intervals were fitted with three exponential components, and four components described the shut intervals, in both control and DEP-modified channels. However, the distribution of shut intervals was obviously different after DEP treatment, consistent with the single-channel current record. After modification, the proportion of long shut states was decreased while the time constants were largely unaffected. Burst kinetics reflected these effects with an increase in the average number of openings/burst from 1.5 (control) to 2.2 (DEP), and a decrease in the average interburst interval from 54.1 to 38.2 ms. These effects were most likely due to histidine modification because other reagents (n-acetylimidazole and 2,4,6-trinitrobenzene 1-sulfonic acid) that are specific for residues other than histidine failed to reproduce the effects of DEP, whereas hydroxylamine could restore channel open probability to control levels. In contrast to these effects on channel gating, DEP had no effect on average single-channel conductance or reversal potential under bi-ionic (Na⁺:Cs⁺) conditions. Inhibition by zinc was also unaffected by DEP. We propose a channel gating model in which transitions between single- and multi-opening burst modes give rise to the channel activity observed under steady state conditions. When adjusted to account for the effects of DEP, this model suggests that one or more extracellular histidine residues involved in channel gating are associated with a single kinetic state.

Address correspondence to Barry S. Pallotta, CB No. 7365 FLOB, Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599-7365.

Janet L. Donnelly’s current address is Department of Neurology, Neuroscience Laboratory Building, University of Michigan Medical Center, Ann Arbor, MI 48104-1687.

J. GEN. PHYSIOL. © The Rockefeller University Press - 0022-1295/95/06/0837/23 $2.00
Volume 105 June 1995 837–859
INTRODUCTION

Glutamate is the primary excitatory neurotransmitter in the mammalian brain and activates several subtypes of receptors (see reviews Monaghan, Bridges, and Cotman, 1989; Seeburg, 1993; Hollmann and Heinemann, 1994; McBain and Mayer, 1994). One subtype, the N-methyl-d-aspartate (NMDA) receptor, plays an important role in normal brain development and synaptic plasticity (Collingridge and Bliss, 1987; McDonald and Johnston, 1990) as well as in pathological processes such as excitotoxicity (Rothman and Olney, 1987; Meldrum and Garthwaite, 1990) and possibly in several types of neurodegenerative diseases (Choi, 1988). When activated by the binding of NMDA and glycine (Kleckner and Dingledine, 1988), the receptor opens a pore that is relatively nonselective among monovalent cations but has high permeability to calcium (MacDermott, Mayer, Westbrook, Smith, and Barker, 1986; Mayer and Westbrook, 1987; Zarei and Dani, 1994). This activity is also subject to modulation by magnesium (Nowak, Bregestovski, Ascher, Herbet, and Prochiantz, 1984), zinc (Westbrook and Mayer, 1987; Peters, Koh, and Choi, 1987; Christine and Choi, 1990), calcium (Clark, Clifford, and Zorumski, 1990; Mayer, Vylický, and Westbrook, 1989; Legendre, Rosenmund, and Westbrook, 1993; Vylický, 1993), and polyamines (Ransom and Stec, 1988; Rock and Macdonald, 1992).

Single-channel studies of NMDA receptor gating reveal complex gating behavior, but they provide no direct information about the conformational changes of the channel protein that underly this complexity. Mutagenesis studies of the NMDA receptor have the potential to identify specific protein structures involved in gating, but have so far focused primarily on the permeation pathway. These studies have identified an asparagine residue in the putative second transmembrane domain that is necessary for magnesium block and calcium permeability. Mutations at this residue also alter the kinetics of the whole-cell and single-channel currents (Burnashev, Schoepfer, Monyer, Ruppersberg, Gunther, Seeburg, and Sakmann, 1992; Mori, Masaki, Yamakura, and Mishina, 1992; Sakurada, Masu, and Nakanishi, 1993; Kawajiri and Dingledine, 1993).

Another way to examine the relationship between the physical structure of a channel and its kinetic or conductance states is through the use of specific chemical modifiers that alter the physical properties of amino acid side chains. One such reagent, diethylpyrocarbonate (DEP), has been used to study the role of histidine residues in a number of proteins, including several ion channels. For example, application of DEP to the quisqualate/kainate receptor reduces the whole-cell current, possibly through action at an inhibitory H⁺ binding site (Christensen and Hida, 1990), while treatment of GABA_A receptors with DEP reduces activity by modifying a histidine in the benzodiazapine binding site (Maksay, 1992). DEP reduces currents from voltage-gated sodium channels of squid axon (Oxford, Wu, and Narahashi, 1978), and slows and decreases voltage-gated potassium channel currents from crayfish (Shrager, 1975).

NMDA receptors have also been modified by DEP. Traynelis and Cull-Candy (1991) showed that DEP treatment of cerebellar granule cells causes an increase in whole-cell current response to NMDA. Reynolds (1992) used the DEP-modified NMDA receptor to study the interaction between the inhibitory zinc binding site and...
the spermine modulatory site. He found that treatment of rat brain membranes with 1.2 mM DEP for 15 min resulted in a fourfold decrease in zinc binding affinity and an increase in the dissociation rate of MK-801 (dizocilpine), suggesting an increase in channel open time. Crilley and Turner (1994) found that treatment of pig forebrain membranes with 1–3 mM DEP for 30 min reduced binding affinity for the NMDA receptor but did not affect the number of binding sites. With respect to macroscopic measurements then, these results suggest that histidine residues may determine a number of receptor properties.

The goal of this study was to examine the role of histidine residues in NMDA receptor function at the single-channel level. We found that treatment of the extracellular side of the NMDA receptor potentiated the whole-cell and single-channel response. This increase occurred through an effect on the closed state kinetics, increasing burst frequency and openings/burst, with little change in open state kinetic or conductance properties. Zinc inhibition of the DEP-modified channel, and voltage-dependent magnesium block, were unchanged. Our evidence suggests that DEP acts at one or more histidine residues on the extracellular side of the receptor that are involved in channel gating. The data are summarized with a kinetic model in which the effects of DEP are reproduced by altering transitions away from one intraburst closed state. Part of this work has been previously described (Fisher and Pallotta, 1993; Donnelly and Pallotta, 1994).

M E T H O D S

Cell Culture

Procedures for neuron cell culture are described by Hoch and Dingledine (1986). Briefly, brain cortical neurons were obtained from fetal (E19) rat pups removed from ether-anesthetized, pregnant females (Sprague-Dawley, Harlan). The cortices were minced in Hanks Balanced Salt Solution (HBSS) + 10 mM HEPES (pH = 7.4), then incubated with 0.3% type XXIII protease (3.5 U/mg) and 0.25% collagenase (425 U/mg) (Sigma Chemical Co., St. Louis, MO) for 45 min at 37°C. The tissue was then rinsed with Modified Eagles Medium (MEM) + 1 mg/ml DNAse, and triturated with a fire-polished Pasteur pipette. The single-cell suspension was then centrifuged and resuspended in MEM (with Eagle's salts without L-glutamine) supplemented with 1 mM sodium pyruvate, 33 mM glucose, 15 mM KCl, 2 mM CaCl₂, 5 μg/ml insulin, 10% fetal bovine serum and 5% horse serum. The cells were plated onto plastic coverslips coated with 1 mg/ml poly-D-lysine and incubated at 37°C, 5% CO₂. After 3–5 d of incubation, half of the media was removed and replaced with serum and insulin-free media.

Recording and Solutions

Recordings were made from neurons and excised outside-out patches 1–2 wk after plating. Patch electrodes were pulled from borosilicate glass (Drummond, Broomall PA), coated with Sylgard (Dow-Corning, Midland, MI), then fire-polished to a resistance of 1–3 MΩ for whole-cell recordings and 3–5 MΩ for outside-out patch recordings. Ultrapure KCl, NaCl, and CaCl₂ were used for all solutions. For outside-out patches the external recording solution contained (in millimolar): 150 NaCl, 10 HEPES, 2.5 KCl, 0.2 CaCl₂, and 0.5 μM TTX (pH = 7.4, 295–305 mOsm). The electrode tips were filled with a solution containing (in millimolar): 150 CsCl, 10 HEPES, 2.5 CaCl₂ (pH 7.4, 295–305 mOsm). The electrode was then backfilled with a pipette solution containing (in millimolar): 145 CsCl, 10 HEPES, 2.5 KCl, 5
EGTA and 0.5 CaCl₂ (pH 7.4, 295–305 mOsm). For whole-cell recordings Ba²⁺ replaced Ca²⁺ in all solutions, and 2 mM MgATP was added to the internal solution in order to reduce rundown (Rosenmund and Westbrook, 1993). Voltage control of the whole-cell recordings was verified by reversal potentials near 0 mV for responses to NMDA and glycine. All test solutions were applied through a U-tube (Krishtal and Pidoplichko, 1980) and were prepared in the external solution. In some experiments 10 μM strychnine and 2 μM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzof(quinoxaline (NBQX) were included in the test solutions to block glycine-activated Cl⁻ currents (Hamill, Bormann, and Sakmann, 1983) and non-NMDA glutamate receptors (Sheardown, Nielsen, Hansen, Jacobsen, and Honoré, 1990).

Diethylpyrocarbonate (DEP) (Sigma Chemical Co.) was diluted in external solution containing 0.5% DMSO at pH 6.5. DEP adds a carbethoxy group to the nitrogen of the histidine imidazole ring (Scheme 1), and at pH < 7, reacts at least 50- to 250-fold faster with histidines than with any other amino acid residues (Miles, 1977).

DEP was applied in the absence of agonist to the external surface of outside-out patches for 15–30 s, and was removed before recording by a continuous flow of fresh extracellular solution (pH 7.4). For whole-cell currents, neurons were exposed for 1–3 min before washout and recording. DEP is unstable in aqueous solution, and we measured a half-life of ~ 3 h from the change in absorbance (230 nm) that occurred during the reaction between DEP and 10 mM imidazole to form N-carbethoxyimidazole (Melchior and Fahrney, 1970). Consequently, the actual concentration of DEP in our solutions was somewhat less than the concentration at which it was prepared (3 mM), but > 1 mM. The presence of 0.5% DMSO had no effect on the breakdown rate of DEP or on channel properties (two experiments), and was included in our solutions to improve solubility.

**Data Recording and Analysis**

Currents were recorded with an EPC-7 patch clamp (Medical Systems, Greenvale, NY) and stored on VHS tape. Data were digitized and analyzed off line with the Acquire and TAC analysis programs (Instrutech, Elmont NY) written for the Atari ST computer. Open (and shut) intervals were measured with respect to a threshold that was set to half the amplitude of the channel main opening level. Actual threshold-crossing times were determined by interpolation between the sample points that bracketed the threshold. All currents were analogue filtered at 5 KHz and sampled at 40 μs/point. Additional programs for burst analysis and model simulations were written in Visual Basic.

**Fitting distributions.** Log-binned interval histograms for burst durations, open, and closed times, were plotted with square root vertical axes and fitted to sums of exponentials that maximized the likelihood of observing the binned data (Sigworth and Sine, 1987). The number of exponential components in each fit was estimated first by eye, with additional components added if the log-likelihood ratio test (Horn, 1987; McManus and Magleby, 1988) demonstrated
significant improvement \( (P < 0.05) \) in the fit. These calculations were restricted to bins that contained intervals with durations > 1.5–2 times the system deadtime (Magleby, 1992). No other corrections for missed events were applied. Distributions of the number of openings/burst were fitted with one or more geometric components based upon maximization of the likelihood calculated from the unbinned observations (Colquhoun and Sigworth, 1983).

**Definition of bursts.** Bursts were defined as groups of open and shut intervals that were bracketed by shut intervals longer than a particular critical time \( (t_c) \). The critical time was calculated for each experiment from the unconditional distribution of all shut intervals, and was the time at which the relative proportion of shut intervals misclassified as intraburst were offset by an equal proportion of shut intervals that were misclassified as inter-burst (Colquhoun and Sakmann, 1985). Because the shut interval distribution contained (usually) four components, the two shortest duration components were assigned as gaps within bursts, with the remaining two components assumed to be gaps between bursts (Howe, Cull-Candy, and Colquhoun, 1991; Gibb and Colquhoun, 1992).

**Patches with multiple channels.** Our ability to perform detailed kinetic analyses was compromised by the presence of more than one channel in all patches. Under these conditions, the mean durations of the longer shut interval components were reduced in proportion to the number of channels in the patch, and were therefore of limited usefulness. Once a patch was treated with DEP, this problem became worse, as channel activity was typically increased to the extent that substantial numbers of overlapping events were observed. Computer simulations showed that this caused artifactually long components in some distributions of open intervals because the threshold-detection method that we used counts the time during which any channel is open (without an intervening closure) as an open duration. As a result, patches with open probabilities (after DEP) > 0.15 were not subjected to detailed kinetic analysis.

**RESULTS**

**DEP Modifies NMDA-activated Channel Behavior**

Traynelis and Cull-Candy (1991) showed that treatment of cultured rat cerebellar granule cells with 1–3 mM DEP for 1–10 min causes a potentiation (186%) of the whole-cell current evoked by micromolar concentrations of aspartate. In two experiments, we found a similar potentiation (202 ± 37%) of the steady state whole-cell current when cortical neurons were challenged with 20 μM NMDA and 10 μM glycine after 1–3 min exposure to DEP (data not shown). This effect might be due to an increase in single-channel conductance, an alteration in the channel open probability, or perhaps the conversion of inactive channels into an active form. To distinguish among these possibilities, we examined the single-channel properties of modified NMDA receptors in excised outside-out patches. The effect of DEP treatment on single channels in an excised outside-out patch is illustrated in Fig. 1. In the presence of 10 μM NMDA and 10 μM glycine, channel openings from normal channels occur infrequently and open probability \( (n_{p_o}) \) is low (0.03 in Fig. 1 A). After 15 s of treatment with 3 mM DEP, openings appeared more frequently and open probability increased to 0.14 (Fig. 1 B). On average, open probability was potentiated 341 ± 37% (mean ± SEM; 25 patches) by treatment with DEP, as was observed with whole-cell currents. It is also apparent in Fig. 1 that DEP had no obvious effect on the amplitudes of the channel currents. As described later, single-channel current-voltage characteristics were unaffected by DEP treatment. Because the apparent effects of DEP on channel open probability might be due to alterations in opening and/or
closing rate, we examined the distributions of open and shut intervals to identify those features of the gating process that were altered.

Open and Shut Interval Distributions

Fig. 2 shows that DEP had significant effects on the distribution of shut intervals. Before treatment (Fig. 2 A), the shut intervals were fitted with four exponential components with mean durations (and relative areas) of 0.05 ms (0.35), 1.33 ms (0.10), 10.74 ms (0.18) and 66.76 ms (0.37). After DEP modification, the distribution was obviously different (Fig. 2 B). The number of components was unchanged, and had mean durations (and relative areas) of 0.07 ms (0.32), 0.97 ms (0.22), 6.86 ms (0.24), and 88.93 ms (0.22). In other words, DEP caused an approximate doubling of the relative number of shut intervals from the ~1-ms component, with a concomitant halving of the relative proportion of long-duration intervals. The results from 9-10 patches in which the duration histograms were fitted with four components are summarized in Table I. On the whole, DEP caused an increase in the relative proportions of intervals from the two briefest components, with relatively fewer shut intervals of longer durations. As shown below, these changes in the shut interval distributions underly alterations in the channel burst kinetics.

In contrast to its effects on shut intervals, DEP had inconsistent effects on the open times. In most experiments, the distribution of open intervals was fitted with the sum of three exponential components. Distributions that contained only two components were excluded from analysis. In Fig. 2 C, the time constants (and relative areas) of these components were 0.07 ms (0.37), 1.18 ms (0.43), and 3.14 ms (0.20). After treatment with DEP, the time constants of the components were little changed (0.05, 0.51, and 2.76 ms) (Fig. 2 D). On the whole, however, fits obtained from DEP-modified channels were highly variable, probably as a result of the (increased) number of overlapping events that occurred after channel activity was potentiated (Table I). For example, the time constant for the longest-duration component ranged from 1.95 to 10.44 ms, whereas the relative areas ranged from 6 to 60%. The relative areas of the three components also varied widely, although there was a statistically
significant (Student's independent t test, $P < 0.05$) decrease in the relative proportion of short open intervals after DEP treatment (Table I). Given the high variability in the fitted constants, and the relative closeness of the two longer open interval time constants to each other, we conclude that DEP had only a minor effect on the distribution of open intervals.

**FIGURE 2.** Open and shut duration histograms from untreated (A and C) and DEP-modified (B and D) channels. (A) Distribution of 955 shut intervals recorded from an outside-out patch exposed to 10 $\mu$M NMDA and 0.5 $\mu$M glycine (membrane potential $-50$ mV). The distribution was fitted with the sum of four exponential components (solid line) with time constants (and relative areas) of 0.054 ms (0.35), 1.33 ms (0.10), 10.74 ms (0.18), and 66.77 ms (0.37) (dashed lines). (B) Distribution of 1,085 shut intervals obtained from the same patch but after treatment with 3 mM DEP. This distribution was fitted with four components with time constants (and relative areas) of 0.073 ms (0.32), 0.97 ms (0.22), 6.9 ms (0.24), and 88.9 ms (0.22). (C) Distribution of 955 open intervals obtained during the control recording period. The distribution was fitted with three exponential components with time constants (and relative areas) of 0.065 ms (0.37), 1.18 ms (0.43) and 3.14 ms (0.20). (D) After modification by DEP, the distribution of 1,085 open intervals was fitted with three components with time constants (and relative areas) of 0.053 ms (0.27), 0.51 ms (0.15), and 2.76 ms (0.58). The intervals plotted in these histograms were measured from currents filtered at 5 kHz and sampled at 40 $\mu$s/pt (see Methods).

**Burst Kinetics**

Openings from NMDA receptors often appear in groups that are separated from each other by relatively long-duration shut intervals. These bursts and clusters of openings appear to arise from multiple openings of the channel in response to a
single activation by agonist. We analyzed such activity by calculating a critical gap from the distribution of shut intervals for each patch that was used to partition openings (and intervening shut intervals) into bursts (see Methods).

Burst durations were measured by summing the open and shut intervals that were bracketed by shut times greater than the critical gap. Fig. 3 A shows the distribution of burst durations from an experiment where the critical gap was 1.07 ms. The distribution was fitted with three exponential components (see legend) that in eight patches averaged 0.17, 1.29, and 8.32 ms (Table II). After treatment with DEP, bursts became longer (Fig. 3 C); in eight patches, the mean burst duration time constants after modification were 0.18, 2.13, and 18.00 ms. In addition, the relative areas for the two longer components in this distribution increased (Table II).

Underlying these changes in the burst durations were increases in the number of openings in each burst. Before modification, two geometric components were found in the distribution of the number of openings in each burst (Fig. 3 B). In this experiment, ~76% of the bursts had a mean length of 1.1 openings, with the remaining bursts averaging 2.6 openings each. After modification with DEP (Fig. 3 D), there was an obvious change in the distribution. There were still two classes of bursts, but the number of openings in the bursts of each type increased to 1.9 openings (63%) and 5.8 openings (37%). Similar results were found in eight patches (Table II): the number of openings/burst in each component was significantly increased, with a much smaller effect on the relative proportions of the components.

These effects on the burst kinetics are consistent with the observed increases in open probability found after DEP treatment. From Table II we find that before DEP treatment, the mean burst consisted of 1.5 openings and lasted 2.50 ms. After DEP,
bursts consisted of 2.2 openings and lasted 6.2 ms. From the average number of openings, the open probability would be increased $2.2 / 1.5 = 1.5$-fold. In addition, we find from Table I that the mean interburst interval, calculated from the two longer components in the distribution of shut intervals, decreased from 54.1 to 38.2 ms after modification. This 1.9-fold increase in burst frequency, coupled with the increase in openings/burst, would result in an average 2.8-fold potentiation of channel open
probability. This is consistent with the average increase in open probability cited earlier (3.4-fold) for all patches treated with DEP.

Modulation by Zinc

At some synapses, zinc is coreleased with glutamate where it can potentially affect receptor function. The NMDA receptor is modulated by zinc at two distinct sites. At low concentrations (< 10 μM), zinc inhibits channel opening in a voltage-independent manner (Westbrook and Mayer, 1987; Peters et al., 1987; Mayer et al., 1989; Legendre and Westbrook, 1990). At higher concentrations, zinc blocks the channel in a voltage-dependent manner (Christine and Choi, 1990; Legendre and Westbrook, 1990). Because histidine residues are common components of zinc binding sites (Higaki, Fletterick, and Craik, 1992), we investigated the effects of zinc on DEP-modified channels.

### Table II

| Burst Properties | Control | DEP |
|------------------|---------|-----|
| Openings/burst   | n = 8   | n = 8 |
| Mean1            | 1.1 ± 0.1 | 1.3 ± 0.1 |
| Area1            | 0.55 ± 0.05 | 0.47 ± 0.05 |
| Mean2            | 1.9 ± 0.1 | 3.0 ± 0.5 |
| Area2            | 0.45 ± 0.06 | 0.53 ± 0.05 |
| Burst duration   |         |     |
| τ1 (ms)          | 0.17 ± 0.04 | 0.18 ± 0.06 |
| Area1            | 0.38 ± 0.05 | 0.26 ± 0.05 |
| τ2 (ms)          | 1.30 ± 0.20 | 2.13 ± 0.75 |
| Area2            | 0.40 ± 0.06 | 0.45 ± 0.05 |
| τ3 (ms)          | 8.32 ± 2.63 | 17.99 ± 5.30 |
| Area3            | 0.23 ± 0.05 | 0.29 ± 0.05 |

Fig. 4 shows the effects of 1 μM extracellular zinc on currents recorded from an outside-out patch (−50 mV) in 20 μM NMDA and 10 μM glycine. In this experiment, zinc caused a reduction in channel open probability from 0.026 to 0.010 (compare Fig. 4 A with B). Similar effects were seen in seven patches, where the average reduction in open probability was 65 ± 6%. Zinc also reduced open probability in DEP-modified channels (Fig. 4 D). In the experiment shown, zinc caused a 66% decrease in open probability (from 0.044 in Fig. 4 C to 0.0154 in Fig. 4 D). Similar results were found in four other experiments (average decrease in open probability was 72 ± 5%). These results are summarized in Fig. 4 E, which shows the relationship between open probability and zinc concentration fitted with the logistic equation (see legend). While the fitted curves imply that DEP-modified channels were more sensitive to zinc than unmodified channels, the standard errors for the fitted constants (see legend) suggest that this difference was not significant. It is noteworthy that zinc inhibits at much lower concentrations in our experiments than other investigators have reported (Peters et al., 1987; Christine and Choi, 1990; Legendre...
and Westbrook, 1990). This might be due to the lower Ca$^{2+}$ concentration (0.2 mM) in our external solution as increasing extracellular Ca$^{2+}$ is known to decrease zinc block, possibly by competing for the binding site (Mayer et al., 1989).

**Is a Histidine Modified to Produce the Effects of DEP?**

It is important to determine which type of residue is responsible for the behavior of DEP-modified channels if we are to draw conclusions about the structures involved in channel gating. Although DEP reacts ~50- to 250-fold faster with histidines than with any other amino acid residue, it can also modify tyrosine, lysine, and cysteine residues (Miles, 1977). Therefore, we compared the effects of other amino acid modifiers to those of DEP, so that possible modifications to these residues might be ruled out.

**N-acetylimidazole (N-AI):** DEP can react with the hydroxyl group on tyrosine residues, and there are 17 such residues on putative extracellular domains of the
NR1 subunit (Moriyoshi, Masu, Ishii, Shigemoto, Mizuno, and Nakanishi, 1991). N-AI, a reagent that primarily modifies tyrosines (Riordan, Wacker, and Vallee, 1965), did not, however, have any effects upon channel gating or permeation. In five outside-out patches treated with 5 mM N-AI for 1-5 min at pH = 7.4, neither channel amplitudes at -50 mV (100 ± 1.6% of unmodified) nor open probabilities (85 ± 10% of unmodified) were affected.

TNBS: we used 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS) to modify lysine residues (Okuyama and Satake, 1960). Treatment of outside-out patches (n = 9) with 1.9 mM TNBS for 0.5–2 min had variable effects. Most often, channel conductance (n = 3) or activity (n = 3) were decreased after TNBS, and no effects were observed in three experiments.

---

**Figure 5.** Hydroxylamine reverses the effect of DEP on channel activity. (A) Current trace from an outside-out patch exposed to 10 μM NMDA and 10 μM glycine at -50 mV. During the control period, channel open probability was 0.03. Same patch as in Fig. 1. (B) Trace obtained after treatment with 3 mM DEP increased open probability 4.7-fold to 0.14. (C) Subsequent treatment with 10 mM hydroxylamine for 1 min returned the open probability to the control level (0.03). (D) Another application of DEP to the same patch for 15 s again potentiated channel activity. Open probability increased 3.7-fold over control. Traces were filtered at 500 Hz and sampled at 100 μs/pt for display.

**Reversal by Hydroxylamine**

An alternative approach to assessing the specificity of DEP is to chemically reverse the modification of histidine and tyrosine residues with hydroxylamine (Miles, 1977) (see Methods, Scheme I). By itself, hydroxylamine (1–10 min) had little effect on channel open probability (npo = 83 ± 15% of control, n = 3). However, when applied to DEP-modified patches, hydroxylamine had two effects. In four patches, hydroxylamine restored channel activity to 85 ± 13% of control (Fig. 5C). In two of these,
reapplication of DEP potentiated the response a second time (213 ± 47%) (Fig. 5 D).

Another effect was observed in four other patches, where hydroxylamine caused a further potentiation of open probability by 1,289 ± 408% (Fig. 6). In these experiments, channel activity was marked by extremely long duration (seconds) bursts of openings that contained mostly short-duration shut intervals. The long continuous burst of activity shown in Fig. 6 C is typical of this effect. In this patch it also appears that the other channels suddenly became inactive during the burst, whereas in other patches, a variety of effects were apparent as the DEP-modified channels independently reacted to hydroxylamine with either attenuation or potentiation of activity. The long bursts of activity are also interesting because they lack the long-duration shut intervals that correspond to desensitized state(s) (Sakmann, Patlak, and Neher, 1980). As a result, macroscopic currents evoked with these chemically modified channels would be expected to desensitize little, if at all.

![Control](image)

**Figure 6.** Hydroxylamine also potentiates the activity observed in DEP-treated patches. (A) Current trace from an outside-out patch exposed to 10 μM NMDA and 10 μM glycine at -50 mV. Open probability was 0.06. (B) DEP modification increased open probability 3.3-fold to 0.20. (C) In contrast to the effect shown in Fig. 5, 10 mM hydroxylamine (1 min) further increased channel open probability in this patch to 0.95, 15.8-fold higher than control (A) and 4.8-fold over the DEP-modified response (B). Traces were filtered at 500 Hz and sampled at 100 μs/pt for display.

In one patch, both reversal and potentiation were observed: hydroxylamine reversed the effect of the initial treatment with DEP, but potentiated the response to a second treatment. The potentiation after hydroxylamine was unaffected by repeated applications of either DEP or hydroxylamine. One explanation for these results is that reversal of DEP-induced potentiation of channel activity (Fig. 5) was due to (expected) removal of the added carbethoxyl by hydroxylamine (Scheme I). The further potentiation that is seen in some patches (Fig. 6) might be due to the reaction of hydroxylamine with an imidazole ring that has been twice modified by DEP (Scheme II). Subsequent reaction with hydroxylamine results in irreversible ring cleavage (Miles, 1977), consistent with our observation that the potentiation induced
by hydroxylamine was also irreversible and insensitive to further treatments with either DEP or hydroxylamine.

\[
H_2C_2O - C \equiv N \equiv C - OC_2H_5
\]

Hydroxylamine

**Scheme II**

**Figure 7.** Single-channel current-voltage relationship for NMDA receptors before and after modification with DEP. Amplitude measurements of openings to the main conductance level were recorded from 14 different patches, before and after treatment with DEP. (Open symbols) Measurements from unmodified channels; (closed symbols) DEP-modified channels. The regression line was drawn through all the data points for each condition. For control channels (dashed line) the conductance was 66.4 pS and the reversal potential 1.9 mV \((r = 0.99)\). For DEP-modified channels (solid line) the conductance was 65.5 pS with reversal potential 4.1 mV \((r = 0.99)\).

Taken together, our results suggest that the effects of DEP on channel activity were due to modification of a histidine residue. Other modifiers could not reproduce the effects of DEP, and hydroxylamine, in some patches, reversed the potentiation by DEP.

**Single-Channel Conductance**

As is evident from the single channel traces in Fig. 1, the increase in channel activity that appeared after DEP was not accompanied by obvious changes in the amplitudes of openings at \(-50\) mV. To verify that the conductance of the channel was unaffected, channel amplitudes were measured over a range of voltages from \(-70\) to +70 mV.
Fig. 7 shows the current-voltage relationships obtained from 14 patches before (open circles) and after (filled circles) modification by DEP. In these experiments, 145 mM Cs+ in the pipette carried outward current and 150 mM Na+ in the extracellular medium carried inward current. Only the main conductance level was used for these measurements as subconductance levels were not observed in most patches. From a linear regression to the measurements from all patches, the average slope conductance of unmodified channel was 66.4 pS ($r = 0.99$), and was unaffected by DEP treatment (65.5 pS; $r = 0.99$). It is also apparent that channel selectivity for sodium (with respect to cesium) was unchanged, as the reversal potential obtained before modification (1.9 mV) was shifted insignificantly by DEP to 4.1 mV. We do not know if the selectivities for other ions were changed by DEP. Additional selectivity measurements, and a quantitative analysis of the voltage-dependence of Mg$^{2+}$ and Zn$^{2+}$ block, would provide additional evidence for (or against) DEP interactions within the channel pore.

**DISCUSSION**

We examined the single-channel properties of NMDA receptors that were treated briefly with the protein-modifying reagent diethylpyrocarbonate (DEP). From the currents in outside-out excised patches, we found an increase in channel open probability that arose from an increase in the number of openings per burst, and in the burst frequency. In contrast to these effects on gating, DEP had no apparent effects on channel conductance or selectivity, or on the binding of an open-channel blocker, magnesium. We concluded that one or more extracellular histidines are involved in channel gating. These histidines must also be accessible to the extracellular medium when the channel is in its resting (unliganded) state(s), because we applied DEP to the channel in the absence of ligands.

Given the number of histidine residues that might have been modified by DEP, it is surprising that effects were observed only upon the gating process. The primary structure of the NR1 subunit contains 22 histidines, 17 of which might be extracellular and one that is within the first putative transmembrane domain (Moriyoshi et al., 1991). While no mutagenesis studies have specifically examined histidine residues, mutants in which channel conductance, selectivity or magnesium block were affected also manifested altered gating (Moriyoshi et al., 1991; Ruppersberg, Mosbacher, Günther, Schoepfer, and Fakler, 1993; Kawajiri and Dingledine, 1993; Burnashev et al., 1992). Our results provide evidence that gating and permeation in NMDA receptors are separable processes, and suggest that histidine residues are molecular targets that might allow the structural changes underlying these processes to be separately studied.

**Involvement of Histidine Residues**

Several lines of evidence support our conclusion that modified histidine residues were responsible for the effects we observed. Patches were treated with DEP at pH 6.5 because at pH < 7, DEP reacts at least 50- to 250-fold faster with histidines than with
any other residues (Miles, 1977). Because there was some chance that DEP might modify lysine and tyrosine residues, we also employed reagents (TNBS, n-acetylimidazole) that modified those residues as well. As described in Results, neither reagent duplicated the effects of DEP. Although DEP can also modify cysteine residues, it is unlikely that cysteine residues contributed to the effects we observed because the alkylating reagent NEM (N-ethylmaleimide) has no effect, by itself, on channel properties (Tang and Aizenman, 1993). We also found that the effects of DEP were reversed, or in some experiments, greatly potentiated, by hydroxylamine. Finally, we believe that the histidines modified by DEP are extracellular because channels in excised inside-out patches reacted to DEP treatment with somewhat inconsistent alterations in both single-channel conductance and open lifetime, unlike the effects we described here from outside-out patches.

**Zinc Binding**

Traynelis and Cull-Candy (1989) found that DEP caused a 50% decrease in zinc (10 μM) inhibition of whole-cell currents from cerebellar granule cells. Consistent with this result, Reynolds (1992) found that DEP treatment for 15 min caused a fourfold decrease in the apparent affinity between zinc and NMDA receptors in rat whole brain membranes. In contrast, we found that zinc inhibition of channel open probability was unaffected by treatment with DEP. We conclude that although a histidine residue may form part of the inhibitory zinc binding site, it is not the same histidine responsible for the potentiation of channel activity after modification by DEP. This discrepancy might arise from differences in the cell types that were used, because NMDA receptors differ in subunit composition and channel properties throughout the brain (Monyer et al., 1994). For example, the presence or absence of the N1 splice site might influence the interactions between the receptor and zinc (Hollmann, Boulter, Maron, Beasley, Sullivan, Pecht, and Heinemann, 1993). Another possibility is that longer exposures to DEP (1–15 min in the above studies), might allow modification of less accessible histidines, or of residues perhaps revealed by structural modifications during the first few minutes of DEP treatment.

**Modeling the Effects of DEP**

As described above, the primary consequences of DEP treatment were an increase in burst length and frequency. To describe these kinetic results, we began with a model that was derived to describe the properties of NMDA receptor bursts of two or more openings (Kleckner and Pallotta, 1995). That study showed that each opening within a burst (excluding single-opening bursts) had the same duration distribution as every other opening, and each shut interval had the same distribution as every other. These results were described by a model in which the channel opened to one of three open states of short, intermediate, or long duration (represented by a single state $O_i$ in Scheme III, for simplicity) from the intra-burst closed state ($C_L$). Each open state was linked to a separate short-duration intra-burst closed state ($C_i$), each of which had the
same lifetime (again simplified to a single state in Scheme III). These states that give rise to multiopening bursts are surrounded by the dotted line in the scheme below.

![Diagram of Scheme III]

To account for the closed times between bursts, two additional shut states ($C_2, C_3$) were added. An additional open state ($O_1'$) was also added to account for the component of bursts that contained approximately one opening (single-opening bursts) (Fig. 3). Because the open lifetime distribution of such openings is composed of three exponential components (Gibb and Colquhoun, 1992; Kleckner and Pallotta, 1995), a detailed model must have three separate open states. For simplicity, however, these are represented in the scheme by the single state $O_1'$, and the transition rates to and from these states are similarly represented by $\alpha_1'$ and $\beta_1'$.

To test the predictions of this model, open and shut intervals were obtained from Scheme III by simulation and analyzed in the same manner as actual intervals. Because the number of channels in our patches were unknown, five channels were

| Rate constant | Control $\text{ms}^{-1}$ | DEP $\text{ms}^{-1}$ |
|---------------|--------------------------|---------------------|
| $k_1$         | 0.2                      | 0.2                 |
| $k_{-1}$      | 0.9                      | 0.5                 |
| $k_2$         | 0.09                     | 0.045               |
| $k_{-2}$      | 0.0009                   | 0.0009              |
| $k_3$         | 0.006                    | 0.006               |
| $k_{-3}$      | 0.005                    | 0.005               |
| $\alpha_1$   | 0.5                      | 0.7                 |
| $\alpha_1'$  | 0.005                    | 0.005               |
| $\beta_1$    | 0.15                     | 0.15                |
| $\beta_1'$   | 11                       | 11                  |
| $\delta_1$   | 0.1                      | 0.1                 |
| $\gamma$     | 16.67                    | 16.67               |
assumed in the simulated patch. The values for the rate constants are given in Table III, and the results of the analysis are summarized in Table IV.

The distribution of simulated shut intervals (control) contained four exponential components (Table IV) and agreed closely with the distribution shown in Fig. 2 A and Table I. From a distribution that contained 65,000 intervals, the interburst critical time ($t_{\text{crit}}$) was calculated (1.37 ms) and the simulated intervals were divided into bursts with the same methods that were applied to the actual intervals. Under the simulated control conditions, channel open probability was 0.018, and bursts that contained an average of 1.6 openings occurred (on average) every 51 ms. The simulated distribution of openings/burst also contained two components, with 55% of the bursts containing only one opening (compare Table II with Fig. 3 B).

**TABLE IV**

*Simulated Distributions for a Five-Channel Patch*

|                    | Control | DEP |
|--------------------|---------|-----|
| **Open intervals** |         |     |
| $\tau_1$ (ms)     | 0.09    | 0.09 |
| $\text{Area}_1$   | 0.37    | 0.18 |
| $\tau_2$ (ms)     | 4       | 4   |
| $\text{Area}_2$   | 0.63    | 0.82 |
| **Shut intervals**|         |     |
| $\tau_1$ (ms)     | 0.06    | 0.06 |
| $\text{Area}_1$   | 0.25    | 0.33 |
| $\tau_2$ (ms)     | 0.74    | 0.82 |
| $\text{Area}_2$   | 0.11    | 0.25 |
| $\tau_3$ (ms)     | 15.90   | 9.72 |
| $\text{Area}_3$   | 0.32    | 0.25 |
| $\tau_4$ (ms)     | 85.42   | 82.79|
| $\text{Area}_4$   | 0.32    | 0.17 |
| **Openings/burst**|         |     |
| Mean$_1$           | 1.0     | 1.0 |
| $\text{Area}_1$   | 0.55    | 0.40 |
| Mean$_2$           | 2.3     | 3.2 |
| $\text{Area}_2$   | 0.45    | 0.60 |

Unlike the actual distribution of open intervals, the distribution of simulated open intervals contained only two components. This occurred because the three open states represented by $O_i$ were treated as one open state with a mean lifetime of 4 ms. The three open states that make up the single-opening bursts were treated as a single open state with mean lifetime 0.09 ms. These assignments are not completely arbitrary, as single openings tend to be short duration, whereas the openings during multiopening bursts tend to be intermediate- and long-duration (Gibb and Colquhoun, 1992; Kleckner and Pallotta, 1995). In addition, these simplifications caused the predicted distributions of burst durations to be inaccurate.

The effects of DEP on channel kinetic characteristics were simulated by changing the three rate constants leading from state $C_L$ (bold type in Table III). With rate constant values as in Table III, the distributions of 55,000 simulated open and shut intervals agreed closely with those obtained from actual patches (Fig. 2, B and D;
Table I). From the distribution of shut intervals a critical gap (1.34 ms) yielded 3,631 bursts. These bursts contained an average of 2.3 openings (40% were single opening) and occurred approximately every 39 ms on average. Simulated channel open probability was 0.047. Simulated DEP also increased the length of the average opening (2.6–3.3 ms), as the relative proportion of short-duration openings fell by 51% compared to (simulated) control. Thus, Scheme III accurately predicts the distributions of open intervals, shut intervals, and openings/burst under both control and treatment conditions.

**Conclusions**

In Scheme III, we proposed a detailed description of normal (and DEP-modified) channel gating. NMDA channel gating is complex, and gives rise to a pattern of multiopening bursts temporally separated from each other by silent periods that are themselves punctuated by lone, brief openings (see traces in Figs. 1, 4, 5, and 6 (this paper); Howe et al., 1991; Gibb and Colquhoun, 1992; Kleckner and Pallotta, 1995). Duration histograms reveal several underlying kinetic open and closed states (Jahr and Stevens, 1987; Howe et al., 1991; Gibb and Colquhoun, 1992), and an appropriately complex model was required to describe their role in channel activity. Although the fully expanded model contains seven closed and six open states, the model is far from complete because it contains no coagonist binding sites. A more complete picture of the NMDA receptor's role in synaptic transmission would be obtained if our model of within- and between-burst activity were combined with macroscopic models that contain the coagonist binding sites (Benveniste, Clements, Vyklický and Mayer, 1990; Lester and Jahr, 1992; Lester, Tong, and Jahr, 1993).

Our model also provides a useful qualitative template with which to interpret the complicated pattern of normal, steady state channel activity. This is more apparent in the abbreviated form of the model that is presented in Scheme III. This form suggests that channel gating can be viewed as the sum of two types of bursting behavior. One set of states gives rise to multiopening bursts, and another set gives rise to long silent periods that are interrupted by single openings. Transitions between these two aggregates give rise to the characteristic alternating pattern of bursts and isolated openings. The frequency of switching between the aggregates is usually fast enough so that the current is not dominated by multiopening or single-opening bursts for any appreciable period, although occasional periods of high open probability have been observed previously (Jahr and Stevens, 1987; Howe et al., 1991). Channel occupancy in one aggregate versus the other(s) might thus be another control point for the modulation of channel function under physiological and pathological conditions. Because the single openings are generally briefer than those that occur during multiopening bursts, their association with the long-lived closed states $C_2$ and $C_3$ also accounts for the correlations observed between long-duration closed intervals and short-duration open states (Gibb and Colquhoun, 1992).

Our results with DEP provide a link between the kinetic and structural states of the receptor. Because the receptor was modified by transient application of DEP in the absence of agonists, the histidine targets must have been accessible to the extracellular bath while the receptor was unliganded. Our kinetic interpretation (Scheme III) of
the consequences of modification were that a single kinetic state was affected by DEP such that openings from C_t to O_t became more favorable. Because the number of openings/burst is independent of agonist concentration, the intraburst states are themselves probably liganded (Gibb and Colquhoun, 1992). Although not explicit in Scheme III, this suggests that state C_t would be liganded during channel gating. Thus, the model implies that the modified residues exert their effects upon conformational changes that occurred after ligand binding. The (kinetic) discreteness of DEP-modification, and the absence of obvious effects on permeation, are also consistent with the view that the effects of DEP modification arose from relatively limited alterations in the channel protein.

This work was performed in partial fulfillment of the requirements for Doctor of Philosophy (J. L. Donnelly). Supported by grant NS 29881 (B. S. Pallotta) and Predoctoral Training Grant GM07040 (Department of Pharmacology) from the National Institutes of Health.

Original version received 20 December 1994 and accepted version received 8 March 1995.

REFERENCES

Benveniste, M., J. Clements, L. Vyklický, Jr., and M. Mayer. 1990. A kinetic analysis of the modulation of N-methyl-D-aspartic acid receptors by glycine in mouse cultured hippocampal neurones. Journal of Physiology. 428:333–357.

Burnashev, N., R. Schoepfer, H. Monyer, J. P. Ruppersberg, W. Gunther, P. H. Seeburg, and B. Sakmann. 1992. Control by asparagine residues of calcium permeability and magnesium blockade in the NMDA receptor. Science. 257:1415–1419.

Choi, D. W. 1988. Glutamate neurotoxicity and diseases of the nervous system. Neuron. 1:623–634.

Christensen, B. N., and E. Hida. 1990. Protonation of histidine groups inhibits gating of the quisqualate/kainate channel protein in isolated catfish cone horizontal cells. Neuron. 5:471–478.

Christine, C. W., and D. W. Choi. 1990. Effect of zinc on NMDA receptor-mediated channel currents in cortical neurons. Journal of Neuroscience. 10:108–116.

Clark, G. D., D. B. Clifford, and C. F. Zorumski. 1990. The effect of agonist concentration, membrane voltage and calcium on N-methyl-D-aspartate receptor desensitization. Neuroscience. 39:787–797.

Collingridge, G. L., and T. V. P. Bliss. 1987. NMDA receptors—their role in long-term potentiation. Trends in Neuroscience. 10:288–293.

Colquhoun, D., and B. Sakmann. 1985. Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. Journal of Physiology. 369:501–557.

Colquhoun, D., and F. J. Sigworth. 1983. Fitting and statistical analysis of single-channel records. In Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York, NY. 191–263.

Crilley, C. T., and A. J. Turner. 1994. Effect of chemical modification of arginyl and histidyl residues on [3H]MK-801 binding to brain synaptic membranes. Biochemical Pharmacology. 47:961–967.

Donnelly, J. L., and B. S. Pallotta. 1994. Properties of NMDA-activated channels treated with the histidine modifier diethylpyrocarbonate (DEP): burst properties, inhibition by zinc, and reversal by hydroxylamine. Biophysical Journal. 66:A379 (Abstr.)

Fisher, J. L., and B. S. Pallotta. 1993. Effects of the histidine modifier diethylpyrocarbonate on the single channel properties of NMDA-activated channels. Biophysical Journal. 64:A324. (Abstr.)

Gibb, A. J., and D. Colquhoun. 1991. Glutamate activation of a single NMDA receptor-channel produces a cluster of channel openings. Proceedings of the Royal Society of London, B. 243:39–45.
Gibb, A. J., and D. Colquhoun. 1992. Activation of N-methyl-D-aspartate receptors by L-glutamate in cells dissociated from adult rat hippocampus. *Journal of Physiology.* 456:143–179.

Hamill, O. P., J. Bormann, and B. Sakmann. 1983. Activation of multiple-conductance state chloride channels in spinal neurones by glycine and GABA. *Nature.* 305:805–808.

Higaki, J. N., R. J. Fletterick, and C. S. Craik. 1992. Engineered metalloregulation in enzymes. *Trends in Biochemical Science.* 17:100–104.

Hoch, D. B., and R. Dingledine. 1986. GABAergic neurons in rat hippocampal culture. *Developmental Brain Research.* 25:53–64.

Hollmann, M., J. Boulier, C. Maron, L. Beasley, J. Sullivan, G. Pecht, and S. Heinemann. 1993. Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. *Neuron.* 10:943–954.

Hollmann, M., and S. Heinemann. 1994. Cloned glutamate receptors. *Annual Review of Neuroscience.* 17:31–108.

Horn, R. 1987. Statistical methods for model discrimination: applications to gating kinetics and permeation of the acetylcholine receptor channel. *Biophysical Journal.* 51:255–263.

Howe, J. R., S. G. Cull-Candy, and D. Colquhoun. 1991. Currents through single glutamate receptor channels in outside-out patches from rat cerebellar granule cells. *Journal of Physiology.* 432:143–202.

Jahr, C. E., and C. F. Stevens. 1987. Glutamate activates multiple single channel conductances in hippocampal neurons. *Nature.* 325:522–525.

Kawajiri, S., and R. Dingledine. 1993. Multiple structural determinants of voltage-dependent magnesium block in recombinant NMDA receptors. *Neuropharmacology.* 32:1203–1211.

Kleckner, N. W., and R. S. Pallotta. 1995. Burst kinetics of single NMDA receptor currents in cell-attached patches from rat brain cortical neurons in culture. *Journal of Physiology.* In press.

Kleckner, N. W., and R. Dingledine. 1988. Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes. *Science.* 241:855–857.

Krishtal, O. A., and V. I. Pidoplichko. 1980. A receptor for protons in the nerve cell membrane. *Neuroscience.* 5:2325–2327.

Legendre, P., and G. L. Westbrook. 1990. The inhibition of single N-methyl-D-aspartate-activated channels by zinc ions on cultured rat neurones. *Journal of Physiology.* 429:429–449.

Legendre, P., C. Rosenmund, and G. L. Westbrook. 1995. Inactivation of NMDA channels in cultured hippocampal neurones by intracellular calcium. *Journal of Neuroscience.* 13:674–684.

Lester, R. A. J., and C. E. Jahr. 1992. NMDA channel behavior depends on agonist affinity. *Journal of Neuroscience.* 12:635–643.

Lester, R. A. J., G. Tong, and C. E. Jahr. 1993. Interactions between the glycine and glutamate binding sites of the NMDA receptor. *Journal of Neuroscience.* 13:1088–1096.

MacDermott, A. B., M. L. Mayer, G. L. Westbrook, S. J. Smith, and J. L. Barker. 1986. NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. *Nature.* 321:519–522.

Magleby, K. L. 1992. Preventing artifacts and reducing errors in single-channel analysis. *Methods in Enzymology.* 207:763–791.

Maksay, G. 1992. Modification of benzodiazepine receptors supports the distinctive role of histidine residues. *European Journal of Pharmacology.* 227:57–62.

Mayer, M. L., and G. L. Westbrook. 1987. Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. *Journal of Physiology.* 394:501–527.
Mayer, M. L., L. Vyklíčky, Jr., and G. L. Westbrook. 1989. Modulation of excitatory amino acid receptors by group IIb metal cations in cultured mouse hippocampal neurones. _Journal of Physiology_. 415:329–350.

McBain, C. J., and M. L. Mayer. 1994. _N_-methyl-d-aspartic acid receptor structure and function. _Physiological Reviews_. 74:723–760.

McDonald, J. W., and M. V. Johnston. 1990. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. _Brain Research Reviews_. 15:41–70.

McManus, O. B., and K. L. Magleby. 1988. Kinetic states and modes of single large conductance calcium-activated potassium channels in cultured rat skeletal muscle. _Journal of Physiology_. 402:79–120.

Melchior, W. B. J., and D. Fahrney. 1970. Ethoxyformylation of proteins. Reaction of ethoxyformic anhydride with α-chymotrypsin, pepsin, and pancreatic ribonuclease at pH 4. _Biochemistry_. 9:251–258.

Meldrum, B., and J. Garthwaite. 1990. Excitatory amino acid neurotoxicity and neurodegenerative disease. _Trends in Pharmacological Sciences_. 11:379–387.

Miles, E. W. 1977. Modification of histidyl residues in proteins by diethylpyrocarbonate. _Methods in Enzymology_. 47:431–442.

Monaghan, D. T., R. J. Bridges, and C. W. Cotman. 1989. The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. _Annual Reviews of Pharmacology and Toxicology_. 29:365–402.

Monyer, H., N. Burnashev, D. J. Laurie, B. Sakmann, and P. H. Seeburg. 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. _Neuron_. 12:529–540.

Mori, H., H. Masaki, T. Yamakura, and M. Mishina. 1992. Identification by mutagenesis of a Mg"²⁺-block site of the NMDA receptor channel. _Nature_. 358:673–675.

Moriyoshi, K., M. Masu, T. Ishii, R. Shigemoto, N. Mizuno, and S. Nakanishi. 1991. Molecular cloning and characterization of the rat NMDA receptor. _Nature_. 354:31–37.

Nowak, L. P., Bregestovski, P. Ascher, A. Herbet, and A. Prochiantz. 1984. Magnesium gates glutamate-activated channels in mouse central neurones. _Nature_. 307:462–465.

Okuyama, T., and K. Satake. 1960. On the preparation and properties of 2,4,6-trinitophenyl-amino acids and -peptides. _Journal of Biochemistry_. 47:454–466.

Oxford, G. S., C. H. Wu, and T. Narahashi. 1978. Removal of sodium channel inactivation in squid giant axons by N-Bromoacetamide. _Journal of General Physiology_. 71:227–247.

Peters, S., J. Koh, and D. W. Choi. 1987. Zinc selectively blocks the action of N-methyl-d-aspartate on cortical neurons. _Science_. 236:589–593.

Ransom, R. W., and N. L. Stec. 1988. Cooperative modulation of [³H]MK-801 binding to the N-methyl-d-aspartate receptor-ion channel complex by l-glutamate, glycine, and polyamines. _Journal of Neurochemistry_. 51:830–836.

Reynolds, I. J. 1992. Interactions between zinc and spermidine on the N-methyl-d-aspartate receptor complex: clues to the mechanism of action of 1,10-bis(guanidino)decane and pentamidine. _Journal of Pharmacology and Experimental Therapeutics_. 263:632–638.

Riordan, J. F., W. E. C. Wacker, and B. L. Vallee. 1965. _N_-Acetylimidazole: a reagent for determination of "free" tyrosyl residues of proteins. _Biochemistry_. 9:1758–1765.

Rock, D. M., and R. L. Macdonald. 1992. The polyamine spermine has multiple actions on N-methyl-d-aspartate receptor single-channel currents in cultured cortical neurons. _Molecular Pharmacology_. 41:83–88.
Rosenmund, C., and G. L. Westbrook. 1993. Rundown of N-methyl-D-aspartate channels during whole-cell recording in rat hippocampal neurons: role of Ca\(^{2+}\) and ATP. *Journal of Physiology.* 470:705–729.

Rothman, S. M., and J. W. Olney. 1987. Excitotoxicity and the NMDA receptor. *Trends in Neuroscience.* 10:299–302.

Ruppersberg, J. P., J. Mosbacher, W. Günther, R. Schoepfer, and B. Fakler. 1993. Studying block in cloned N-methyl-D-aspartate (NMDA) receptors. *Biochemical Pharmacology.* 46:1877–1885.

Sakmann, B., J. Patlak, and E. Neher. 1980. Single acetylcholine-activated channels show burst-kinetics in presence of desensitizing concentrations of agonist. *Nature.* 286:71–73.

Sakurada, K., M. Masu, and S. Nakanishi. 1993. Alteration of Ca\(^{2+}\) permeability and sensitivity to Mg\(^{2+}\) and channel blockers by a single amino acid substitution in the N-methyl-D-aspartate receptor. *Journal of Biological Chemistry.* 268:410–415.

Seeburg, P. H. 1993. The TIPS/TINS lecture: the molecular biology of mammalian glutamate receptor channels. *Trends in Pharmacological Science.* 14:297–303.

Sheardown, M. J., E. O. Nielsen, A. J. Hansen, P. Jacobsen, and T. Honoré. 1990. 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia. *Science.* 247:571–574.

Shrager, P. 1975. Specific chemical groups involved in the control of ionic conductance in nerve. *Annals of the New York Academy of Science.* 264:293–303.

Sigworth, F. J., and S. M. Sine. 1987. Data transformations for improved display and fitting of single-channel dwell time histograms. *Biophysical Journal.* 52:1047–1054.

Tang, L.-H., and E. Alzenman. 1993. The modulation of N-methyl-D-aspartate receptors by redox and alkylating reagents in rat cortical neurones in vitro. *Journal of Physiology.* 465:303–323.

Traynelis, S. F., and S. G. Cull-Candy. 1991. Pharmacological properties and H\(^{+}\) sensitivity of excitatory amino acid receptor channels in rat cerebellar granule neurones. *Journal of Physiology.* 433:727–763.

Vyklický, L., Jr. 1993. Calcium-mediated modulation of N-methyl-D-aspartate (NMDA) responses in cultured rat hippocampal neurones. *Journal of Physiology.* 470:573–600.

Westbrook, G. L., and M. L. Mayer. 1987. Micromolar concentrations of Zn\(^{2+}\) antagonize NMDA and GABA responses of hippocampal neurons. *Nature.* 328:640–643.

Zarei, M. M., and J. A. Dani. 1994. Ionic permeability characteristics of the N-methyl-D-aspartate receptor channel. *Journal of General Physiology.* 103:231–248.