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

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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.

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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, Vyklický, and Westbrook, 1989; Legendre, Rosenmund, and Westbrook, 1993; Vyklický, 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).

METHODS

Cell Culture

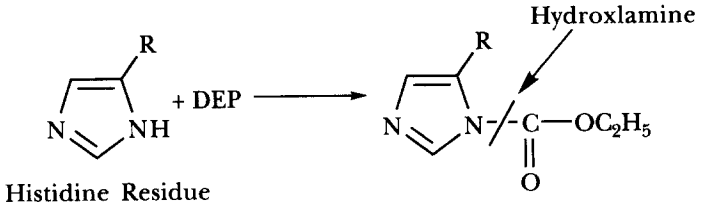
Procedures for neuron cell culture are described by Hoch and Dingledine (1986). Briefly, brain cortical neurons were obtained from fetal (E19) rat pups removed from ether-anesthetized, pregnant females (Sprague-Dawley, Harlan). The cortices were minced in Hanks Balanced Salt Solution (HBSS) + 10 mM HEPES (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 $CaCl_2$, 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% CO_2. 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 $CaCl_2$ 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 $CaCl_2$, 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 $CaCl_2$ (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 $CaCl_2$ (pH 7.4, 295–305 mOsm). For whole-cell recordings Ba^{2+} replaced Ca^{2+} 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-benzo(f)quinoxaline (NBQX) were included in the test solutions to block glycine-activated Cl^- currents (Hamill, Bormann, and Sakmann, 1983) and nonNMDA 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).



SCHEME 1

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 (np_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

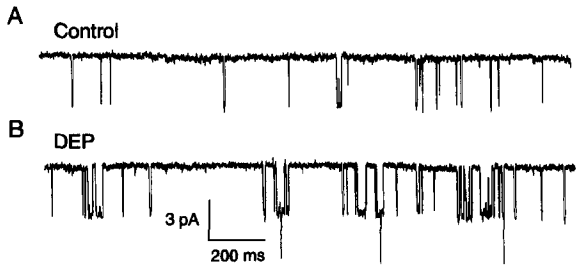


FIGURE 1. Currents from single NMDA receptors before (*A*) and after (*B*) modification with DEP. Currents were recorded from an outside-out patch exposed to 10 μM NMDA and 10 μM glycine (membrane potential −50 mV). Both currents were filtered at 500 Hz and sampled at 100-μs intervals for display. In this and all subsequent figures, inward currents are drawn downward. (*A*) 1.8-s trace recorded under control conditions. Channel open probability (np_o) was 0.03 during the control recording period. (*B*) Single-channel currents recorded after 15 s treatment with 3 mM DEP. Channel open probability increased to 0.14.

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.

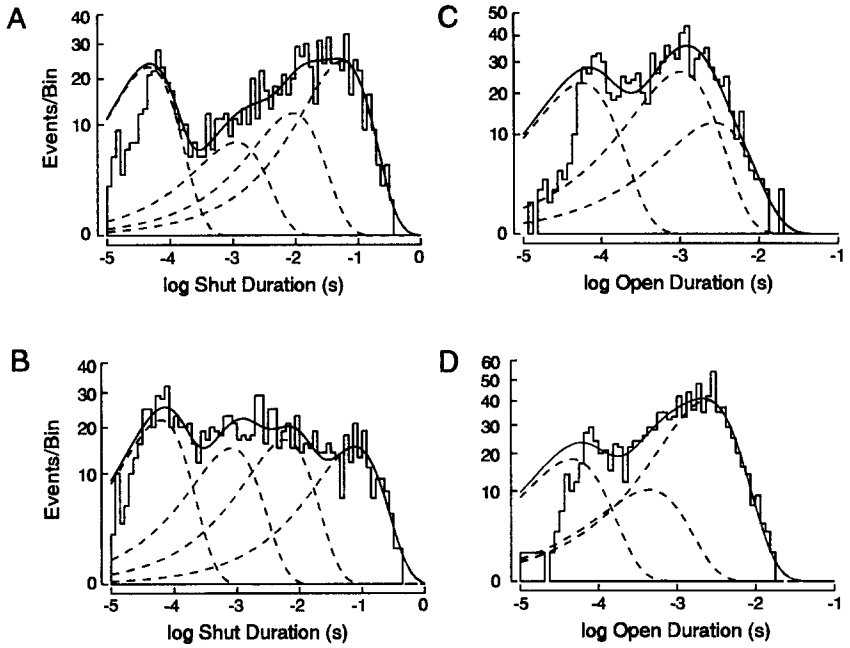


FIGURE 2. Open and shut duration histograms from untreated (*A* and *C*) and DEP-modified (*B* and *D*) channels. (*A*) Distribution of 955 shut intervals recorded from an outside-out patch exposed to 10 μM NMDA and 0.5 μM glycine (membrane potential −50 mV). The distribution was fitted with the sum 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 μs/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).

TABLE I

Distributions of Open and Shut Intervals

	Control	DEP
Open intervals	$n = 7$	$n = 9$
τ_1 (ms)	0.11 ± 0.02	0.09 ± 0.01
Area_1	0.32 ± 0.02	0.22 ± 0.02*
τ_2 (ms)	1.03 ± 0.12	1.53 ± 0.32
Area_2	0.47 ± 0.06	0.41 ± 0.08
τ_3 (ms)	3.77 ± 0.56	4.86 ± 0.99
Area_3	0.22 ± 0.08	0.37 ± 0.08
Shut intervals	$n = 10$	$n = 9$
τ_1 (ms)	0.05 ± 0.01	0.06 ± 0.01
Area_1	0.29 ± 0.03	0.36 ± 0.04
τ_2 (ms)	0.82 ± 0.19	0.66 ± 0.07
Area_2	0.11 ± 0.01	0.21 ± 0.01*
τ_3 (ms)	14.81 ± 2.37	8.61 ± 1.5*
Area_3	0.26 ± 0.05	0.24 ± 0.02
τ_4 (ms)	89.8 ± 30.8	81.8 ± 19.70
Area_4	0.33 ± 0.03	0.19 ± 0.04*

*Significantly different from control values, $P < 0.05$, Student's independent t test

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,

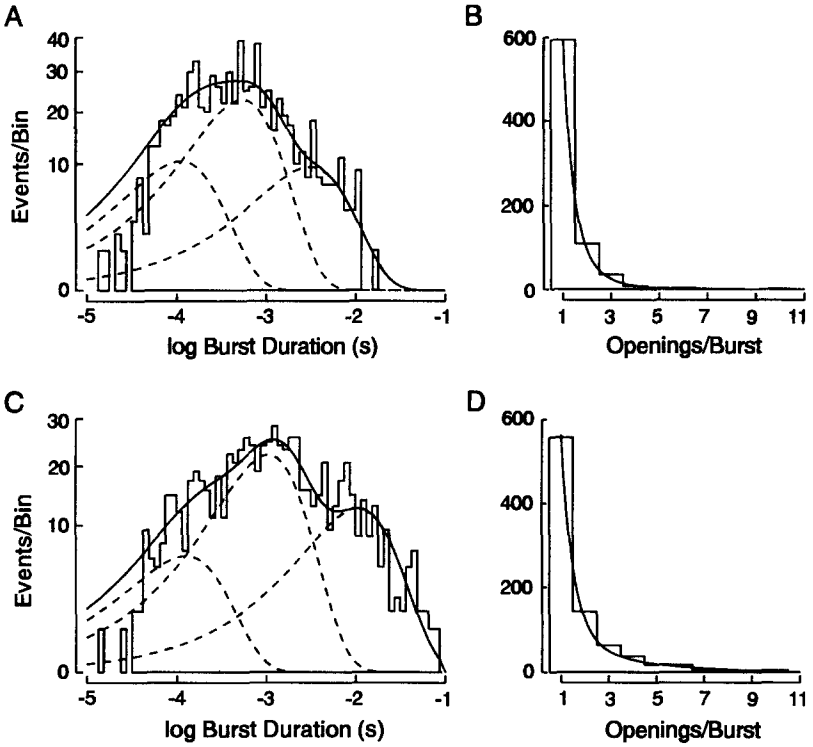


FIGURE 3. Burst kinetics from NMDA receptors in outside-out patches before (*A* and *B*) and after (*C* and *D*) modification by DEP. (*A*) Burst duration distribution obtained before treatment with DEP. The critical gap for dividing channel openings into bursts was 0.86 ms under these conditions (see Methods). The distribution of 733 burst durations was fitted with three exponential components with mean durations (and relative areas) of 0.13 ms (0.24), 0.62 ms (0.54), and 3.59 ms (0.22). (*B*) Distribution of openings/burst obtained from the same patch during the control recording period. The distribution was fitted with two geometric components with means (and relative areas) of 1.1 openings/burst (0.64) and 1.7 openings/burst (0.36). (*C*) Burst duration distribution after modification with DEP (same patch as in A). The critical gap under these conditions was 1.07 ms, and 860 burst durations were measured. The three fitted components have mean durations (and relative areas) of 0.15 ms (0.15), 1.21 ms (0.54), and 12.24 ms (0.31). (*D*) Distribution of openings/burst after modification by DEP. The distribution was fitted with two geometric components with means (and relative areas) of 1.1 openings/burst (0.64) and 3.3 openings/burst (0.36). The open probability in this patch was increased fivefold by 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	$n = 8$	$n = 8$
Mean_1	1.1 ± 0.1	1.3 ± 0.1
Area_1	0.55 ± 0.05	0.47 ± 0.05
Mean_2	1.9 ± 0.1	3.0 ± 0.5
Area_2	0.45 ± 0.06	0.53 ± 0.05
Burst duration		
τ_1 (ms)	0.17 ± 0.04	0.18 ± 0.06
Area_1	0.38 ± 0.05	0.26 ± 0.03
τ_2 (ms)	1.30 ± 0.20	2.13 ± 0.75
Area_2	0.40 ± 0.06	0.45 ± 0.05
τ_3 (ms)	8.32 ± 2.63	17.99 ± 5.30
Area_3	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

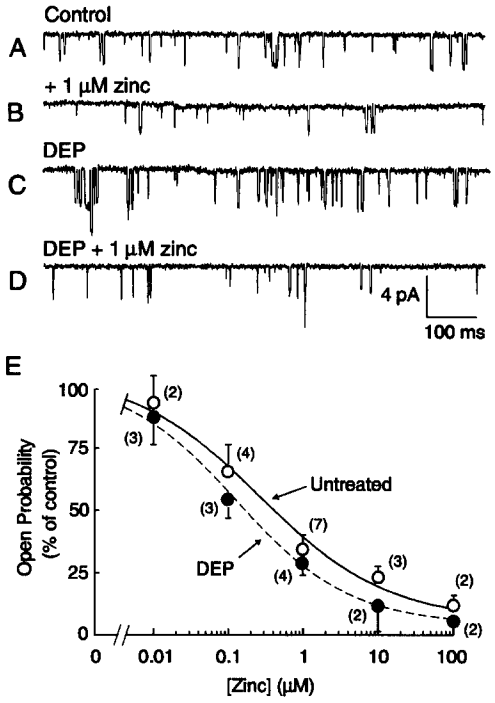


FIGURE 4. Zinc inhibition of NMDA channel activity. (*A*) Current trace from an outside-out patch (−50 mV) exposed to 20 μM NMDA and 10 μM glycine. Channel open probability was 0.03. All traces in this figure were obtained from the same patch. Currents were filtered at 500 Hz (for display) and digitized at 10 kHz. (*B*) In the presence of 1 μM zinc, open probability decreased to 0.01. (*C*) After zinc washout, treatment with 3 mM DEP increased channel open probability to 0.04. (*D*) Subsequent addition of 1 μM zinc to the DEP-modified channels decreased open probability to 0.02. (*E*) Zinc concentration dependence of channel open probability. Open probability was plotted as a percent of the response in the absence of zinc (mean ± SEM). The numbers in parentheses indicate the number of patches contributing to each point. The data were fit with a logistic equation $y = a_1/(1 + (x/a_2)^{a_3} + a_0$, where y is the response (% of control) at x concentration of zinc. a_1 is the maximal response and was constrained to 100 (%). a_0 is the response that remains at very high zinc concentration, a_2 is a measure of efficacy, and a_3 is a slope factor. For unmodified channels the fit parameters (±SE) were $a_0 = 5.4 \pm 4.9$, $a_2 = 0.25 \pm 0.16$, and $a_3 = 0.51 \pm 0.09$ (*solid line*). After DEP modification, the values were $a_0 = 2.6 \pm 2.6$, $a_2 = 0.14 \pm 0.04$, and $a_3 = 0.57 \pm 0.06$ (*dashed line*).

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.

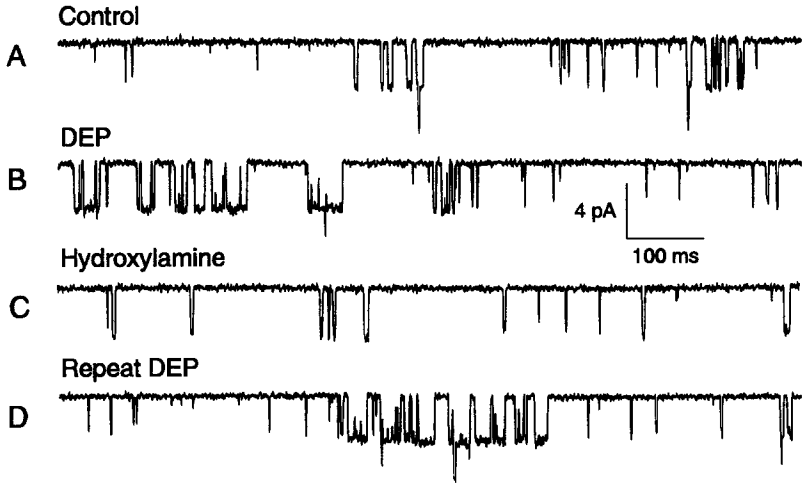


FIGURE 5. Hydroxylamine reverses the effect of DEP on channel activity. (*A*) Current trace from an outside-out patch exposed to 10 μM NMDA and 10 μM glycine at −50 mV. During the control period, channel open probability was 0.03. Same patch as in Fig. 1. (*B*) Trace obtained after treatment with 3 mM 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 (np_o = 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. 5 *C*). 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.

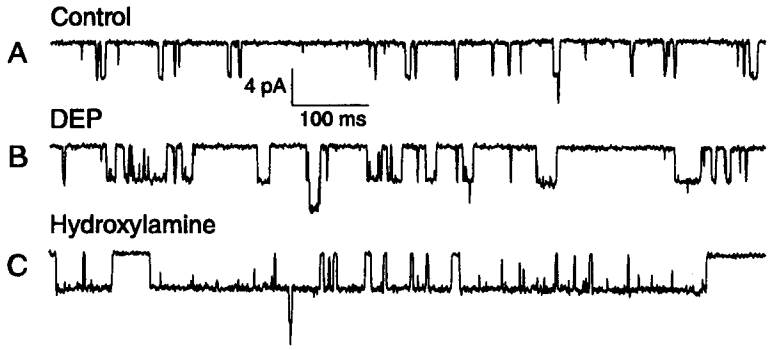
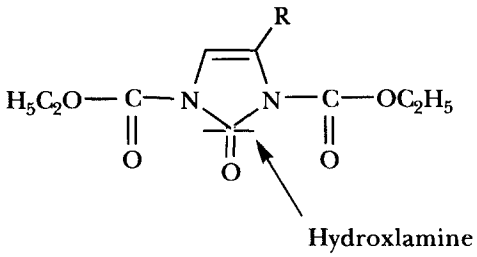


FIGURE 6. Hydroxylamine also potentiates the activity observed in DEP-treated patches. (*A*) Current trace from an outside-out patch exposed to 10 μM NMDA and 10 μM glycine at −50 mV. Open probability was 0.06. (*B*) DEP modification increased open probability 3.3-fold to 0.20. (*C*) In contrast to the 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 hydroxlyamine 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.



SCHEME II

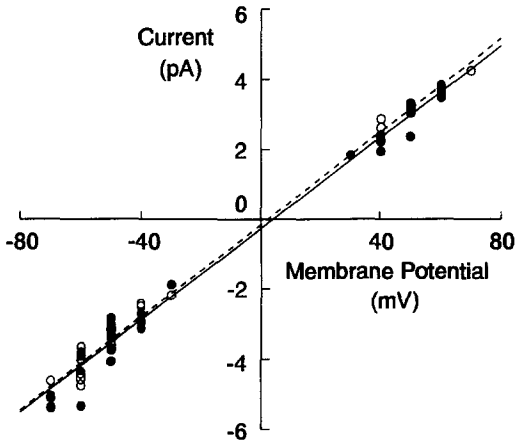


FIGURE 7. Single-channel current-voltage relationship for NMDA receptors before and after modification with DEP. Amplitude measurements of openings to the main conductance level were recorded from 14 different patches, before and after treatment with DEP. (*Open symbols*) Measurements from unmodified 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.

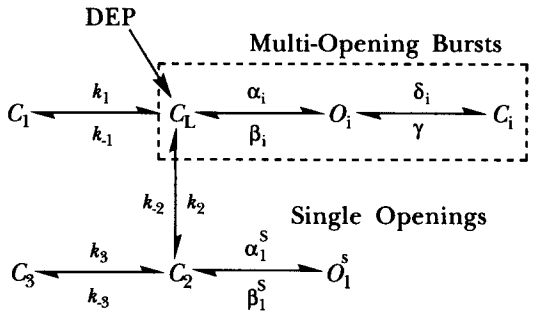
Zinc Binding

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.



SCHEME III

TABLE III

Rate Constants for Scheme III

Rate constant	Control ms^{-1}	DEP 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
α_i	0.5	**0.7**
α_i^s	0.005	0.005
β_i	0.15	0.15
β_i^s	11	11
δ_i	0.1	0.1
γ	16.67	16.67

To account for the closed times between bursts, two additional shut states (C_2, C_3) were added. An additional open state (O_i^s) 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_i^s, and the transition rates to and from these states are similarly represented by α_i^s and β_i^s.

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

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_{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		
τ_1 (ms)	0.09	.09
$Area_1$	0.37	0.18
τ_2 (ms)	4	4
$Area_2$	0.63	0.82
Shut intervals		
τ_1 (ms)	0.06	0.06
$Area_1$	0.25	0.33
τ_2 (ms)	0.74	0.82
$Area_2$	0.11	0.25
τ_3 (ms)	15.99	9.72
$Area_3$	0.32	0.25
τ_4 (ms)	85.42	82.79
$Area_4$	0.32	0.17
Openings/burst		
$Mean_1$	1.0	1.0
$Area_1$	0.55	0.40
$Mean_2$	2.3	3.2
$Area_2$	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_L to O_i 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_L 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.

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