A Slow Fraction of Mg\(^{2+}\) Unblock of NMDA Receptors Limits Their Contribution to Spike Generation in Cortical Pyramidal Neurons

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Vargas-Caballero, Mariana and Hugh P. C. Robinson. A slow fraction of Mg\(^{2+}\) unblock of NMDA receptors limits their contribution to spike generation in cortical pyramidal neurons. J Neurophysiol 89: 2778–2783, 2003; 10.1152/jn.01038.2002. The timing of voltage-dependent removal of Mg\(^{2+}\) block of N-methyl-D-aspartate receptors (NMDARs) is potentially critical for determining their nonlinear contribution to excitability. Here, we measure the kinetics of NMDAR unblock in nucleated patch and whole cell recordings of rat cortical pyramidal neurons during depolarizing voltage steps. At room temperature, the unblock showed a very fast component (\(\tau < 1\) ms) and a slower component (\(\tau = 14–23\) ms in nucleated patches). The slow component accounted for half of the current at +40 mV and its amplitude and time constant showed some voltage dependence. Blocking with hyperpolarization was very fast (\(\tau < 200\) ms). Voltage-clamp with action potential waveforms, at both room temperature and at 33°C, showed that the rising phase of single fast action potentials unblocks far less NMDAR current than expected from the stationary voltage dependence, while a large amplitude of current is uncovered during the upstroke of slow calcium action potentials. The repolarization of fast sodium action potentials uncovers an NMDAR tail current, much bigger than the stationary level of current.

INTRODUCTION

N-methyl-D-aspartate receptors (NMDARs) provide a long-lasting component of elevated conductance at glutamatergic synapses, which follows a very rapid conductance transient, mediated by \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (AMPARs) (Forsythe and Westbrook 1988; Robinson et al. 1991; Stern et al. 1992). The relatively slow decay of the NMDA phase (Lester et al. 1990) means that activation of NMDARs may easily accumulate to high or saturating levels, even at low firing rates. However, opening of the channel is also subject to a voltage-dependent block by external magnesium ions (Mayer et al. 1984; Nowak et al. 1984), which is relieved only with depolarization from rest. In the subthreshold range of membrane potentials, this block produces a negative slope conductance, or positive feedback between depolarization and inward current through activated NMDARs, which might contribute significantly to membrane excitability. Recent pharmacological evidence suggests that NMDARs are required for the initiation of dendritic spikes in basal dendrites of cortical pyramidal neurons (Schiller et al. 2000), which might provide an amplification mechanism for clustered synaptic input (Schiller and Schiller 2001). It is assumed in all modeling studies so far that the channel has an instantaneous voltage dependence.

The timing of Mg\(^{2+}\) unblock is critical in determining the contribution of NMDARs to spike generation. To have an appreciable effect, unblock must keep pace with the rate of depolarization, leading into and during spikes—a small difference in the speed of unblock at the millisecond timescale could have a large effect on spike generation. Very few experimental studies have looked at the responses of NMDARs to voltage jumps in physiological external magnesium concentration, and early studies did not remark any slow component of unblock (Mayer and Westbrook 1987). However, Spruston et al. (1995) described slow and fast components of unblock of NMDARs in outside-out patches isolated from hippocampal pyramidal cells. In this paper, we confirm that a large component of NMDAR unblock is delayed, using nucleated patches from layer two/thirds rat cortical pyramidal neurons. We characterize this effect quantitatively and demonstrate that NMDARs consequentely contribute much less inward current than previously assumed during fast depolarizations such as the rising phase of sodium action potentials (APs). We find that slower depolarizations lasting at least several milliseconds, for example, calcium APs, are required to fully unblock the NMDAR. Some of these results have been presented previously in abstract form (Vargas-Caballero and Robinson 2001).

METHODS

Electrophysiological recordings

Using UK Home Office approved procedures, brains were removed from 8-to 14-day-old Wistar rats killed by cervical dislocation. Sagittal slices 300 \(\mu\)m thick were cut on a vibrating slicer (Campden Instruments, Leicester, UK). During the slicing procedure, tissue was kept in the following ice-cold low sodium solution (in mM): 254 sucrose, 2.5 KCl, 26 NaHCO\(_3\), 10 glucose, 1.25 NaH\(_2\)PO\(_4\), 2 CaCl\(_2\), and 1 MgCl\(_2\). Slices were then incubated in Ringer solution at room temperature. The Ringer solution contained 125 mM NaCl, 2.5 mM KCl, 25 mM NaHCO\(_3\), 25 mM glucose, 1.25 mM NaH\(_2\)PO\(_4\), 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 10 \(\mu\)M glycine. Both slicing and recording solutions were bubbled with a 95% \(O_2\), 5% \(CO_2\) gas mixture, giving a pH of 7.4.

For recording, a single slice was transferred to a recording chamber. The preparation was continuously perfused with oxygenated Ringer solution containing 100 mM tetrodotoxin (Sigma) to block voltage-dependent Na channels. Recordings were carried out at room temperature (20–23°C, Figs. 1–4) or at 31–35°C (Fig. 5, E–G). Slices were viewed using an upright microscope (Olympus BWS50WI, Olympus

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UK, London) with a water-immersion objective (Olympus LUMPlanFl, 60X, N.A. 0.90) and infrared differential interference contrast optics. Whole-cell and nucleated patch recordings were obtained using standard techniques (Hamill et al. 1981; Sather et al. 1992) from layer II/III cortical pyramidal cells in the occipital cortex. The shanks of pipettes used for nucleated patch recordings were coated with dental wax to reduce the pipette capacitance and heat-polished.

Pipettes were filled with one of two intracellular solutions, a potassium-based solution that contained 20 mM phosphocreatine-Na₂, 4 mM MgCl₂, 0.3 mM GTP, 4 mM Na₃-ATP, 100 mM K-glutamate, 20 mM KCl, 10 mM HEPES, and 5 U/ml creatine phosphokinase, balanced to pH 7.3 with KOH, or a caesium-based solution that had the following composition (in mM): 20 phosphocreatine-Na₂, 4 Mg-ATP, 0.3 GTP, 50 Cs-methane sulfonate, 30 CsCl, 10 HEPES, and 10 EGTA, balanced to pH 7.3 with CsOH. All presented results were obtained with the Cs-based solution, except Figs. 2A and 4B, as stated.

To activate NMDAR currents, cells or nucleated patches were perfused locally with the bath Ringer solution containing agonist in concentrations between 20 and 25 μM for NMDA and 3 and 5 μM for glutamate, in the latter case adding 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) both to the perfusate and to the bath solution. To minimize cable filtering of currents in whole cell recordings, superfusion with agonist was confined to the somatic region of the cell. For local perfusion we used a second pipette (tip diameters between 4 and 6 μm) connected to a computer-controlled pressure ejector. Voltage steps were delivered during the agonist perfusion to study the voltage-dependent activation of the channels when equilibrated with agonist. The perfusion was started 500 to 800 ms prior to the voltage step to ensure complete replacement of external solution at the membrane by perfusate before the voltage step. Membrane potentials were corrected for prenullled liquid junction potential, which was measured directly (Neher 1992). Recordings were made with an Axopatch 200A amplifier (Axon Instruments. Foster City, CA) in voltage-clamp mode; the built-in series resistance compensation circuitry was used in most recordings. Signals were filtered at 5 kHz (–3 dB, four-pole Bessel) and sampled with 12-bit resolution at 20 kHz. The NMDAR current responses to voltage steps were corrected for associated capacitative and leak currents by recording control and agonist-induced responses 7 to 10 times at intervals of 5–10 s, averaging and then subtracting the control from the agonist-induced ensemble average. For fitting of time constants, current responses were digitally filtered (Gaussian filter at 1 kHz cutoff frequency) and fitted by least squares. The step response of the digital filter, which dominated the total step response of the system, had a 10–90% risetime of 0.3 ms. Where indicated, recordings have been plotted without digital filtering to best expose the speed of current responses.

**RESULTS**

**Equilibrium voltage-dependence**

To study the kinetics of voltage-dependent block, NMDAR currents were elicited by local application of agonists to isolated nucleated patches or to the somatic region of whole cells in layers II/III of slices of young rat cortex. NMDA (25 μM) superperused through the perfusing pipette evoked currents with the typical nonlinear NMDAR current-voltage (I-V) relation and with reversal potentials around −3 mV (Fig. 1A).

We fitted a Boltzmann distribution (Wollmuth et al. 1998; Woodhull 1973) to the conductance during 1- to 3-s ramps of

![Figure 1](http://jn.physiology.org/)

**FIG. 1.** Stationary N-methyl-D-aspartate receptor (NMDAR) current. A: NMDAR current evoked by superfusion of a nucleated patch with 25 μM NMDA, plotted as a function of membrane potential during a 3-s ramp command potential. B: fraction of the maximal slope conductance at highly depolarized potentials is plotted as a function of membrane potential (omitting data near the reversal potential to exclude large fluctuations), fitted to a Boltzmann distribution. (Vₐₛ = −15 mV, δ = 0.97 (see Eq. 1)), E₂glyc = −5 mV). C: agonist was perfused by a computer-controlled pressure ejector, whose timing is indicated by the upper trace. Dotted lines show the timing adopted later for the voltage steps. Application of agonist (in this case 5 μM glutamate) elicited a current, which became stationary after a few hundred milliseconds and remained constant for about a second. Holding potential as indicated. Black traces: with agonist; gray traces: without agonist.

**voltage (−70 to 40 mV)**

\[ F(V) = \frac{1}{1 + \exp(-(V - V_{0.5})\delta F)/RT} \]  

This showed a voltage for half-maximal block (V₀.₅) of −13 mV ± 2.45 and a fraction of the membrane potential sensed by the blocking site (δ) of 0.96 ± 0.01 (n = 5 nucleated patches; Fig. 1B). At a constant membrane potential, NMDAR current was stationary for several hundred milliseconds, indicating that the receptors were at equilibrium over this period. Desensitization during each record was minimal, as reported by Nahum-Levy et al. (2001) for similar agonist and glycine concentrations. In some recordings, no sign of desensitization was evident over the period of 2–3 min while a set of NMDA-induced control responses were recorded for the same voltage step, while in others, desensitization was detectable but small (Fig. 1C). Therefore it is expected that the current transient following a voltage step reflects predominantly the relaxation in response to the voltage step and not unrelated changes in availability of receptors or transmitter. In subsequent experiments, voltage steps were applied during this stationary period of the response (Fig. 1C). Responses to voltage steps were corrected for leak and capacitative currents and other non-agonist-dependent voltage-activated currents by subtraction of
control responses in the absence of agonist (Fig. 2A, see METHODS).

Responses to depolarizing voltage steps: slow and fast components of unblock

Responses of NMDA-induced current to voltage steps showed a clear asymmetry both in nucleated patch recordings (Figs. 2, A–C and 3A) and in whole cell recordings (Fig. 3B).

![Image](image_url)

**FIG. 2.** NMDA-induced currents with depolarizing voltage steps from rest. A: leak and capacitative current subtraction. Individual current responses of a nucleated patch are shown superimposed with (black, 8 sweeps) and without (gray, 8 sweeps) agonist (25 μM NMDA) during voltage steps from −70 to 40 mV (top). Averaging and subtracting control from total current isolates the NMDAR current (bottom). B: voltage steps to +40 mV starting from −70 and 0 mV elicit currents with different kinetics in the presence of 1 mM Mg2+. Amplitude is normalized to the maximum (steady state) level reached. A large slow component is observed when stepping from −70 mV (gray), while responses to steps from 0 mV are dominated by a fast component (black). C: short depolarizations (5 ms, black trace) only partially relieve block (approximately 50%) compared with the response to a long depolarization (500 ms, gray trace). The maintained depolarization has no effect on the amplitude of a subsequent short response. D: in nominally zero [Mg2+]i, the same nucleated patch as in B shows a fast symmetric block and unblock in response to a 500-ms voltage step, and 5-ms depolarizations recover >90% of the stationary current.

FIG. 3. Families of NMDAR responses to voltage jumps in nucleated patches and whole cell recordings. Leak corrected and averaged currents, see METHODS and Fig. 2. A: nucleated patch responses. Currents during voltage steps from −70 mV to voltages indicated. Top: outward responses. Bottom: inward responses. Right: expanded view of the current at the times of onset and offset of the voltage step. B: whole cell NMDA-induced current responses to depolarizing voltage steps from rest to the voltages indicated. with much faster block than unblock. In whole cell recordings, cancellation of the fast capacity transient was not always complete, but the transient appeared to be restricted to the first 2–3 ms. The slow component of current was dependent on the presence of Mg2+, since no slow relaxation was observed in nominally Mg2+-free solution in nucleated patches (Fig. 2D) (see also Mayer and Westbrook 1987; Spruston et al. 1995). Note that, without Mg2+, a much larger stationary inward current is obtained at −70 mV. A predominantly fast current activation occurred following voltage steps from 0 mV to a more depolarized potential (n = 6 nucleated patches), reflecting the restoration of driving force to already unblocked channels. The slow component is associated with the range of membrane potentials over which Mg block is removed (see Fig. 1A).

The asymmetry of on and off relaxations in the current during a depolarizing step from −70 mV, with an extremely fast block phase, indicated that incomplete compensation of series resistance was not responsible for the slow unblock phase. As an additional check of the quality of space-clamp in nucleated patches, we stepped from 0 to −70 mV in nominally Mg2+-free external solution while applying NMDA (not shown). This produced an effectively instantaneous onset of a steady level of inward current, indicating that the voltage step was applied rapidly and constantly to the membrane. The slow phase of current activation is therefore ascribed to Mg2+-unblock of the NMDAR.

The unblock phase could be well fitted by a sum of two exponential functions with fast and slow time constants

\[ I(t) = I_s[A_1 [1 - \exp(-t/\tau_1)] + A_2 [1 - \exp(-t/\tau_2)] \]  

(2)

where \( A_1 + A_2 = 1 \) and \( I_s \) is the stationary current level (see Fig. 4A). For voltage pulses from −70 to +40 mV (n = 5, nucleated patches), the following values were obtained: \( \tau_1 = 0.68 \pm 0.21 \) ms, \( \tau_2 = 22.7 \pm 2.3 \) ms, \( A_1 = 0.53 \pm 0.06 \) and \( A_2 = 0.47 \pm 0.06 \). Thus, for this voltage step, each component accounts for approximately half of the current. However these fractions showed some dependence on the test voltage (see Fig. 4B), with a linear decline in \( A_1 \) and a rise in \( A_2 \) with increasing depolarization. For K-based solution the linear fit was \( A_1(V) = 0.49 - 0.0043 V \) \((r^2 = 0.93, P < 0.001)\) and for Cs-based solution the linear fit was \( A_1(V) = 0.51 - 0.0028 V \) \((r^2 = 0.79, \)
delayed component of unblock of NMDA receptors

FIG. 4. Kinetics of NMDA currents after depolarizing step. A: unblock kinetics of NMDA currents (25 μM NMDA, Cs-based intracellular solution) in a nucleated patch after a depolarizing step from −70 mV to the potentials indicated in each panel. Double exponential fits with parameters as shown. B: voltage dependence of the fast-unblocking fraction of current (see Eq. 2). C: rapid block of NMDAR at the end of a depolarizing step. NMDAR conductance in a nucleated patch (K-based solution) during voltage steps as indicated at the top, from the voltages shown to −70 mV. Exponential fits to the decays with time constants between 120 and 150 μs. Current is reploted as chord conductance, for a reversal potential of −5 mV, to facilitate comparison of inward and outward responses. No digital filter applied. Dotted curve: scaled step response of analog filter/recording system combination.

NMDAR current during AP waveforms

To assess the functional consequences of the noninstantaneous block and unblock of NMDARs, we recorded NMDAR current while voltage clamping nucleated patches with AP waveforms (Fig. 5). First, to check the consequences of the glutamate, including CNQX in the perfusate to block AMPA receptor current. In some patches we observed some long-term changes in the reversal potential of the current (a shift to −10 or −15 mV), apparently due to additional activation of an inwardly rectifying K/Cs permeability (not shown). This presumably was a consequence of activation of metabotropic glutamate receptors. However, we observed similar time courses of block and unblock in experiments with glutamate as in experiments with NMDA. Likewise there was no difference in kinetics when using Cs- or K-based internal solution.
room temperature kinetics measured above, we clamped nucleated patches with spontaneous APs recorded in the same cells at room temperature (Fig. 5, B–D). Under these conditions, there was little or no NMDAR current during the upstroke of fast APs, implying that both slow and fast phases of unblock occur too slowly to react to the AP initiation (3 patches). In contrast, the expected current calculated from the measured steady-state $I-V$ relation under the assumption of instantaneous voltage dependence (red solid line) shows a substantial contribution of the NMDAR to inward current during the onset of the upstroke, i.e., to excitability. By the peak of the AP, however, NMDARs have sufficiently unblocked to conduct an outward current positive to the reversal potential and a large and longer-lasting inward current during repolarization, which in fact exceeds the level expected from the stationary $I-V$ relation (see DISCUSSION).

To examine the impact of noninstantaneous unblock under more physiological conditions, we used a near physiological temperature (31–35°C) and clamped with waveforms recorded in the same temperature range by Larkum et al. 2001. This showed the same basic effects, with only slight differences ($n = 8$ nucleated patches). No inward current is detectable during depolarization, while during repolarization the current matches or exceeds that predicted from the stationary $I-V$ relation of the patch (Fig. 5E). Unlike for the fast sodium AP, activated NMDARs made a strong contribution during the upstroke of calcium AP (Fig. 5F) and therefore do contribute substantially to the excitability of Ca$^{2+}$ APs. During a dendritic Na spikelet or boosted excitatory postsynaptic potential, the NMDAR current follows the expected equilibrium current quite closely (Fig. 5G).

**DISCUSSION**

*Slow unblock of NMDA receptors*

A slow component of unblock has not been remarked on previously in the literature, with the exception of the study by Spruston et al. (1995). There are probably several reasons for this. First, as we have shown, whole cell recording obscures the effect because of the impossibility of achieving complete leak and capacitance transient cancellation. In addition, we found that whole cell, but not nucleated patch recordings, showed an additional very slow component of increasing current in response to depolarization, with a time constant in the range of hundreds of milliseconds. This was particularly evident for outward currents (Fig. 3B). It is likely that Ca$^{2+}$ buffering by the pipette solution was more effective in nucleated patches than in whole cells and especially in their dendrites. It is possible therefore that this effect could be due to unbuffered calcium rises in the dendrites, activating nonspecific cation or K-selective permeabilities (despite the presence of intracellular Cs) produced by calcium influx through the NMDAR. Second, very few studies have applied voltage steps during activation of NMDARs. Third, current models of NMDAR gating (Ascher and Nowak 1988; Lester and Jahr 1992; Sobolevsky and Yelshansky 2000), based on agonist/blocker concentration jump data at stationary potentials and low concentrations of Mg$^{2+}$, do not explain the slow fraction of unblock (Vargas-Caballero and Robinson 2001; unpublished observations) observed with voltage jump recordings.

Mayer and Westbrook (1985) applied voltage steps using dual sharp electrode voltage-clamp in spinal cord neurons in the presence of 1 mM [Mg$^{2+}$]$_e$. They observed a symmetrical onset and offset of responses with time constants of 3 ms. Their results are similar to those shown in Fig. 3C for steps to −20 mV. As in our whole cell recordings, it is probable that the technique used did not allow resolution of the fast component of unblock or did not cancel completely the capacitance artifacts by leak subtraction. D’Angelo et al. (1994) applied voltage steps from 0 to 40 mV in cerebellar granule cells and obtained a very fast block and unblock, similar to our experimental results in Fig. 2B. In these conditions, receptors appear to be substantially unblocked at 0 mV; the fast activation of current reflects primarily the introduction of the driving force. Benveniste and Mayer (1995), in their Fig. 3C, demonstrated fast current activation when depolarizing a nucleated patch 200 ms after a 20-ms application of 50 μM Mg$^{2+}$ and 200 μM glutamate. However, owing to the short application of Mg$^{2+}$, and the time allowed between its application and the voltage step, it is again likely that the rapid onset of outward current after depolarization reflects mainly the change in driving force and not the unblocking kinetics of Mg$^{2+}$. As mentioned above, the measurements in dendritic patches of hippocampal pyramidal neurons made by Spruston et al. (1995), who applied voltage jumps after brief pulses of glutamate in a physiological concentration of Mg$^{2+}$, are consistent with our findings. Their records (in their Fig. 13) clearly show a slow phase of unblock. Its extent and time course is only apparent in the example shown of a long depolarization (50 ms) to +40 mV; at the time scale used for their plots, it is much less obvious for their brief (5 ms) pulses. It is also notable, although they do not comment on it, that their Fig. 13C shows that brief 5-ms pulses recover only about half of the current obtained for continuous depolarization, analogous to our Fig. 2C.

Our results on the kinetics of unblock go beyond those of Spruston et al. (1995) in several respects. We show the effect at a faster time scale and quantitate the contributions and time courses of fast and slow components at different voltages. We demonstrate that both components depend on the presence of Mg$^{2+}$ (Fig. 2D) and that the slow unblock occurs in the range of potentials over which the steady-state Mg$^{2+}$ block is steeply voltage dependent, i.e., about −60 to +20 mV. Finally, we point out the potential functional significance of this time dependence in limiting excitability and demonstrate this using an AP waveform clamp.

NMDARs in these cells are expected to be mostly composed of NR1, NR2A, and NR2B subunits, a combination that results in high magnesium-sensitive voltage dependence (Monyer et al. 1994). It is possible that the NR2 subunit type could affect the time course of Mg unblock, so that heterogeneity among channels might result in several components of unblock in the population average. This possibility will require testing, for example, by subunit-specific pharmacological block (Kirson et al. 1999) or by single-channel recording.

*Function of NMDARs during APs*

We have shown that the contribution of NMDARs to excitability during fast Na spikes, for example, back-propagating APs in cortical pyramidal cells, is small. NMDARs become much more involved during the slower upstroke of Ca$^{2+}$ APs.
and so could contribute strongly to Ca\(^{2+}\) excitability. The large inward current during repolarization reflects the unblock that has occurred during the preceding depolarization. However, the fact that, particularly at near-physiological temperatures, this current is much larger than the steady-state prediction suggests that it is essentially a tail current. It is particularly prominent in the potential range from −20 to 50 mV and appears before the channels reequilibrate to the change in voltage-dependent rates. The consequence of this large current pulse during slow repolarization is that NMDARs will help to depolarize the membrane toward a succeeding spike and also contribute additional Ca\(^{2+}\) influx about 2–3 ms after the initiation of the AP. This could be important in determining the time window of spike-timing–dependent plasticity (Markram et al. 1997).

**Final remarks**

We have shown that the time dependence of NMDARs is critically important for their function in excitable cells. The amplitude and timing of the current and Ca\(^{2+}\) influx that they contribute is strongly shaped by this time dependence, which leads to a pattern of activation quite unlike that predicted by steady-state measurements of voltage-dependent Mg\(^{2+}\) block. Future studies should provide a kinetic model that accounts for the gating of this complex receptor channel in conditions of rapidly changing membrane potential.

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