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 ($\tau < 200$ $\mu$s). 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.

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**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.

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**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 nM 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 BW50WI, Olympus).
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) superfused 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 voltage (-70 to 40 mV)

\[
F(V) = \frac{1}{1 + \exp(-(V - V_{50})/\delta F)/RT}
\]

This showed a voltage for half-maximal block \( (V_{50}) \) of -13 mV ± 2.45 and a fraction of the membrane potential sensed by the blocking site \( \delta \) 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 and 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

\[ \text{FIG. 1. Stationary N-methyl-D-aspartate receptor (NMDAR) current. A: } \text{NMDAR current evoked by superfusion of a nucleated patch with } 25 \text{ μM NMDA, plotted as a function of membrane potential during a 3-s ramp command potential. B: fraction of the maximal slope conductance at highly depolarized potentials is plotted as a function of membrane potential (omitting data near the reversal potential to exclude large fluctuations), fitted to a Boltzmann distribution.} \]

\[ (V_{50}) = -15 \text{ mV}, \delta = 0.97 \text{ (see Eq. 1)}, E_{rev} = -5 \text{ mV}. \text{ C: agonist was perfused by a computer-controlled pressure ejector, whose timing is indicated by the upper trace. Dotted lines show the timing adopted later for the voltage steps. Application of agonist (in this case 5 μM glutamate) elicited a current, which became stationary after a few hundred milliseconds and remained constant for about a second. Holding potential as indicated. Black traces: with agonist; gray traces: without agonist.} \]
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), 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 Mg$^{2+}$, since no slow relaxation was observed in nominally Mg$^{2+}$-free solution in nucleated patches (Fig. 2D) (see also Mayer and Westbrook 1987; Spruston et al. 1995). Note that, without Mg$^{2+}$, 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 Mg$^{2+}$-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 Mg$^{2+}$ unblock of the NMDAR.

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

\[ I(t) = I_\text{st} \left[ A_1 \left(1 - \exp(-t/\tau_1)\right) + A_2 \left(1 - \exp(-t/\tau_2)\right) \right] \tag{2} \]

where \(A_1 + A_2 = 1\) and \(I_\text{st}\) 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 \(A_1(V) = 0.49 - 0.0043V\) (\(r^2 = 0.93\), \(P < 0.001\)) and for Cs-based solution the linear fit was \(A_1(V) = 0.51 - 0.0028V\) (\(r^2 = 0.79\), \(P = 0.0012\)).
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 replotted 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.

P < 0.003). There appeared to be an increase in the slow time constant of unblock with depolarization, for which the following values were measured (in ms): 14.2 ± 1.8 at −40 mV, 17.7 ± 3.5 at −30 mV, 18.3 ± 1.5 at +20 mV, 19.4 ± 1.4 at +30 mV, and 22.9 ± 2.9 at +40 mV (n = 4–7 nucleated patches for each voltage). However, in the range of −20 to +10 mV it was not possible to measure the time constant accurately because of the low signal-to-noise ratio.

With repolarization to −70 mV, complete reblock occurred very rapidly (Fig. 4C). No digital filter was applied to these records. After a few hundred microseconds, the conductance was close to zero. In some nucleated patch recordings, clear fast tail currents were observed at the end of depolarizing pulses. Distortion of the signal observed immediately after repolarization was probably due to imperfect leak subtraction of capacitance transients, and exponential fits excluded this period of distortion. The time course of conductance change (E_rev = −5 mV) during block was a little slower (τ = 120–150 μs) than the step response of the combination of analog filter and recording system (indicated by dotted curve).

We used NMDA as an agonist to specifically activate NMDARs and avoid contribution from other glutamate receptors, particularly metabotropic receptors, for which highly specific and completely effective blockers are not available and which can affect other voltage-dependent currents. However, since the receptor has a slower dissociation rate constant for glutamate (Painet and Mayer 1990), the kinetics of unblock with glutamate activation could differ slightly from those obtained using NMDA. We carried out several experiments using 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.

**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

FIG. 5. NMDAR current activation during action potential (AP) waveforms. Nucleated patch recordings. A: stationary NMDAR current during a 3-s voltage ramp at room temperature and its fit. B: voltage was clamped to a train of APs waveform, in the presence and absence of 25 μM NMDA. Each leak subtracted NMDAR current is shown below the corresponding voltage command. C and D: detailed views of the segments in B. The expected current from an instantaneous block/unblock as fitted in A is superimposed in red. E–G: recordings at 33°C, using APs from Larkum et al. 2001. E: a burst of fast somatic Na APs (Larkum et al. 2001, Fig. 2D). F: dendritic Ca²⁺/Na AP corresponding to the somatic waveform in E. G: a dendritic Na spikelet (boosted excitatory postsynaptic potential), smaller and slower than the somatic spikes in E (Larkum et al. 2001, Fig. 2B). No digital filter was applied in any of these records.
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 Ca2+ 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 Ca2+ 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 Mg2+, 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 [Mg2+]o. 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.

Venieniste 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 Mg2+ and 200 μM glutamate. However, owing to the short application of Mg2+, 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 Mg2+. 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 Mg2+, are consistent with our findings. Their records (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 Mg2+ (Fig. 2D) and that the slow unblock occurs in the range of potentials over which the steady-state Mg2+ 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.

NMDA receptors 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 Ca2+ 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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