Permeant ion regulation of \textit{N}-methyl-\textit{D}-aspartate receptor channel block by \textit{Mg}^{2+}

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Block of the channel of \textit{N}-methyl-\textit{D}-aspartate (NMDA) receptors by external \textit{Mg}^{2+} (\textit{Mg}^{2+}) has broad implications for the many physiological and pathological processes that depend on NMDA receptor activation. An essential property of channel block by \textit{Mg}^{2+} is its powerful voltage dependence. A widely cited explanation for the strength of the voltage dependence of block is that the \textit{Mg}^{2+}-binding site is located deep in the channel of NMDA receptors; \textit{Mg}^{2+} then would sense most of the membrane potential field during block. However, recent electrophysiological and mutagenesis studies suggest that the blocking site cannot be deep enough to account for the voltage dependence of \textit{Mg}^{2+} block. Here we describe the basis for this discrepancy: the magnitude and voltage dependence of channel block by \textit{Mg}^{2+} are strongly regulated by external and internal permeant monovalent cations. Our data support a model in which access to the channel by \textit{Mg}^{2+} is prevented when permeant ion-binding sites at the external entrance to the channel are occupied. \textit{Mg}^{2+} can block the channel only when the permeant ion-binding sites are unoccupied and then can either unblock back to the external solution or permeate the channel. Unblock to the external solution is prevented if external permeant ions bind while \textit{Mg}^{2+} blocks the channel, although permeation is still permitted. The model provides an explanation for the strength of the voltage dependence of \textit{Mg}^{2+} block and quantifies the interdependence of permanent and blocking ion binding to NMDA receptors.

\textit{N}-methyl-\textit{D}-aspartate (NMDA) receptors are ligand-gated ion channels that exhibit remarkably strong voltage dependence. In contrast to the voltage-gated family of ion channels, the voltage dependence of NMDA receptor-mediated conductance changes depends on a channel-blocking ion, external \textit{Mg}^{2+} (\textit{Mg}^{2+}). Current flow through the channel of NMDA receptors is blocked when \textit{Mg}^{2+} enters and binds in the channel, and block becomes much more effective as membrane potential (\textit{V}_m) is hyperpolarized. A question fundamental to NMDA receptor function is how the block by \textit{Mg}^{2+} achieves its powerful voltage dependence. The hypothesis first proposed to explain the voltage dependence of \textit{Mg}^{2+} block was that \textit{Mg}^{2+} binds to a site located deep in the channel (1, 2). \textit{Mg}^{2+} then would need to traverse part of the channel and, therefore, enter the membrane voltage field, to block; as a result, \textit{Mg}^{2+}-blocking rate would increase with hyperpolarization (3). \textit{Mg}^{2+} unblocking rate similarly would decrease with hyperpolarization if \textit{Mg}^{2+} cannot permeate the channel. Both of these effects of \textit{V}_m on blocking kinetics have been observed (4).

This direct influence of \textit{V}_m on \textit{Mg}^{2+}-blocking kinetics because of the electrical location of the \textit{Mg}^{2+}-binding site undoubtedly contributes to the voltage dependence of block. However, starting with the observation that the voltage dependence of the blocking rate appears anomalously high (4), data from numerous studies have suggested that other mechanisms amplify blocking voltage dependence. For the voltage dependence of channel occupation by \textit{Mg}^{2+} to be explained exclusively by blocking site location, the required site depth has been estimated to be about 80% through the membrane voltage field [range of estimates, 53–107% (4–12)]. However, internal \textit{Mg}^{2+} also has been found to block the channel of NMDA receptors at a site estimated to be 55–71% through the membrane voltage field from the outside of the channel (13–16). It appears implausible that \textit{Mg}^{2+} on the way to its blocking site could pass by the site at which internal \textit{Mg}^{2+} blocks. In addition, there is evidence that \textit{Mg}^{2+} may be able to permeate the channel of NMDA receptors under some conditions (17, 18); if this happens under physiological conditions, it would require that the blocking site be even deeper in the voltage field to explain the voltage dependence of block (4). Finally, amino acids crucial to block by \textit{Mg}^{2+} are located near the tip of the M2 region of NMDA receptor subunits (12, 19–22). The M2 region is a pore loop that enters and exits the membrane intracellularly and is believed to form a large portion of the channel of NMDA receptors. A physical location of the \textit{Mg}^{2+}-blocking site near the external tip of the M2 region appears inconsistent with an electrical location near the internal extreme of the channel.

One mechanism by which the voltage dependence of block by \textit{Mg}^{2+} might be amplified is through a voltage-dependent influence on block of ions other than \textit{Mg}^{2+} (4). Changes in the concentrations of intracellular and extracellular ions other than \textit{Mg}^{2+} have been shown to influence block by \textit{Mg}^{2+} (8, 17, 23) and by some organic channel blockers of NMDA receptors (24–26). Mutation of amino acids in several well-separated regions of NR2 subunits have been shown to influence \textit{Mg}^{2+} block (9), leaving open the possibility that block may be affected by multiple binding sites on NMDA receptors. There is evidence for \textit{Ca}^{2+}-binding sites deep in the channel (19) and on the external side of the channel (10, 27) and for permeant monovalent cation-binding sites on both sides of the channel (26). We recently demonstrated that occupation of the permeant monovalent cation-binding sites has a profound effect on the blocking kinetics of two adamantane derivatives that block the channel of NMDA receptors (26). Here, we examine how occupation of these permeant monovalent cation-binding sites influences block by \textit{Mg}^{2+}.

**Experimental Procedures**

**Preparation and Solutions.** Primary cultures of rat cortical neurons were prepared from 16-day embryos as described (28). Neurons were used for experiments after 15–30 days in culture. Recordings of single-channel currents were performed by using outside-out patches (29) at room temperature (21–23°C). Pipettes were filled with one of three solutions. The control (130

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Abbreviations: \textit{k}_{\text{app}} blocking rate constant; \textit{k}_{\text{unapp}} unblocking rate constant; \textit{C}_\text{m} inner \textit{Mg}^{2+}; \textit{C}_\text{o} internal \textit{Mg}^{2+}; \textit{C}_{\text{m}} external \textit{Mg}^{2+}; \textit{t}_\text{m} mean duration of block; \textit{t}_\text{o} external \textit{Na}^{+}; \textit{NMDA}, \textit{N}-methyl-\textit{D}-aspartate; \textit{V}_\text{m} channel mean open time; \textit{V}_\text{m} membrane potential.

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mM Cs\(^+\) pipette solution contained 120 mM CsF, 10 mM CsCl, 10 mM EGTA, and 10 mM Hepes. pH was adjusted to 7.2 with CsOH. The 25 mM Cs\(^+\) solution contained 25 mM CsF, 105 mM NMDG, 10 mM EGTA, and 10 mM Hepes, and 1 mM tetraethyl-ammonium (TEA). The 8 mM Cs\(^+\) solution contained 8 mM CsF, 122 mM NMDG, 10 mM EGTA, 10 mM Hepes, and 1 mM TEA. pH of the 25 and 8 mM Cs\(^+\) solutions was adjusted to 7.2 with HCl. Osmolality of each solution was approximately 260 mosmol. Three different external solutions were used. The control (140 mM Na\(^+\)) external solution contained 140 mM NaCl, 2.8 mM KCl, 1 mM CaCl\(_2\), and 10 mM Hepes. The 105 mM Na\(^+\) solution contained 105 mM NaCl, 2.8 mM KCl, 0.75 mM CaCl\(_2\), 10 mM Hepes, and 70 mM sucrose. The 70 mM Na\(^+\) solution contained 70 mM NaCl, 2.8 mM KCl, 0.5 mM CaCl\(_2\), 10 mM Hepes, and 140 mM sucrose. Sucrose was used as the external NaCl substitute because all ionic substitutes considered would either block or permeate the channel of NMDA receptors. All external solutions contained 0.2 mM tetrodotoxin. pH of all external solutions was adjusted to 7.2 with NaOH. Osmolality was approximately 270 mosmol. Although external Ca\(^{2+}\) binds sites on the NMDA receptor have been described (10, 27), external Ca\(^{2+}\) does not bind significantly to the external permeant monovalent ion binding sites at the external Ca\(^{2+}\) to monovalent cation concentration ratios used here (26). This result was confirmed in one experiment in which Ca\(^{2+}\) was omitted from the external solution with the 105 mM Na\(^+\) solution; the apparent rates of Mg\(^{2+}\) block and unblock with 0 added Ca\(^{2+}\) was within the range of values measured in the presence of 0.75 mM Ca\(^{2+}\). Voltages in all experiments were corrected for junction potentials (8 mV with the 140 mM Na\(^+\) and 130 mM Cs\(^+\) solution combination, and −2 mV with all other solution combinations).

Data Recording and Analysis. Data recording and analysis were performed as described (26, 28). NMDA receptor-mediated, single-channel currents were activated at 1 mV values from −150 to −20 mV by application of 5 or 10 μM NMDA plus 10 μM glycine. Single-channel currents were low-pass-filtered and recorded on videotape. Recorded currents were played back off-line, low-pass-filtered at 3 or 4 kHz, and sampled at 40 kHz with a TL-1 interface and pclamp 6.02 (Axon Instruments). For most data the effective filter frequency resulting from cascaded filters (f\(_c\)) was 3.7 kHz; events shorter than 96 μs (two times the system dead time) were deleted from histograms and omitted from histogram fits.

Open-time distributions were well-fit by a sum of two exponential components under control conditions and by one or two components in the presence of Mg\(^{2+}\). When two components were present, the channel mean open time (τ\(_o\)) was estimated from the much larger slow component (26). The apparent blocking rate constant (k\(_{app}\)) was estimated as the slope of a regression line fit to a linear plot of 1/τ\(_o\) vs. Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_o\)) (30). A separate value of k\(_{app}\) was estimated at each V\(_m\) in each patch by using values of τ\(_o\) in control conditions and at two or more Mg\(^{2+}\) concentrations. Measurements of τ\(_o\) were not likely to be in error due to missed brief closures because correction of τ\(_o\) for missed closures under similar conditions did not significantly change mean open-time values (28).

Closed-time histograms in the absence and presence of Mg\(^{2+}\) were well-fit by a sum of three or four exponential components. A large closed-time component appeared in the presence of Mg\(^{2+}\) that was not present in the absence of Mg\(^{2+}\). The mean duration of this component was used to estimate the mean duration of block (τ\(_b\)). Although this component generally obscured one of the control closed-time components, τ\(_b\) could be accurately estimated as long as the closed-time component induced by Mg\(^{2+}\) was much larger than any overlapping control closed-time components (26). This was the case in all experiments except when 70 mM Na\(^+\) was used; in this external solution, τ\(_o\) could be measured accurately in only one patch (used for points in Fig. 3d). A separate value of the apparent unblocking rate constant (k\(_{-app}\)) was estimated at each V\(_m\) in each patch by averaging the values of 1/τ\(_o\) measured in at least two Mg\(^{2+}\) concentrations. To prevent error in measurement of τ\(_o\) because of missed brief openings we used [Mg\(^{2+}\)]\(_o\) in which τ\(_o\) ≥ 0.4 ms (26, 28).

Curve fitting and simulations were performed by using SIGMAPLOT (SPSS, Chicago). Optimal values of k\(_{app}\), b, K\(_{Na}\), K\(_{Cs}\), and a were determined first by finding values that minimized the sum of squared errors (SSE) when fitting Eq. 2 to values of k\(_{-app}\) measured in 140 mM Na\(^+\)/130 mM Cs\(^+\). Small parameter adjustments then were made to provide optimal fits to all five data sets shown in Fig. 2 as judged by eye. Optimal values of k\(_{-app}\), c, k\(_{-app}\), and d were determined by first finding values that minimized the SSE when fitting Eq. 3 to values of k\(_{-app}\) measured in 140 mM Na\(^+\)/K\(_{Na}\) fixed at 34.4 mM, the value determined by fits to all of the data in Fig. 2. Small parameter adjustments then were made to provide optimal fits to three data sets shown in Fig. 3d as judged by eye.

Each plotted point in the figures is the mean of measurements from n = 1–8 patches; error bars representing SD are plotted when larger than the symbol size and n > 2.

Results

Effects of Permeant Ions on Rate of Mg\(^{2+}\) Block. We investigated the influence of permeant monovalent cations on Mg\(^{2+}\) block of single-channel currents through native NMDA receptors of cultured cortical neurons, which express predominantly the NR1, NR2A, and NR2B receptor subunits (26, 31). Rates of channel block and unblock by Mg\(^{2+}\) were measured from the single-channel current “flicker” induced by Mg\(^{2+}\) (Fig. 1a). τ\(_o\) (Fig. 1b) depended linearly on [Mg\(^{2+}\)]\(_o\) (Fig. 1c and d) under all conditions, as expected for an open-channel blocker, permitting measurement of the rate constants of block (4, 6, 28, 30). Under control ionic concentrations at −50 mV, the blocking rate constant was consistent with previous measurements (4, 6, 30). However, when the internal Cs\(^+\) concentration ([Cs\(^+\)]\(_i\)) was decreased from 130 to 8 mM, τ\(_o\) decreased more than 4-fold (Fig. 1a and b), reflecting a large increase in the Mg\(^{2+}\)-blocking rate (Fig. 1c).

When [Cs\(^+\)] was held at 25 mM while the external Na\(^+\) concentration ([Na\(^+\)]\(_o\)) was decreased, a similar increase in the blocking rate of Mg\(^{2+}\) was observed (Fig. 1d). These observations indicate that Mg\(^{2+}\) does not act via the classical model of open channel block (30). Because the measurable rate constant of channel block depends on the concentrations of multiple ions rather than just the blocking ion, we refer to k\(_{app}\) as an “apparent” blocking rate constant.

To gain insight into the mechanisms by which permeant ions influence block by Mg\(^{2+}\), we investigated the voltage dependence of the effect of permeant ion concentrations on k\(_{app}\). In control ionic concentrations and at V\(_m\) values from −40 to −90 mV, k\(_{app}\) exhibits steep, single-exponential voltage dependence (−fold change in 18 mV), in agreement with earlier studies (4, 6). However, the expected exponential dependence of k\(_{app}\) on voltage (3, 4, 6, 30) was not observed over the entire range of V\(_m\) studied (Fig. 2); at V\(_m\) values negative of −90 mV, the voltage dependence became much weaker (−fold change in 55 mV). The increase in k\(_{app}\) induced by decreasing [Cs\(^+\)]\(_i\) (Fig. 1c) was observed only at depolarized V\(_m\) values (Fig. 2a). Because Cs\(^+\) efflux through the channel increases progressively with depolarization, this observation suggests that Cs\(^+\) must partially or completely permeate the channel before it can slow the rate of Mg\(^{2+}\) block. In contrast, the increase in k\(_{app}\) induced by decreasing [Na\(^+\)]\(_o\) (Fig. 1d) was largely voltage independent (Fig. 2b).
2b), suggesting that Na$^+$ slows the rate of Mg block by acting at an external site.

**Effects of Permeant Ions on Rate of Mg$^{2+}$ Unblock.** Permeant ions also affect the rate at which Mg$^{2+}$ unblocks from the channel of NMDA receptors. Fig. 3a shows single-channel currents obtained in 140 mM Na$^{+}$ (Fig. 3a, upper traces) and 105 mM Na$^{+}$ (Fig. 3a, lower traces). At both [Na$^{+}$], values, block by Mg$^{2+}$ induced an additional component in the closed-time distributions (Fig. 3b) that was not present in the absence of Mg$^{2+}$. The time constant of the additional component corresponds to $\tau_{M}$; the unblocking rate constant was calculated as $1/\tau_{M}$ (4, 6, 28, 30). The block duration induced by 30 $\mu$M Mg$^{2+}$ was clearly briefer in 105 mM Na$^{+}$ than in 140 mM Na$^{+}$ (Fig. 3a and b). Because the measurable rate constant of channel unblock depends on [Na$^{+}$], we refer to $k_{\text{app}}$ as an “apparent” unblocking rate constant.

We next examined the voltage dependence of the effect of permeant ions on $k_{\text{app}}$. Although changing [Cs$^{+}$], had no effect on $k_{\text{app}}$ at $V_m$ values from $-40$ to $150$ mV (Fig. 3c), $k_{\text{app}}$ was strongly affected by [Na$^{+}$], over nearly this entire voltage range (Fig. 3d). In 140 mM Na$^{+}$ at $V_m$ values from $-40$ to $-90$ mV $k_{\text{app}}$ exhibited a nearly exponential voltage dependence similar to that reported previously (4, 6). However, further hyperpolarization from $-90$ to $-140$ mV did not further decrease $k_{\text{app}}$. This could reflect permeation by Mg$^{2+}$ at very hyperpolarized $V_m$ values, as was proposed previously based on whole-cell experiments (17). Decreasing [Na$^{+}$], to 105 mM induced an increase in $k_{\text{app}}$ at $V_m$ values from $-40$ to $-110$ mV that was not obviously voltage-dependent. This suggests that Na$^{+}$ prevents Mg$^{2+}$ unblock to the external solution by acting at an external site. From $-120$ to $-140$ mV, where the decreased voltage dependence of $k_{\text{app}}$ suggests that Mg$^{2+}$ frequently permeates the channel, the dependence of $k_{\text{app}}$ on [Na$^{+}$], is weak (Fig. 3d). Na$^{+}$ therefore is unlikely to affect the permeation rate of Mg$^{2+}$ that is blocking the channel.

**Quantitative Model of the Effects of Permeant Ions on Mg$^{2+}$ Block.** Recently, we reported (26) that occupation of external permeant monovalent cation-binding sites on the channel of NMDA receptors prevents channel block and unblock by two adamantane derivatives, IEM-1754 and IEM-1857. To determine whether a similar mechanism can explain the unexpected dependence of Mg$^{2+}$ block on $V_m$, [Na$^{+}$], and [Cs$^{+}$], we developed a quantitative model of block by Mg$^{2+}$. In this model Mg$^{2+}$ can enter the channel only when external cation-
binding sites are unoccupied, and so $k_{\text{app}}$ depends on $[\text{Na}^+]_o$ and $[\text{Cs}^+]$, (Fig. 2). The strong dependence of $k_{\text{app}}$ on $[\text{Na}^+]_o$ (Fig. 2b) requires a model with two sites to which Na$_o$ can bind. The voltage independence of the effect of $[\text{Na}^+]_o$ on $k_{\text{app}}$ suggests that these sites are located in a region of the outer vestibule of the channel external to the voltage field. The alternative possibility that Na$_o$ affects Mg$_o^{2+}$-blocking rate by screening surface charges on NMDA receptors seems improbable (26), because the surface charges are screened effectively by quite low concentrations of monovalent cations (23). Because the effect of $[\text{Cs}^+]$ on $k_{\text{app}}$ exhibits relatively weak concentration dependence but strong voltage dependence (Fig. 2a), the model permits Cs$_o$ to bind to only one of the sites after permeating the channel. The decrease in $k_{\text{app}}$ that results from increasing $[\text{Na}^+]_o$ at all but the most hyperpolarized voltages (Fig. 3d) is modeled through a “lock-in” effect (32): if Na$_o$ binds to one or both permeant ion-binding sites while Mg$_o^{2+}$ blocks the channel, Mg$_o^{2+}$ cannot unblock to the external solution. Cs$_o^{+}$ does not affect $k_{\text{app}}$ (Fig. 3c) because Cs$_o^{+}$ cannot reach its external binding site while Mg$_o^{2+}$ blocks the channel. Finally, the weakened dependence of $k_{\text{app}}$ on $V_m$ and $[\text{Na}^+]_o$ at very hyperpolarized voltages (Fig. 3d) is accounted for in the model by voltage-dependent Mg$_o^{2+}$ permeation that is unaffected by Na$_o^{+}$ binding.

The resulting model is diagrammed in Fig. 4 and below in Scheme 1:

$$
\begin{align*}
\text{Na}_o^{2+} + 2 \text{Mg}_{o}^{2+} & \rightarrow \text{Na}_o^{+} \text{Mg}_o^{2+} \\
\downarrow k_{-a} & \downarrow k_{-a} & k_{+} \mid k_{-a} + k_{-o}
\end{align*}
$$

$$
\begin{align*}
\text{Na}_o^{2+} \text{O} & \rightarrow \text{Na}_o^{+} \text{O} \\
\downarrow 2K_{\text{Na}} & \downarrow 0.5K_{\text{Na}}
\end{align*}
$$

$$
\begin{align*}
\text{Na}_o^{+} \text{Cs}_o^{+} \text{O} & \rightarrow \text{Cs}_o^{+} \text{O} \\
\downarrow K_{\text{Na}} & \downarrow K_{\text{Na}}
\end{align*}
$$

O represents the fully liganded NMDA receptor with its channel open and permeant ion-binding sites unoccupied. When Na$_o$ or Cs$_o^{+}$ is bound to O, Mg$_o^{2+}$ cannot enter the channel (Fig. 4a). From state O, Mg$_o^{2+}$ can block the channel (Fig. 4b) to create state Omg$_o^{2+}$. Mg$_o^{2+}$ then can either unblock to the external solution or can permeate the channel (Fig. 4c). If Na$_o$ ion(s) occupy the external cation-binding sites while Mg$_o^{2+}$ is blocking the channel, Mg$_o^{2+}$ cannot unblock to the external solution, but still can permeate the channel (Fig. 4d).

To aid in the derivation of equations to describe Scheme 1, we made several assumptions. Because the data suggest that the cation-binding sites are located outside of the voltage field, the equilibrium dissociation constant for Na$_o^{+}$ ($K_{\text{Na}}$) was made voltage-independent. $K_{\text{Na}}$ was assumed to be the same whether or not Mg$_o^{2+}$ is bound, as was found with the IEM blockers (26). Cs$_o^{+}$ must cross the entire membrane voltage field to bind, and so $K_{\text{Cs}}$ must be strongly voltage-dependent. To limit adjustable parameters, we used the simplest plausible equation (Table 1)
model the voltage-dependence of Cs⁺ binding (26). Note that $K_{Cs}$ is not an equilibrium constant, because it involves Cs⁺ permeation of the channel. The true rates (rates in the absence of permeant ions) of Mg⁰⁺⁺ channel block ($k_+$), unblock to the internal solution ($k_-o$), and unblock to the external solution ($k_-e$) also were assumed to vary exponentially with $V_m$ (3) (Table 1). Based on these assumptions, we derived the following equations for $k_+{app}$ and $k_-{app}$ from Scheme 1:

$$k_+{app} = k_+/[ (1 + [Na⁺]/K_{Na})(1+[Na⁺]/K_{Na} + [Cs⁺]/K_{Cs})]$$

$$K_-{app} = k_-o/(1 + [Na⁺]/K_{Na})^2 + k_-j$$

(2)

(3)

The model provides excellent fits to the data (Figs. 2 and 3d). Parameter values derived from fitting Scheme 1 to data are shown in Table 1. The value of $K_{Na}$ is in good agreement with its previous estimate (26); the small values of $K_{Cs}(0)$ and $a$ found here may result from an oversimplified approximation of the voltage dependence of Cs⁺ permeation.

**Discussion**

These results reveal that occupation of the permeant ion-binding sites has enormous effects on the rates of Mg⁰⁺⁺ block and unblock. At −60 mV with control ion concentrations, occupation of cation-binding site(s) prevents Mg⁰⁺⁺ from blocking 99.4% of the time. In the absence of permeant ion binding, the estimated Mg⁰⁺⁺-blocking rate (1.10 · 10⁹ M⁻¹s⁻¹ at 0 mV) is extraordinarily high: it is close to the predicted diffusion limit (33). This suggests that the highest energy barrier that Mg⁰⁺⁺ must cross to block the channel of the NMDA receptor is very low. Unblock of Mg⁰⁺⁺ to the external solution is prevented by Na⁺ lock-in 96.1% of the time with control ion concentrations; as a result, the fraction of blocking Mg ions that permeate is 22-fold greater than it would be if lock-in did not occur. The permeant ion-binding sites might play a role in the unusual permeation properties of the NMDA receptor, such as its high Ca²⁺ permeability (4, 17, 34, 35) and the ability of Mg⁰⁺⁺ to permeate rapidly under some experimental conditions (17, 18).

It is likely that under physiological conditions occupation of the permeant ion-binding sites has a powerful effect on equilibrium block by Mg⁰⁺⁺ as well as on the kinetics of block. According to the model developed here, the true Mg⁰⁺⁺ IC₅₀ (the IC₅₀ in the absence of permeant ions, calculated as $(k_-o + k_-j)/k_+$) at −60 mV is 10.9 μM. With 140 mM Na⁺ and 130 mM Cs⁺, the predicted IC₅₀ is 95.4 μM, nearly 10-fold higher. This increase in IC₅₀ is predicted to be predominantly caused by the lowering of $k_+$. By Cs⁺; the effects of Na⁺ on IC₅₀ are predicted to be small because increasing [Na⁺]o lowers both $k_+$ and $k_-o$. Consistent with these predictions, in whole-cell experiments and with [Mg²⁺]o up to 3 mM, decreasing [Cs⁺]i causes a large and voltage-dependent decrease in the Mg⁰⁺⁺ IC₅₀ (36). Decreasing [Na⁺]o has much smaller and less voltage-dependent effects (37).

An effect of permeant ion-binding sites on equilibrium block by Mg⁰⁺⁺ is supported further by two additional observations: decreasing internal [K⁺] strengthens inhibition of macroscopic NMDA responses by Mg⁰⁺⁺ (8), and the voltage dependence of the Mg⁰⁺⁺ IC₅₀ becomes greater as the voltages over which measurements are made become more depolarized (9, 12, 38). The latter observation is predicted by the curve we observed in semilog plots of $k_+{app}$ (Fig. 2) and $k_-{app}$ (Fig. 3) as a function of voltage. The sensitivity of the voltage dependence of block to the voltage range over which measurements are made also may contribute to the 2-fold variability in estimates of the electrical location of the Mg⁰⁺⁺-blocking site (4–12). Additional possible causes of this variability include differences in the permeant monovalent cation concentrations used and difference in the subunit composition of the NMDA receptors present in the cells studied. Subunit composition is important because the voltage dependence of block is NR2 subunit-specific (9) and because inaccuracies in macroscopic measurements could be introduced by potentiation of some NMDA receptor subtypes by Mg⁰⁺⁺ (39).

Our data suggest that the permeant ion-binding sites are in or near the single-file region of the channel and, therefore, are likely to play a role in ion permeation. If they were the only, or the highest-affinity, monovalent permeant ion-binding sites in the channel, then half-saturation of NMDA-receptor single-channel current should occur when half these sites are occupied. Previous estimates of the permeant monovalent cation concentrations at which half-maximal saturation of NMDA-receptor single-channel currents occurs range from 0.5 to 129 mM (8, 10, 23, 40). The variation in these measurements, which were made under differing conditions and with different permeant ions, prevents their use in assessing the potential role of the permeant ion-binding sites in channel permeation. It nevertheless seems likely that other ion-binding sites, such as a site near the Q/R/N sites (19) or an intracellular permeant ion-binding site (26), are involved in monovalent cation permeation.

In the model developed here, occupation of either permeant ion-binding site prevents access of Mg⁰⁺⁺ to the channel. This, coupled with the observation that Na⁺ binding to either site is voltage-independent, appears to imply that the sites are in close proximity. However, the $K_{Na}$ of either permeant ion-binding site is the same whether or not the other site is occupied. This appears to imply, in contrast, that the sites are relatively distant. Previous work suggests that the sites indeed could be in close proximity: when one of two neighboring cation-binding sites in the pore of cyclic nucleotide-gated channels binds a proton, the proton affinity of the other site is unaffected (41). Occupation of a permeant ion-binding site on NMDA receptors may affect Mg⁰⁺⁺ more strongly than Na⁺ or Cs⁺ because of the larger charge on Mg²⁺, its greater hydrated radius, its slower exchange of waters of hydration, or a combination of these factors. It also is possible that occupation of one permeant ion-binding site has a moderate effect on monovalent cation binding to the second site. To test this possibility, we fit to the

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**Table 1. Equations and parameter values for rate and equilibrium constants**

<table>
<thead>
<tr>
<th>Parameter and equation</th>
<th>Value at $V_m = 0$</th>
<th>$V_m$ dependence</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{Na}$</td>
<td>$K_{Na} = 34.4$ M</td>
<td>None</td>
</tr>
<tr>
<td>$K_{Cs} = K_{Cs}(0)exp(V_m/a)$</td>
<td>$K_{Cs}(0) = 0.270$ M</td>
<td>$a = -21.0$ mV</td>
</tr>
<tr>
<td>$k_+ = k_+o(0)exp(V_m/b)$</td>
<td>$k_+o(0) = 1.10 \cdot 10^9$ M⁻¹s⁻¹</td>
<td>$b = -55.0$ mV</td>
</tr>
<tr>
<td>$k_-o = k_-o(0)exp(V_m/c)$</td>
<td>$k_-o(0) = 1.10 \cdot 10^9$ M⁻¹s⁻¹</td>
<td>$c = 52.7$ mV</td>
</tr>
<tr>
<td>$k_-j = k_-j(0)exp(V_m/d)$</td>
<td>$k_-j(0) = 61.8$ s⁻¹</td>
<td>$d = -50.0$ mV</td>
</tr>
<tr>
<td>$K_{Mg} = K_{Mg}(0)exp(V_m/e)$</td>
<td>$K_{Mg}(0) = 101$ μM</td>
<td>$e = 26.9$ mV</td>
</tr>
</tbody>
</table>

The equations for $k_+$, $k_-o$, $k_-j$, and $K_{Mg}$ can be used to calculate the rate or equilibrium constant value in the absence of permeant monovalent cations. $K_{Mg}$, which equals $k_-o/k_+$, takes into account unbinding of Mg²⁺ to the external solution but not permeation. Its voltage dependence therefore can be used to calculate the $\delta$ of the Mg⁰⁺⁺ blocking site, but its value cannot be used to predict the Mg⁰⁺⁺ IC₅₀.
Fig. 2 data a modified model in which the binding affinity of the second Na\textsuperscript{+} could be equal to or lower than the binding affinity of the first Na\textsuperscript{+}. The best fits still were achieved when each Na\textsuperscript{+} bound with the same affinity.

The model of Mg\textsuperscript{2+} block developed here permitted us to quantify the voltage dependence of the Mg\textsuperscript{2+} blocking and unblocking rates independent from the influence of permeant ions. As modeled here, these “true” blocking and unblocking rates satisfy the assumptions of the Woodhull model (3): most importantly, that the voltage dependence of the rates depends only on the electrical locations of the barrier to Mg\textsuperscript{2+} entry, the Mg\textsuperscript{2+}-binding site, and the barrier to Mg\textsuperscript{2+} permeation. If the model is correct, then we can now determine accurately the electrical locations of the channel structures that govern block by Mg\textsuperscript{2+}. The voltage dependence of \( k_+ \) places the peak of the barrier to Mg\textsuperscript{2+} entry at a fractional electrical depth of 0.23 through the membrane field; the additional voltage dependence of \( k_- \) places the Mg\textsuperscript{2+}-binding site (6) at 0.47; the additional voltage dependence of \( k_{-\text{app}} \) places the peak of the barrier to permeation at 0.73. These locations are much shallower than estimated from previous data or from our data by using traditional approaches: based on the approximately exponential voltage dependence of \( k_{+\text{app}} \) and \( k_{-\text{app}} \) from −40 to −90 mV, the peak of the barrier to Mg\textsuperscript{2+} entry is at 75%, and the Mg\textsuperscript{2+}-binding site at 89%, through the membrane voltage field. Traditional approaches provide exaggerated estimates of electrical depth because the voltage-dependent binding of Cs\textsuperscript{+} inflates the voltage dependence of \( k_{+\text{app}} \) at \( V_m \) values positive of −100 mV (note in Fig. 2 the low voltage dependence of \( k_{+\text{app}} \) at very negative \( V_m \) values, where Cs\textsuperscript{+} has little effect on \( k_{+\text{app}} \).

Our conclusion that Mg\textsuperscript{2+} traverses only 47% of the membrane voltage field to reach its blocking site appears consistent with evidence that two asparagine residues (12, 19, 20) near the tip (22) of the M2 region of NR2 subunits help form the Mg\textsuperscript{2+}-blocking site. This electrical location of the Mg\textsuperscript{2+}-blocking site also leaves room for a nearby but distinct internal Mg\textsuperscript{2+}-binding site 55–71% through the membrane voltage field (13–16). The estimated depth of the internal Mg\textsuperscript{2+}-binding site is somewhat discordant with our location of the permeation barrier at 75% through the field; one possible explanation is that the electrical depth of block by internal Mg\textsuperscript{2+} has been misestimated because of occupation of an internal binding site for permeant ions (26). Finally, our results are consistent with the observation (9) that structural domains outside the M2 region have a strong influence on channel block by Mg\textsuperscript{2+}, because the M2 region is unlikely to contribute to the external permeant ion-binding sites (22, 42).

In summary, the data and model presented here provide an explanation for the high voltage dependence of block by Mg\textsuperscript{2+}, give a corrected location for the Mg\textsuperscript{2+}-blocking site, demonstrate a lock-in effect of Na\textsuperscript{+} on Mg\textsuperscript{2+} during block, and provide a quantitative estimate of the rate of Mg\textsuperscript{2+} permeation.

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