Voltage-dependent block by intracellular Mg^{2+} of *N*-methyl-D-aspartate–activated channels

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ABSTRACT The *N*-methyl-D-aspartate (NMDA)–activated channel, which is known to be blocked by extracellular Mg ions, is shown also to be blocked by intracellular Mg ions. The block by intracellular Mg can be explained by assuming that Mg ions from the intracellular side enter the membrane electrical field before binding to the blocking site. The dissociation constant of the binding site for intracellular Mg is 8 mM at 0 mV, which is close to the value previously calculated for the extracellular Mg blocking site. The unbinding rates of intracellular and extracellular Mg are different, and their effects are additive, suggesting that the corresponding binding sites are distinct. Both blocks occur at physiological concentrations of Mg, making the NMDA-activated channel a bidirectional rectifier.

INTRODUCTION

The neuronal receptor–channel complex specifically activated by *N*-methyl-D-aspartate (NMDA) is blocked in a voltage-dependent manner by extracellular Mg ions (Nowak et al., 1984; Mayer et al., 1984). At voltages near resting potential, current flow is nearly fully prevented by physiological concentrations of extracellular Mg; depolarization relieves the block. Whereas the functional importance of this blockade is well established (see Johnson and Ascher, 1988), its mechanism is only partly understood. The voltage dependence of the block has been interpreted by assuming that Mg binds to a site situated in the NMDA channel, and the magnitude of the voltage dependence has been used in an attempt to localize the Mg site by evaluating the fraction (δ) of the transmembrane field sensed by Mg (Mayer and Westbrook, 1985; Ascher and Nowak, 1988; Mayer et al., 1989).

In their initial description of the extracellular Mg block, Nowak et al. (1984) reported that the single channel current recorded in outside-out patches was reduced at positive potentials when Mg was present in the pipette solution at a concentration of 2 mM. This suggested a voltage-dependent blockade of the NMDA response by *intracellular* Mg ions. In the present study we have developed these initial observations to evaluate more precisely the mechanism of the block by intracellular Mg, the localization of the Mg binding site involved in this block, and the relation of this binding site to the site involved in the block by extracellular Mg. Some of our initial observations were reported at the MBL Centennial Symposium (Ascher and Johnson, 1988).

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METHODS

Experiments were performed on primary cultures of cortical and diencephalic neurons from 15–16-d-old mouse embryos (see Ransom et al., 1977). Cells were used after 1–10 wk in culture. Outside-out patches were formed using conventional patch clamp techniques (Hamill et al., 1981) and single channel currents recorded using a model EPC-7 patch clamp amplifier (List-Electronic, 6100 Darmstadt, Eberstadt, FRG). Data were stored on tape using a Racal tape recorder operated at 7.5 ips. Current records were played back through an eight-pole Bessel filter with a cutoff frequency of 2 kHz, digitized at 5 kHz, and analyzed with a Minc PDP 11/23 computer. Single channel current was determined by measuring current in sections of the records with clear single channel transitions. Gaussian curves were fit by eye to current amplitude histograms. Fitting of Eq. 1 (see Results) was performed with FORTRAN programs based on the Simplex algorithm (Caceci and Carcheris, 1984). Best fit was defined as the combination of free parameter values that maximized the correlation coefficient between the equation and the data points.

NMDA channel openings were induced by fast perfusion of 10 μM NMDA + 1 μM glycine (Johnson and Ascher, 1987). Extracellular solutions contained (in millimolar): 140 NaCl, 2.8 KCl, 1 $CaCl_2$, 10 Hepes-Na (pH 7.2). $MgCl_2$ (0.2 mM) was added in some experiments. The pipette solution contained (in millimolar): 140 CsCl and 10 Hepes-Cs (pH 7.3). $MgCl_2$ was usually added to this solution without any Ca buffer. In a few experiments, EGTA (10 mM) was added to complex possible traces of Ca, and the total concentration of Mg had then to be raised to achieve the same concentration of free Mg as in the control experiments. This adjustment was done using the complexation constants of Steinhardt et al. (1977). We calculated that in 10 mM EGTA, a total Mg concentration of 2 mM was required to achieve a free Mg concentration of 1 mM, and a total Mg concentration of 14.8 mM to achieve a free concentration of 10 mM. At $MgCl_2$ concentrations over 1

mM, the intracellular solution was diluted before addition of $MgCl_2$ to maintain a constant osmolarity.

The experiments were conducted at room temperature (20–26°C).

RESULTS

When single channel currents recorded in the presence of intracellular Mg were compared with those obtained in the absence of intracellular Mg, the most obvious effect was a reduction in current size observed at positive potentials. This is illustrated in Fig. 1, which shows records obtained with $[Mg]_i = 0$ and $[Mg]_i = 1$ mM. The reduction of the single channel current in 1 mM Mg is quite marked at positive potentials, and barely detectable at negative potentials. The reduction of the single channel current produced by intracellular Mg occurs without detectable flickering (contrary to what is observed with extracellular Mg; see Fig. 3).

The simplest explanation of the intracellular Mg block is a rapid interaction of Mg with the open state of the channel

$$R^* + Mg \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} RMg,$$

where R* is the open state and RMg the blocked state. The fact that the channel conductance appears to decrease can be explained if the association and dissociation rates of Mg (k_{on}, k_{off}) are too rapid for flicker to be detected with the limited bandwidth of our recording apparatus. Because the block is voltage dependent the affinity of Mg for its blocking site is assumed to depend on voltage. We found that i-V curves measured in the presence of intracellular Mg are well fit if Mg affinity is assumed to vary exponentially with membrane voltage. Noting that the single NMDA channel i-V curve is linear in the absence of Mg (Nowak et al., 1984; Ascher et al., 1988), we can describe such a model with the equation:

$$i(V) = \frac{g(V - V_r)}{1 + [Mg]_i/K_o[\exp(-V/V_o)]}, \quad (1)$$

where $i(V)$ is the single channel current at any membrane voltage V in the presence of intracellular Mg concentration $[Mg]_i$, g is the single channel conductance in the absence of Mg, V_r is the reversal potential, K_o is the apparent dissociation constant of Mg at $V = 0$, and V_o is a parameter that reflects the voltage dependence of the Mg block. The four parameters g, V_r, K_o, and V_o were determined by the fitting procedure described in Methods.

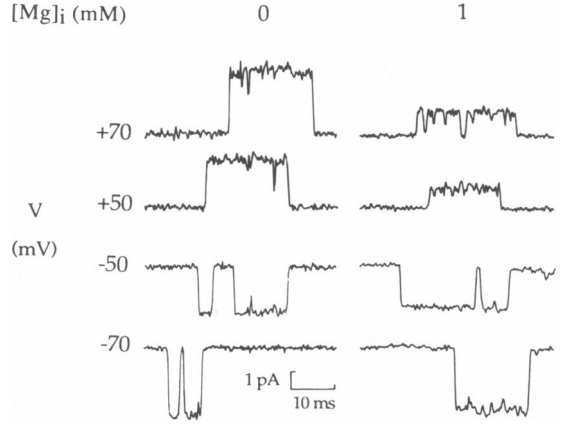

FIGURE 1 Voltage-dependent block by intracellular Mg of NMDA-activated single channel currents. Data are from two outside-out patches that were perfused with an extracellular solution containing 10 μM NMDA, 1 μM glycine, and no Mg. In the experiment of the left-hand column no Mg was present in the intracellular (pipette) solution, whereas for the right-hand data the intracellular Mg concentrations was 1 mM.

Although V_o is an empirically determined parameter used to characterize the block, one possible interpretation of its value is provided by the model of Woodhull (1973). In this model the binding of a single blocking ion to a site within the membrane field blocks the channel, and no interactions can take place between the blocking ion and other ions present in the solution and/or in the channel. With these assumptions, the fraction δ of the membrane field felt by the blocking ion can be calculated from:

$$V_o = RT/z\delta F, \quad (2)$$

where R, T, z, F have their usual significance. Because in these experiments Mg blocks from the intracellular solution, δ will reflect the fraction of the membrane field measured from the intracellular side of the membrane.

The i-V data were well fitted by Eq. 1 over a range of $[Mg]_i$ from 0.3 to 30 mM. Fig. 2 *A* illustrates the effects observed in three experiments in which $[Mg]_i$ was fixed at 0, 0.3, and 1 mM. Data points more negative that −20 mV are not shown because the block at these Mg concentrations is only visible at more depolarized voltages. Fig. 2 *B* illustrates the effects of higher concentrations of Mg_i. In this concentration range the block can be seen to extend smoothly down to relatively hyperpolarized voltages, as expected from the chosen model.

The values of the four parameters obtained from all experiments have been grouped in Table 1. This table shows that there is no significant difference between the values obtained at various Mg concentrations. Thus, over the voltage range and Mg concentration range investigated, there appears to be one dominant mechanism of block by Mg_i that is well described by Eq. 1. It was therefore possible to calculate a mean value of V_o and a mean value of K_o from the 17 experiments of Table 1. The

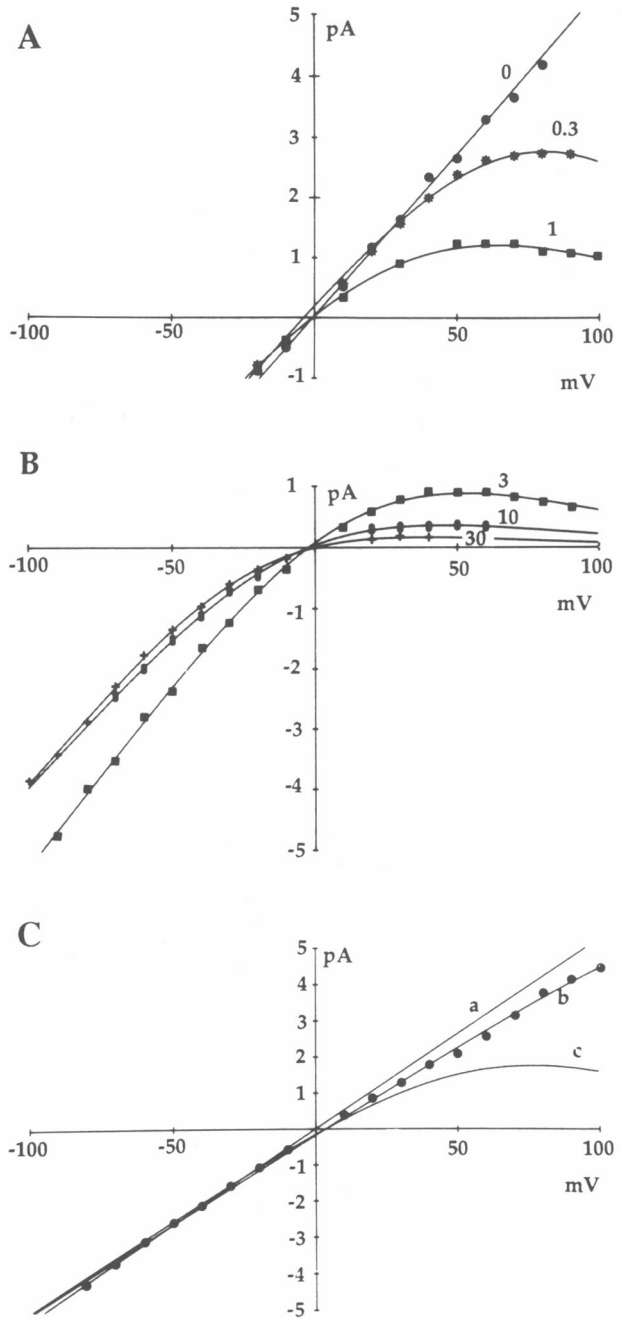

FIGURE 2 *i-V* relations of NMDA-activated single channel currents in the presence of intracellular Mg or intracellular Ca. Single channel currents were measured over a range of voltages under the same conditions as described in Fig. 1. (*A* and *B*). Each *i-V* curve corresponds to measurements from a single patch. The concentration of Mg (in millimolar) inside the pipette is indicated on the figure. The continuous lines represent fits to the data of Eq. 1 (see Methods) with all four parameters left free, except for the data with $[Mg]_i = 0$, for which a linear least squares fit was used. (*C*) The data is from a patch with 0 Mg and 1 mM Ca inside the pipette. Curve *a*, a linear extrapolation of a least squares fit to single channel currents measured at voltages negative to 0 mV (g = 49.3 pS), shows that 1 mM Ca decreases outward currents in the patch. Curve *c* corresponds to the fit of Eq. 1 using for the single channel conductance the same value as for curve *a* and for V_0 and K_0 the mean values calculated from the mean of all experimental data, i.e., 36.8 mV and 8 mM. The effects on outward current of 1 mM Ca_i are clearly far less than those of 1 mM Mg_i. Curve *b* is a fit obtained from Eq. 1 with V_0 set to 36.8 mV and the other parameters left free. A good fit was achieved with a K_0 for Ca of 232 mM (see text).

TABLE 1 Fitted values for the parameters of Eq. 1 at different Mg concentrations

$[Mg]_i$	n	V_0	K_0	g	V_r
mM		*mV*	*mM*	*pS*	*mV*
0.3	2	35.4	4.3	57.5	−2.7
1.0	6 (5)	38.7	7.5	47.2	−0.1
3.0	1	38.7	4.6	56.7	−2.2
10.0	5 (3)	37.0	9.9	48.3	−4.8
30.0	3	33.3	9.3	45.8	2.6

Fitted values of the four parameters of Eq. 1 at various intracellular Mg concentrations. The concentration indicated is the "free" Mg concentration, which equalled the total concentration in all experiments except for four in which 10 mM EGTA had been added. In those experiments the total Mg concentration was raised accordingly to give a free concentration of 1 mM in two cases, and of 10 mM in the two others. n is the number of experiments fitted at each Mg concentration. The four parameters were left free in all but three experiments in which the best fit occurred with a combination of extreme parameter values when all four parameters were left free. In these cases (one at 1 mM Mg_i and two at 10 mM Mg_i) the single channel conductance was fixed at 50 pS and the other three parameters were left free. The number of measurements (n) used to calculate mean conductance (g) at 1 and 10 mM Mg are indicated in parentheses. In one experiment performed with 0.1 mM Mg_i, the data were consistent with the average results of the other experiments. However, the curvature at positive voltages was so small that determination of parameter values by fitting the data was not feasible.

values obtained were V_0 = 36.8 ± 2.4 mV (mean ± SEM) and K_0 = 8.0 ± 0.9 mM. The value of V_0 corresponds to a value of δ = 0.35. The values of δ and K_0 are slightly different from those that we reported earlier (Ascher and Johnson, 1988) on the basis of a smaller number of experiments.

The effect of intracellular Mg was not mimicked by intracellular Ca. In six experiments in which the intracellular Ca concentration was raised to 1 mM, the single channel currents recorded at positive potentials were smaller than in the absence of intracellular Ca, but always larger than the currents recorded at the same potentials with 1 mM intracellular Mg (Fig. 2 *C*). A possible explanation for these effects is that intracellular Ca mimicks the effects of intracellular Mg, but has a much lower affinity for the blocking site. Indeed, the average values of the currents recorded in six experiments using $[Ca]_i$ = 1 mM were well fitted (Fig. 2 *C*) by using Eq. 1. Assuming a value of V_0 equal to the mean value found for Mg, i.e., V_0 = 36.8 mV, the corresponding value

for K_{Ca} ($V = 0$) was found to be 232 mM. However, the change produced by 1 mM Ca can also be explained by a screening of intracellular surface charges (Ascher and Nowak, 1988) (see Discussion).

Finally, the possibility was considered that Mg ions both from the intracellular and from the extracellular solutions block the NMDA channel at the same site. If this were the case, the rate at which a Mg ion leaves the blocking site at a given voltage should be the same regardless of the side of origin of the Mg. At −40 mV, the off rate for extracellular Mg ions is $\sim 2 \times 10^3\ s^{-1}$, an off rate slow enough to produce full channel closures (flicker) without a reduction in the open channel current at the recording bandwidth used here (Ascher and Nowak, 1988). In contrast, 30 mM intracellular Mg causes a decrease in the apparent single channel current (Fig. 2 *B*) without significant channel flicker. Thus, at −40 mV, the off rate of the intracellular Mg from its blocking site appears to be much faster than the off rate of the extracellular Mg. This difference suggests that extracellular and intracellular Mg bind at different sites. The same conclusion was reached by observing the effects of intracellular and extracellular Mg on a single patch. In Fig. 3, full channel closures induced by 200 μM extracellular Mg at potentials between −40 and −70 mV are shown both in the absence of intracellular Mg and with an intracellular Mg concentration of 30 mM. It is clear that at these negative potentials intracellular Mg ions occupy their blocking site without causing discernible full channel closures, while under the same conditions extracellular Mg ions cause full channel closures when occupying their blocking site. Thus, under identical conditions the off rate for intracellular Mg is much faster than for extracellular Mg. This implies that there must be at least two separate mechanisms of block, probably mediated by Mg occupation of two different blocking sites in the NMDA channel.

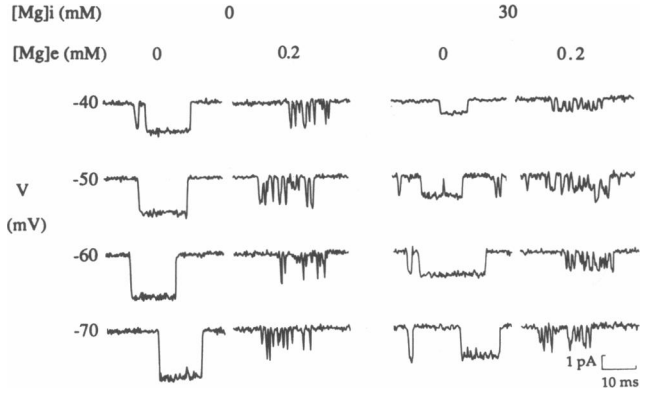

FIGURE 3 Blockade by extracellular Mg can be superimposed on blockade by intracellular Mg. Single channel openings from a patch with 0 intracellular Mg (left two columns) and from a second patch with 30 mM intracellular Mg (right two columns) were recorded in the absence and presence of 0.2 mM extracellular Mg.

DISCUSSION

The data presented above are consistent with a simple model in which intracellular Mg ions cause a rapid and reversible block of the NMDA channel. The dissociation constant of Mg (8 mM at 0 mV) decreases *e*-fold for a 36.8 mV depolarization. If this voltage dependence is assumed to result solely from the position of the blocking site within the membrane field, then Mg ions blocking from the intracellular side of the membrane enter about one-third of the field.

Application of Eq. 1 requires the assumption that any nonlinearity in the single channel *i-V* curve is due to the voltage-dependent channel block by Mg. The validity of this assumption depends on the interpretation and ionic dependence of the small effects of intracellular Ca illustrated in Fig. 2 *C*. As already mentioned, this slight voltage-dependent block can be explained by a low-affinity binding of Ca to the Mg site, as shown in curve *b* of Fig. 2 *C*. However, these results are also compatible with an effect of intracellular Ca on the surface potential that appears to influence ion permeation through the NMDA channel (Ascher and Nowak, 1988). If the latter interpretation is correct, then intracellular Mg may also be able to change the internal surface potential. The Goldman-Hodgkin-Katz equations, modified to account for surface potential (Frankenhaeuser, 1960; Lewis, 1979; Ascher and Nowak, 1988), predict that a positive shift in the intracellular surface potential will decrease the slope of the outward arm of the *i-V* curve without changing the reversal potential. In addition, at very negative potentials, a parallel shift to the left of the inward arm of the *i-V* curve would occur. A similar effect is predicted by a model in which the change of surface charge is assumed to be localized on the channel protein (MacKinnon et al., 1989). Thus, a change of surface potential can result in *i-V* curve nonlinearity. If such effects were induced by high intracellular Mg concentrations, a misestimate of the Mg affinity at 0 mV for the blocking site and voltage dependence of the block could result. However, if internal Mg ions induced a large change in surface potential, both V_0 and K_0 would be expected to vary with $[Mg]_i$, an effect that was not apparent. Because of their relatively low precision the data cannot be used to estimate the magnitude of the influence of $[Mg]_i$ on surface potential, but the results suggest that the effect is not large enough to significantly bias our estimates of V_0 and K_0.

Comparison of the position of the blocking site for

intracellular Mg estimated here with the previously estimated location of the blocking site for extracellular Mg (near the inner membrane surface; Ascher and Nowak, 1988) presents a serious problem: a Mg ion from either side of the membrane would have to pass by one blocking site without effect to reach its own blocking site. As such a "crossing of the δ's" is highly unlikely, it seems necessary to elaborate a model more complete than the one used up to now. Ascher and Nowak (1988) had already noted that the simple model of channel block that they used did not explain the very high voltage dependence of the forward blocking rate (e-fold for 17 mV). Armstrong et al. (1982) described a somewhat similar situation in their analysis of the blocking of squid axon K channels by Ba ions. The voltage dependence of the block by intracellular Ba at positive voltages suggested a blocking site near the extracellular surface, whereas the block by extracellular Ba at negative voltages required that Ba enter two thirds of the field. One solution proposed to resolve this apparent contradiction was to assume that the K channels are multi-ion pores (Hille and Schwarz, 1978) and that the voltage dependence of the block results from the fact that the transition from the unblocked to the blocked state involves a movement of both the blocker and of the permeant ions inside the field. It seems possible to account for our data by a similar hypothesis, which could pull out the extracellular blocking site and possibly pull in the intracellular blocking site. Armstrong et al. (1982) also considered another hypothesis, in which there was only one site of Ba block in the K channel, the position of which changed with membrane potential. One of the main arguments in favor of this hypothesis was that the dissociation rate of Ba from its site at a given voltage was the same whether Ba had entered the channel from the inside or from the outside. In contrast, the rate of exit of Mg from the NMDA channel appears much faster during block by intracellular Mg than during block by extracellular Mg at the same voltage. This observation leads us to consider as the most plausible hypothesis that there are two distinct Mg binding sites that account for the two types of block. This does not exclude some interaction between the sites. More careful analysis of the data of Fig. 3 may reveal some difference between the rates of block by extracellular Mg in the absence and in the presence of 30 mM intracellular Mg. Such a difference could reflect an interaction between the two sites, but it could also be due to a reduction of the intracellular surface potential by Mg, which would reduce the absolute value of the potential at the extracellular Mg binding site.

The fact that the intracellular Mg concentration at which the NMDA channel is half blocked at 0 mV (8.0 mM) is close to the value giving a half block with extracellular Mg (8.8 mM, Ascher and Nowak, 1988), may suggest a similarity between the two sites. It should be noted, however, that the association and dissociation rates for the two sites are very different. This could reflect a difference in the characteristics of the binding sites, or of the barrier(s) that Mg ions must pass in order to reach their binding sites.

The presence of more than one Mg blocking site on the NMDA channel makes it somewhat analogous to the nicotinic ACh channel. Imoto et al. (1988) have localized in the protein sequence of this channel three anionic rings, an intracellular one that interacts preferentially with intracellular Mg, an extracellular one that interacts preferentially with extracellular Mg ions, and an intermediate ring that interacts with Mg from either side. However, the voltage dependence of the Mg block of the NMDA channel is very different from the Mg-induced decrease in the conductance of the nicotinic channel. Over an extended voltage range, neither intracellular nor extracellular Mg induce a negative resistance region in the nicotinic i-V relation; the i-V curve remained relatively linear even in the voltage range in which the effects of Mg were apparent (Dani and Eisenmann, 1987; Imoto et al., 1988; Cachelin and Neuhaus, 1989). The differences between the effects of Mg on the NMDA and nicotinic channels are probably in part explained by the high permeability to Mg of the nicotinic channel, which can pass significant inward current with Mg as the sole charge carrier (Dani and Eisenmann, 1987).

Intracellular Mg is known to produce a voltage-dependent block of at least four other channel types: the ATP-dependent K channel (Horie et al., 1987; Findlay, 1987; Ciani and Ribalet, 1988), the K inward rectifier (Matsuda et al., 1987; Vandenberg, 1987; Matsuda, 1988), the muscarinic K channel (Horie and Irisawa, 1989), and the voltage-dependent Na channel (Pusch et al., 1989). The voltage dependencies of the blocks and the affinities of Mg at 0 mV vary greatly among these channel types. The relative voltage dependencies of the block by intracellular Mg, approximated from the literature, appear to be: inward rectifier, muscarinic K channel > ATP-sensitive K channel, NMDA channel > voltage-dependent Na channel. The Mg affinities at 0 mV are difficult to compare. In the case of the inward rectifier, for example, whereas Mg affinity appears to be very high, it depends on K concentration as well as on voltage. The inward rectifier also exhibits a higher frequency of equally spaced subconductance states in the presence of Mg, suggesting that there are three similar subunits each of which can be blocked by Mg. The data for all four channel types are consistent, however, with a general model in which the reversible occupation by one or more Mg ions of a binding site within the membrane field results in the blockage of current flow through the channel.

The physiological intracellular concentration of Mg is in the same range as the extracellular concentration (1–3 mM) (see, e.g., Alvarez-Leefmans et al., 1986). Thus both the extracellular and intracellular voltage-dependent Mg blocks are likely to occur during the normal functioning of neurons. The NMDA receptor therefore acts as a bidirectional rectifier under physiological conditions; it passes current well in a voltage range around 0 mV, and progressively less well as absolute membrane voltage is increased. The physiological consequences of the intracellular Mg block have not been explored, but it appears possible that it reduces the shunting of the action potential at positive potentials that would otherwise occur during activation of the NMDA response.

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