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Inhibition by Intracellular Mg^{2+} of Recombinant *N*-Methyl-D-aspartate Receptors Expressed in Chinese Hamster Ovary Cells[1]

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ABSTRACT

Intracellular Mg^{2+} (Mg_i^{2+}) inhibits the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors in cultured cortical neurons. To examine the effects of Mg_i^{2+} on recombinant NMDA receptors composed of subunit combinations found in cortical neurons, we expressed heteromeric receptors composed of NR1/NR2A and of NR1/NR2B subunits in Chinese hamster ovary (CHO) cells. We recorded whole-cell currents from the recombinant receptors in the absence and presence of Mg_i^{2+}. The voltage dependence of control (0 Mg_i^{2+}) NMDA-activated currents obtained from CHO cells transfected with NR1/NR2A and with NR1/NR2B receptors showed outward rectification, a property that has been observed previously in native cortical NMDA receptors. The magnitude and voltage dependence of inhibition by Mg_i^{2+} of NMDA-activated currents were similar in CHO cells transfected with NR1/NR2A receptors, CHO cells transfected with NR1/NR2B receptors, and in cultured neurons expressing native NMDA receptors. These observations suggest that Mg_i^{2+} has uniform effects on the native NMDA receptors expressed in cortical neurons. Furthermore, inhibition by Mg_i^{2+} must not depend on intracellular factors or post-translational receptor modifications that are specific to neurons. Finally, the results indicate that the previously observed differences between whole-cell and outside-out patch measurements of Mg_i^{2+} inhibition could not result from poor control of voltage or Mg_i^{2+} concentration in the dendrites of neurons. The most likely alternative explanation is that patch excision causes an alteration in NMDA receptors that results in more effective inhibition by Mg_i^{2+}.

The *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors is thought to be of fundamental importance in brain physiology and pathology. Probably because of its intimate involvement in the function and dysfunction of the brain, NMDA receptors are under tight regulation by multiple endogenous factors (for review, see McBain and Mayer, 1994). One type of regulation, the voltage-dependent block by extracellular Mg^{2+} (Mg_e^{2+}; Mayer et al., 1984; Nowak et al., 1984; Jahr and Stevens, 1990), is well established to be critical for many of the roles that NMDA receptors play (McBain and Mayer, 1994).

The channel of NMDA receptors also can be blocked by intracellular Mg^{2+} (Mg_i^{2+}). Unlike block by Mg_e^{2+}, block by Mg_i^{2+} increases with membrane depolarization (Johnson and Ascher, 1990). Mg_e^{2+} and Mg_i^{2+} block the channel of NMDA receptors at two distinct sites on opposite sides of the selectivity filter (Johnson and Ascher, 1990; Li-Smerin and Johnson, 1996a). Because its binding site is located at a position of critical importance within the channel, Mg_i^{2+} has become an important tool for study of the structure and function of recombinant NMDA receptors (Kupper et al., 1996, 1998; Wollmuth et al., 1998).

In this study, we had two main goals. First, we compared inhibition by Mg_i^{2+} of recombinant NMDA receptors composed of NR1/NR2A and of NR1/NR2B subunits. Although there is extensive evidence that the external channel-blocking site is similar in these two subunit combinations, there have been no studies of the NR2 subunit dependence of block at the Mg_i^{2+} blocking site. Much of the previous work on block by Mg_i^{2+} (Johnson and Ascher, 1990; Li-Smerin and Johnson, 1996a,b) has been performed on native NMDA receptors in cultured cortical neurons. NMDA receptors in this preparation are composed predominantly of NR1 subunits coassembled with NR2A and/or NR2B subunits (Stern et al., 1992, 1994; Zhong et al., 1994; Béhé et al., 1995; Blanpied et al., 1997). Comparisons of the Mg_i^{2+} effects on NR1/NR2A

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CHO, Chinese hamster ovary; GFP, green fluorescent protein; FC, fractional current; I-V, current-voltage.

and NR1/NR2B receptors permitted us to test the validity of the assumption that Mg_i^{2+} has homogenous effects on NMDA receptors expressed in cultured cortical neurons.

A second goal of this study was to examine possible explanations for the observation that Mg_i^{2+} inhibits steady-state NMDA responses less effectively in whole-cell experiments than in excised-patch experiments (Li-Smerin and Johnson, 1996b). Understanding of this discrepancy is necessary to determine the effects of Mg_i^{2+} on NMDA responses under physiological conditions. Steady-state current in excised patches was quantified as "mean patch current," a measurement of the integral of NMDA-activated current flow across the patch. The discrepancy between Mg_i^{2+} inhibition of whole-cell and mean patch current suggests a difference between the preparations either in an experimental condition important to Mg_i^{2+} block, or in NMDA receptor properties. A difference in experimental conditions that might explain this discrepancy results from the presence of dendrites on neurons. If membrane potential and/or the concentration of Mg_i^{2+} ($[Mg^{2+}]_i$) of dendrites during whole-cell recording were not adequately controlled, an artifactual determination that whole-cell currents are inhibited less effectively than mean patch current could result. To test this possibility, we expressed recombinant NMDA receptors in Chinese hamster ovary (CHO) cells, which are spatially compact. If voltage and/or $[Mg^{2+}]_i$ in the dendrites of cultured neurons were inadequately controlled, then the results obtained from CHO cells should resemble the results obtained from excised patches rather than whole neurons. By comparing the Mg_i^{2+} inhibition of transfected CHO cells, whole neurons, and excised patches, we determined whether the dendrites of neurons influence the measured effects of Mg_i^{2+}. Our results support the alternative explanation that patch excision affects Mg_i^{2+} inhibition by inducing a change in NMDA receptor properties.

Materials and Methods

CHO Cell Culture and Transfection of NMDA Receptors. CHO-K1 cells (ATTC CCL61) were chosen for expression studies because they are compact, do not form syncytia, and are easily transfected (Boeckman and Aizenman, 1994, 1996). CHO-K1 cells were grown in Ham's F-12 nutrient medium with 10% fetal bovine serum and 1 mM glutamine (CHO medium) and passaged approximately every 2 days. The cDNAs encoding NR1-1a, NR2A, and NR2B subunits were subcloned into mammalian expression vectors as described previously (Boeckman and Aizenman, 1994, 1996). CHO cells were transiently transfected with the indicated combination of NMDA receptor subunit constructs with LipofectAMINE (Gibco-BRL, Paisley, Scotland). To facilitate recognition of positively transfected cells used for whole-cell recordings, a marker protein, green fluorescent protein (GFP), was cotransfected with NMDA receptors in CHO cells. The expression vector for GFP (Chalfie et al., 1994) was generated as described previously (Blanpied et al., 1997). Approximately 24 h before transfection, cells were seeded in CHO medium at a density of 3×10^5 cells/35-mm culture dish. Transfections were accomplished by addition of 1.3 μg of total DNA and 6 μl of LipofectAMINE in 1 ml of serum-free CHO medium per dish, followed by a 4- to 5-h incubation at 37°C. The marker plasmid (pCI/GFP) and total DNA were transfected at a ratio of 1:4.3, and NR1 and NR2 subunits were transfected at a ratio of 1:3 (Cik et al., 1993). Twenty-four hours after transfection, cells were trypsinized and replated at a 1:2 dilution onto 12-mm-diameter glass coverslips in 35-mm plastic Petri dishes. 5,7-Dichlorokynurenic acid (1 mM) was added to the medium to prevent the cell death due to the toxic effects of NMDA receptor expression (Cik et al., 1994; Anegawa et al., 1995; Boeckman and Aizenman, 1996). Cells were used ~40 to 50 h after the start of transfection.

Whole-Cell Patch-Clamp Recordings. The conventional whole-cell configuration was used to record membrane currents with pipettes pulled from borosilicate thin-walled glass with filaments (Clark Electromedical, Reading, England) and an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Partial compensation for series resistance (50–80%) was performed in some experiments. The pipettes had a resistance of 2 to 4 MΩ when filled with either the control solution, which contained 0 $MgCl_2$, 125 mM CsCl, 10 mM HEPES, and 10 mM EGTA, or the Mg^{2+} solution, which contained 15 mM $MgCl_2$, 105 mM CsCl, 10 mM HEPES, and 10 mM EGTA. The pH was adjusted to 7.2 with CsOH. The concentration of free Mg^{2+} ($[Mg^{2+}]_i$) in the Mg^{2+} solution was calculated as 10 mM with a program that subtracted Mg^{2+} bound to EGTA from total Mg^{2+} using apparent affinity constants of EGTA for Mg^{2+} (Li-Smerin and Johnson, 1996a). However, the $[Mg^{2+}]$ in the Mg^{2+} solution was found actually to be 6.2 mM when it was measured with the Mg^{2+}-sensitive fluorescent dye mag-indo-1 (Li-Smerin et al., 1996). The value of 6.2 mM was therefore used as the $[Mg^{2+}]$ in the Mg^{2+} solution in this study. The control extracellular solution contained 140 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, and 10 mM HEPES. pH was adjusted to 7.2 with NaOH. To activated NMDA receptors, stocks of NMDA (10 mM), and glycine (10 mM) were diluted into the control extracellular solution to achieve 30 μM NMDA and 10 μM glycine (NMDA solution). All the chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

The extracellular solutions were controlled with a five-barrel fast perfusion system (Blanpied et al., 1997). Briefly, barrels made of square capillary tubing were arrayed in parallel and connected to solution reservoirs. After formation of the whole-cell configuration, the barrel perfusing control solution was positioned ~0.2 mm away from the cell under study. Subsequent changes of the perfusion solution were achieved by a lateral movement of the array of barrels. For each measurement of whole-cell NMDA-activated current, the cell was superfused with NMDA solution for typically 10 to 15 s. This application was preceded and followed by superfusion of the control solution. Whole-cell NMDA-activated currents were recorded at membrane potentials ranging from −60 to +60 mV in the following order: −60, +60, −60, −40, +40, −60, −20, +20, and −60 mV. Currents recorded at −60 mV were used to monitor the consistency in the magnitude of NMDA responses during an experiment. Any cell in which the current recorded at −60 mV varied during an experiment by >25% from the first measurement was excluded. Data were discarded if voltage drift at the end of an experiment was >4 mV.

Whole-cell currents were low-pass filtered at 500 Hz and recorded on chart paper (Thermal Arraycorder WP 7700; Western Graphtec, Irvine, CA). Currents also were low-pass filtered at 10 kHz, sampled at 44 kHz with a Neuro-Corder (DR-890; Neuro Data Instruments Corp., New York, NY) and stored on magnetic tapes. All experiments were performed at room temperature (20–25°C).

Calculations and Statistics. Steady-state whole-cell currents under control conditions and during applications of NMDA plus glycine were measured from chart paper records. The difference between the responses in the absence and the presence of the agonists was calculated as the amplitude of NMDA-activated current. To pool data from different cells, current measurements at each membrane potential were normalized for each cell to current measured at −60 mV, a voltage at which inhibition by Mg_i^{2+} is minimal. Mg_i^{2+} does weakly inhibit NMDA responses at −60 mV; at the $[Mg^{2+}]_i$ used herein (6.2 mM), the strongest action of Mg_i^{2+} quantified herein would be a 15% inhibition of mean patch current (Li-Smerin and Johnson, 1996b). The maximal inaccuracy of 15% introduced by normalization would not affect interpretation of any of the data presented herein.

Results are presented as means ± S.E. Systat for Windows, ver-

sion 5, was used to perform statistical comparisons between means (Figs. 1 and 2). The level of statistical significance was set as $P < .05$. To analyze quantitatively the magnitude of inhibition by Mg_i^{2+}, fractional current (FC) was calculated as the mean normalized currents in the presence of Mg_i^{2+} (I_{Mg}) divided by the mean normalized currents in the absence of Mg_i^{2+} (I_{con}) (FC = I_{Mg}/I_{con}; Fig. 3). The significance of the differences among fractional whole-cell currents of CHO cells and cultured neurons and fractional mean patch current of cultured neurons was tested by adapting the procedures used by Kupper et al. (1996). The variance of the factional current [s^2(FC)] was first obtained as follows: s^2 (FC) = $(I_{Mg}/I_{con})^2 \cdot [s^2{}_{Mg}/(n_{Mg} \cdot I^2{}_{Mg}) + s^2{}_{con}/(n_{con} \cdot I^2{}_{con})]$, where s^2 is variance and n is the number of cells in the presence ($_{Mg}$) and absence ($_{con}$) of Mg_i^{2+}. A two-tailed Student's t test, with Bonferroni corrections for multiple pairwise comparisons, was then used to examine the significance of the differences between fractional currents. The t value for the difference between any two fractional currents being compared (FC_x versus FC_y) was calculated as $t = (FC_x - FC_y)/[s^2(FC_x) + s^2(FC_y)]^{0.5}$.

Results

Inhibition by Mg_i^{2+} of Whole-Cell NR1/NR2A Receptor-Mediated Currents. To investigate the effects of Mg_i^{2+} on the NMDA receptor subunit combinations that predominate in native cortical neurons, we used CHO cells transfected with NR1 and NR2A or with NR1 and NR2B subunits. Figure 1A shows examples of current records obtained in 0 Mg_i^{2+} and in 6.2 mM Mg_i^{2+} from CHO cells transfected with NR1 and NR2A subunits. The NMDA-activated currents at −60 and +60 mV were of similar amplitude with a $[Mg^{2+}]_i$ of zero, but current amplitude is smaller at +60 mV than at −60 mV with a $[Mg^{2+}]_i$ of 6.2 mM. This suggests that at positive potentials Mg_i^{2+} inhibits whole-CHO cell currents mediated by NR1/NR2A receptors.

The relation between whole-cell NMDA-activated current and membrane potential is presented in Fig. 1B. In the absence of Mg_i^{2+}, the current-voltage (I-V) relation shows outward rectification: the ratio of current at +60 mV to that at −60 mV was 1.11 ± 0.05 ($n = 6$). A similar nonlinear I-V relation of NMDA-activated currents of native receptors has previously been described and attributed to a voltage-dependent change in the channel open probability (Nowak and Wright, 1992; Li-Smerin and Johnson, 1996b). In contrast, inward rectification was observed in the presence of 6.2 mM Mg_i^{2+} ($n = 5$). Compared with the mean normalized current in the absence of Mg_i^{2+}, the mean normalized current in the presence of 6.2 mM Mg_i^{2+} was reduced by 14% at +20 mV, 29% at +40 mV, and 42% at +60 mV. The differences between 0 and 6.2 mM Mg_i^{2+} at +40 and +60 mV are statistically significant ($P < .05$, two-tailed t test). The amplitude of current recorded at +20 mV and negative potentials was not significantly affected by Mg_i^{2+}.

A central goal of this study is to determine whether Mg_i^{2+} inhibits NMDA responses similarly in transfected CHO cells and in neurons. We addressed this goal by predicting Mg_i^{2+} inhibition of CHO cell currents based on previous whole-cell measurements in cultured cortical neurons (Li-Smerin and Johnson, 1996b; see Fig. 1 legend). As shown in Fig. 1B, the predicted I-V relation based on Mg_i^{2+} action in cultured neurons (dotted line) closely resembles the currents measured in CHO cells in the presence of Mg_i^{2+} (squares and solid lines). These data are consistent with two conclusions: first, Mg_i^{2+} inhibits native NMDA receptors in cultured cortical neurons and recombinant NR1/NR2A receptors via quantitatively similar mechanisms; and second, the complex geometry of cultured cortical neurons did not interfere with accurate measurement of the action of Mg_i^{2+} in whole-cell experiments. However, because cultured cortical neurons are likely to express NR1/NR2B as well as NR1/NR2A receptors, these conclusions require further experiments on NR1/NR2B receptors.

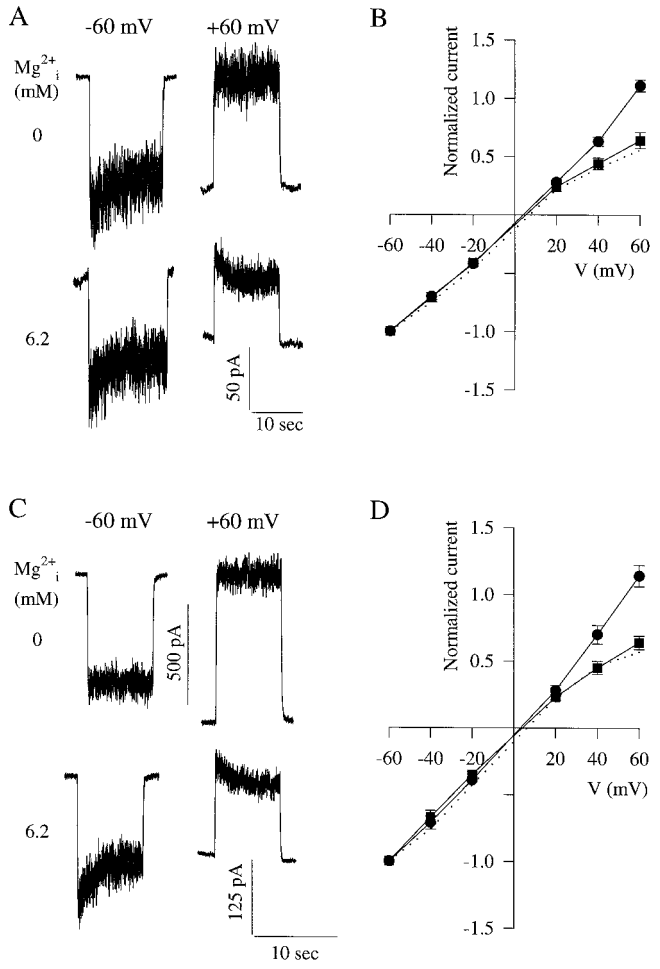

Fig. 1. Voltage-dependent inhibition by Mg_i^{2+} of whole-cell current recorded in CHO cells transfected with NR1/NR2A or with NR1/NR2B subunits. A and C, examples of whole-cell current recorded at the indicated membrane potential and $[Mg^{2+}]_i$ from NR1/NR2A (A) or NR1/NR2B (C) receptors. The records come from four different cells; a single cell was used for each pair of records at 0 Mg_i^{2+} and each pair of records at 6.2 mM Mg_i^{2+}. All the traces were filtered at 100 Hz for display. The ratio of the current at +60 mV to that at −60 mV is: NR1/NR2A receptors, 1.13 with 0 Mg_i^{2+} and 0.70 with 6.2 mM Mg_i^{2+}; NR1/NR2B receptors, 1.24 for 0 Mg_i^{2+} and 0.82 for 6.2 mM Mg_i^{2+}. B and D, I-V relations in 0 (●) and 6.2 mM (■) Mg_i^{2+} from NR1/NR2A (B) or NR1/NR2B (D) receptors. The amplitude of each current measurement was normalized to current amplitude measured at −60 mV in the same cell. Symbols represent the mean of data from: NR1/NR2A receptors, six cells in 0 Mg_i^{2+} and five cells in 6.2 mM Mg_i^{2+}; NR1/NR2B receptors, five cells in 0 Mg_i^{2+} and six cells in 6.2 mM Mg_i^{2+}. For both NR1/NR2A and NR1/NR2B receptors, the normalized current in 0 Mg_i^{2+} and in 6.2 mM Mg_i^{2+} are significantly different ($P < .05$, two-tailed t test) at +40 and +60 mV. Data points were connected with solid lines. The dotted lines represent the normalized current in 6.2 mM Mg_i^{2+} predicted from previous whole-cell experiments on cultured cortical neurons (see Fig. 5 in Li-Smerin and Johnson, 1996b). The prediction was generated by multiplying the normalized 0 Mg_i^{2+} current plotted herein (●) by the fractional current in 6.2 mM Mg_i^{2+} derived from cultured neurons. The value of fractional current was calculated as mean normalized current in 6.2 mM Mg_i^{2+} (indicated as 10 mM in Li-Smerin and Johnson, 1996b; see *Materials and Methods*) divided by the mean normalized current in 0 Mg_i^{2+}.

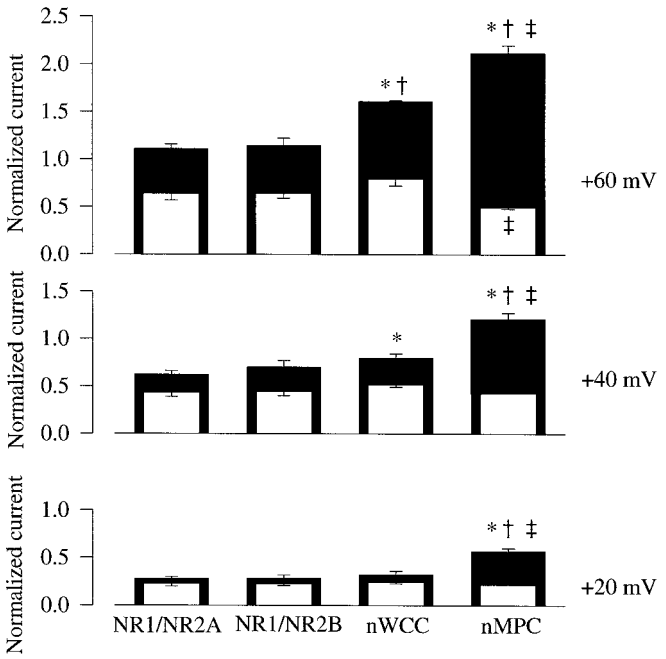

Fig. 2. Comparison among preparations of normalized NMDA-activated currents measured in the absence and the presence of Mg_i^{2+}. Normalized whole-cell currents of CHO cells transfected with NR1/NR2A subunits (NR1/NR2A) and NR1/NR2B subunits (NR1/NR2B) were replotted from Fig. 1. Normalized neuronal whole-cell current (nWCC) and neuronal mean patch current (nMPC) also are plotted for comparison; they are taken from Li-Smerin and Johnson (1996b). At each indicated membrane potential, the solid wide column represents the current recorded in 0 Mg_i^{2+} and the open narrow column in 6.2 mM Mg_i^{2+}; current amplitude was normalized to that measured at −60 mV in each cell or patch. Statistically significant differences ($P < .05$, ANOVA followed by Tukey tests) are indicated as follows: ∗, different from corresponding NR1/NR2A current; †, different from corresponding NR1/NR2B current; ‡, different from corresponding nWCC current. Symbols above the solid wide column refer to statistical differences between currents recorded in 0 Mg_i^{2+}; symbol within the open narrow column refers to statistical difference between currents recorded in 6.2 mM Mg_i^{2+}. There was no significant difference between NR1/NR2A and NR1/NR2B in the current recorded in the absence or presence of Mg_i^{2+} at any voltage.

Inhibition by Mg_i^{2+} of Whole-Cell NR1/NR2B Receptor-Mediated Currents. Fig. 1C shows examples of current measurements from CHO cells transfected with NR1 and NR2B subunits obtained at −60 and +60 mV with 0 Mg_i^{2+} and with 6.2 mM Mg_i^{2+}. Figure 1D presents I-V relations of whole-cell NMDA-activated current in the absence and the presence of 6.2 mM Mg_i^{2+} recorded from CHO cells transfected with NR1 and NR2B subunits. Consistent with the results obtained with the NR1/NR2A receptors (Fig. 1, A and B), the I-V relation in the absence of Mg_i^{2+} shows outward rectification: the ratio of current at +60 mV to that at −60 mV was 1.14 ± 0.08 ($n = 5$). This nonlinear relation of current and voltage once again resembles that observed in native NMDA receptors. The current amplitude in the presence of 6.2 mM Mg_i^{2+} ($n = 6$) also was reduced in NR1/NR2B receptors at positive potentials. Compared with the mean of currents measured in the absence of Mg_i^{2+}, the mean of normalized currents measured in the presence of 6.2 mM Mg_i^{2+} was reduced by 18% at +20 mV, 36% at +40 mV, and 44% at +60 mV. The differences between normalized currents measured in 0 and in 6.2 mM Mg_i^{2+} at +40 and +60 mV are statistically significant ($P < .05$, two-tailed t test). Thus, NR1/NR2B receptors also appear to be subject to voltage-dependent inhibition by Mg_i^{2+}.

We compared inhibition by Mg_i^{2+} of NR1/NR2B receptors in CHO cells with inhibition of native receptors in cultured neurons with the procedures described in the previous section (see Fig. 1 legend). The predicted I-V relation in the presence of Mg_i^{2+} (dotted line in Fig. 1D) was nearly identical with the measured relation (squares and solid lines). These results support the conclusions that Mg_i^{2+} inhibits NR1/NR2B receptors and native NMDA receptors by quantitatively similar mechanisms, and again that the dendrites of neurons did not influence measurements of the effects of Mg_i^{2+}.

Comparison among Preparations of NMDA Response Rectification and Mg_i^{2+} Inhibition. The above-mentioned data permit comparison in transfected CHO cells and cultured neurons both of control current rectification and of inhibition by Mg_i^{2+}. In Fig. 2, four types of normalized currents recorded at three positive potentials in the absence and presence of Mg_i^{2+} are compared: whole-cell currents of NR1/NR2A receptors, whole-cell currents of NR1/NR2B, whole-cell currents of native NMDA receptors, and mean patch currents of native NMDA receptors. Data from native NMDA receptors are taken from Li-Smerin and Johnson (1996b).

The normalized control (0 Mg_i^{2+} whole-cell current was

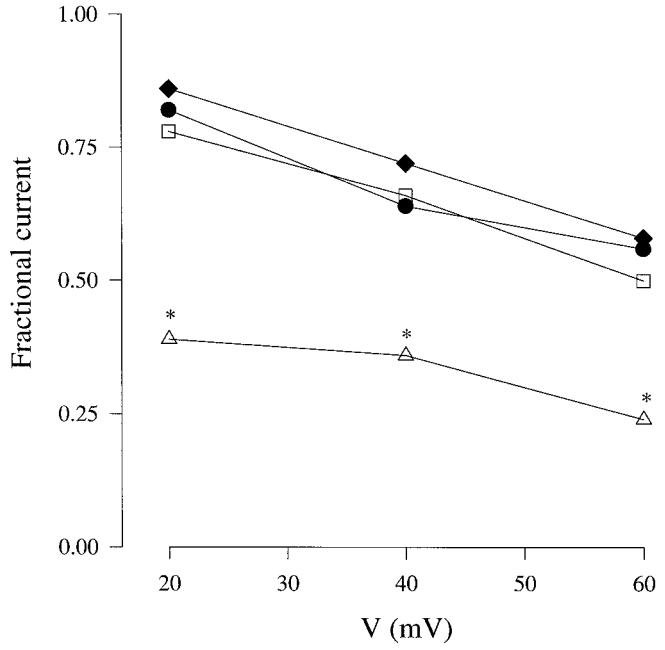

Fig. 3. Comparison among preparations of fractional NMDA-activated current measured in the presence of Mg_i^{2+}. Fractional whole-cell current of CHO cells transfected with NR1/NR2A (♦) or NR1/NR2B (●) receptor subunits were transformed from the data presented in Fig. 1. Fractional neuronal whole-cell (□) and mean patch current (Δ) were calculated from data in Li-Smerin and Johnson (1996b) and also are plotted for comparison. The fractional current was calculated as I_{Mg}/I_{con} where I_{Mg} is the mean value of normalized currents recorded from cells or patches in the presence of 6.2 mM Mg_i^{2+} and I_{con} the mean value of normalized currents recorded in 0 Mg_i^{2+}. Data points were connected with solid lines. There was no significant difference in fractional whole-cell current between native receptors and either NR1/NR2A or NR1/NR2B receptors, or between NR1/NR2A and NR1/NR2B receptors at any potential ($P > .05$; see *Materials and Methods*). The fractional mean patch current at each potential was significantly different from the fractional whole-cell currents of NR1/NR2A, NR1/NR2B, and native receptors (indicated by $*P < .05$ with Bonferroni corrections for pairwise comparisons; see *Materials and Methods*).

nonlinear in transfected CHO cells, as previously observed in cultured neurons (Nowak and Wright, 1992; Li-Smerin and Johnson, 1996b) and in patches from *Xenopus* oocytes expressing NMDA receptors (Kupper et al., 1998). However, outward rectification of control whole-cell currents was significantly greater in neurons than in CHO cells transfected with either NR1/NR2A (at +60 and +40 mV) or NR1/NR2B (at +60 mV) receptors (Fig. 2). In addition, all control whole-cell currents exhibited significantly less rectification than control mean patch current (Fig. 2). Whole-cell and mean patch currents depend on single-channel current amplitude and on channel open probability. Under the recording conditions used herein, single-channel current amplitudes of both native and heterologously expressed NMDA receptors depend nearly linearly on voltage. It therefore is likely that the differences between rectification of transfected CHO whole-cell, neuronal whole-cell, and neuronal mean patch control currents reflect differences in the voltage dependence of channel open probability (Nowak and Wright, 1992).

Mg_i^{2+} inhibited whole-cell currents mediated by NR1/NR2A and by NR1/NR2B receptors in a voltage-dependent manner, similar to its effect on native receptors of cultured neurons. The only significant difference among normalized currents in the presence of Mg_i^{2+} was between neuronal whole-cell and mean patch current at +60 mV (Fig. 2). However, normalized currents in the presence of Mg_i^{2+} reflects both rectification of control current and the inhibitory effects of Mg_i^{2+}. We therefore quantified fractional inhibition by Mg_i^{2+} of each type of NMDA-activated current (Fig. 3).

To compare the effects of Mg_i^{2+} on NMDA responses from each of the preparations shown in Fig. 2, we plotted the fractional current (normalized current in the presence of Mg_i^{2+} divided by the normalized current in the absence of Mg_i^{2+}) as a function of voltage (Fig. 3). Consistent with the data of Fig. 1, B and D, the effect of Mg_i^{2+} on whole-cell currents measured from CHO cells transfected with either subunit combination and on cultured cortical neurons are similar. There was no significant difference at any potential in fractional current among these three preparations. In contrast, the fractional mean patch current was significantly different from fractional current in each of the three whole-cell preparations at each voltage ($P < .05$).

Discussion

In cultured cortical neurons, the preparation in which we previously examined inhibition by Mg_i^{2+} (Li-Smerin and Johnson, 1996a,b), the predominant NMDA receptor subunits are NR1, NR2A, and NR2B. This conclusion is supported by the similarity of the single-channel conductance and kinetics of NMDA receptors in cultured cortical neurons (Antonov and Johnson, 1996; Li-Smerin and Johnson, 1996a) and in recombinant NR1/NR2A or NR1/NR2B receptors (Stern et al., 1992, 1994; Béhé et al., 1995; Brimecombe et al., 1997). In contrast, the single-channel properties of NR1/NR2C (Stern et al., 1992) and NR1/NR2D (Wyllie et al., 1996) receptors differ considerably from those of cultured cortical neurons. Furthermore, mRNA for NR1, NR2A, and NR2B, but not NR2C, is found in cultured cortical neurons (Zhong et al., 1994). Therefore, to further our understanding Mg_i^{2+} inhibition of the NMDA receptors in cortical neurons, we studied recombinant NR1/NR2A and NR2/NR2B receptors.

Control I-V Relation of Recombinant NMDA Receptors Expressed in CHO Cells. In the absence of Mg_i^{2+}, the outward rectification previously observed in cultured cortical neurons also is exhibited by NR1/NR2A and NR1/NR2B receptors expressed in CHO cells (Fig. 1). However, the magnitude of the rectification is smaller in recombinant NMDA receptors of either subunit composition than in native NMDA receptors. This difference could result from neuron-specific molecules that influence the degree of NMDA receptor current rectification. The lack of this factor in CHO cells would result in less I-V relation nonlinearity of recombinant NMDA receptors. Alternatively, post-translational modifications of NMDA receptors that affect current rectification may differ in neurons and CHO cells.

In neurons, control (0 pipette Mg^{2+}) mean patch currents rectify more strongly than do whole-cell currents. The conclusion that this difference is not due to residual intracellular Mg^{2+} during whole-cell recording with Mg^{2+}-free pipette solutions (Li-Smerin and Johnson, 1996b) is supported by the even weaker rectification observed herein of control CHO whole-cell currents. Patch excision may induce a change in NMDA receptor properties that results in an increase in the voltage dependence of channel open probability (Nowak and Wright, 1992).

Inhibition by Mg_i^{2+} of Recombinant NMDA Receptors Expressed in CHO Cells. Whole-cell NMDA-activated currents of transfected CHO cells were reduced at positive potentials by Mg_i^{2+}. The magnitude of the voltage-dependent inhibition by Mg_i^{2+} of recombinant NR1/NR2A or NR1/NR2B receptors were similar to each other and to inhibition of native NMDA receptors expressed in cultured neurons (Fig. 3). The characteristics of Mg_i^{2+} inhibition of whole-cell currents thus appear to be independent of the type of cell in which NMDA receptors are expressed.

Subunits NR2A and NR2B of NMDA receptors have identical amino acid sequences in the region (M2) that has been proposed to line the channel (McBain and Mayer, 1994). The effects of Mg_i^{2+} on the single-channel current of NMDA receptors are influenced by residues located in the M2 region of NR1/NR2A receptors (Kupper et al., 1996, 1998; Wollmuth et al., 1998). The similar effects of Mg_i^{2+} on NR1/NR2A and NR1/NR2B receptors presented here further support the notion that these effects result from binding of Mg_i^{2+} to the pore-forming region.

The nonselective effects of Mg_i^{2+} on NR1/NR2A and NR1/NR2B receptors suggests that Mg_i^{2+} has a homogeneous effect on native NMDA receptors in preparations in which only these subunits are expressed. We did not examine the effects of Mg_i^{2+} on CHO cells transfected with NR1, NR2A, and NR2B receptors; we therefore cannot exclude the possibility that native neurons express NR1/NR2A/NR2B receptors, and that Mg_i^{2+} has a distinct effect on such receptors. There is considerable evidence that NR1/NR2A/NR2B receptors can assemble and that their properties are intermediate between NR1/NR2A and NR1/NR2B receptors (Sheng et al., 1994; Brimecombe et al., 1997; Luo et al., 1997; Vicini et al., 1998; Hawkins et al., 1999; Tovar and Westbrook, 1999). For Mg_i^{2+}, we observed indistinguishable effects on NR1/NR2A, NR1/NR2B, and native receptors. Thus, it is likely that the effects of Mg_i^{2+} on NR1/NR2A/NR2B receptors would be similar to its effects on NR1/NR2A and NR1/NR2B receptors. Models in which Mg_i^{2+} is assumed to interact with a homogeneous

population of receptors, as used in Li-Smerin and Johnson (1996b), should be applicable to cortical neurons.

Differential Effects of Mg_i^{2+} on Steady-State Responses of Whole Cells and Patches. In cultured cortical neurons, Mg_i^{2+} inhibits whole-cell NMDA-activated currents less effectively than currents measured in patches. Mg_i^{2+} inhibits single-channel currents recorded from cultured cortical neurons (Johnson and Ascher, 1990; Li-Smerin and Johnson, 1996a) with relatively high affinity. Mg_i^{2+} inhibition of patch currents is somewhat less effective (~1.4-fold lower affinity) because Mg_i^{2+} binding affects channel gating of NMDA receptors (Li-Smerin and Johnson, 1996b). The large and surprising preparation dependence of Mg_i^{2+} action was observed when inhibition of NMDA-activated whole-cell and mean patch currents from cultured neurons were compared (Li-Smerin and Johnson, 1996b). This difference implied that Mg_i^{2+} action differed in the two recording configurations. Interpretation of these data was hindered, however, by a possible lack of control of $[Mg^{2+}]_i$ or membrane potential in neuronal whole-cell measurements. No comparison of Mg_i^{2+} inhibition of whole-cell and patch responses have previously been made in any other system.

Combined with previous work, the data reported herein indicate that the difference between Mg_i^{2+} inhibition in whole-cells and in patches applies to NMDA responses in heterologous expression systems as well as in cultured neurons. We did not measure patch currents because of the low yield of patch recordings in transfected CHO cells (Brimecombe et al., 1997). However, previous work in heterologous expression systems has demonstrated that the effect of Mg_i^{2+} on single-channel currents of NMDA receptors is not preparation-dependent. Inhibition by Mg_i^{2+} of single-channel currents of NR1/NR2A receptors expressed in *Xenopus* oocytes (Kupper et al., 1998; Wollmuth et al., 1998) and in human embryonic kidney-293 cells (Wollmuth et al., 1998) is quantitatively very close to inhibition of single-channel currents in cultured cortical neurons. Mg_i^{2+}binding affects the gating of some mutant NMDA receptors expressed in *Xenopus* oocytes (Kupper et al., 1998), but no evidence for an effect on channel gating was observed in wild-type receptors expressed in oocytes (Kupper et al., 1998; Wollmuth et al., 1998). Thus, our observation herein of nearly identical inhibition by Mg_i^{2+} of whole-cell currents from transfected CHO cells and from neurons (Fig. 3) indicates that the effects of patch excision are not exclusive to neuronal preparations.

One possible explanation for the patch versus whole-cell difference observed in neurons was inadequate control of voltage and/or $[Mg^{2+}]_i$ in dendrites during whole-cell recordings, which could cause underestimation of the magnitude of inhibition. If this were the case, then the magnitude of Mg_i^{2+} inhibition in CHO whole-cell recordings should resemble inhibition in excised patches but should differ from inhibition in whole neurons. Our data fully contradict this prediction. Therefore, control of dendritic voltage and $[Mg^{2+}]_i$ in whole-cell experiments was adequate to permit accurate measurement of Mg_i^{2+} inhibition.

The differential effects of Mg_i^{2+} on patch- and whole-cell currents also could have resulted from a difference in the Mg_i^{2+} sensitivity of NR1/NR2A and NR1/NR2B receptors; if one receptor subtype were enriched at the neuron soma, then patch- and whole-cell experiments would sample NMDA receptor subtypes with different Mg_i^{2+} sensitivity. This possibility can be rejected because there was no difference between the effect of Mg_i^{2+} on NR1/NR2A and on NR1/NR2B receptors.

Thus, in heterologous expression systems as well as in neurons, Mg_i^{2+} inhibits whole-cell NMDA responses less effectively than patch responses. The differential effects of Mg_i^{2+} cannot be explained by differences between configurations in control of experimental conditions, nor by heterogeneous effects of Mg_i^{2+} on NMDA receptor subtypes. Our results suggest that patch excision modifies NMDA receptors in a way that weakens their sensitivity to inhibition by Mg_i^{2+}. Several other properties of NMDA receptors are modified by patch excision, including glycine-insensitive desensitization (Sather et al., 1990), mechanosensitivity (Paoletti and Ascher, 1994), channel open probability (Rosenmund et al., 1995), and single-channel conductance (Clark et al., 1997). It is possible that some or all of these changes result from disruption of an interaction between NMDA receptors and a cytoplasmic protein. NMDA receptors are known to be capable of binding to many cytoplasmic proteins, including PSD-95 (postsynaptic density-95) and related proteins that contain the PDZ domain (a protein binding domain first found in **P**SD-95, **D**iscs large, and **Z**O-1), α-actinin, yotiao, neuronal intermediate filaments, calmodulin, and phospholipase C-γ (Gurd and Bissoon 1997; Sheng and Wyszynski, 1997; O'Brien et al., 1998). In some cases, the interactions can affect NMDA receptor activity (Krupp et al., 1999; Yamada et al., 1999). If patch excision does affect Mg_i^{2+} inhibition through disruption of interactions between NMDA receptors and intracellular proteins, the data presented herein suggest that the cytoplasmic protein is ubiquitous and can interact with both NR1/NR2A and NR1/NR2B receptors.

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