NMDA Channel Gating Is Influenced by a Tryptophan Residue in the M2 Domain but Calcium Permeation Is Not Altered

D. P. Buck,* S. M. Howitt,† and J. D. Clements†

*John Curtin School of Medical Research and †Biochemistry and Molecular Biology, Australian National University, Canberra, ACT 0200, Australia

ABSTRACT *N*-Methyl-D-aspartate (NMDA) receptors are susceptible to open-channel block by dizolcipine (MK-801), ketamine and Mg^{2+} and are permeable to Ca^{2+}. It is thought that a tryptophan residue in the second membrane-associated domain (M2) may form part of the binding site for open-channel blockers and contribute to Ca^{2+} permeability. We tested this hypothesis using recombinant wild-type and mutant NMDA receptors expressed in HEK-293 cells. The tryptophan was mutated to a leucine (W−5L) in both the NMDAR1 and NMDAR2A subunits. MK-801 and ketamine progressively inhibited currents evoked by glutamate, and the rate of inhibition was increased by the W−5L mutation. An increase in open channel probability accounted for the acceleration. Fluctuation analysis of the glutamate-evoked current revealed that the NMDAR1 W−5L mutation increased channel mean open time, providing further evidence for an alteration in gating. However, the equilibrium affinities of Mg^{2+} and ketamine were largely unaffected by the W−5L mutation, and Ca^{2+} permeability was not decreased. Therefore, the M2 tryptophan residue of the NMDA channel is not involved in Ca^{2+} permeation or the binding of open-channel blockers, but plays an important role in channel gating.

INTRODUCTION

Excitatory synaptic transmission in the central nervous system is mediated primarily by ionotropic glutamate receptors (iGluRs) (Collingridge and Lester, 1989). The iGluR family is divided into several groups based on pharmacology. Receptors that are activated by *N*-methyl-D-aspartate (NMDA) are permeable to Ca^{2+} (Mayer and Westbrook, 1987; Schneggenburger et al., 1993) and are blocked by Mg^{2+} at hyperpolarized membrane potentials (Mayer et al., 1984; Kutsuwada et al., 1992). In contrast, most receptors activated by α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) or kainate are Ca^{2+} impermeable (Iino et al., 1990) and none are blocked by Mg^{2+}. These important functional properties are determined by amino acid residues at key locations in the M2 region, which is believed to line the channel pore of iGluRs. One of these locations is termed the "Q/R/N" site, because it is occupied by an asparagine residue in NMDA receptors, and a glutamine or arginine in non-NMDA receptors (Moriyoshi et al., 1991; Mori et al., 1992; Burnashev et al., 1992a,b; Jonas and Burnashev, 1995). When the Q/R/N-site of an NMDA receptor is mutated from an asparagine to a glutamine, Ca^{2+} permeability and open-channel block are reduced (Burnashev et al., 1992b; Mori et al., 1992). When the opposite mutation is applied to the Q/R/N site of a Ca^{2+}-permeable AMPA receptor, changing the glutamine to an asparagine, Ca^{2+} permeability is increased (Dingledine et al., 1992).

The divalent cation permeability of non-NMDA receptors is also influenced by another site in the M2 region, five residues toward the amino terminal from the Q/R/N site. This is labeled the −5 position using a convention that numbers the amino acid residues of iGluRs relative to the Q/R/N site, with the amino-terminal direction being negative (Kuner et al., 1996). NMDA receptors have a tryptophan residue at the −5 position, while AMPA receptors have a leucine. When the AMPA receptor GluR1 subunit has the −5 leucine mutated to a tryptophan (L−5W) it becomes permeable to divalent cations, and susceptible to block by the NMDA open-channel blocker, phencyclidine (PCP) (Ferrer-Montiel et al., 1996). Sensitivity to PCP can also be introduced by the Q0N mutation, but is greatly increased by the additional L−5W mutation. However, the mutant channel remains insensitive to Mg^{2+} block. When the opposite mutation (W−5L) is applied to the NMDAR2B subunit, NMDA channels have a reduced sensitivity to Mg^{2+} block (Williams et al., 1998). The NMDAR2B W-8L mutation also reduces sensitivity to Mg^{2+} block, but the same mutation of NMDAR2A has little effect (Williams et al., 1998). The effects of the W−5L mutation of the NMDAR2A subunit have not yet been investigated. It also remains to be determined whether the −5 site is involved in Ca^{2+} permeation and open-channel block of NMDA channels, as it is in AMPA channels.

The present study assessed the role of the −5 tryptophan residue of NMDA receptors in calcium permeability and open-channel block. NMDAR1 and NMDAR2A subunits were coexpressed and the −5 tryptophan was mutated to leucine (W−5L), on either or both subunits. The mutation altered channel gating and increased the rate of progressive inhibition produced by MK-801 and ketamine. However, the W−5L mutation did not significantly affect the equilibrium affinity of open-channel blockers and had little effect on Ca^{2+} permeability.

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Address reprint requests to John Clements, Biochemistry and Molecular Biology, Australian National University, Canberra, ACT 0200, Australia. Tel.: 61-2-6249-3465; Fax: 61-2-6249-0313; E-mail: john.clements@anu.edu.au.

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METHODS

Mutagenesis and plasmid preparation

Mouse cDNA clones of the NMDAR subunits NMDAR1 and NMDAR2A were ligated into the mammalian expression vector pCDNA3(−) (Invitrogen, San Diego, CA). In this plasmid, an enhancer and the human cytomegalovirus promoter precede the cloning site. Mutations were introduced using a Pfu-polymerase based double-stranded DNA site-directed mutagenesis system (Quikchange, Stratagene, La Jolla, CA). The sequences of all wild-type and mutant subunit cDNAs were confirmed using a dye-terminator sequencing method (ABI Prism Big Dye, PE Applied Biosystems, Rofkreuz, Switzerland). Plasmids for the transformation of HEK-293 cells were prepared by purification on a cesium chloride gradient or by using an ion-exchange column based plasmid purification procedure (Jetstar Maxipreps, Genomed, Bad Oeynhausen, Germany, and Qiagen Megapreps, Qiagen, Hilden, Germany).

Expression system

HEK-293 cells were grown to 80% confluence in MEM (Dulbecco's minimal essential medium supplemented with 10% fetal calf serum) and harvested with trypsin. Harvested cultures were resuspended in BME (Eagle's basal medium supplemented with 10% fetal calf serum) and placed in a 0.4-cm electroporation cuvette together with plasmid coding for the CD4 cell surface antigen (0.5 μg) and plasmid coding for NMDAR1 and NMDAR2A (5 μg each). The cells were electroporated at 250 V using a 960 μF capacitance extender (Gene Pulser, BioRad, Hercules, CA), which yielded pulse decay time constants of between 11.5 and 13.5 ms.

Cells were seeded onto 35-mm glass cover slips and incubated in BME supplemented with the NMDA receptor antagonists D-AP5 (1 mM, Tocris) and kynurenate (3 mM, Sigma) for between 12 and 24 h. BME was used in preference to MEM because it contained a lower concentration of glutamate. Preliminary studies revealed that when transfected cells were maintained in MEM very few cells expressed NMDA channels, even when supplemented with antagonist (data not shown). Cells expressing NMDA receptors may have been killed by the excitotoxic effects of glutamate in the MEM out-competing the antagonists. The cover slips were treated with 1.4×10^5 anti-CD4 beads (Dynabeads M-450 CD4 T-helper/inducer, Dynal, Oslo, Norway) and placed on a platform rocker for 10 min. Dynabeads preferentially adhered to transformed cells permitting them to be identified for electrophysiological recording.

Solutions

The pipette solution contained 140 mM CsCl, 10 mM BAPTA, 10 mM HEPES and was adjusted to pH 7.2 with CsOH and 285 mOsm with glucose. All extracellular solutions contained 10 mM HEPES and were adjusted to pH 7.2 and 285 mOsm. The extracellular solutions for the ketamine and MK-801 inhibition experiments, and for the fluctuation analysis study contained 150 mM NaCl and 1 mM $CaCl_2$. The extracellular solution for the Mg^{2+} inhibition experiments contained 140 mM NaCl and 2 mM $CaCl_2$. Mg^{2+} concentration was adjusted by mixing in a solution containing 110 mM $MgCl_2$. Calcium permeability was determined using three different extracellular solutions containing 150 mM CsCl, 110 mM $CaCl_2$, 150 mM *N*-methyl-D-glucosamine ($NMDG^+$). Different Ca^{2+} concentrations were achieved by mixing these solutions in the appropriate proportions. Where glutamate was added to the external solution to activate NMDA receptors, glycine (10 μM) was included as a co-agonist.

Electrophysiology

Recordings were made from HEK-293 cells in whole-cell or outside-out patch configuration using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Cells were patched using 2–3 MΩ pipettes pulled from borosilicate hematocrit tubes. They were held at −60 mV unless otherwise stated. Cells and patches were lifted away from the cover slips and solutions were applied using a multi-barrel perfusion system. The solution exchange time constant for HEK-293 cells was 39 ± 5 ms ($n = 15$) as measured by the inhibition of NMDA currents with 150 mM Mg^{2+} (Lester et al., 1990). Current-voltage (I-V) curves were constructed by stepping the membrane potential from −100 mV to +140 mV in 10-mV increments. Leak currents were recorded before and after the agonist application. They were averaged and subtracted offline.

Results were collected using AxoData (Axon Instruments, Foster City, CA) or Chart 3.2 (AD Instruments, NSW, Australia) data acquisition software. Current-voltage and fluctuation analysis data were sampled at 2 kHz and low-pass filtered at 1 kHz. MK-801 and ketamine data were sampled at 4 Hz and low-pass filtered at 2 Hz. Analysis was performed off line using AxoGraph (Axon Instruments, Foster City, CA). All results are given as mean ± SE. In the figures, error bars represent ± SE and * represents a significant difference based on one-way analysis of variance (ANOVA, $p < 0.05$), unless otherwise stated.

Fluctuation analysis

The steady-state currents recorded during a prolonged drug application was converted to a power spectrum via a fast Fourier transform. The spectrum was fit with the equation for a single Lorenzian equation (Mayer et al., 1988) of the form,

$$S(f) = \frac{S(0)}{1 + (f/f_c)^2} \tag{1}$$

$S(f)$ is the power density at the frequency of f, $S(0)$ is the zero frequency asymptote and f_c is the corner frequency at which $S(f) = S(0)/2$. The mean open time (MOT) were estimated from f_c with the equation

$$\mathrm{MOT} = \frac{1}{2\pi f_c} \tag{2}$$

Relative permeability

The permeability properties of NMDA receptors do not follow the predictions of the Goldmann-Hodgkin-Katz equation (Ascher and Nowak, 1988; Wollmuth and Sakmann, 1998), even though the channels appear to have an ionic occupancy of one under many recording conditions (Zarei and Dani, 1994). This discrepancy arises because of ion-ion interactions at or near the channel pore (Schneggenburger and Ascher, 1997; Premkumar and Auerbach, 1996; Sharma and Stevens, 1996). We therefore used a method for investigating Ca^{2+} permeability that was designed to minimize ion-ion interactions (Wollmuth and Sakmann, 1998).

To quantify the relative Ca^{2+} permeability $P_{Ca^{2+}}/P_{Cs^+}$ the difference between the Ca^{2+} and Cs^+ solutions reversal potentials (ΔE_{rev}) was determined. This value was substituted into a modified Lewis equation (Wollmuth and Sakmann, 1998), of the form,

$$\frac{P_{Ca^{2+}}}{P_{Cs^+}} = \frac{[Cs^+]_o}{4[Ca^{2+}]_o}\left(1 + \exp\left(\frac{E_{rev,Ca}}{RT/F}\right)\right)\exp\left(\frac{\Delta E_{rev}}{RT/F}\right) \tag{3}$$

Tip potentials offsets were measured and used to correct the observed reversal potentials. For simplicity, and to allow a direct comparison with other results using this method (Wollmuth and Sakmann, 1998), calculations were based on concentrations without correction for activity coefficients. Under our recording conditions, activity coefficients show negligible variation with Ca^{2+} concentration, and their inclusion in the calculation would approximately double the permeability ratio at all concentrations (data not shown).

Estimation of the IC_{50} for Mg^{2+} block

Dose-inhibition curves were recorded at a holding potential of −100 mV to enhance Mg^{2+} inhibition, and were fit with a Hill-type equation of the form,

$$\frac{I_{Mg^{2+}}}{I} = \frac{1}{1 + ([Mg^{2+}]_o/IC_{50})^n} \quad (4)$$

where $I_{Mg^{2+}}$ is the normalized current at −100 mV, IC_{50} is the Mg^{2+} concentration for which the inhibition at −100 mV is 50%, and n is the Hill coefficient.

RESULTS

MK-801 block

Currents were evoked in HEK-293 cells expressing NMDA receptors by applying glutamate (1 mM). When the evoked current had reached steady-state (>10 s), a rapid step (exchange time constant ~40 ms) was made into a solution containing both MK-801 (1 μM) and glutamate. The current was progressively inhibited with a time course that had distinct fast (<0.3 s) and slow (>10 s) components (Fig. 1 *A*). Theory and modeling suggest that the fast component of MK-801 inhibition is due to a rapid reduction in the MOT of NMDA channels, while the slow component is due to progressive elimination of the channels as they are irreversibly blocked by MK-801 (Rosenmund et al., 1993). The relative magnitude of the fast block is determined by the MOT of the channels in the absence of MK-801, and by the binding rate of MK-801 to open channels. The time constant of the slow progressive inhibition is not sensitive to the MOT, but is determined by the open probability of the

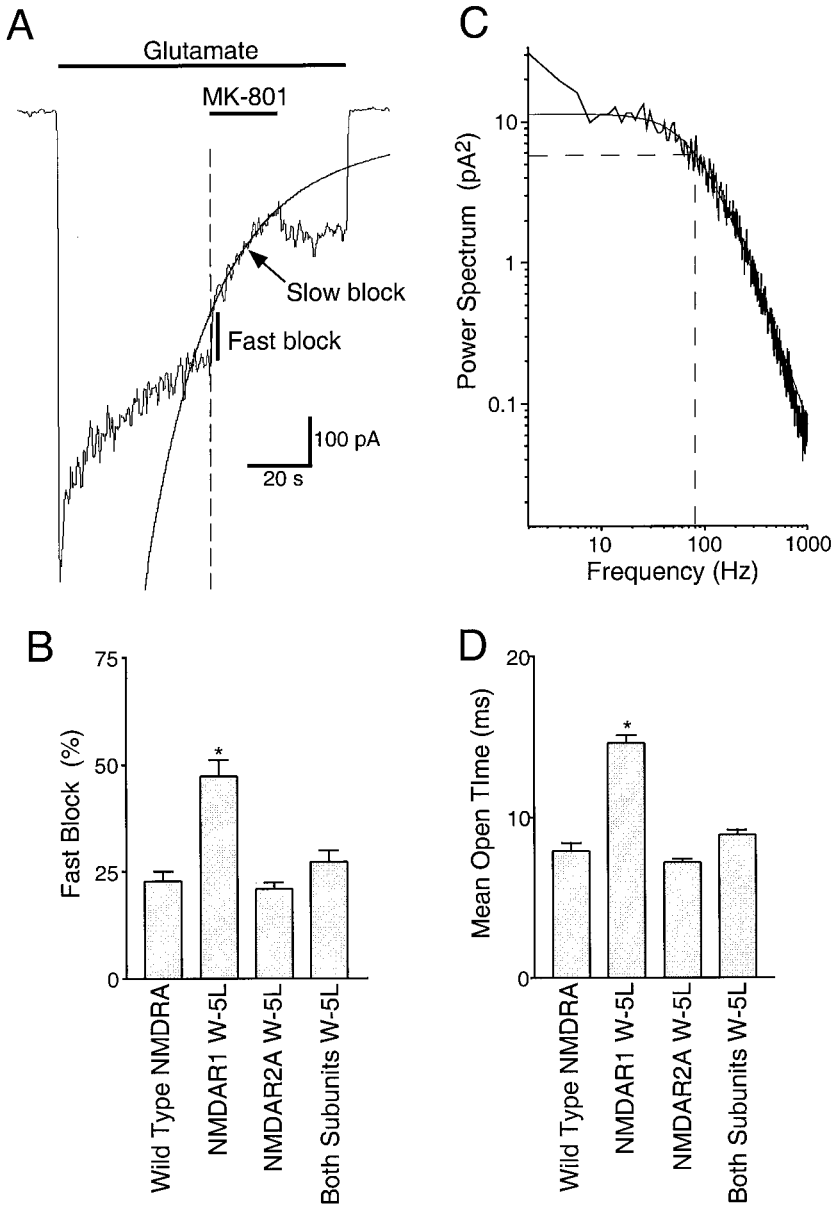

FIGURE 1 Fast and slow components of inhibition by MK-801 (1 μM) of whole-cell NMDA currents. (*A*) The slow, progressive inhibition was fit with a single exponential. Fast block was defined as the difference between the steady-state current amplitude immediately before MK-801 application, and the amplitude of the fitted exponential immediately after MK-801 application. Exponential fits to the fast component of the inhibition had time constants of less than 0.3 s (data not shown), which is consistent with the solution exchange rate in this system. (*B*) The fast block was increased significantly by the NMDAR1 W−5L mutation, but not by the NMDAR2A W−5L mutation or the double mutation ($n = 8$). (*C*) Noise analysis of whole-cell NMDA current. The power spectrum recorded in the absence of glutamate was subtracted from the spectrum recorded in glutamate (1 mM). The spectrum was fit with a Lorenzian equation to estimate the channel mean open time. (*D*) The MOT was significantly increased by the NMDAR1 W−5L mutation, but not by the NMDAR2A W−5L mutation or the double mutation ($n = 6$).

NMDA channel, and also by the binding rate of MK-801 (Rosenmund et al., 1993). The fast component of the MK-801 block reduced the steady-state NMDA current by 23 ± 2% (n = 8) in wild-type receptors. The magnitude of the fast block was approximately doubled by the NMDAR1 W−5L mutation to 47 ± 4% (n = 8) (Fig. 1 *B*). In contrast, the NMDAR2A W−5L mutation had no effect on the fast block. Surprisingly, when both subunits contained the mutation the fast block was not significantly altered (Fig. 1 *B*).

The enhancement of the fast block of NMDAR1 W−5L channels by MK-801 may be due to an increased MOT, or to a faster MK-801 binding rate to open channels, or both. To distinguish between these possibilities, we estimated the MOT using fluctuation analysis of steady-state NMDA currents (Mayer et al., 1988). A current was evoked by prolonged application of glutamate (1 mM, 250 s). The power spectrum of the noise in the absence of agonist was subtracted from the power spectrum of the evoked steady-state current. A single Lorenzian equation was optimally fit to the difference spectrum (Fig. 1 *C*). The corner frequency for the result shown in Fig. 1 *C* was 80 Hz, which was typical for wild-type NMDA receptors. The MOT for these receptors (from Eq. 2) was 2 ± 0.1 ms (n = 8). This is comparable to the MOT value of 4 ms obtained for native NMDA receptors from cultured neurons using the fluctuation analysis technique (Mayer et al., 1988), and a value of 3 ms obtained from single-channel recordings (Stern et al., 1994). The NMDAR1 W−5L mutation approximately doubled the channel MOT to 4 ± 0.1 ms (n = 5) (Fig. 1 *D*). The similarity between the increase in MOT, and the increase in the magnitude of the fast MK-801 block (Fig. 1B, D) suggests that the NMDAR1 W−5L mutation alters channel gating, but has little effect on the binding rate of MK-801 to open channels. Neither the NMDAR2A W−5L mutation nor the double mutation had a detectable effect on the channel's MOT or on the fast MK-801 block. Together, these data suggest that the −5 tryptophan is not directly involved in forming the MK-801 binding site in the NMDA channel, but it does have an important role in channel gating. This hypothesis was further investigated by analyzing the time course of the progressive inhibition in MK-801.

The slow component of MK-801 progressive inhibition was fit with a single-exponential equation (Fig. 1 *A*), and the time constant was 48 ± 10 s (n = 10) for wild-type NMDA receptors. The time constant was approximately 3 times faster at 14 ± 2 s (n = 7) for channels containing the NMDAR1 W−5L mutant subunit (Fig. 2 *A*). The acceleration of the progressive inhibition must be due to an increase in the open probability of the channel. The only alternative explanation would be an increase in the binding rate of MK-801 to open channels, but the results presented in the previous paragraph rule out this possibility. The increase in the open probability of channels containing the NMDAR1 W−5L subunit can be largely attributed to their longer MOT (Fig. 1 *D*). Channels containing the NMDAR2A W−5L subunit were inhibited more rapidly by MK-801, but the decrease in time constant was not significant. When the W−5L mutation was present in both subunits, the time constant of inhibition was 21 ± 4 s (n = 12), a significant decrease compared with the wild-type channels (Fig. 2 *A*). In summary, NMDA receptors containing a W−5L mutation in either or both subunits generally exhibited an increase in their MOT and open probability.

Another way to test for alteration of the binding site for an open-channel blocker is to examine the rate of recovery from inhibition. Following wash out of MK-801 in the continued presence of glutamate, wild-type NMDA channels did not recover from block (data not shown). This is consistent with previous results for native NMDA channels where MK-801 block is irreversible on the time scale of several minutes (Rosenmund et al., 1993). In contrast, MK-801 unbound from NMDAR1 W−5L and NMDAR2A

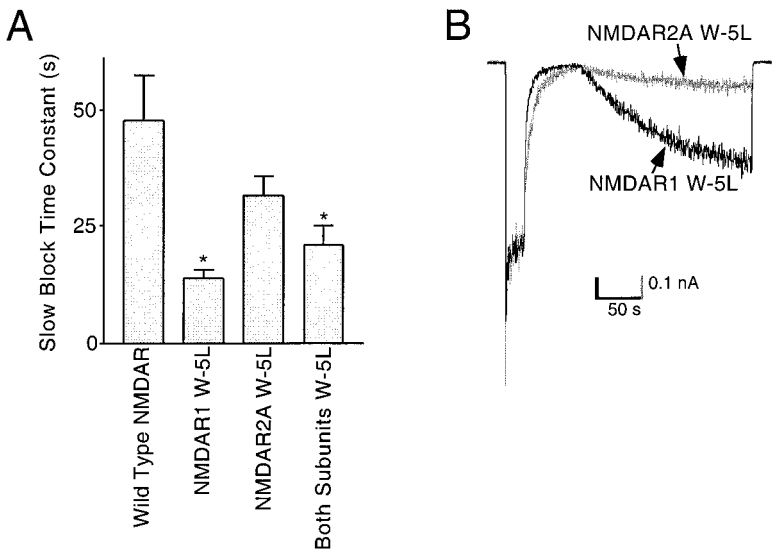

FIGURE 2 Time course of progressive inhibition by MK-801 and recovery from inhibition. (*A*) The time constant of the slow, progressive component of MK-801 inhibition. The W−5L mutation decreased the time constant of inhibition, but the decrease was not significant for NMDAR2A W−5L receptors (n = 6). (*B*) MK-801 can unbind from NMDAR1 W−5L and NMDAR2A W−5L receptors on the time scale of several minutes. In contrast, the MK-801 block of wild-type receptors is irreversible on this time scale (data not shown).

W−5L receptors on this time scale. One possible interpretation is that the increased unbinding rate reflects increased open-channel probability. It was difficult to reliably estimate the unbinding rates due to rundown of the NMDA response on this long time scale. To overcome this problem, we studied the kinetic properties of the open-channel blocker, ketamine, which is known to dissociate from wild-type NMDA receptors much more rapidly than MK-801. We first examined the inhibition rates and compared these with the MK-801 results, then measured the recovery from ketamine inhibition.

Ketamine block

NMDA receptor-mediated currents were evoked by glutamate, and then a rapid step was made into a solution containing both ketamine (10 μM) and glutamate (Fig. 3 *A*). The current was inhibited with a time course that had distinct fast (<0.3 s) and slow (>4 s) components (Fig. 3 *A*). The time constant of the slow component was 9 ± 2 s (n = 16) for the wild-type receptor, and this was reduced to 4 ± 1 s (n = 11) by the NMDAR1 W−5L mutation (Fig. 3 *B*). This is consistent with the MK-801 results, and supports the suggestion that NMDA channel open probability is increased by the W−5L mutation. Channels containing the NMDAR2 W−5L subunit appeared to be inhibited more rapidly by ketamine, but the rate increase was not significant. Channels with the W−5L mutation on both subunits were inhibited by ketamine with a time constant of 2 ± 0.4 s (n = 11), which was significantly faster than for the wild-type

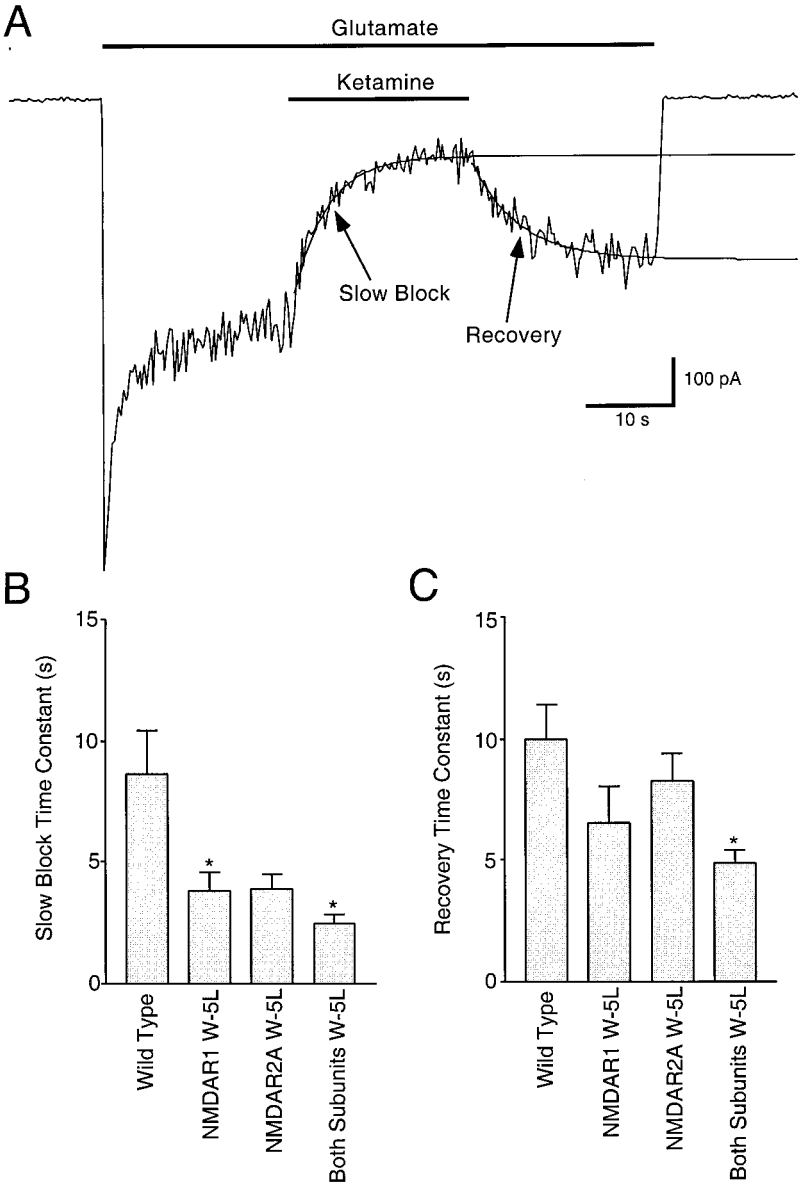

FIGURE 3 Time course of progressive inhibition by ketamine and recovery from inhibition. (*A*) The current evoked by glutamate (1 mM) in wild-type NMDA receptors was inhibited by ketamine (10 μM). The slow component of the inhibition, and the recovery from inhibition were both fit with an exponential equation. (*B*) The time constants of progressive inhibition in ketamine for wild-type and mutant NMDA receptors. The W−5L mutation decreased the time constant of inhibition, but the decrease was not significant for NMDAR2A W−5L receptors (n = 6). (*C*) The time constants for recovery from ketamine inhibition. The ketamine dissociation rate was significantly increased when both subunits contained the W−5L mutation (n = 6).

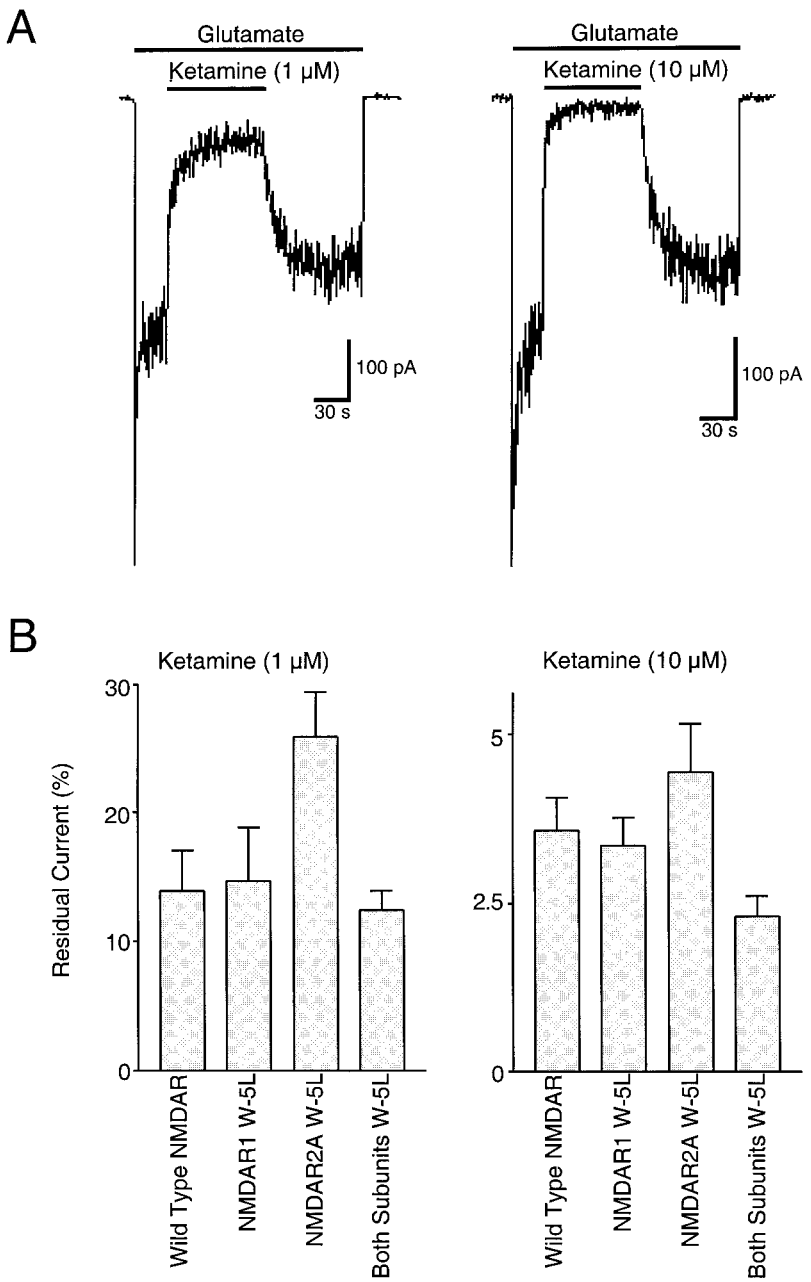

FIGURE 4 Equilibrium inhibition by ketamine. (*A*) The current evoked by glutamate (1 mM) in wild-type NMDA receptors was inhibited by ketamine (1 or 10 μM). Ketamine was applied for 60 s, and the steady-state current was measured when the inhibition had reached equilibrium. (*B*) The residual current at equilibrium was calculated as a fraction of the steady-state current immediately before the application of ketamine. The W−5L mutation had no significant effect on the equilibrium inhibition produced by ketamine (ANOVA, $n = 5$).

receptors (Fig. 3 *B*). These results confirm that the W−5L mutation increases NMDA channel open probability.

Following the rapid removal of ketamine, the response recovered as ketamine unbound from the NMDA channels (Fig. 3 *A*). The recovery time constant is determined by the dissociation rate of ketamine. For wild-type receptors, the current recovered with a time constant of 10 ± 1 s ($n = 21$). Introducing the W−5L mutation to both NMDAR1 and NMDAR2 reduced the recovery time constant (Fig. 3 *C*). The ketamine dissociation rate was approximately doubled in these channels. The recovery time constant appeared to be reduced when the mutation was introduced to either subunit alone, but the reduction was not significant. It has been suggested that open-channel blockers become trapped when the channel enters its closed state, and can only dissociate when the channel is in the open state (Benveniste and Mayer, 1995). If this is true for ketamine, then the faster dissociation rate from channels carrying the W−5L mutation may simply reflect the increased open probability of these channels. In this case, the faster dissociation rate would be coupled with a faster binding rate, and no overall change in the steady-state affinity of ketamine would be expected.

The affinity of ketamine at its binding site in the NMDA channel pore was studied by measuring the equilibrium inhibition of the glutamate-evoked current during a 60 s application of ketamine (1 μM) (Fig. 4 *A*). There was no significant

difference between the equilibrium inhibition of wild-type channels and channels containing the W−5L mutation on one or both subunits (Fig. 4B). These results support the suggestion that the −5 residue does not form part of the binding site for open-channel blockers. This leads to the prediction that the W−5L mutation will have little effect on the affinity with which Mg^{2+} inhibits the NMDA channel, because Mg^{2+} competes with MK-801 for a binding site in the channel pore (Huettner and Bean, 1988).

Mg^{2+} block

NMDA receptor-mediated currents were evoked in HEK-293 cells and Mg^{2+} was applied at a range of concentrations. Cells were clamped at −100 mV to enhance the voltage-dependent Mg^{2+} block. A dose-inhibition plot was constructed, and the IC_{50} was calculated by fitting a Hill equation to the plot (Fig. 5 *A*). For the wild-type receptor, the IC_{50} was 9.6 ± 0.9 μM (n = 17), which is consistent with the value obtained in other studies using recombinant NMDA receptors (Burnashev et al., 1992a,b). As predicted, the IC_{50} for Mg^{2+} was similar for the NMDAR1 W−5L. However, the NMDAR2A W−5L mutation produced a small but significant increase in IC_{50} to 26 ± 3 μM (n = 21) (Fig. 5 *B*). This effect was much smaller than the 20-fold increase in the IC_{50} for Mg^{2+} produced by the NMDAR2A N0Q mutation (Burnashev et al., 1992b), but very similar to the increase recorded for the NMDAR2B W−5L mutation (Williams et al., 1998).

These results confirm that the −5 residue plays little or no role in forming the binding site for open-channel blockers, contrary to previous suggestions (Ferrer-Montiel et al., 1996). It has also been suggested previously that the −5 residue is involved in Ca^{2+} permeation of AMPA and NMDA channels. We next studied the Ca^{2+} permeability of wild-type and mutant NMDA receptors.

Ca^{2+} permeability

The reversal potential of the glutamate-evoked currents was recorded in a range of different solutions containing either Ca^{2+} or Cs^{+} as the only permeable ion (Fig. 6 *A*). The impermeable organic cation *N*-methyl-D-glucosamine ($NMDG^{+}$) was used to maintain the ionic strength of the solutions. The difference between the reversal potential in Cs^{+} and Ca^{2+}, ΔE_{rev} (Fig. 6 *C*), was plotted as a function of Ca^{2+} concentration (Fig. 6 *B*). This experimental approach was used because it minimizes the number of ion-ion interactions in and near the channel pore. The Lewis equation was used to estimate the relative Ca^{2+} permeability at each Ca^{2+} concentration (Wollmuth and Sakmann, 1998) (Fig. 6C). The Ca^{2+} permeability was dependent on the Ca^{2+} concentration, with a maximum at 1 mM for wild-type receptors. This is consistent with the previous finding of a permeability maximum at 0.8–1.0 mM Ca^{2+} (Wollmuth and Sakmann, 1998). Note that if the activity coefficients of Ca^{2+} and Cs^{+} had been taken into account, the values calculated for the Ca^{2+}/Cs^{+} permeability ratio would be approximately doubled (see Methods). At most Ca^{2+} concentrations, there was no difference between the Ca^{2+} permeability of wild-type receptors or receptors containing the W−5L mutation. A small increase in the relative permeability for Ca^{2+} was seen at 0.35 mM for NMDAR1 W−5L channels (Fig. 6 *C*) but was not statistically significant. These results demonstrate that the −5 tryptophan residue does not confer Ca^{2+} permeability to NMDA receptors, as had previously been suggested (Ferrer-Montiel et al., 1996).

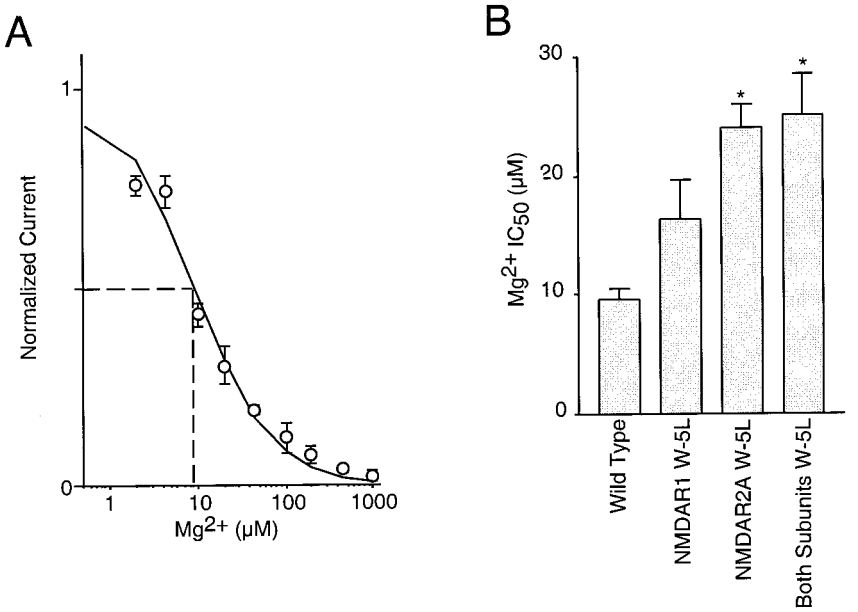

FIGURE 5 Mg^{2+} block. (*A*) Normalized whole-cell current evoked by glutamate at −100 mV plotted against Mg^{2+} concentration (n = 6). The Hill equation was fit to the data (*solid line*) giving an estimate IC_{50} for Mg^{2+}. (*B*) The IC_{50} for Mg^{2+} was increased in receptors containing the NMDAR2A W−5L subunit (n = 17).

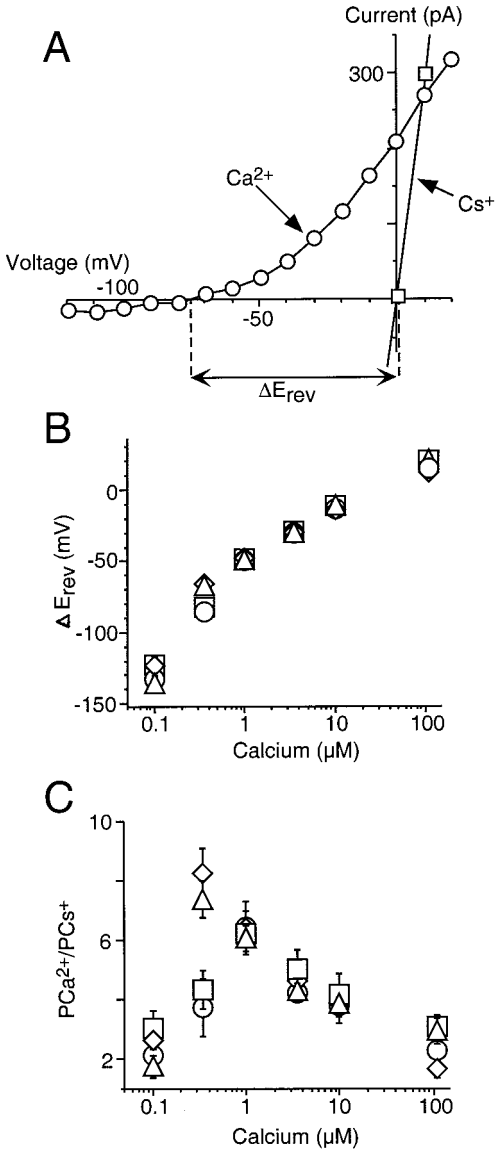

FIGURE 6 Relative Ca^{2+} permeability. (*A*) The current-voltage relationship for wild-type NMDA receptors recorded in 0.35 mM Ca^{2+} with no added Cs^+ (*open circles*), and in 143.5 mM Cs^+ with no added Ca^{2+} (*open squares*). In all Ca^{2+} solutions, ionic strength was maintained with $NMDG^+$. The difference was measured between the reversal potentials in these two solutions (ΔE_{rev}). (*B*) The value of ΔE_{rev} observed at a range of different Ca^2 concentrations for wild-type NMDA receptors (*circles*), NMDAR1 W−5L (*diamonds*), NMDAR2A W−5L (*squares*), and double mutant (*triangles*) ($n = 6$). For some data points, the error bars are obscured by the symbols. (*C*) ΔE_{rev} was used to calculate the relative permeability to Ca^{2+} at a range of different Ca^{2+} concentrations. There was no difference between the permeability of wild-type and mutant channels (two-way ANOVA, $p > 0.05$).

DISCUSSION

Recombinant NMDA receptors containing a W−5L mutation in either or both subunits exhibited an increased open channel probability, but little or no change in their permeability to divalent cations, or in the efficacy of open-channel blockers. These results rule out any major role for the −5 tryptophan residue in enabling or facilitating divalent ion permeation of NMDA channels. The −5 position was a strong candidate for such a role because the L−5W mutation of the GluR1 subunit confers divalent cation permeability to AMPA receptors (Ferrer-Montiel et al., 1996). The L−5W mutation also confers sensitivity to block by PCP, which suggests that the −5 site may form part of the binding site for NMDA receptor open-channel blockers. However, the results of the present study are not consistent with such a role. Instead they are consistent with the results of a cysteine scanning mutagenesis study of NMDA receptors, which suggested that the −5 position is not directly exposed to the channel pore (Kuner et al., 1996; Beck et al., 1999).

The major effect of the NMDAR1 W−5L mutation was on channel gating. An increase in the open probability of channels carrying the mutation can account for the increased rates of progressive inhibition in the presence of MK-801 or ketamine. A parallel increase in the MOT of the mutant channels supports this interpretation. In contrast, a change in the binding rate for these blockers is unlikely because the change in the magnitude of the fast MK-801 block could be entirely attributed to the change in MOT. An increased open probability could also explain the faster dissociation of ketamine from channels carrying the W−5L mutation. This interpretation relies on the assumption that ketamine is trapped when the channel is in the closed state (Benveniste and Mayer, 1995). A stabilization of the open state of the NMDAR1 W−5L mutant channel could explain the increase in their MOT and open probability.

Channels carrying the W−5L mutation on the NMDAR1 subunit had an increased MOT, but channels carrying the mutation on the NMDAR2A subunit were not affected. When channels carried the mutation in both subunits, this unexpectedly appeared to nullify the effect induced by the mutation in NMDAR1 alone (Fig. 1). In contrast, when other channel properties were examined, this nullifying effect was not seen (Fig. 2, 3, and 5). Several interpretations of these results are possible. For example, the changes in channel properties produced by the W−5L mutation might have been simpler and more consistent than our data suggests, but some changes were not detected at the $p < 0.05$ level due to experimental uncertainties. Alternatively, our data may accurately reflect a complex pattern of alterations in channel properties that arises from subunit-subunit interactions.

Ketamine was used to explore the affinity of the open-channel block site of wild-type and mutant NMDA channels. It is more suitable than MK-801 for this purpose because it dissociates on the time scale of several seconds from wild-type channels. The W−5L mutation produced no significant change in the equilibrium block by ketamine. The progressive inhibition in the presence of ketamine and the recovery following its removal, were both accelerated to a similar extent by the mutation. Although these results do not rule out an alteration of the ketamine binding site, they are most simply explained by an increase in open channel probability.

The 2-fold decrease in the Mg^{2+} affinity produced by the W−5L mutation of the NMDAR2A subunit was an order of magnitude smaller than the decrease produced by the N0Q mutation (Burnashev et al., 1992b). Interestingly, the effect of these mutations on Mg^{2+} block was confined to the 2A subunit. These results suggest that the −5 tryptophan residue influences the Mg^{2+} binding site indirectly, possibly by altering the position of key residues such as 0 asparagine.

It has been suggested that the −5 tryptophan residue contributes to the Ca^{2+} permeability of NMDA channels. This hypothesis predicts that channels carrying the W−5L mutation should have a reduced Ca^{2+} permeability. However, the mutation did not reduce Ca^{2+} permeability at any of the Ca^{2+} concentrations tested in this study, suggesting that the site is not directly involved in Ca^{2+} permeation of the channel. The mutation appeared to produced an increase in permeability at 0.35 mM Ca^{2+} but the change was not significant (ANOVA, $p > 0.05$).

In summary, NMDA receptors containing a W−5L mutation in either or both subunits generally exhibited an increased open channel probability, but little or no change in their permeability to divalent cations, or in the efficacy of open-channel blockers. These results suggest that the −5 tryptophan residue has little influence on divalent ion permeation and open-channel block, but does play a significant role in channel gating.

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