Site within *N*-Methyl-D-aspartate Receptor Pore Modulates Channel Gating

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ABSTRACT

N-methyl-D-aspartate-type glutamate receptors (NMDARs) are ligand-gated ion channels activated by coagonists glutamate and glycine. NMDARs play a critical role in synaptic plasticity and excitotoxicity, largely because of their high calcium permeability and slow deactivation and desensitization kinetics. NR1 is an obligate subunit in all NMDAR complexes, where it combines with NR2A, 2B, 2C, and/or 2D. NR1 binds glycine, and residue Asn598 in the re-entrant membrane loop M2 largely determines NMDAR calcium permeability. In contrast, NR2 subunits bind glutamate and contain regions that regulate receptor desensitization and deactivation. Here, we report that mutations of NR1(Asn598) in combination with wild-type NR2A, expressed in human embryonic kidney 293 cells, exhibit altered glycine-independent desensitization. In the absence of extra-cellular calcium, substitution of Arg for Asn598 (NR1R) slowed desensitization by 2- to 3-fold compared with wild-type NR1/NR2A, and glutamate-evoked peak current EC_{50} and deactivation rate were also affected. Replacement of Asn by Gln (NR1Q) produced two distinct rates of calcium- and glycine-independent desensitization. Moreover, in the presence of extracellular calcium, the voltage-dependent pore block by calcium for the NR1Q mutant mimicked the effects of the positively charged Arg at this site in NR1R on slowing desensitization and deactivation. A kinetic model of the NMDA receptor-channel suggests that these results can be explained by altered gating and not ligand binding. Our data increase understanding of the role that amino acids within the NMDAR pore play in channel gating.

The highly Ca^{2+} permeable *N*-methyl-D-aspartate (NMDA) subclass of ionotropic glutamate receptors plays a critical role in synaptic plasticity as well as neuronal death in stroke and neurodegenerative disorders (Dingledine et al., 1999). Previous studies have shown that glutamate receptor desensitization is protective against excitotoxicity (Raymond et al., 1996 and references therein). Although NMDA receptors (NMDARs) desensitize more slowly and less extensively than α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors, NMDAR activity is limited during sustained glutamate stimulation by a variety of processes, including Ca^{2+}- and glycine-independent desensitization (reviewed by McBain and Mayer, 1994). N-terminal regions of the NR2 subunit that flank the S1 segment of the agonist-binding domain (Stern-Bach et al., 1994) are involved in this

form of desensitization, including the leucine/isoleucine/valine-binding protein-like domain and four amino acids immediately preceding the first membrane domain (M1) (Krupp et al., 1998; Villarroel et al., 1998). In addition, Ca^{2+}- and glycine-independent desensitization, as well as deactivation, of NMDAR-mediated current is modified by a point mutation of NR1 in the highly conserved motif (SYTANLAAF) (Kohda et al., 2000) that connects M3 with S2, which is also involved in agonist binding (Stern-Bach et al., 1994). In both cases, regions identified as modifying desensitization gating connect agonist-binding regions to membrane domains involved in pore formation, and these connector regions have been proposed to couple ligand binding and channel gating (Krupp et al., 1998; Villarroel et al., 1998; Jones et al., 2002).

On the other hand, residues within the NMDAR M2 segment of the pore region are generally viewed as sites for control of ion permeability. The Asn/Gln/Arg site, at the apex of the re-entrant M2 loop, contributes to forming the narrowest region of the glutamate receptor pore and is critical to controlling block and permeability of divalent ions (Burnashev et al., 1992, 1996; Mori et al., 1992; Sakurada et al.,

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; NR1Q, NR1(N598Q)/NR2A; NR1R, NR1(N598R)/NR2A; WT, wild type; HEK, human embryonic kidney; TPEN, *N,N,N′,N′*-tetrakis-[2-pyridylmethyl]-ethylenediamine; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid.

1993; Wollmuth et al., 1996) as well as single channel conductance (Behe et al., 1995). NMDARs have asparagine at this site and relatively high Ca^{2+} permeability, whereas non-NMDA-type glutamate receptors have either glutamine or arginine and lower or negligible Ca^{2+} permeability, respectively. Asn598 in NR1 makes the largest contribution to determining the high Ca^{2+} permeability of NMDARs. Interestingly, one study using single-channel recording showed that substitution of Gln at this site [NR1(N598Q)], combined with NR2A, could alter channel gating under bi-ionic conditions because of differential binding of monovalent ions in the permeation pathway (Schneggenburger and Ascher, 1997). Moreover, recent evidence from studies using the scanning cysteine accessibility method and trapping blockers suggests that Asn598, along with two amino acids in M3—Val626 and Thr630—in NR1 lie very near the channel activation gate, which is postulated to be formed by the M2 loop (Sobolevsky et al., 2002).

To further investigate a role for the Asn/Gln/Arg site deep within the pore in modulating NMDAR channel gating, we re-examined properties of receptors composed of NR2A together with NR1A containing Asn (wild-type), Gln, or Arg at position 598. Recombinant receptors were expressed in HEK293 cells to allow analysis of mutant receptors and facilitate ultra-fast solution exchange during whole-cell patch clamp recording. We report experimental and modeling evidence to support that the Asn/Gln/Arg site plays a critical role in channel closing and desensitization of the NMDAR.

Materials and Methods

Cell Culture and Transfection. Culture and transfection of HEK293 cells (American Type Culture Collection, Manassas, VA) were performed as described previously (Chen et al., 1997). Briefly, cells were passaged every 2 to 4 days. For Ca^{2+} phosphate transfection, cells were plated at a density of 1×10^6 cells/ml in 10-cm culture dishes (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). Cells were transfected with cDNAs encoding NR2A and either wild-type (WT) NR1A, NR1A(N598R), or NR1A(N598Q) at a ratio of 1:1. A total of 10 μg of plasmid cDNA was used for transfection of a 10-cm culture plate. After transfection, 1 mM (+)-2-amino-5-phosphonopentanoic acid (RBI/Sigma, Natick, MA) was added to the culture media and the cells were transferred onto glass coverslips in 35-mm culture plates (Falcon).

Electrophysiology. The whole-cell, patch-clamp recording technique and recording solutions for HEK293 cells were essentially the same as described previously (Chen et al., 1997). Twenty-four to 36 h after the start of transfection, the HEK293 cells were transferred to the recording chamber on the stage of an inverted microscope (Aviovert 100; Carl Zeiss, Thornburg, NY). Agonist-evoked currents were recorded in the whole-cell mode under voltage clamp ($V_H = -60$ mV) at room temperature ($\sim22°C$). Electrodes with open tip resistances of 1 to 5 MΩ were used. After establishing the whole-cell mode, cells were lifted from the coverslip. Ultrafast application of agonists was achieved by a piezo-driven θ-shaped tube (Hilgenburg, Malsfeld, Germany) (Chen et al., 1997). Control and agonist solutions were continuously gravity-fed through the two sides of the θ-shaped tube. Solution exchange time (10-to-90% rise time) across the open tip of the recording electrode was 0.2 ms. The extracellular recording solution contained 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 11 mM glucose, and 10 mM HEPES, titrated to pH 7.35 with NaOH. EGTA or *N,N,N′,N′*-tetrakis-[2-pyridylmethyl]-ethylenediamine (TPEN) was added to the external solution in some experiments to avoid contamination by zinc, as indicated. In all experiments, the external solution contained no added Mg^{2+} to enhance

NMDAR-mediated currents at negative holding potentials, and 50 μM glycine was added to both control and glutamate-containing solutions. The intracellular recording solution contained 145 mM KCl, 10 mM HEPES, 5.5 mM BAPTA, 4 mM MgATP, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, and 2 mM tetraethylammonium, titrated to pH 7.2 with NaOH. Currents were sampled at 2 kHz and acquired and analyzed using pCLAMP software and the Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Results are expressed as the mean $\pm$ S.E.M. For calculation of EC_{50} values for both experiments and simulations, the amplitude of the glutamate-evoked current was measured at the peak of the response. Peak response EC_{50} was calculated from the equation: $I = I_{max} \times [1 / (1 + (EC_{50}/[A])^{n_H})]$, where I is the measured peak current amplitude, I_{max} is the maximum peak current amplitude (evoked by 1 mM glutamate), [A] is the agonist concentration, and n_H is the Hill coefficient.

Site-Directed Mutagenesis. Mutant NR1A cDNAs were generated by site-directed mutagenesis of wild-type NR1A cDNA as described previously (Raymond et al., 1996). The antisense primers 5′-CCCAATGCCGGACTGGAGCAGGACGCCC-3′ and 5′-CCCAAT-GCCGGGACCGGAGCAGGACGCCC-3′ were used to generate NR1(N598Q) and NR1(N598R), respectively (single letter amino acid code, mature protein).

Simulation and Modeling. For simulation of NMDAR activation, we began by modifying previous kinetic models (Clements and Westbrook, 1991; Hessler et al., 1993; Destexhe et al., 1998; see also Chen et al., 2001). Because there was no Mg^{2+} in our extracellular recording solutions, Mg^{2+} block of NMDARs was not taken into consideration in the modeling. The NMDAR current was described by the product of maximal conductance (g_{NMDA}), the driving force, and open probability P_o: $I_{NMDA} = g_{NMDA} \times P_o \times (V - E_{NMDA})$. The maximal conductance g_{NMDA} was set to 0.05 nS in all simulations, and the reversal potential E_{NMDA} was 0 mV (Destexhe et al., 1998). The model assumed two (independent) glutamate binding sites that must both be occupied for the channel to open [giving three different closed states (C0, C1, and C2), one desensitized form of the receptor (D1), and an open state (O)], as we proposed previously (Chen et al., 2001) (Fig. 4A). In this model, R_b is the binding rate of glutamate to the NMDAR, R_u is the unbinding rate of glutamate, R_o is the opening rate of the double-liganded receptor, R_c is the closing rate, R_d is the desensitization rate, R_r is the resensitization rate, and [A] indicates agonist concentration. R_d and R_r were set based on experimentally measured values; R_u, R_b, and R_o were assigned values based on work published previously (Chen et al., 2001); and R_c was adjusted until the resulting model current response matched the peak glutamate EC_{50}, 10-to-90% rise time, I_{ss}/I_p, and deactivation time constant measured for the NMDAR mutant with a 3-s application of glutamate. We performed our simulation using NEURON (version 4.1.1) (Hines and Carnevale, 1997) on a PII-450 PC, using a 5- to 100-μs time step (depending on the kinetic model used).

Results

NMDARs composed of NR2 subunits combined with mutant NR1A containing Gln or Arg instead of Asn at position 598 in the pore exhibit decreased or negligible Ca^{2+} permeability, respectively (Burnashev et al., 1992; Sakurada et al., 1993). These mutant receptors have been used in previous studies to discern the role of NMDAR-mediated Ca^{2+} influx in a variety of cellular processes as well as to investigate the stoichiometry of the NMDAR complex (Behe et al., 1995). In this study, we re-examined the properties of NMDARs composed of NR2A and NR1A compared with NR2A and mutant NR1A(N598Q) or NR1A(N598R) (referred to as NR1Q or NR1R, respectively) to determine whether mutations at this site in the pore could affect other receptor channel properties, such as gating.

Wild-type NR1/NR2A (WT)-mediated currents evoked by a 3-s application of 1 mM glutamate (in the presence of saturating glycine of 50 μM) showed significant desensitization when recorded in 1.8 mM extracellular Ca^{2+} (Fig. 1A). Ca^{2+}-dependent inactivation (reviewed by McBain and Mayer, 1994) and the acceleration of agonist-induced desensitization by ambient zinc (Chen et al., 1997; Zheng et al., 2001) both may contribute to the rapid rate and large extent of current decay during sustained application of this saturating agonist concentration. When the same cell was superfused with external solution containing no added Ca^{2+} and 1 μM TPEN or 5 mM EGTA to chelate residual Ca^{2+} and zinc, desensitization was characterized by a single exponential decay with a time constant of 1.6 s and high steady state-to-peak current
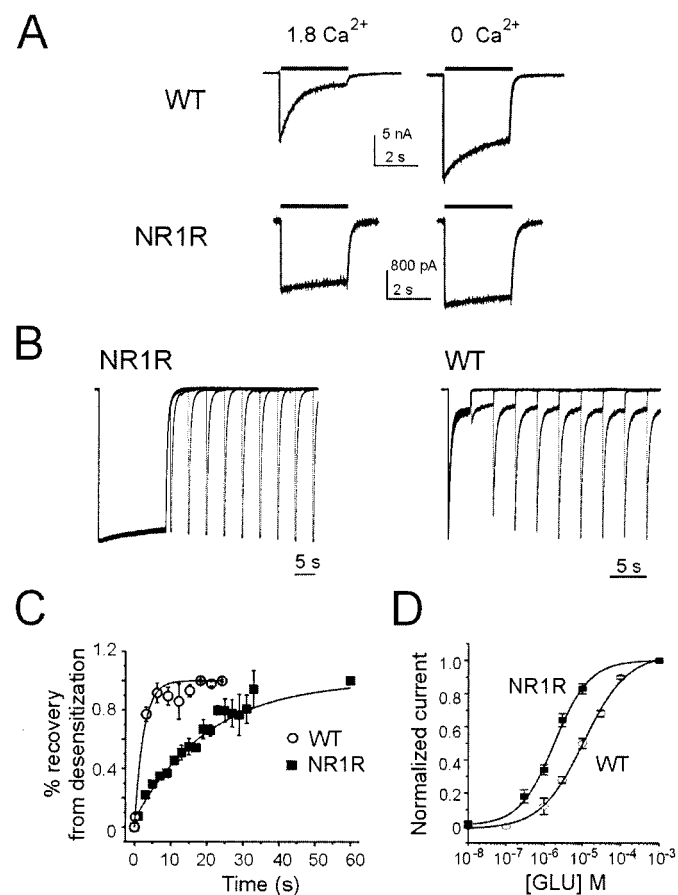

Fig. 1. NR1R shows altered gating and agonist potency. A, currents evoked by 1 mM glutamate with 50 μM glycine (indicated by bars) for WT (top) and NR1R (bottom) in the presence or absence of external Ca^{2+}, as indicated. Traces shown at right were recorded in presence of 1 μM TPEN. B, currents evoked by paired pulses of glutamate recorded from NR1R or WT illustrating time course of recovery from desensitization. [Glu] and [Gly] same as in A. Recordings were made in absence of Ca^{2+} and with 1 μM TPEN in extracellular solution. Test pulses were applied at intervals ranging from 1 s up to 60 s after initial (control) pulse. C, plot of time course for recovery from desensitization, where 0% represents current amplitude at end of desensitizing (control) pulse and 100% indicates recovery to initial current amplitude for control pulse. Points indicate mean $\pm$ S.E.M. from $n = 8$ and 5 different cells transfected with NR1R and WT, respectively. All recordings were made under conditions described in B. Data fitted to a single exponential. D, concentration-response curve for peak glutamate-evoked current. [Gly] was 50 μM. Recordings were made in absence of Ca^{2+} and with zinc chelator (see *Materials and Methods*) in extracellular solution. Points are mean $\pm$ S.E.M. for $n = 4$ to 7 different cells. Data fitted to the Hill equation (see *Materials and Methods*).

ratio (I_{ss}/I_p) at the end of the agonist application (Fig. 1A; Table 1), similar to previously published data (Zheng et al., 2001). This process represents agonist-induced Ca^{2+}-, Zn^{2+}-, and glycine-independent desensitization (Krupp et al., 1998; Zheng et al., 2001).

N598R Mutation Slows Agonist-Induced Desensitization, Deactivation, and Shifts the Glutamate EC_{50}. In contrast to wild-type receptors, currents recorded from cells expressing the Ca^{2+}-impermeable NR1R showed no Ca^{2+}-dependent inactivation (Fig. 1A). Strikingly, these currents also showed minimal agonist-induced desensitization during the 3-s application of 1 mM glutamate in the presence of a zinc chelator and a saturating glycine concentration (Fig. 1A; Table 1). Furthermore, recovery from desensitization for this mutant receptor was also dramatically slowed (Fig. 1, B and C; Table 1; $P < 0.0001$ by two-way analysis of variance). On the other hand, currents recorded in the absence of a zinc chelator exhibited substantial desensitization (I_{ss}/I_p of 0.68 $\pm$ 0.06, τ_D of 1.4 $\pm$ 0.3 s, $n = 4$; data not shown); this increase in rate and extent of desensitization was qualitatively similar to effects seen for wild-type NR1A/NR2A (Chen et al., 1997; Zheng et al., 2001) caused by contamination of the external solution by nanomolar zinc. This effect could be explained by the allosteric interaction between the zinc and glutamate binding sites (Zheng et al., 2001), independent of desensitization gating. Taken together, these results suggest that the positively charged arginine residue within the pore of the NR1R mutant channels might directly interfere with the desensitization gate.

In addition to the decreased rate and extent of desensitization found for the mutant receptor NR1R, we observed a marked slowing of the 10-to-90% rise time to peak and of current deactivation with offset of the glutamate pulse (Table 1). These results suggested that the NR1 pore mutation might also alter the EC_{50} for glutamate (measured using 3-s agonist applications). To test this possibility, we constructed concentration-response curves comparing NR1R with wild type. The mutant receptors showed higher sensitivity to glutamate with a peak current EC_{50} of 1.9 μM compared with 7.7 μM for wild type (Fig. 1D; Table 1). Because the potency of glutamate for activating NMDAR currents reflects the combined processes of receptor binding and channel gating (Colquhoun, 1998), the leftward shift in EC_{50} indicates that the pore mutation alters glutamate binding affinity and/or influences the gating process.

Distinct Two-Component Macroscopic Desensitization of NR1Q. The idea that a mutation at Asn598 of NR1 alters channel gating was further supported by analysis of currents mediated by the partially Ca^{2+}-permeable mutant NR1Q. Unlike either wild type or NR1R, these currents showed an apparently rapidly desensitizing component in addition to the slower desensitization when recorded in the presence of a zinc chelator (1–2 μM TPEN) and saturating glycine (Fig. 2A). The rapidly decaying component of the current response to sustained agonist application was consistent with a model for the NR1Q proposed by Schneggenburger and Ascher (1997), in which extracellular sodium favors rapid transition from the main to the subconductance state upon channel opening, leading to apparent macroscopic desensitization (see *Discussion*). Interestingly, the slowly decaying component of macroscopic desensitization was modulated by extracellular Ca^{2+} in an unexpected manner. In the

absence of extracellular Ca^{2+}, this component resembled desensitization of wild-type receptors ($P > 0.05$ by unpaired t test), whereas in the presence of Ca^{2+} (1.8 mM), the rate and extent were strikingly attenuated and resembled those of NR1R ($P > 0.05$ by unpaired t test)(Fig. 2A; Table 1). The rate of current deactivation upon glutamate withdrawal was also influenced by extracellular Ca^{2+}—slow, as for NR1R, in 1.8 mM $CaCl_2$ and more rapid, similar to WT, in the absence of Ca^{2+} (Fig. 2B; Table 1). Unlike the NR1R, however, in the presence of external Ca^{2+}, the glutamate peak current dose-response curve as well as the 10-to-90% rise-time to peak current for NR1Q were similar to those measured for wild-type receptors (Table 1). On the other hand, recovery from slow desensitization for NR1Q occurred at a rate significantly slower than that measured for WT ($P < 0.0001$, 2-way ANOVA), even in the absence of extracellular Ca^{2+} (Fig. 2, C and D; Table 1).

Pore Block by Ca^{2+} Influences Desensitization and Deactivation Rates for NR1Q. It is known that extracellular Ca^{2+} can bind within the pore and block ion flux through NR1Q in a voltage-dependent manner (Premkumar and Auerbach, 1996). Because onset of slow agonist-induced desensitization, as well as deactivation, in the absence of external Ca^{2+} was similar to that of wild type NR1/NR2A but was not statistically different from that of NR1R in the presence of Ca^{2+}, we hypothesized that Ca^{2+} binding to a site within the pore of NR1Q interfered with channel gating. The effects on gating would only occur after channel opening, which may, in part, explain the lack of effect of external Ca^{2+} on the NR1Q rise time and peak current EC_{50}. This hypothesis could be extended to suggest that the slowed desensitization and deactivation of NR1R currents result from the positively charged arginine residue mimicking the effect of Ca^{2+} binding in the pore of NR1Q.

To test whether Ca^{2+} entry into and/or flux through the pore of NR1Q was necessary to reduce the rate of slow-onset agonist-induced desensitization, we analyzed desensitization of current responses to glutamate at a series of holding potentials ranging from -100 to $+100$ mV in the presence of 1.8 mM $CaCl_2$ and 1 to 2 μM TPEN (Fig. 3A). These channels show voltage-dependent block by external Ca^{2+} (Fig. 3A) and anomalous whole-cell current reversal at holding potentials near 0 mV (not shown), as described previously (Premkumar

and Auerbach, 1996; Schneggenburger and Ascher, 1997). As expected, the mean time constant for slow desensitization calculated from current responses at hyperpolarized potentials (-100 to -40 mV) was similar to the mean time constant measured previously from current responses at a holding potential of -60 mV (Fig. 3, A and B). However, the rate of slow, agonist-induced desensitization was significantly accelerated at depolarized holding potentials ($+40$ to $+100$ mV), where Ca^{2+} influx and pore block are dramatically diminished, although it was not as fast as that observed at -60 mV for wild-type receptors or NR1Q in the absence of external Ca^{2+} (Fig. 3, A and B; Table 1). These results support the hypothesis that Ca^{2+} binding to a site within the pore of NR1Q (Premkumar and Auerbach, 1996) contributes to the slowing of agonist-induced desensitization.

Results of Modeling Consistent with Effect of Pore Mutations on Channel Gating. We reasoned that the effects of the pore mutations on glutamate-evoked current deactivation and steady-state to peak ratio, as well as that of NR1R on the rise-time and glutamate concentration-response curve, could all be accounted for by altered rates of channel gating without any changes in ligand unbinding rate. Because the kinetic scheme for gating of NR1Q was complicated by asymmetric gating under bi-ionic (external Na^+ and internal K^+) conditions, which was previously modeled by Schneggenburger and Ascher (1997), we tested this hypothesis only for NR1R, using a standard model to describe NMDAR ligand binding and channel gating (Fig. 4A; see *Materials and Methods*). For this model, the rates of desensitization and resensitization were set to the values measured experimentally for NR1R (Table 1), and we initially set the glutamate binding and unbinding rates to the values used to model wild-type NR1/NR2A (Chen et al., 2001). We then varied channel opening and closing rates from the rates used for WT (Chen et al., 2001) until the model traces fit our data for the shifted glutamate EC_{50}, extent of desensitization at the end of a 3-s pulse of 1 mM glutamate, and the slowed 10-to-90% rise time to peak and rate of current decay upon withdrawal of glutamate. We found that a ~10-fold reduction in the channel closing rate, with no change in channel opening rate (Table 2), resulted in a model current response that closely approximated the experimental data (Fig. 4, B and C): the model current showed a rise-time of 13 ms, deactivation

TABLE 1
Biophysical properties of NR1R, NR1Q and WT receptor currents

Results from 3-s applications of 1 mM glutamate/50 μM glycine with 1–2 μM TPEN. n = 6 to 15 different cells for all kinetic parameters; n = 4–7 different cells for EC_{50} measurements.

	NR1R[a]	WT[b]	NR1Q 0 Ca^{2+}	NR1Q 1.8 mM Ca^{2+}
10–90% Rise time (ms)	13.4 ± 0.3	6.4 ± 0.2	6.7 ± 0.4	6.8 ± 0.3
τ_D (ms)				
τ_{Df}			54 ± 6.5 (32%)	29 ± 3 (67%)
τ_{Ds}	4040 ± 680 (100%)	1610 ± 110 (100%)	1400 ± 110 (68%)	5770 ± 1580 (33%)
τ_{resens} (s)	20.2 ± 0.70	2.42 ± 0.27	14.3 ± 0.54	N.D.
I_{ss}/I_{peak}	0.93 ± 0.01	0.74 ± 0.03	0.54 ± 0.04	0.63 ± 0.03
τ_{off} (ms)				
$\tau_{off,f}$	170 ± 17 (61%)	56 ± 9 (89%)	89 ± 11 (100%)	122 ± 13 (62%)
$\tau_{off,s}$	611 ± 42 (39%)	309 ± 49 (11%)		539 ± 45 (38%)
EC_{50} (μM; peak current):	1.9 ± 0.2	7.7 ± 0.8	N.D.	8.1 ± 0.5

N.D. not determined.
[a] Time course of glutamate-evoked response was not different for individual NR1R-expressing cells switched between external solutions containing no added extracellular Ca^{2+} versus 1.8 mM $CaCl_2$, therefore these data were pooled.
[b] Results from experiments with no added extracellular Ca^{2+} for WT.

time constant of 360 ms (the weighted average time constant from our data was 342 ms), steady-state to peak current ratio of 95%, and EC_{50} of 1.75 μM (see Table 1 for comparison with experimental values).

To determine whether a change in ligand unbinding rate, without any alteration in channel opening or closing rates, could also reproduce our data, we reduced the unbinding rate by 5- to 8-fold. Compared with the experimental values for NR1R, a 5-fold rate reduction produced a similar shift in EC_{50} but a deactivation rate that was markedly faster (220 ms), whereas an 8-fold reduction reproduced the deactivation rate (350 ms) but shifted the EC_{50} to less than 1 μM; both

manipulations resulted in a steady state-to-peak current ratio of 72%, indicating markedly more extensive desensitization than that observed experimentally. For comparison, we also modeled WT, using the rates of desensitization and resensitization measured experimentally in the absence of external Ca^{2+} or zinc; the binding/unbinding as well as channel opening/closing rates were the same as we had used previously (Chen et al., 2001) (Fig. 4, B and C; Table 2). Again, the rise-time (7 ms), deactivation time constant (45 ms), steady state-to-peak current ratio (63%), and EC_{50} (7 μM) were all similar to our experimental data (see Table 1). In all, these results are consistent with a model in which the positively charged arginine residue near the external mouth of the pore of NR1R interferes with both closing and desensitization gating of the channel. Moreover, the leftward shift in the glutamate concentration-response curve could be explained by these effects on channel gating, without any modification in ligand binding or unbinding rates.

Discussion

In this study, we have demonstrated that mutation of NR1 Asn598, a pore residue controlling ion permeation and open channel block, modulates channel gating of NMDA receptors. Furthermore, our data strongly suggest that slowing of desensitization and deactivation gating for the NR1 mutants is
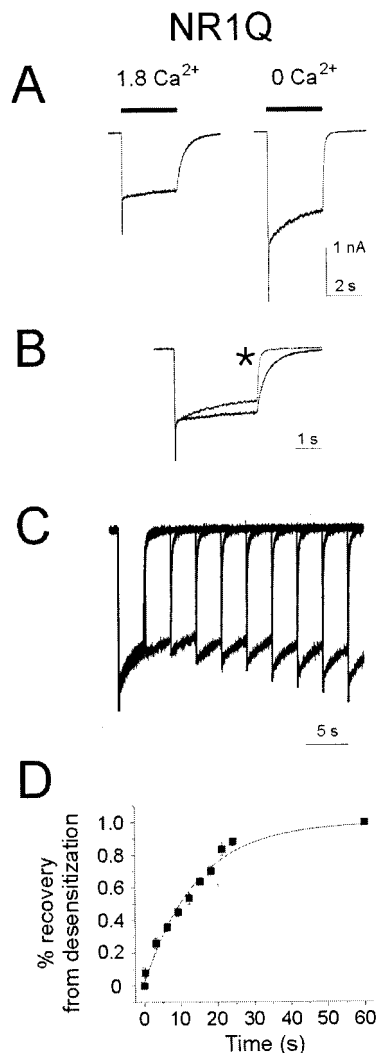

Fig. 2. NR1Q shows altered gating that is modulated by external Ca^{2+}. A, current evoked by 1 mM glutamate plus 50 μM glycine in presence or absence of external Ca^{2+} recorded from same NR1Q-transfected cell. B, same traces shown in A but scaled to match peak amplitude, better illustrating difference in rate of desensitization and deactivation in presence versus absence (*) of external Ca^{2+}. C, currents evoked by paired pulses of glutamate (1 mM, plus 50 μM glycine) recorded from NR1Q-transfected cell in absence of external Ca^{2+}. Test pulses were applied at intervals ranging from 1 s up to 60 s after initial (control) pulse. D, plot of time course of recovery from desensitization for $n = 11$ different NR1Q-transfected cells, recorded in absence of external Ca^{2+}. 0% represents current amplitude at the end of the desensitizing (control) pulse, and 100% indicates recovery to initial current amplitude for control pulse. Data fitted to a single exponential. All recordings in A to D were made with zinc chelator in extracellular solution (see *Materials and Methods*).
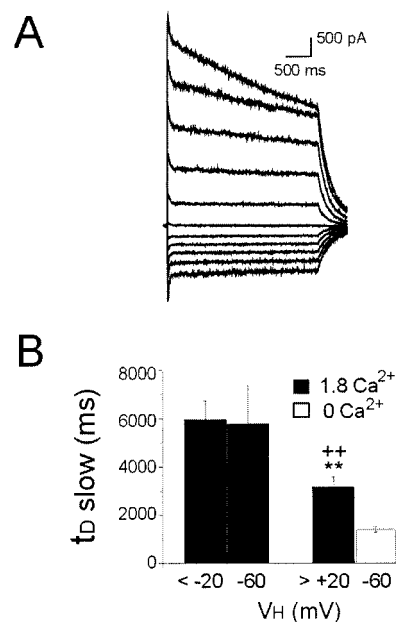

Fig. 3. Ca^{2+}-mediated pore block slows desensitization of NR1Q. A, Representative current-voltage relation recorded from NR1Q-transfected cell in presence of 1.8 mM $CaCl_2$. Holding potential was -60 mV, with jumps to voltages ranging from -100 to $+100$ mV in increments of 20 mV during 3-s applications of 1 mM glutamate in presence of 50 μM glycine. Note increasing peak of outward current (relative to driving force), as well as rate of desensitization, with depolarization to positive potentials. B, mean desensitization time constant of glutamate-evoked currents recorded in presence of external 1.8 mM $CaCl_2$ is significantly smaller for pooled data from holding potentials of $+40$ to $+100$ mV compared with -100 to -40 mV ($n = 4$ different cells; **, $P < 0.01$), and approaches, though is still significantly higher than (††, $P < 0.01$), that measured for currents recorded in the absence of $CaCl_2$ at $V_H = -60$ mV ($n = 11$ different cells). Note that mean time constant in 1.8 mM $CaCl_2$ determined from pooled data at holding potentials ranging from -100 to -40 mV matches that measured at -60 mV ($n = 15$ different cells; $P = 0.92$). Recordings were made in presence of extracellular 1 to 2 μM TPEN.

caused by the presence of a positively charged ion at the NR1 Asn598 site. In addition, we have shown a leftward shift of the glutamate concentration-response for NR1R, consistent with previous studies of NR1 pore mutations N598R or A635T expressed with NR2B or NR2A, respectively (Kashiwagi et al., 1997; Kohda et al., 2000). Using a kinetic model, we confirm that this effect results from an alteration in channel gating rather than a more global change in receptor conformation that affects ligand binding.

There is strong evidence indicating that Asn598 in NR1 contributes to controlling the narrow pore constriction involved in determining selective ion permeability and block and single channel conductance (Burnashev et al., 1992; Mori et al., 1992; Sakurada et al., 1993; Behe et al., 1995; Wollmuth et al., 1996); however, the influence of NR1 Asn598 on NMDAR macroscopic desensitization or channel closing has not been explored. On the other hand, a variety of data indicates that residues lining the pore are involved in con-

Fig. 4. A kinetic model suggests changes in channel desensitization and closing rate account for differences in macroscopic current kinetics and agonist potency between NR1R and WT. A, state diagram used to model NMDAR channel behavior. B, traces generated using parameters shown in Table 2 to model current response to 3-s application of 1 mM glutamate (+ 50 μM glycine) for NR1R or WT NMDAR. C, concentration-response curve for peak glutamate-evoked current (in 50 μM glycine) based on model simulation and fitted to Hill equation.

TABLE 2
Parameters used to model NMDAR current

Receptor	R_b	R_u	R_d	R_r	R_o	R_c
	$\mu M^{-1}s^{-1}$	s^{-1}	s^{-1}	s^{-1}	s^{-1}	s^{-1}
N598R	5	25	0.25	0.05	150	12
WT	5	25	0.62	0.41	150	150

formational changes that couple agonist binding to opening of the channel activation gate. For example, the region in and around amino acids within the NR1 C-terminal segment of M3 (M3$_c$), including the highly conserved SYTANLAAF (Lurcher) motif, contributes to lining the channel in the wide, external vestibule (Beck et al., 1999) and largely determines the effect of protons on channel gating (Low et al., 2003). As well, recent data suggest that the walls of this external vestibule constrict in the channel's closed state but do not form the activation gate (Sobolevsky et al., 2002). Moreover, the Lurcher mutation in the SYTANLAAF motif of NR1 (A635T − NR1^{Lc}) when coexpressed with wild-type NR2A shows marked slowing of deactivation and glutamate-induced desensitization, a 10-fold leftward shift in the glutamate dose-response curve, and a markedly slower channel closing rate (by noise analysis) compared with wild-type NR1/NR2A (Kohda et al., 2000), results that closely match our data for NR1R.

Results of previous studies have also suggested a role for M2 in channel gating. State-dependent accessibility of a substituted cysteine in NR1(N598C) supports a role for this site in gating (Kuner et al., 1996). Another study also used the cysteine accessibility method to compare modification rates of NR1 residues in the external vestibule as well as deeper in the pore (including N598C in M2), in the ligand-bound versus unbound state, with or without the presence of a channel blocking agent; from these data, the authors proposed that the activation gate lies deep within the pore, near the level of Asn598 (Sobolevsky et al., 2002). Consistent with this idea, mutation of the tryptophan five residues toward the N terminus from the Asn/Gln/Arg site (and closer to the internal mouth of the channel) in M2 of both NR1 and NR2A resulted in a change in NMDA channel open probability (Buck et al., 2000). Our data, combined with a kinetic model, demonstrate that mutation of NR1 Asn598 to the positively charged arginine, or Ca^{2+} bound in the pore with a glutamine substitution at this site, significantly slows onset of desensitization as well as channel closing. It may be that the activation gate is formed by the M2 loop in NMDARs, similar to the role of the P loop in gating for cyclic nucleotide-gated channels (Sun et al., 1996).

It has frequently been noted that ionotropic glutamate receptors show significant structural homology with voltage-gated potassium channels in the pore region (MacKinnon, 1995; Wo and Oswald, 1995; Wood et al., 1995); in both families, the pore is formed by a re-entrant loop (M2 for glutamate receptors and the P loop for potassium channels) along with contributions from transmembrane segments (M1, M3, and M4 for NMDARs and S5 and S6 for K$^+$ channels). Previous studies have shown that pore mutations in *Shaker* K$^+$ channels can alter rates of channel closing and C-type inactivation (Molina et al., 1998; Rasmusson et al., 1998). Notably, the latter is thought to be mediated by direct constriction (or "collapse") of the pore itself, and this process can be slowed by occupation of the pore by permeant ions (Kiss and Korn, 1998; Molina et al., 1998; Rasmusson et al., 1998).

Similar to the data for C-type inactivation of potassium channels, previous studies have indicated that occupation of the NMDAR pore by organic cations or other channel-blocking agents alters macroscopic gating. NMDARs composed of NR2A and mutant NR1 in which cysteine was substituted at
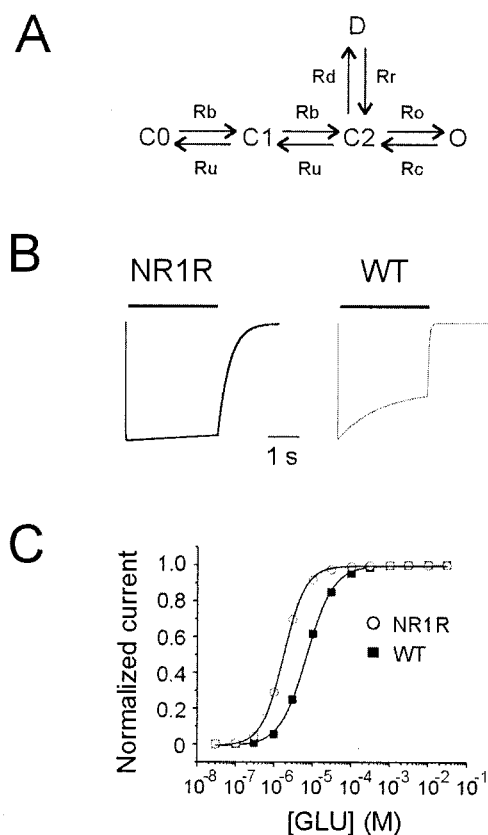
Ala634 (one residue before the Lurcher site) exhibited markedly slowed deactivation after modification with methanethiosulfonate ethylammonium (Jones et al., 2002). In other experiments using a series of organic ions of different sizes that bind to and produce rapid block of the open NMDAR channel, it was shown that the activation/deactivation and desensitization gates are near the external entrance of the narrow constriction of the pore (i.e., at the level of Asn598), because some of these agents prevented desensitization and/or deactivation (Sobolevsky et al., 1999). Similarly, occupation by adamantane derivatives of a binding site in the external vestibule of NMDARs prevented channel closure (Antonov and Johnson, 1996). These studies together with our data are consistent with a model in which the presence of a cation or blocking molecule at or near the narrow pore constriction of NMDARs markedly slows or prevents channel closing and/or desensitization.

An elegant study by Schneggenburger and Ascher (1997) demonstrated that NR1Q shows gating asymmetry under bi-ionic conditions. Specifically, in recordings of agonist-evoked current responses in solutions with Na^+ as the primary external monovalent cation and Cs^+ as the internal cation, they observed preferential cycling of channels from closed to main conductance to subconductance and back to closed states. If we assume that internal Cs^+ and K^+ act similarly in the model proposed by these authors, then the rapidly decaying macroscopic current we observed in response to sustained agonist application could represent initial opening of channels to the main conductance state followed by a shift toward preferential occupation of the subconductance state as Na^+ replaces K^+ at a binding site internal to the narrow constriction within the pore (Schneggenburger and Ascher, 1997). Therefore, the appearance of two distinct components of macroscopic desensitization that we observed probably represents a rapid gating shift from main to subconductance states of the channel, followed by entry into the desensitized state.

It is interesting to note that although glycine-independent desensitization is determined largely by extracellular structural regions in NR2 subunits (Krupp et al., 1998; Villarroel et al., 1998), mutations of a pore residue in NR1 can modify this process as well as deactivation gating. This result is consistent with a model in which extracellular domains of glutamate receptors involved in ligand binding adopt distinct conformations in the closed, open, and desensitized states (Sun et al., 2002), but movement of these extracellular domains is transduced by connector regions to alter the conformation of regions deep within the pore that gate ion flux. The precise nature of the conformational changes associated with gating that occur around the level of Asn598 in the pore remains to be determined.

Acknowledgments

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