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Probing *N*-Methyl-D-aspartate Receptor Desensitization with the Substituted-Cysteine Accessibility Method

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ABSTRACT

Several forms of macroscopic *N*-methyl-D-aspartate (NMDA) receptor desensitization affect the amplitude and duration of postsynaptic responses. In addition to its functional significance, desensitization provides one means to examine the conformational coupling of ligand binding to channel gating. Segments flanking the ligand binding domain in the extracellular N terminus of the NMDA receptor NR2 subunit influence the glycine-independent form of desensitization. The NR2A pre-M1 region, the linker between the glutamate binding domain and the channel pore, plays a critical role in desensitization. Thus, we used the substituted-cysteine accessibility method to scan the accessibility of residues in the pre-M1 region and the first transmembrane domain (M1) of NR2A. Cysteine mutants were expressed with NR1 in human embryonic kidney 293 cells and were assayed by whole-cell recording. With activation of the receptor by glutamate and glycine, only a single mutant, V557C, which is located at the beginning of M1, led to irreversible inhibition by the methanethiosulfonate derivative methanethiosulfonate ethyltrimethylammonium (MTSET). The NR2 ligand glutamate was insufficient on its own to induce modification of V557C by MTSET, suggesting that the change in accessibility required channel gating. The rate of MTSET modification of the homologous residue on NR1 (NR1-1a$_{L562C}$/NR2A) was much slower than V557C. We also substituted cysteine in the V557 site of mutant subunits that exhibit either enhanced or reduced desensitization. Modification by MTSET correlated with the degree of desensitization for these subunits, suggesting that V557C is a sensitive detector of desensitization gating.

The kinetics of NMDA receptors play an important role in shaping postsynaptic responses (Lester and Jahr, 1992; Jones and Westbrook, 1996; Qian and Johnson, 2002). Although intrinsically silent, desensitized states can have significant actions on receptor gating (Jones and Westbrook, 1996). NMDA receptor desensitization can be divided into three forms with distinct underlying mechanisms: calcium-dependent, glycine-dependent, and glycine-independent (McBain and Mayer, 1994). Depending on the kinetics, desensitization can either accelerate or prolong the duration of a synaptic response. We focus here on the glycine-independent form. NMDA receptor channels are believed to desensitize directly from the closed, agonist-bound state (Colquhoun and Hawkes, 1995); thus, glycine-independent desensitization represents a separate closed, bound conformation. This form of desensitization contributes to the decay of excitatory postsynaptic currents and reduces NMDA receptor-mediated responses during high-frequency synaptic stimulation (Lester and Jahr, 1992). Thus, understanding the conformational changes associated with desensitization is ultimately important for understanding synaptic signaling.

NMDA receptors are tetramers composed of two glycine-binding NR1 and two glutamate binding NR2 (A–D) sub-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; M1, first transmembrane domain; MTSET, methanethiosulfonate ethyltrimethylammonium; HEK, human embryonic kidney; LIVBP, leucine/isoleucine/valine-binding protein; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SCAM, substituted-cysteine accessibility method; MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; ANOVA, analysis of variance; TPentA, tetrapentylammonium; 9-AA, 9-aminoacridine; CaN, calcineurin.

units, possibly aligned in a 1-1-2-2 order (Clements and Westbrook, 1991; Schorge and Colquhoun, 2003; Inanobe et al., 2005). In some cell types, NR3 subunits can be incorporated into NMDA receptors (Chatterton et al., 2002). The topology of NMDA receptor subunits consists of a large, N-terminal extracellular domain, four hydrophobic domains (M1–M4), and a cytoplasmic C-terminal domain (Mayer and Armstrong, 2004; Wollmuth and Sobolevsky, 2004). M2 is a re-entrant P-loop that lines the channel. The first 400 amino acids of the N-terminal domain are homologous to the leucine/isoleucine/valine-binding protein (LIVBP-like domain) followed by the agonist-binding S1 domain and a short stretch of amino acids linking the S1 and M1 domains—the pre-M1 region. Based on crystal structures of NMDA receptor subunits, ligand-binding sites of NMDA receptors operate like two lobes of a clamshell composed of the S1 domain on the N-terminal domain and the S2 domain in the extracellular loop between M3 and M4 (Furukama et al., 2005) A short segment linking M3 with the S2 domain is highly conserved among ionotropic glutamate receptors and influences channel gating (Kohda et al., 2000; Jones et al., 2002). Recent crystal structures have confirmed a clamshell arrangement for the glycine-binding pocket of the NR1 subunit (Furukawa and Gouaux, 2003). Studies using the substituted-cysteine accessibility method (SCAM) on NR1 and NR2C subunits suggest that the structure of the pore resembles an inverted potassium channel with a large extracellular vestibule surrounding the pore (Beck et al., 1999; Kuner et al., 2003). It has also been suggested that the extracellular vestibule is involved in activation and desensitization (Sobolevsky, 1999; Sobolevsky et al., 1999). However, little is known about the conformational changes that couple ligand binding to channel gating.

Kinetic models indicate that when bound by two molecules of glutamate and two molecules of glycine, NMDA receptor channels have two main options (other than unbinding): they can open or desensitize. The glycine-independent form of NMDA receptor desensitization is prominent in receptors containing the NR2A or NR2B subunit. Sobolevsky et al. (1999) proposed a physical model in which glycine-independent desensitization occurs because the channel contains a desensitization "gate" that is physically distinct from the activation "gate" (Sobolevsky et al., 1999). However, mutations in several regions of the NR2A subunit alter desensitization in NR1/NR2A-containing receptors. These regions include the LIVBP-like domain, the pre-M1 region, the lurcher site in the third transmembrane domain, and a methionine (Met823) in the fourth transmembrane domain (Krupp et al., 1998; Villarroel et al., 1998; Kohda et al., 2000; Zheng et al., 2001; Ren et al., 2003). Similar results have been obtained by introducing mutations in the P-loop (Asn598) or the lurcher site of NR1 (Kohda et al., 2000; Chen et al., 2004). The pre-M1 region of NR2A is a particularly attractive candidate for coupling ligand binding to channel gating because it links the glutamate-binding S1 domain to the channel. Studies showing that the pre-M1 region influences desensitization are consistent with this idea (Krupp et al., 1998; Villarroel et al., 1998; Sobolevsky et al., 2002).

We used substituted-cysteine mutagenesis to screen the accessibility of amino acids located in and around the pre-M1 region. Cysteine-substituted NR2A subunits were coexpressed in HEK293 cells with NR1. Whole-cell recordings were used to determine the accessibility of the substituted cysteines. Of the residues screened, MTS reagents modified two mutant NR2A subunits: $NR2A_{A548C}$ and $NR2A_{V557C}$. $NR2A_{V557C}$ modification required the presence of glutamate and glycine; thus, it detected channel gating. Using modified NR2 subunits with varying amounts of desensitization, we found that the accessibility of V557C correlated with desensitization.

Materials and Methods

Molecular Biology. All cDNAs encoding NMDA receptor subunits were cloned in pCDNA1/amp (Invitrogen, Carlsbad, CA). Clones used were NR2A (accession number D13211; Ishii et al., 1993) and NR1-1a (accession number U0826; Hollmann et al., 1993). The $NR1\text{-}1a_{stop838}$ (Krupp et al., 1998) and $NR2A_{stop844}$ (Krupp et al., 2002) truncation mutants and NR2 chimeras $2C_0A$, AD1, and D001/AD1 have been described previously (Krupp et al., 1998). Point mutants were generated using gene splicing by overlap-extension polymerase chain reaction with Pfu Polymerase (Stratagene, La Jolla, CA; Horten et al., 1989). DNAs generated by polymerase chain reaction were sequenced. Amino acids are numbered in accordance with the methods used by Ishii et al. (1993). Lymphocyte CD4 receptor cDNA was cloned in a JPA vector kindly provided by Dr. John Adelman (Vollum Institute, Oregon Health and Science University, Portland, OR).

Cell Culture and Transfection. HEK293 cells (American Type Culture Collection, Manassas, VA; or Invitrogen) grown in Dulbecco's modified Eagle's medium (Invitrogen), 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 3 mM kynurenic acid (Sigma, St. Louis, MO), 1% glutamine, and 1% penicillin-streptomycin (Invitrogen; 37°C, 5% CO_2) were plated onto 35-mm poly(lysine)-coated glass coverslips 3 to 6 h before transfection. Cells were transfected for 12 to 18 h in the presence of kynurenic acid and DL-AP5 (1 mM; Tocris, Ballwin, MO) using the Ca^{2+}/phosphate method (Invitrogen) at an NR1/NR2/CD4 cDNA ratio of 4:4:1. Alternatively we used the Polyfect method (QIAGEN, Valencia, CA) for 6 to 12 h at an NR1/NR2/CD4 cDNA ratio of 8:8:1. We stopped transfections by replacing the media with fresh media containing AP5 and FUDR (0.2 mg/ml 5′-fluoro-2-deoxyuridine and 0.5 mg/ml uridine; Sigma). Anti-CD4 receptor-coated beads (Dynal, Oslo, Norway) were used to identify transfected cells.

Electrophysiology. We made whole-cell voltage-clamp recordings 12 to 48 h after transfections. The recording chamber was continuously superfused at room temperature (~20°C) with extracellular solution containing 162 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, and 1 mM $CaCl_2$, pH 7.2, 325 mOsm NaOH. Patch pipettes (2–5 MΩ) were pulled from thin-walled borosilicate glass (TW150F-6; World Precision Instruments, Sarasota, FL) and filled with 115.5 mM $CsCH_4SO_3$, 10 mM HEPES, 6 mM $MgCl_2$, 4 mM Na_2ATP, 20 mM phosphocreatine, 50 U/ml creatine phosphokinase, 0.1 mM leupeptin, 10 mM BAPTA, and 1 mM $CaCl_2$, pH 7.2, 310–320 mOsm CsOH. Where noted, the intracellular solution contained 0.1 mM EGTA and no $CaCl_2$. Solutions were prepared with high-performance liquid chromatography-grade water. Data were acquired with an Axopatch-1C amplifier and Axograph 4.5 software (Axon Instruments, Union City, CA). Unless noted, the membrane potential was clamped at −50 mV. Currents were filtered at 2 kHz and digitized at 5 kHz. Short −10 mV voltage steps before each agonist application were used to monitor cell input resistance (400–3000 MΩ). Drugs were applied by a fast microperfusion system. Unless noted, all solutions contained 100 μM glycine, and agonist applications were made in 0 mM calcium. Percentage of block was calculated according to the following equation: (1 − (peak amplitude after MTS)/(peak amplitude before MTS)) × 100. The rate constant of MTSET modification was calculated from the equation derived by Wilson and

Karlin (1998): (1/modification time constant) × (1/concentration of MTSET in molar concentrations). Percentage of desensitization was calculated by the following equation: (1 − (steady-state current amplitude/peak current amplitude)) × 100. Data were expressed as mean ± S.E.M. ANOVA and Student's *t* test were used as appropriate with statistical significance set at $p < 0.05$.

Results

Screening Accessibility in NR2A. Analysis of NR2A/NR2C chimeric mutant subunits indicates that the last four residues of the NR2A pre-M1 region, residues 553 to 556, influence glycine-independent desensitization (Krupp et al., 1998; Villarroel et al., 1998). We mutated residues in the pre-M1 and M1 domains to cysteines to examine their accessibility during channel gating (Fig. 1A). The mutants were coexpressed in HEK293 cells with a C-terminal truncation mutant of NR1-1a (NR1-1a$_{stop838}$), which, unless noted, we refer to as NR1. This NR1 truncation eliminates calcium-dependent inactivation, thus simplifying the analysis of desensitization (Fig. 1A) (Krupp et al., 1999). We examined the accessibility of each mutated residue using whole-cell recordings of NMDA receptor currents. Test pulses of glutamate and glycine were delivered before and after three 5-s applications of glycine with MTSET or glutamate and glycine with MTSET (Fig. 1B). MTSET had no effect on NR1/NR2A receptors in the absence of glutamate, indicating that modification of endogenous cysteines does not alter channel gating. However MTSET caused a rapid but completely reversible inhibition of NR1/NR2A currents (data not shown). This was attributable to open-channel block by the charged MTSET rather than cysteine modification and thus did not interfere with analysis of the mutants.

Of the 12 residues we analyzed, cysteine substitution altered the control responses of receptors containing NR2A$_{F549C}$, NR2A$_{L550C}$, NR2A$_{F553C}$, or NR2A$_{W558C}$ subunits. These currents were very small or had abnormal kinetics, and thus they were not further analyzed. Of the remaining eight residues, only a single residue, V557C, was modified by MTSET during gating. As shown in Fig. 1B, NR1/NR2A$_{V557C}$ currents showed irreversible inhibition of NMDA receptor inward current after three applications of MTSET plus glutamate and glycine (percentage block, 48 ± 3.1%, $n = 19$). The fast decaying peak during MTSET applications was due to reversible block of open channels as also seen with NR1/NR2A channels. The tail current that immediately followed the end of each MTSET application represented the unblock of open channels. NR1/NR2A$_{A548C}$ currents were irreversibly inhibited by MTSET alone (78.4 ± 6.8%, $n = 7$, in the presence of glycine), indicating that the accessibility of A548C was not linked to channel gating. We subsequently focused our experiments on the modification of V557C during channel gating.

Substitution of MTSET with methanethiosulfonate ethylammonium, a smaller positively charged MTS reagent, or hydroxyethylmethanethiosulfonate, a smaller uncharged MTS reagent, only slightly increased the irreversible inhibition of NR1/NR2A$_{V557C}$ currents (61.98 ± 3.9%, $n = 7$ and 56.76 ± 2.4%, $n = 5$), suggesting that the size and charge of MTSET did not impede accessibility (Karlin and Akabas, 1998). To examine whether glutamate binding alone could alter the accessibility of V557C, we blocked the glycine site on NR1 with 7-chlorokynurenic acid, thus preventing channel activation (Fig. 2). Under these conditions, glutamate application in the presence of MTSET did not cause a signif-

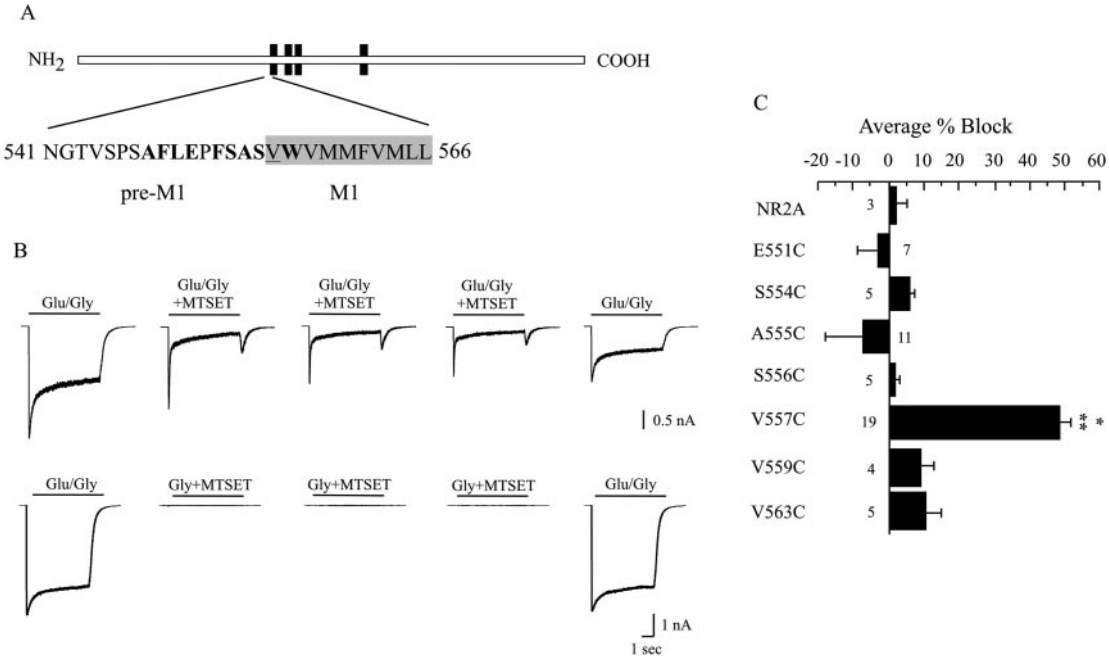

Fig. 1. MTSET modification of NR2A$_{V557C}$ occurred in a state-dependent manner. A, diagram of the NR2A subunit. Residues around the pre-M1/M1 border (enlarged) were individually mutated to cysteine (boldface lettering). Valine 557 is underlined. B, MTSET modification screening protocols. Top and bottom recordings are from two different HEK293 cells expressing the NR1-1a$_{stop838}$/NR2A$_{V557C}$ subunit combination. Test pulses of glutamate (1 mM) and glycine (100 μM) were recorded before (extreme left) and after (extreme right) three applications of glutamate, glycine, and MTSET (2 mM, top traces) or glycine and MTSET (bottom traces). The test pulses are averages of three to four responses. C, the irreversible inhibition by MTSET was plotted for each cysteine-substituted NR2A subunit using the protocol in B. Data represent mean ± S.E.M. ∗, significant compared with wild-type NR2A; ANOVA with Bonferroni/Dunn post hoc test, $p < 0.0005$; ∗∗, significantly different from the percentage of block for V557C tested with glycine and MTSET, unpaired *t* test, $p < 0.0001$.

icant irreversible inhibition of NR1/NR2A$_{V557C}$ currents (14.9 ± 4.2%, n = 10).

Modification Rates of V557C and Its Homologous Residue in NR1. We determined the MTSET modification rate of NR2A$_{V557C}$ by recording NR1/ NR2A$_{V557C}$ currents before and after single glutamate, glycine, and MTSET applications of increasing durations (5–60 s; Fig. 3). Irreversible MTSET inhibition increased exponentially, reaching 88.3 ± 2.7% block after 60 s. The MTSET inhibition curve was fitted with a single exponential resulting in a time constant of 18.6 s that corresponds to a rate constant of 26.88 s^{-1} M^{-1} (Wilson and Karlin, 1998). A 15-s application of MTSET blocked a similar amount of current (53.86 ± 7.2%, n = 7) as three 5-s applications. Thus, the accessibility of the V557C residue had reached equilibrium during the 5-s applications (Horn, 1998).

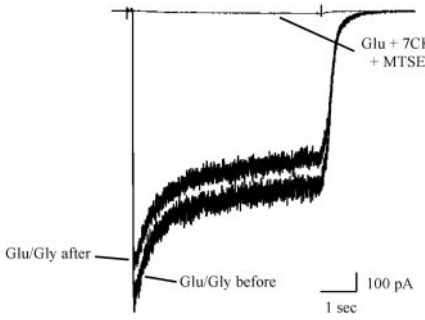

Fig. 2. MTSET modification of NR2A$_{V557C}$ requires channel gating. Currents mediated by NR1-1a$_{stop838}$/NR2A$_{V557C}$ receptors were recorded in the presence of glutamate and glycine immediately before and after three applications of glutamate (1 mM), 7-chlorokynurenic acid (100 μM, 7CKA), and MTSET (2 mM). The glycine antagonist 7CKA completely blocked the evoked current and glutamate- and glycine-dependent modification by MTSET. The irreversible inhibition after three applications of 7CKA and MTSET (8.8 ± 2.7%, n = 6; unpaired t test) was the same as for glutamate, 7CKA, and MTSET (14.9 ± 4.2%, n = 10). The small reduction was caused by the gradual rundown of current amplitudes.

Using SCAM analysis of the NR1 subunit expressed with a C-terminally truncated NR2C subunit containing an NR2A M1 domain (NR2C^{M1}), Beck et al. (1999) reported that leucine 562 (leucine 544 in their nomenclature) on NR1-1a was modified in the presence of glutamate and glycine (~67%, 2 min, 3 mM MTSET). Leucine 562 on NR1-1a is homologous to valine 557 on NR2A. However, MTSET did not irreversibly inhibit NR1-1a$_{L562C}$/NR2A currents in the presence of glutamate and glycine using the protocol shown in Fig. 1. As shown in Fig. 3C, NR1-1a$_{L562C}$/NR2A currents were only modified after long applications of glutamate, glycine, and MTSET (40 ± 3%, 2 min, 2 mM MTSET, n = 6). Thus, the accessibility of L562C on NR1-1a seemed to be slightly slower when expressed with NR2A subunits than with NR2C^{M1} (Beck et al., 1999). Applications longer than 2 min can be damaging to cells; thus, we did not attempt to determine a modification rate for the L562C mutant.

Desensitization and Accessibility of V557C. Krupp et al. (1998) reported a series of modified NR2 constructs that demonstrate enhanced or reduced NMDA receptor desensitization. We introduced the V557C mutation into these constructs and tested for MTSET modification. NR2C$_0$A$_{V557C}$ and NR2D001/AD1$_{V557C}$ are NR2C/NR2A chimeras in which all or most of the N-terminal sequence is from NR2C, whereas the rest of the sequence is taken from NR2A (Krupp

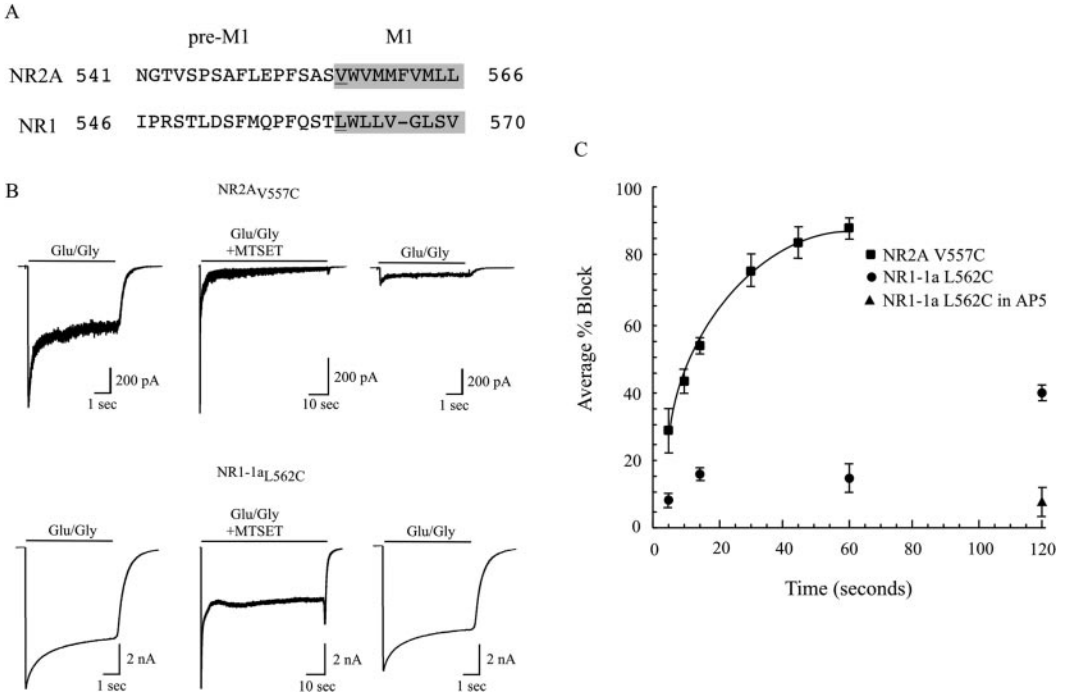

Fig. 3. MTSET modification rates for NR2A$_{V557C}$ and NR1-1a$_{L562C}$. A, the sequences of the pre-M1 and M1 regions of NR2A and NR1 are shown. B, example responses from cells transfected with NR1-1a$_{stop838}$/NR2A$_{V557C}$ (top) and NR1-1a$_{L562C}$/NR2A (bottom). Test pulses were recorded before (left-hand traces) and after (right-hand traces) a 60-s application of glutamate, glycine, and MTSET (2 mM, middle traces). Test pulses are averages of three to four traces. C, MTSET modification rates for each clone, derived from the percentage of irreversible block observed after different MTSET application lengths. Each point is the average of at least six cells. The NR1-1a$_{stop838}$/NR2A$_{V557C}$ averages were fit with a single exponential. MTSET inhibition of NR1-1a$_{stop838}$/NR2A$_{V557C}$ responses recorded after 30-, 45-, and 60-s applications of glutamate, glycine, and MTSET were not significantly different from each other (ANOVA and a Bonferroni/Dunn post hoc test). For NR1-1a$_{L562C}$/NR2A responses, only a 120-s application of glutamate, glycine, and MTSET caused significant irreversible inhibition ($p < 0.0001$).

et al., 1998). These subunits, when expressed with NR1-1a$_{stop838}$, did not desensitize and were not irreversibly inhibited by MTSET (Fig. 4). Conversely, the truncation mutant NR2A$_{stop844V557C}$, which showed significant desensitization, had more irreversible inhibition than full-length NR2A. These results suggested that there was a correlation between desensitization and V557C accessibility. However, NR2AD1$_{V557C}$, which has three mutations, F553Y, A555P, and S556A, that convert the pre-M1 region to the NR2C sequence, seemed to behave anomalously. It desensitized but was not irreversibly blocked by MTSET. This probably reflects the modification of desensitization by domains other than pre-M1 (Krupp et al., 1998; Villarroel et al., 1998).

Modifying Desensitization and Accessibility. If V557C accessibility occurs only when the channel is desensitized, then preventing or enhancing desensitization should alter the modification by MTSET. We took two approaches to address this prediction. Tetrapentylammonium (TPentA) and 9-aminoacridine (9-AA) block open NMDA channels (Costa and Albuquerque, 1994; Benveniste and Mayer, 1995; Sobolevsky, 1999; Sobolevsky et al., 1999) and thus have been reported to block entry into desensitized states. We tested whether their presence during channel gating might prevent MTSET modification of NR1/NR2A$_{V557C}$ receptors. Coapplication of TPentA (1 mM) with hydroxyethylmethanethiosulfonate, glutamate, and glycine did not reduce the irreversible inhibition of NR1/NR2A$_{V557C}$ currents (47.7 ± 10.5%, $n = 5$). However, TPentA only slightly prevented desensitization (36.1 ± 2.2% desensitization in control compared with 21.6 ± 4% desensitization in TPentA, $n = 7$). 9-Aminoacridine (100 μM) reduced irreversible MTSET inhibition in two cells (24 and 22%). However, the effect of 9-AA was overcome when we used 10 mM MTSET (66.3 ± 5.6%, $n = 4$). If one assumes that 9-AA prevents desensitization, then the result with 10 mM MTSET suggests that 9-AA and MTSET may compete for binding within the pore.

Another way to alter desensitization is by altering intracellular calcium. Influx of calcium through NMDA receptors increases desensitization as a result of calcineurin (CaN) activation (Krupp et al., 2002). Thus, we tested whether enhancing desensitization with calcium influx would increase the accessibility of V557C. After 5-s applications of glutamate, glycine, and calcium (2 mM) to activate CaN, we measured MTSET modification of V557C in calcium-free solutions (Fig. 5). Calcium influx increased the desensitization and the irreversible inhibition of currents mediated by NR1/NR2A$_{V557C}$. Furthermore, calcium influx induced the modification of NR1/NR2AD1$_{V557C}$ receptors. This protocol did not affect the accessibility of the homologous L562C residue in NR1. Calcium influx significantly increased desensitization of NR1-1a$_{L562C}$/NR2A, but there was no detectable irreversible inhibition (72.3 ± 3.7% desensitization, 8.8 ± 7.8% MTSET block, $n = 4$; data not shown). The correlation between desensitization and the modification of V557C for the NR2A constructs is plotted in Fig. 6 ($r = 0.86$, $p < 0.01$).

Discussion

Validity of Method and Comparison with Prior Results. The substituted-cysteine accessibility method has been used to infer conformational movements of several ion channels and ligand-gated receptors (Karlin and Akabas, 1998). The general assumptions of the method are that cysteine substitution does not alter channel properties, that any changes in channel properties reflect the modification of the substituted cysteine rather than native cysteines, and that

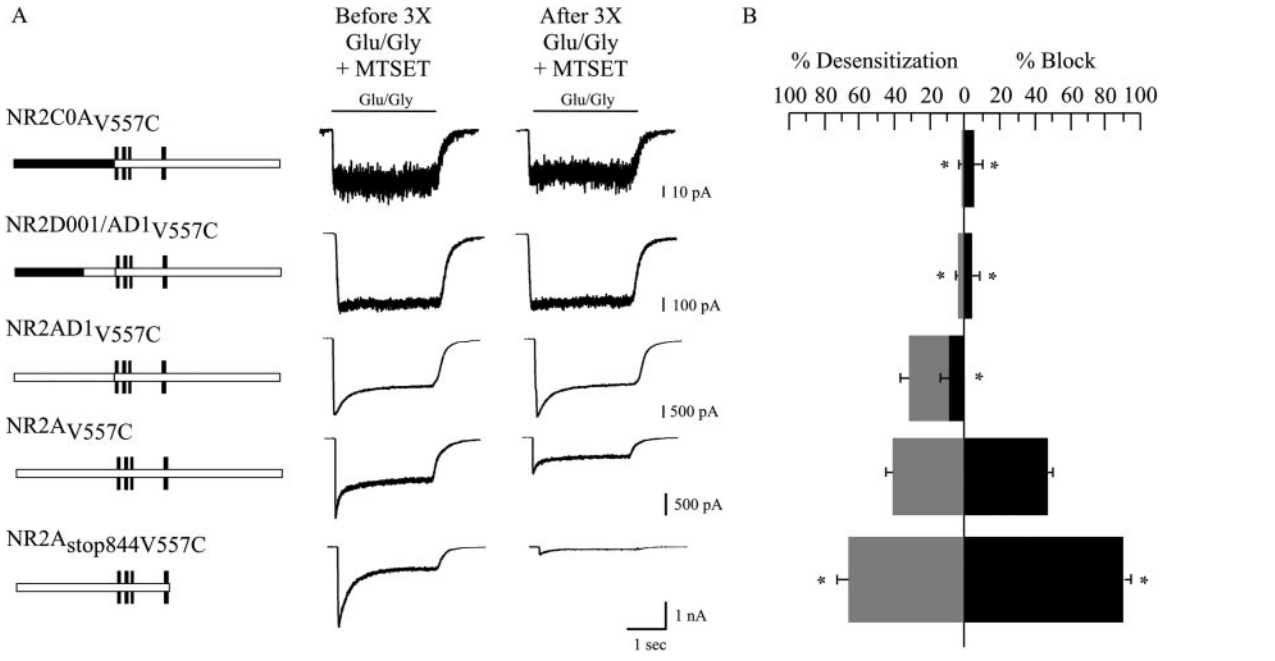

Fig. 4. MTSET modification of NR2A$_{V557C}$ correlates with increased desensitization. A, left-hand column, diagrams of NR2A/NR2C chimeric, NR2A wild-type, and NR2A truncated subunits containing the V557C mutation. ■, NR2C sequence; □, NR2A sequence. Middle and left-hand columns are representative test pulse responses from each construct, expressed with NR1-1a$_{stop838}$. Each was recorded before (middle column) and after (right-hand column) three 5-s applications of glutamate, glycine, and MTSET. B, summary of the average percentage of desensitization and percentage of MTSET irreversible inhibition (% Block). ANOVA with a Bonferroni/Dunn post hoc test was used to analyze differences (NR2C0A$_{V557C}$, $n = 3$; NR2D001/AD1$_{V557C}$, $n = 7$; NR2AD1$_{V557C}$, $n = 5$; NR2A$_{V557C}$, $n = 19$; NR2A$_{stop844V557C}$, $n = 7$). *, significantly different from NR2A$_{V557C}$, $p < 0.0001$ for the percentage of desensitization; $p < 0.0005$ for the percentage of block.

the sufhydryl modification is irreversible. It should also be noted that MTS reagents could modify substituted cysteines without altering receptor function, thus escaping detection by SCAM. In many cases, these studies have been directed at the relatively constrained environment of the channel pore. Within the pore, the validity of the SCAM assumptions is relatively easy to establish. Changes in cysteine accessibility with channel activation have also been used to define residues involved in channel gating (Yang and Horn, 1995; Liu et al., 1996). We applied this method to the desensitization of NMDA receptors. Given the location of the pre-M1 region near the NMDA receptor extracellular vestibule and its importance in glycine-independent desensitization, we used SCAM to examine 12 residues in pre-M1 and the first transmembrane domain of NR2A. Four cysteine mutants yielded nonfunctional or abnormal currents when coexpressed with NR1-1a$_{stop838}$. The eight other cysteine mutants had normal current amplitudes and kinetics, suggesting that cysteine substitution did not significantly alter their secondary structure. Although MTSET blocked open NMDA channels, this effect was reversible and thus did not represent the modification of native or substituted-cysteine residues.

SCAM has been used to define the structure of the pore and the extracellular vestibule of the NMDA receptor channel. In these studies, the number of modified residues in the pore was sensitive to the size of MTS reagents (Kuner et al., 1996). This indicated that the pore is a narrow structure formed by M2 domains centered on the magnesium-binding site. M3 domains occupy most of the extracellular vestibule, whereas portions of pre-M1, M1, and M4 contribute to the vestibule. MTS reagents modify many residues in the vestibule in the absence of channel activation (Beck et al., 1999). In our experiments, MTSET irreversibly inhibited currents mediated by receptors containing NR2A$_{A548C}$ or NR2A$_{V557C}$, but only the modification of NR2A$_{V557C}$ required channel gating. Both glutamate and the coagonist glycine were required for the modification of V557C, suggesting that the change in accessibility involved a concerted action of NR1 and NR2 subunits rather than a direct effect of glutamate binding to the NR2 subunit.

The changes in accessibility of V557C with gating are unique within the pre-M1/M1 region. Previous studies reported that the homologous residue in NR1 also is modified by MTSET (Beck et al., 1999). In those experiments, NR1-1a$_{L562C}$ (L544 in the terminology used by Beck et al., 1999) was expressed with a modified NR2C subunit in *Xenopus laevis* oocytes. When we expressed the same construct with wild-type NR2A, the modification was less. This result suggests that NR2 subunits influence L562C accessibility on NR1. The different rates of accessibility for V557C on NR2 and L562C on NR1 may also reflect asymmetrical gating of NR1 and NR2 subunits (Wollmuth and Sobolevsky, 2004) or simply differences in the specific environments of the cysteines in NR1$_{L562C}$ and NR2$_{V557C}$ subunits.

Channel Blockers and NMDA Channel Gating. Using channel blockers of varying sizes, Sobolevsky et al. (1999) reported that small blockers, such as tetraethylammonium, were trapped in the open channel without affecting channel closure or desensitization, whereas a large blocker, TPentA, prohibited channel closure and desensitization. On this basis, they proposed a physical model of NMDA receptor channel gating that contains distinct activation and desensitization "gates" with the activation gate placed closer to the extracellular surface. This idea was based on the fact that the intermediate size blocker, tetrabutylammonium, prevented desensitization but not channel closure. However, placement of the activation gate near the extracellular surface is incompatible with the fact that many residues in the vestibule are accessible to MTS reagents in the absence of agonist (Beck et al., 1999). The SCAM analysis of nondesensitizing NR1/NR2C receptors suggests that the activation gate is deep within the pore formed by M3 segments, whereas the pre-M1 and M4 segments form the more superficial lining of the extracellular vestibule. In this model, channel closure in-

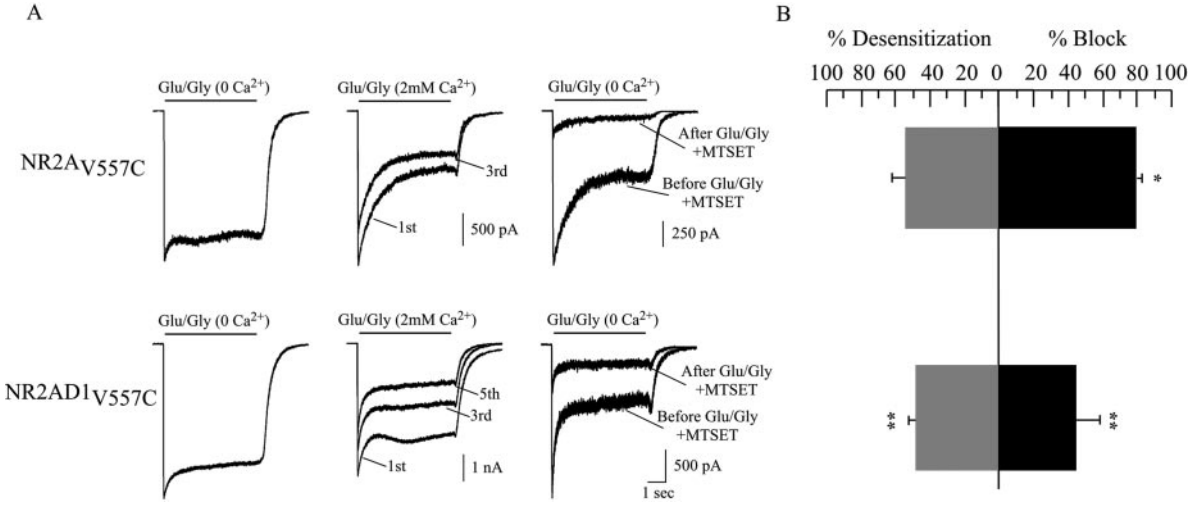

Fig. 5. Calcium influx increases desensitization and MTSET irreversible inhibition in NR2A$_{V557C}$- and NR2AD1$_{V557C}$- containing receptors. A, examples of recordings from NR1-1a$_{stop838}$/NR2A$_{v557c}$- and NR1-1a$_{stop838}$/NR2AD1$_{v557c}$- transfected cells (top and bottom traces, respectively). Initial glutamate and glycine test pulses (left) showed some desensitization. Subsequent glutamate and glycine applications in the presence of calcium (2 mM, middle traces) decreased the current amplitude and increased desensitization. Test pulses recorded again in calcium-free conditions before and after applying glutamate, glycine, and MTSET three times (right-hand traces). B, the percentage of desensitization and the percentage of MTSET irreversible inhibition (% Block) were measured after calcium treatment (Ca^{2+}Tx) for each clone. Unpaired *t* tests were used to compare NR2A$_{V557C}$ with NR2A$_{V557C}$ after Ca^{2+}Tx averages (*, $p < 0.0001$ for the percentage of block) and NR2AD1$_{V557C}$ with NR2AD1$_{V557C}$ after Ca^{2+}Tx averages (**, $p < 0.02$ for the percentage of block and $p < 0.01$ for the percentage of desensitization).

volves constriction of the deep part of the vestibule, thus trapping certain channel blockers (Sobolevsky et al., 2002).

This revised model leaves open the possibility that residues involved in desensitization might be located in the superficial parts of the vestibule. We expected that channel blockers which purportedly block desensitization would be useful to examine changes in the accessibility of residues in the pre-M1/M1 region. Unfortunately, TPentA did not completely block desensitization in our experiments, and the effects of 9-aminoacridine were not interpretable because of its competition with MTSET, which is itself an NMDA channel blocker. Thus, the use of channel blockers was not sufficient in our hands to define the domains involved in NMDA receptor desensitization.

Modification of V557C and Desensitization. Different combinations of NMDA receptor subunits have striking differences in desensitization. The amino acid sequence differences between desensitizing NR2A subunits and nondesensitizing NR2C subunits were used to show that the pre-M1 region and the LIVBP-like domain in the N terminus are involved in NMDA receptor desensitization (Krupp et al., 1998; Villarroel et al., 1998). Our results provide further evidence that residues in the pre-M1 region are sensitive to gating steps associated with desensitization. The overall correlation between desensitization and MTSET modification suggests that V557C, the first residue in M1, was only modified when the receptor was in the desensitized state. This correlation was supported by the increases in MTSET modification resulting from calcium-induced increases in desensitization of NR1/NR2A$_{V557C}$ currents. These last results also support the idea that intracellular regulation of NR2A modifies desensitization at a distance by affecting regions at or surrounding V557C. This action of the intracellular domain of NR2A, possibly influenced by an intracellular protein-protein interaction, could alter the conformation of the N-terminal domain (Krupp et al., 2002).

In addition to the pre-M1 region, the LIVBP-like domain also influences desensitization. Thus, it is reasonable to consider whether the LIVBP-like domain could explain the apparently anomalous behavior of NR2AD1$_{V557C}$. This mutant is identical with NR2A, except for an NR2C pre-M1 region. The LIVBP-like domain affects glycine-independent desensitization through an allosteric effect (Zheng et al., 2001; Erreger and Traynelis, 2005), yet desensitization of NR1/NR2AD1 receptors persists in the absence of zinc (Hu and Zheng, 2005). We did not test the mutant in the presence of zinc chelators; thus, further experiments are necessary to fully understand desensitization of NR1/NR2AD1$_{V557C}$ receptors. However, when desensitization in this mutant was enhanced by CaN activation, the receptor was modified by MTSET. Thus accessibility of V557C is a reliable detector of desensitization.

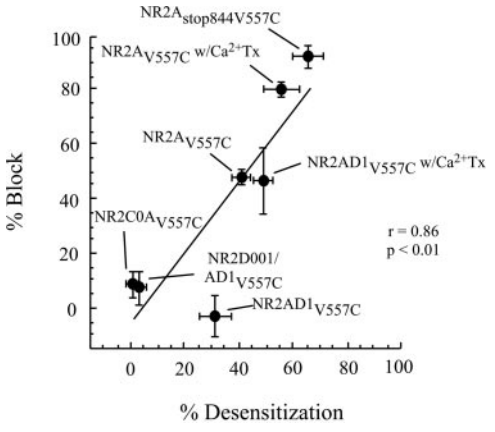

Fig. 6. MTSET modification correlates with desensitization. Data from Figs. 4 and 5 were combined and plotted as a correlation. A line fit of the data yielded a correlation coefficient of 0.86, $p < 0.01$.

A Structural View of Desensitization Gating. Our results indicate that modification of V557C correlates with NMDA receptor desensitization. The proximity of valine 557 to the pre-M1 region fits with a critical role of that region in desensitization, but, as discussed above, other domains, including the LIVBP-like domain and regulatory effects in the C terminus, also contribute to desensitization. Models based primarily on studies of the channel pore cannot account for these more distant effects (Sobolevsky et al., 1999), nor can they account for the coupling between ligand binding and channel gating. Thus, it is necessary to incorporate a model of conformational changes associated with ligand binding to understand desensitization. Such information largely comes from studies of AMPA receptors.

The crystal structures of the ligand-binding pocket of AMPA receptors provide a framework to consider how ligand binding leads to channel gating, including desensitization. Structures with and without ligands have provided information on the conformational movements triggered by ligand binding that have been tested in functional studies (Sun et al., 2002; Mayer and Armstrong, 2004). These studies indicate that movements in ligand-binding domains directly cause changes in gating. Several basic features emerge that are probably shared among ionotropic glutamate receptors. Each AMPA receptor is a tetramer composed of two subunit pairs. The subunits within each pair noncovalently interact with each other through their S1 domains. Glutamate initially binds to the S1 pocket of an open S1–S2 clamshell, causing the displacement of S2 away from the plasma membrane as it closes around the ligand. This S2 movement causes channel gating by pulling the M3 domains up and away from each other. The open channel conformation puts tension on the interactions between a pair of S1 domains. Desensitization occurs when S1–S1 interactions rearrange into a more stable conformation, causing transmembrane domains to relax and close the channel (Horning and Mayer, 2004).

Recent structural information on NMDA receptor subunits shows there are similarities with AMPA receptors. NMDA and AMPA receptors share similar kinetic schemes; they have very similar secondary structures; the crystal structure of the NR1 and NR2A ligand-binding domain are similar to that of AMPA subunits; NR2A requires a portion of the LIVBP-like domain near the S1 of NR1 for surface expression; and S1 domains mediate negative cooperativity between NR1 and NR2 subunits (Meddows et al., 2001; Regalado et al., 2001; Furukawa et al. 2005; Wollmuth and Sobolevsky, 2004; Inanobe et al., 2005). Assuming that NMDA receptors are NR1/NR2 dimers of dimers arranged in 1-1-2-2 tetramers, (Schorge and Colquhoun, 2003), desensitization may involve a relaxation in S1–S1 interfaces. Our results suggest that this relaxation is sensed by the linker connecting S1 to M1, resulting in the accessibility of V557C in NR2A (the glutamate binding subunit). The homologous

NR1 residue was actually more accessible in a nondesensitizing receptor (Beck et al., 1999). The change in accessibility of V557C is consistent with a specific role of the pre-M1 region in NMDA receptor desensitization. Our data cannot resolve whether the putative relaxation of the S1–S1 interface simply reveals V557C, making it a sensitive detector of the desensitized state, or if V557C is the desensitization "gate". Even if the latter is true, our data confirm that the LIVBP-like domain can influence desensitization independently and thus presumably the S1–S1 relaxation. The modulation by CaN further suggests that intracellular domains can induce conformational changes in ligand binding domains.

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