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Intracellular Domains of NR2 Alter Calcium-Dependent Inactivation of *N*-Methyl-D-aspartate Receptors

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ABSTRACT

At central excitatory synapses, the transient elevation of intracellular calcium reduces *N*-methyl-D-aspartate (NMDA) receptor activity. Such 'calcium-dependent inactivation' is mediated by interactions of calcium/calmodulin and α-actinin with the C terminus of NMDA receptor 1 (NR1) subunit. However, inactivation is also NR2-subunit specific, because it occurs in NR2A- but not NR2C-containing receptors. We examined the molecular basis for NR2-subunit specificity using chimeric and mutated NMDA receptor subunits expressed in HEK293 cells. We report that the intracellular loop immediately distal to the pore-forming P-loop M2 (M2–3 loop), as well as a short region in the C terminus, are involved in NR2-subunit specificity. Within the M2–3 loop, substitution of residue 619 in NR2A (valine) for the corresponding NR2C residue (isoleucine) reduced inactivation without affecting calcium permeability of the channel. In contrast, a Q620E mutation in NR2A reduced the relative calcium permeability without altering inactivation. Mutation of three serine/threonine residues in the M2–3 loop also reduced inactivation, as did substitution of the intracellular C terminus of NR2A for NR2C. We speculate that the M2–3 loop of NR2 modulates calcium-dependent inactivation by interacting with the NR1 C terminus, a region known to be essential for inactivation.

NMDA receptors are heteromers of NMDA receptor 1 (NR1), NR2, and, in some cases, NR3 subunits. Several characteristics of native NMDA channels, including magnesium sensitivity, glycine affinity, and desensitization, depend on both NR1 and NR2 (for review see McBain and Mayer, 1994; Dingledine et al., 1999). The molecular mechanisms responsible for NR2-subunit specific differences in receptor function are complex and not well characterized. For example, block by magnesium ions is largely determined by residues in the NR2 pore domain (M2) (Burnashev et al., 1992; Mori et al., 1992; Sakurada et al., 1993; Williams et al., 1998; Wollmuth et al., 1998), but regions outside of M2 are modulatory (Kuner and Schoepfer, 1996). Likewise, calcium-dependent inactivation (Legendre et al., 1993; Rosenmund and Westbrook, 1993) also depends on NR1 and NR2. Whereas the interactions of two intracellular proteins, calcium/calmodulin (Ehlers et al., 1996) and α-actinin (Wyszynski et al., 1997), with the C-terminal region of NR1 are necessary for inactivation (Zhang et al., 1998; Krupp et al., 1999), the coexpressed NR2 subunit is permissive (Krupp et al., 1996). This effect of the NR2-subunit is not caused by the small differences in the calcium permeability between the different NR1/NR2 heteromers (Krupp et al., 1996), suggesting that domains within NR2 influence inactivation.

To determine domains in NR2 that influence inactivation, we expressed NR1/NR2 heteromers in HEK293 cells. We found that the valine residue at position 619 in the M2–3 loop of NR2A is critical for NR2 subunit specificity. A point mutation that switched this residue in NR2A (valine) for NR2C (isoleucine) reduced inactivation without affecting calcium permeability. Mutagenesis of serine/threonine residues in the M2–3 loop of NR2 also affected inactivation. In addition, the proximal portion of the intracellular NR2 C terminus had a small modulatory effect.

Materials and Methods

Molecular Biology. The following NMDA subunit cDNAs were used: NR1–1a (GenBank accession number U08261), NR1–4a (GenBank accession number U08267) (Hollmann et al., 1993), NR2A (GenBank accession number D13211), and NR2C (GenBank accession number D13212) (Ishii et al., 1993). Most NR2A/2C chimeras have been described previously (Krupp et al., 1998), except for $2C_{2+i}A$ ($NR2C_{M1–I630}A_{Q620–V1464}$), $2C_{2+ii}A$ ($NR2C_{M1–E631}A_{N621–V1464}$), and $2C_3A$ ($NR2C_{M1–L643}A_{V633–V1464}$). Chimeras and mutants were generated using the strategy of gene splicing by overlap extension polymerase chain reaction (Horten et al., 1989) using *Pfu* polymerase (Stratagene, La Jolla, CA). All NMDA subunit cDNAs and chimeras

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; NR, *N*-methyl-D-aspartate receptor; HEK, human embryonic kidney; M*x*, membrane region, where *x* is 1, 2, or 3; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

were cloned into pCDNA1/amp (Invitrogen, Carlsbad, CA). Truncation mutants were generated by introduction of a stop codon at the appropriate position. Amino acid numbers for mutations in wild-type subunits are as given in Ishii et al. (1993). For mutations in chimeras, we used the amino acid numbering of the embedding wild-type subunit. All clones were confirmed by restriction analysis and sequence analysis. To identify cells expressing NMDA receptors, HEK293 cells were cotransfected with cDNA coding for the lymphocyte CD4 receptor. The CD4 cDNA, kindly provided by Dr. John Adelman (Vollum Institute, Portland, OR), was inserted into the JPA vector. For detection of successfully transfected cells, 1 μl of Dynabeads M-450 CD4 (Dynal, Norway) was added in 1 ml of medium to each 35-mm dish. The dish was then gently swirled for 15 to 20 min before recording.

Transfection and Handling of HEK293 cells. HEK293 cells were plated 6 to 12 h before transfection in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (Hyclone, Logan, UT), 1% glutamine (Invitrogen), and 1% penicillin-streptomycin (Invitrogen; 37°C, 5% CO_2). Cells were plated on 31-mm polylysine-coated glass coverslips placed in 35-mm dishes. The cDNAs for NR1/NR2/CD4 were mixed in a 4:4:1 ratio and added to HEK293 cells as a calcium-phosphate complex (Calcium Phosphate Transfection System; Invitrogen). Kynurenic acid (3 mM; Sigma, St. Louis, MO) and D,L-2-amino-5-phosphonopentanoic acid (1 mM; Tocris Cookson, St. Louis, MO) were routinely added to prevent NMDA receptor-mediated excitotoxic cell death. The transfection mixture was removed after 12 to 18 h by exchanging with fresh culture medium containing kynurenic acid and D,L-2-amino-5-phosphonopentanoic acid. 5′-Fluoro-2-deoxyuridine (0.2 mg/ml) and 0.5 mg/ml uridine (Sigma) was added to inhibit cell proliferation.

Recording, Solutions, and Drug Application. Whole-cell voltage-clamp recordings were performed 12 to 48 h after transfection. The recording chamber was continuously superfused at room temperature (~ 20–22°C) with an extracellular solution: 162 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM dextrose, and 1 mM $CaCl_2$, pH 7.25 (NaOH), 325 mOsm. High-performance liquid chromatography–grade water was used to avoid contaminating amounts of glycine or other amino acids. Patch pipettes were pulled from thin-walled borosilicate glass (TW150F-6; World Precision Instruments, New Haven, CT) and had resistances between 2 and 5 MΩ. The intracellular solution included an ATP-regenerating system: 115.5 mM $CsCH_4SO_3$, 10 mM HEPES, 6 mM $MgCl_2$, 4 mM Na_2ATP, 20 mM phosphocreatine, 500 U/ml creatine phosphokinase, 0.1 mM leupeptin, and 0.1 mM EGTA, pH 7.2 (CsOH), 320 mOsm (sucrose). Patch solutions were prepared daily from frozen stocks and kept on ice until use. Data were acquired using pClamp6 software in combination with an Axopatch-1B amplifier (Axon Instruments, Union City, CA). The membrane voltage was clamped at −50 mV unless otherwise indicated. Currents were low-pass filtered at 0.2 kHz and digitized at 1 kHz. Series resistance was routinely compensated (60 to 90%). Cell input resistances (range, 400-3000 MΩ) were continuously monitored by a short −10 mV voltage step just before each agonist application.

For measurements of calcium permeability, cells were superfused with a high-calcium, sodium-free solution: 50 mM $CaCl_2$, 100 mM *N*-methyl-D-glutamine, 10 mM HEPES, and 15 mM dextrose, pH 7.25 (HCl), 330 mOsm. The internal solution in these experiments contained 150 mM CsCl, 10 mM HEPES, 10 mM EGTA, and 4 mM MgATP, pH 7.25 (CsOH), 300 mOsm. Current-voltage relationships were constructed from peak current amplitudes evoked by 1-s applications (1 mM glutamate, 100 μM glycine). A small junction potential of 1 to 3 mV was corrected off-line. Reversal potentials were obtained by fitting a linear regression to the data. The permeability ratio pCa/pCs was calculated from the reversal potential (E) according to the rearranged constant-field equation $pCa/pCs = ([Cs^+]_i / [Ca^{2+}]_o) \exp(EF/RT)(\exp(EF/RT) + 1) / 4$, where F is Faraday's constant, R is the gas constant, and T is temperature (20°C) (Iino et al., 1990). There are at least two binding sites each for divalent and monovalent cations in the NMDA channel (Johnson and Ascher, 1990; Premkumar and Auerbach, 1996; Antonov et al., 1998), and occupancy of these sites influences ion permeation. Although the constant field theory is an oversimplified description of NMDA channel permeation, the constant-field equation provides a reasonable approximation of the relative calcium permeability for the ionic conditions used here (Mayer and Westbrook, 1987; Schneggenburger, 1998).

NMDA (10 μM–1 mM; Tocris) or L-glutamate (1 mM; Sigma) were applied by a fast microperfusion system (Rosenmund and Westbrook, 1993). NMDA (1 mM) is a saturating agonist concentration at all wild-type and chimeric NMDA receptors tested (see Krupp et al., 1998, and references in Dingledine et al., 1999). Glycine (100 μM) was added to the control and drug solutions to prevent glycine-dependent desensitization. Unless otherwise noted, agonist was applied for 5 s at 30-s intervals. When used, the membrane-permeable kinase inhibitors staurosporine (dimethyl sulfoxide [1:10,000 final dimethyl sulfoxide dilution]; Calbiochem, San Diego, CA) or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; ethanol [1:2,000 final ethanol-dilution]; Calbiochem) were added to bath and agonist-containing solution at least 15 min before recording.

Data Analysis and Statistics. The extent of inactivation was measured as the percentage reduction in current amplitude at the end of the 5-s application compared with its initial peak (see Legendre et al., 1993). The onset of inactivation was evaluated by fitting a monoexponential function with the time constant τ. This provided satisfactory fits in most cases. However, responses from some mutants had variable kinetics, including biphasic inactivation with a very slow second component. This slow component was often too slow ($\tau > 5$ s) to be fitted in a meaningful way during a 5-s agonist application. For such mutants, we calculated time constants only for responses that could be adequately fitted by a monoexponential function. Responses with less than 15% inactivation were not fitted. We analyzed responses obtained during the first 5 min after whole-cell access.

Data are expressed as mean ± S.E.M. For statistical comparisons, Student's *t* test and analysis of variance with subsequent Bonferroni test for multiple comparisons were used as appropriate. Statistical significance was set at $p < .05$.

Results

Molecular Determinants for the NR2-Subunit Specificity of Calcium-Dependent Inactivation. Calcium-dependent inactivation is NR2-subunit specific (Krupp et al., 1996). During long (5 s) whole-cell applications of NMDA in 2 mM extracellular Ca^{2+}, responses from NR1–1a/2A heteromers showed prominent inactivation that reached steady-state by the end of the application (Fig. 1B, top trace). The onset of inactivation could usually be adequately described by a monoexponential fit (47.6 ± 2.6%; $n = 28$; $\tau = 2.1 \pm 0.3$ s; $n = 22$). In contrast, NR1–1a/2C responses did not inactivate (−3.5 ± 2.3%; $n = 5$; Fig. 1B, bottom trace). To explore the molecular basis of this NR2 specificity, we constructed a series of chimeras in which progressively larger segments of NR2A were exchanged for NR2C (Fig. 1). The N terminus of the NR2 subunit controls one form of NMDA receptor desensitization, glycine-independent desensitization (Krupp et al., 1998; Villaroel et al., 1998). Because the chimeras contained the N terminus of the nondesensitizing NR2C subunit, we were able to use saturating concentrations of agonist (1 mM NMDA or glutamate). Thus the relaxation in the chimeras exclusively reflects inactivation.

Neither the exchange of the N terminus (Fig. 1B; $2C_0A$; $\tau = 2.1 \pm 0.3$ s; $n = 5$) nor the additional exchange of the first transmembrane region (M1) in NR2A for NR2C ($2C_1A$; 41.7 ± 6.4%; $\tau = 2.2 \pm 0.5$ s; $n = 4$) affected inactivation. A chimera in

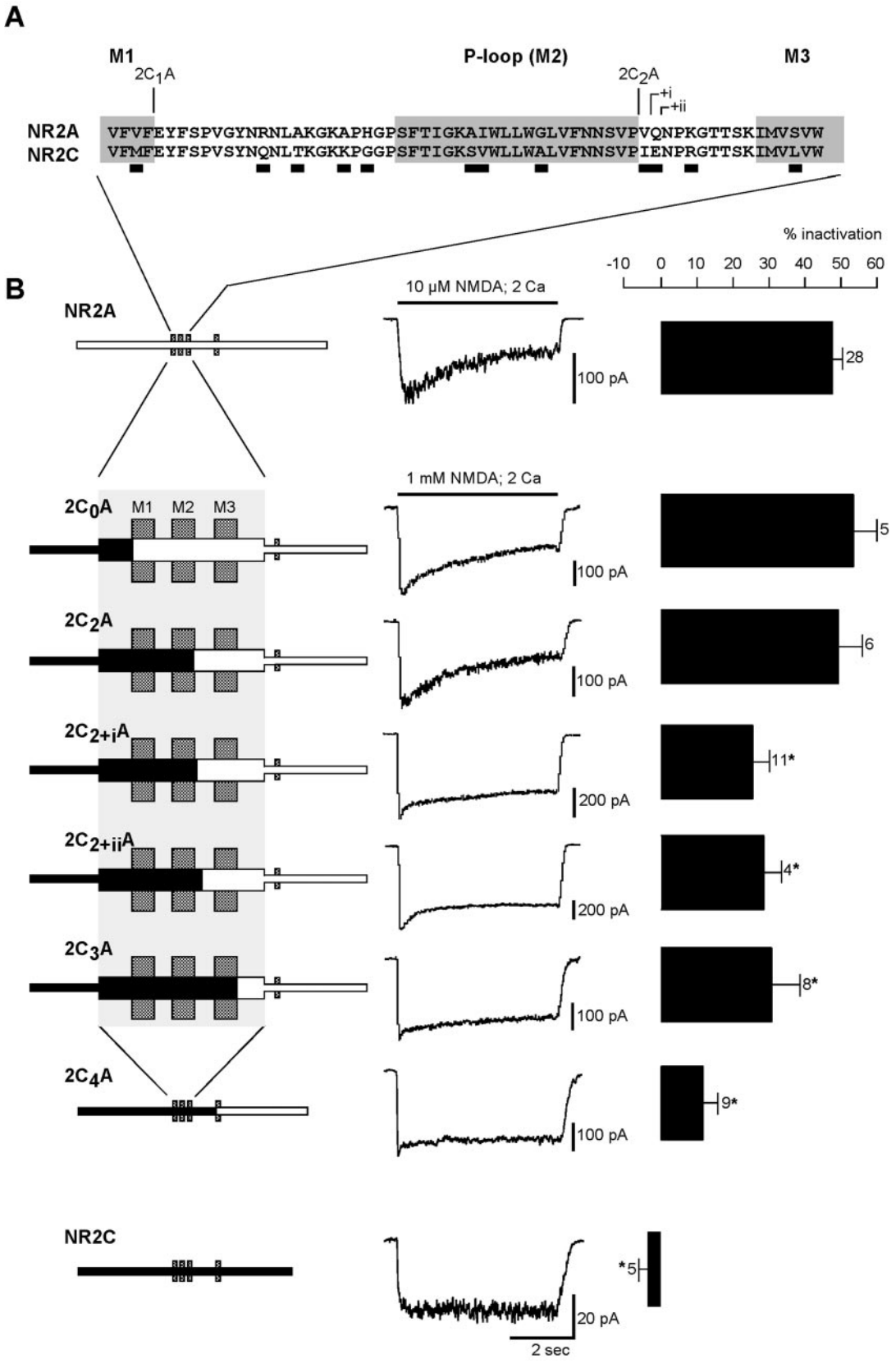
which the switch from NR2A to NR2C was at the end of M2 ($2C_2A$; $\tau = 2.1 \pm 0.2$ s; $n = 6$) also showed normal inactivation (Fig. 1B). However, the exchange of one additional residue in the intracellular loop distal to M2 ($2C_{2+i}A$; Fig. 1) markedly reduced inactivation (25.5 ± 4.7%, $n = 11$). The exchange of the subsequent NR2A residue (chimera $2C_{2+ii}A$) or a switch from the NR2C to the NR2A sequence at the end of the third membrane region ($2C_3A$) had no additional effect, suggesting that the proximal M2–3 loop is a determinant of inactivation. Furthermore, exchange of this region also influenced the kinetics of inactivation, which became highly variable. In chimera $2C_{2+i}A$, 8 of 11 cells had inactivation that was biphasic or too small to be fitted (Fig. 1B). Onsets in the remaining three cells were monoexponential ($\tau = 1.6 \pm 0.6$ s), similar to NR2A. Three of four cells expressing chimera $2C_{2+ii}A$ and four of eight cells expressing chimera $2C_3A$ also had biphasic inactivation. The onset of inactivation in the other cells was monoexponential with time constants of $\tau = 1.8$ s ($n = 1$; $2C_{2+ii}A$) and $\tau = 1.6 \pm 0.6$ s ($n = 4$; $2C_3A$). These values were not significantly different from those of NR2A. In all chimeras, the current relaxations were caused by calcium-dependent inactivation, because they were abolished in calcium-free solution or when the chimeras were cotransfected with $NR1_{stop838}$ (not shown), which lacks the C0 domain essential for inactivation (Zhang et al., 1998; Krupp et al., 1999).

These results implicate the intracellular M2–3 loop in the NR2-subunit specificity of inactivation. However, other domains in NR2 also influenced inactivation. For example, inactivation was further reduced when the extracellular M3–4 loop was also exchanged ($2C_4A$; 11.5 ± 4.1%; $n = 9$). The residual inactivation in this chimera was too small to allow adequate fits to the onset. The difference between $2C_3A$ and $2C_4A$ indicates a small effect of the extracellular M3–4 loop on inactivation. However, substitution of the NR2C M3–4 loop into NR2A did not affect inactivation ($2A_3C_4A$; 44.1 ± 7.2%; $\tau = 1.8 \pm 0.2$ s; $n = 5$; monoexponential decay in all cells), suggesting that this effect was indirect, perhaps through the spatial arrangements of M3, M4, and/or the M2–3 loop with respect to the channel pore. Finally, the difference between $2C_4A$ and 2C suggests that M4 or the intracellular C terminus of NR2 has a small effect on inactivation. Consistent with this interpretation, $2A_4C$, the reverse chimera to $2C_4A$, had reduced inactivation (32.9 ± 3.9%; $\tau = 2.3 \pm 0.6$ s; $n = 7$; monoexponential decay in all cells) compared with NR2A. Based on the results with these chimeras, we examined the role of the M2–3 loop and the C terminus using site-directed mutagenesis.

NR2A Positions 619 and 620 Differentially Affect Inactivation and Calcium Permeability. The chimeras indicate that the first residue immediately distal to M2 affects inactivation. Consistent with this observation, a mutation of valine 619 in NR2A to the corresponding isoleucine in NR2C reduced inactivation (33.9 ± 4.9%; $n = 12$) to a similar degree as chimera $2C_{2+i}A$ (Fig. 2A). We also examined other neutral amino acid exchanges at position 619. A V619A mutation did not reduce inactivation (53.1 ± 2.3%; $n = 5$), whereas V619F and V619W mutations did (Fig. 2B). Mutation of the immediately adjacent residue 620 (glutamine) to the corresponding NR2C residue (glutamate) did not change inactivation (Fig. 2C).

These mutations potentially could affect inactivation by a secondary change in calcium permeability. Indeed, longer applications of NMDA (10 s) restored full inactivation (Fig. 2B). Thus, to determine the relative calcium permeability of the point mutants, glutamate (1 mM, 1 s) was applied at different holding potentials where Ca^{2+} and Cs^+ were the only charge carriers. The permeability ratio (pCa/pCs) was then calculated from the reversal potential (see *Materials and Methods*). Examples for NR1–1a heteromers with NR2A, $NR2A_{(V619I)}$ and $NR2A_{(Q620E)}$ are shown in Fig. 3, A-C. NR2A and $NR2A_{(V619I)}$ reversed near +20 mV, whereas $NR2A_{(Q620E)}$ reversed at +15 mV. The pCa/pCs for NR2A was 4.8 ± 0.2 ($n = 9$; Fig. 3D), comparable with values published previously for native NMDA receptors (Mayer and Westbrook, 1987; Iino et al., 1990) and NR1/2A (Burnashev et al., 1995; Schneggenburger, 1998, Zhang et al., 1998). The pCa/pCs value for $NR2A_{(V619I)}$ was similar to that of NR2A, whereas that of $NR2A_{(Q620E)}$ was significantly smaller (3.8 ± 0.3; $n = 5$). Consistent with a strong effect of residue 620 on calcium permeability, the permeability ratio of the chimera $2C_{2+i}A$ (4.4 ± 0.2; $n = 6$) was similar to that of NR2A, whereas $2C_{2+ii}A$ had a significantly lower pCa/pCs (3.8 ± 0.5; $n = 4$). Thus, for $NR2A_{(V619I)}$ and $NR2A_{(Q620E)}$, pCa/pCs does not predict the degree of inactivation (Figs. 2 and 3, D and E). However, mutations of valine 619 to phenylalanine or tryptophan did reduce the relative calcium permeability as well as inactivation (Fig. 3, D and E). If reduced inactivation in V619F and V619W results from reduced calcium influx, the onset of inactivation should be slower than in NR1–1a/2A, because the onset is determined by intracellular calcium accumulation (Legendre et al., 1993). This was not the case. The responses to 5-s applications could always be fitted adequately with monoexponential functions (Fig. 2A), although some cells had biphasic response to longer agonist applications (not shown). The time constants for monoexponential fits to currents induced by 10 μM NMDA in 2 mM $[Ca^{2+}]_o$ were 2.0 ± 0.6 s for $NR2A_{(V619F)}$ ($n = 6$) and 1.8 ± 0.4 s for $NR2A_{(V619W)}$ ($n = 5$), similar to that of NR2A (2.1 ± 0.3 s; $n = 22$). In contrast, reducing calcium influx through NR2A by lowering $[Ca^{2+}]_o$ to 1 mM not only produced a reduction in inactivation (~37%; see Krupp et al., 1996) but also slowed inactivation to such a degree that a monoexponential fit to the onset was not meaningful. Thus, the reduced inactivation with some mutations at position 619 cannot be explained by calcium influx. This interpretation is further supported by the lack of a significant correlation between inactivation and pCa/pCs for the point mutations (Fig. 3E; $r = 0.41$; nondirectional $p = 0.42$). Finally, the chemical characteristics of the substituted amino acids at position 619 showed different correlation patterns with pCa/pCs compared with inactivation. For example, the relative hydrophilicity of the residue at position 619 correlated negatively with pCa/pCs ($r = 1.00$), but not with inactivation ($r = 0.50$). The relative hydrophobicity of the substituted amino acid was negatively correlated with inactivation ($r = 0.93$), but to a lesser degree with pCa/pCs ($r = 0.74$). Inactivation and pCa/pCs were negatively correlated with the volume ($r = 0.88$ and 0.84, respectively), the accessible surface area ($r = 0.82$ and 0.90, respectively), and the mass ($r = 0.78$ and 0.92, respectively) of the substituted amino acid.

Mutation of Serine/Threonine Residues in the M2–3 Loop of NR2 Affects Inactivation. Residues 619 and 620 are immediately C-terminal to the pore-forming M2-region and thus could have nonspecific effects on pore architecture.



Because nonspecific perturbations might also be expected from the exchange of charged residues in other regions close to the cytoplasmic face of the pore, we individually mutated other negatively charged residues in the intracellular loops of NR1 and NR2A. None of the mutations in NR1 significantly affected inactivation, including alanine substitutions of four serine residues (Fig. 4, A and B). Likewise, neither the neutralization of three sequential glutamic acids immediately before M2 nor their charge inversion affected inactivation (Fig. 4, A and B). Inactivation was also unaffected by phenylalanine substitutions of two tyrosines in the M1–2 loop of NR2 (Fig. 4C). In contrast, there was a significant reduction of inactivation when three adjacent serine/threonine residues in the M2–3 loop of NR2 were mutated to alanine ($NR2A_{(TTS625–7A)}$: 23.7 ± 5.1%; n = 11; Fig. 4D). A similar reduction was observed when any two of these three residues were mutated to alanine. Single point mutations of these residues did not reduce inactivation (Fig. 4D). The reduced inactivation with $NR1–1a/2A_{(TTS625–7A)}$ was not caused by decreased calcium permeability (pCa/pCs: 6.0 ± 1.0; n = 4).

These results raise the possibility that phosphorylation modulates inactivation. We therefore examined whether kinase inhibitors affected inactivation. To avoid phosphorylation of residues in the C1 exon in NR1 (Tingley et al., 1997), we used NR1–4a, which lacks C1. Consistent with previous data (Krupp et al., 1996), the broad-spectrum kinase inhibitor staurosporine (500 nM) did not change inactivation in NR1–4a/2A heteromers (Fig. 5C). Staurosporine also had no effect on $NR1–4a/2A_{(TTS625–7A)}$, $NR1–4a/2A_{(T625A)}$, and $NR1–4a/2A_{(S627A)}$ but did reduce inactivation in the single alanine-mutations $NR2A_{(T626A)}$ (22.9 ± 5.1%; n = 6; Fig. 5A). At the concentrations we used, staurosporine affects most serine/threonine kinases, but not casein kinase II (Meggio et al., 1995). Inhibition of casein kinase II by DRB (50 μM) reduced inactivation in NR1–4a/2A but did not affect $NR1–4a/2A_{(TTS625–7A)}$ heteromers. Because DRB reduced inactivation of $NR1–4a/2A_{(T625A)}$ and $NR1–4a/2A_{(T626A)}$ heteromers but not of $NR1–4a/2A_{(S627A)}$ heteromers, the affected residue may be S627 (Fig. 5, B and C). Consistent with that possibility, cotreatment with staurosporine and DRB did not reduce inactivation in $NR1–4a/2A_{(S627A)}$.

The NR2 C Terminus Affects Calcium-Dependent Inactivation. We addressed the potential role of the NR2 C terminus in inactivation using a series of C-terminal truncations in NR2A expressed with NR1–1a. Inactivation was normal for $NR2A_{stop1029}$ (44.9 ± 3.6%; n = 5), $NR2A_{stop905}$ (46.5 ± 3.6%; n = 9), and $NR2A_{stop874}$ (Fig. 6A). As expected for a process dependent on calcium influx, inactivation was absent at positive holding potentials. In contrast, responses from $NR2A_{stop844}$ showed a prominent relaxation not only at a holding potential of −50 mV but also at +50 mV (Fig. 6A). Likewise, when coexpressed with $NR1_{stop838}$, a construct that prevents inactivation, a relaxation of ~40% was present at all holding potentials in $NR1_{stop838}/2A_{stop844}$ but not $NR1_{stop838}/2A_{stop874}$ (Fig. 6A).

To circumvent this calcium-independent desensitization, we re-engineered the $NR2A_{stop874}$ and $NR2A_{stop844}$ truncations into the nondesensitizing NR2 chimera D001/AD1 (see Krupp et al., 1998). D001/AD1 (48.4 ± 2.5%, n = 5) and $D001/AD1_{stop874}$ (51.6 ± 0.8%, n = 3) showed prominent inactivation that was absent at positive holding potentials (Fig. 6B), or when inactivation was abolished by substituting $NR1_{stop838}$ for NR1–1a (not shown). In contrast, inactivation was reduced in $D001/AD1_{stop844}$ (31.9 ± 4.3%; n = 4; Fig. 6B). As expected, there was no current relaxation at positive holding potentials in $NR1–1a/D001/AD1_{stop844}$ (8.1 ± 2.9%; n = 3), confirming the absence of macroscopic glycine-independent desensitization. These results indicate that a short C-terminal region in NR2A distal to M4 is required for the expression of full calcium-dependent inactivation. This segment in NR2A also influences the peak currents of NMDA responses through tyrosine dephosphorylation involving residues 842 in NR2A and 837 in NR1 (Vissel et al., 2001). However, $NR1–1a_{(Y837F)}/2A_{(Y842F)}$ had normal inactivation (43.4 ± 5.9%; n = 5). To test whether the effects of residue 619 and the C terminus of NR2 were additive, we constructed the chimera $NR2A_{(V619I)4}C$, which incorporates an isoleucine at position 619 and the C terminus of NR2C. $NR1–1a/2A_{(V619I)4}C$ heteromers inactivated to 30.8 ± 12.8% (n = 5). This was not different from the inactivation observed in $NR1–1a/2A_{(V619I)}$ or $NR1–1a/2A_4C$.

Discussion

Complexity of NMDA Channel Structure and Function. Our results show that multiple structural elements contribute to the NR2-subunit specificity of inactivation. This highlights the impact of the complex structure of large proteins such as the NMDA channel on their function. Such complexity is also evident from studies of receptor properties that might be expected to be confined to a single domain. The structural determinants responsible for NMDA channel block by extracellular magnesium are a case in point. The main determinants for extracellular magnesium block are within the pore-forming M2-loop of all NR2 subunits (Burnashev et al., 1992; Mori et al., 1992; Sakurada et al., 1993; Williams et al., 1998; Wollmuth et al., 1998). However, three NR2 regions outside of M2 modulate the efficacy of extracellular magnesium (Kuner and Schoepfer, 1996) and complicate the picture substantially. Even a combined exchange of these regions in NR2C for NR2B is insufficient to produce magnesium block identical to NR2B, indicating that small structural differences outside the pore affect the channel. A similar conclusion has been reached by studies of single point mutations in the NR1 subunit (Kawajiri and Dingledine, 1993). It is not surprising that a similar complexity prevails

Fig. 1. Several molecular elements contribute to NR2-subunit specificity of calcium-dependent inactivation. A, sequence alignment (one-letter code) is shown for the region between the first (M1) and third hydrophobic region (M3) for NR2A and NR2C. Divergent residues are underlined. Vertical lines mark the site of the switch from NR2C to NR2A for chimeras $2C_1A$, $2C_2A$, $2C_{2+i}A(+ i)$, and $2C_{2+ii}A(+ ii)$. B, the left column illustrates the structure of the chimeras with black indicating NR2C and white indicating NR2A sequence. A typical response from each chimera is shown in the middle column, the percentage inactivation in the right column. Except for NR2A, all chimeras contained the NR2C N terminus, which prevented macroscopic glycine-independent desensitization. Thus we were able to use a saturating concentration of agonist without contaminating the measurements of inactivation. As NR2C sequence was incorporated into the first residue after the P-loop, the extracellular M3–4 loop, and the M4/C terminus, inactivation progressively decreased. Asterisks indicate significant difference to NR2A.

when the molecular structures associated with the dynamics of channel gating are studied.

Such complexity is likely to be increased by the environment surrounding the protein. Besides modification of gating by extracellular modulators (for review, see McBain and Mayer, 1994; Dingledine et al., 1999), glycosylation (Everts et al., 1997), or the lipid composition of the membrane (Casado and Ascher, 1998), interactions with intracellular proteins also modulate NMDA channel gating. Such an interaction could explain why the C terminus of NR2 affects inactivation, potentially similar to the protein-protein interactions of the C0 domain of NR1 (Ehlers et al., 1996; Wyszynski et al., 1997; Zhang et al., 1998; Krupp et al., 1999). For example, we have recently proposed that the proximal C-terminal region in NR2A interacts with the nonreceptor type tyrosine kinase Src and the clathrin adaptor protein AP-2 (Vissel et al., 2001).

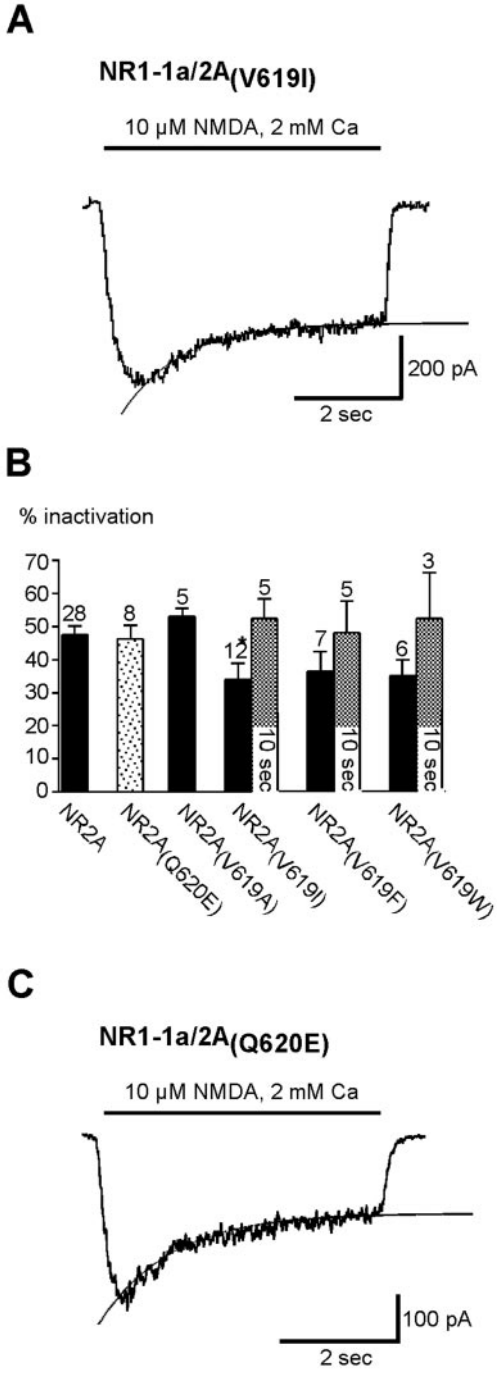


Fig. 2. Residue 619 of NR2A is a codeterminant of the NR2-subunit specificity of inactivation. A, a V619I mutation in NR2A reduced inactivation (5-s NMDA application). The onset of inactivation was fitted by a monoexponential function (τ = 1051 ms). B, replacement of valine 619 with isoleucine, phenylalanine or tryptophan all reduced inactivation (5-s application, ■), whereas longer applications (10-s, ▨) restored full inactivation. Asterisks indicate significant differences to NR2A. C, A Q620E mutation in NR2A did not affect inactivation (onset fitted with a monoexponential function: τ = 1224 ms).

Calcium-Dependent Inactivation and Calcium Permeability: Coexistence or Codependence? Our results show that the M2–3 loop affects two prominent features of the NMDA channel: calcium permeability and calcium-dependent inactivation. Thus, both characteristics could be related functionally and structurally. Although calcium influx through NMDA channels is a prerequisite for calcium-dependent inactivation (Legendre et al., 1993; Krupp et al., 1996), the modulation of both characteristics does not covary with the NR2-subunit. For example, the calcium permeability of NR1/2C heteromers is only 30% lower than NR1/2A heteromers (Burnashev et al., 1995), yet expression of NR2C completely prevents inactivation by a mechanism that cannot be explained by changes in calcium influx (Krupp et al., 1996). In addition, the calcium-permeability of NR1/2A and NR1/2B is slightly higher than NR1/2C and NR1/2D (Monyer et al., 1992, 1994). In contrast, calcium-dependent inactivation occurs in NR1/2A, NR1/2D, and possibly NR1/2B but is absent in NR1/2C (Medina et al., 1995; Krupp et al., 1996). Our findings provide an explanation for the lack of correlation between calcium permeability and calcium-dependent inactivation. A residue that affects calcium permeability (residue 620 in NR2A) is a glutamine in NR2A and NR2B, but a glutamate in NR2C and NR2D. Likewise, the position that codetermines the NR2-subunit specificity of inactivation (residue 619 of NR2A) is a valine in NR2A, NR2B, and NR2D, but an isoleucine in NR2C.

The reduced calcium permeability with exchange of a polar glutamine at position 620 in NR2A to an acidic glutamate of NR2C is reminiscent to findings by Schneggenburger (1998), showing that a charge inversion of residue 621 in NR1 affects calcium permeability. Interestingly, glutamate 621 of NR1 is at a position homologous to an aspartate in AMPA receptor subunits that affects permeation (Dingledine et al., 1992). Thus, although alignments (Ishii et al., 1993) show residue 620 in NR2A displaced by one position from E621 of NR1, our results suggest that the two positions are functionally equivalent. A similar discrepancy exists five residues further toward the N terminus, where the N + 1 site of NR2 is functionally equivalent to the N/Q/*R*-site of NR1 (Burnashev et al., 1992; Kuner et al., 1996; Wollmuth et al., 1996, 1998).

Both NR2C (Burnashev et al., 1995) and the Q620E mutation in NR2A reduce calcium permeability by 20 to 30% compared with receptors containing NR2A. This similarity suggests that position 620 is the major determinant of NR2-subunit specific differences in calcium permeability. Because this effect is small, it is unlikely to be caused by a direct physical interaction with permeating ions. An alternative explanation may be an electrostatic interaction with the N/Q/R-site, as proposed for the corresponding E621K-mutation in NR1 (Schneggenburger, 1998). However, the mutations in

NR2A and NR1 are opposite in sign yet produce the same phenotype. Tikhonov et al. (1999) suggested that the acidic residue at the end of M2 can interact with an arginine at the N/Q/R-site, potentially through a salt bridge, as proposed for AMPA receptors (Dingledine et al., 1992). However, if the N/Q/R-site does not contain an arginine, the acidic residue at the end of M2 can also form a hydrogen bond with a tryptophan in M2 (Tikhonov et al., 1999). In this case, a nucleophilic ring is formed that could affect permeability of the channel. The tryptophan in the proposed hydrogen bond has been reported to affect permeability of NMDA channels (Williams et al., 1998; but see Buck et al., 2000). A polar glutamine instead of the acidic glutamate at the end of M2 would interfere with this interaction and the formation of the nucleophilic ring. However, this residue in NR2C is not modulated by methanethiosulfonate reagents (Kuner et al., 1996), suggesting that the residue might not face the channel lumen.

In contrast, the position that codetermines the NR2 subunit-specificity of inactivation, position 619, is accessible from the cytoplasmic side for modification by small methanethiosulfonate reagents. Methanethiosulfonate ethylammonium, with a headgroup of ~0.38 nm size, could access this position from the intracellular side, but currents were not modified after exposure to the larger methanethiosulfonate ethyltrimethylammonium with a headgroup size of ~0.58 nm (Kuner et al., 1996). Thus, residue 619 may be part of a constriction at the cytoplasmic side of the channel, possibly providing a structural basis for its role in inactivation. Although mutations V619F and V619W had effects on calcium permeability, this could be caused by structural restraints imposed by these large amino acids on the spatial localization of residue 620. In addition, residues with aromatic sidechains such as phenylalanine or tryptophan can also directly influence the permeation properties of ion channels (Yool and Schwarz, 1991; Williams et al., 1998).

Inactivation and the Role of the M2–3 Loop of NR2. Our results show that several residues in the M2–3 loop of

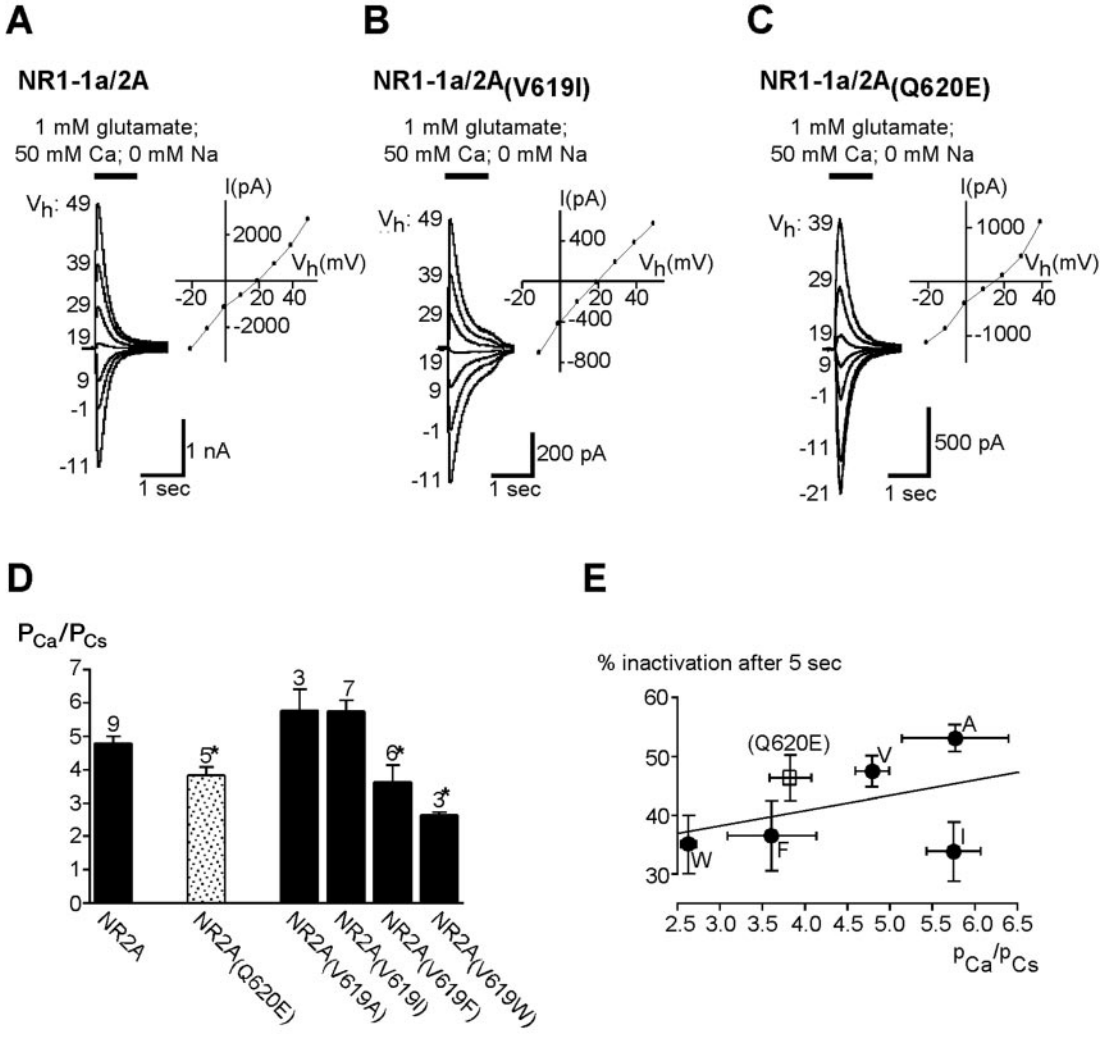


Fig. 3. The effect of the M2–3 loop on inactivation could not be attributed to changes in calcium permeability. A-C, shown are currents evoked at different holding potentials by 1 mM glutamate from cells expressing NR1–1a/2A, NR1–1a/2A$_{(V619I)}$, and NR1–1a/2A$_{(Q620E)}$, respectively. With 50 mM Ca^{2+} in the extracellular solution and 150 mM Cs^{+} in the intracellular solution as the only permeating ions, NR2A and NR2A$_{(V619I)}$ reversed close to +20 mV, whereas NR2A$_{(Q620E)}$ reversed close to +15 mV. The I-V plots show that under these ionic conditions the current-voltage relationships were quasilinear over the voltage range investigated. D, mutation of glutamine 620 in NR2A to the corresponding glutamate of NRC (Q620E) significantly reduced the relative calcium permeability. Mutation of valine 619 to phenylalanine (V619F) or tryptophan (V619W) also reduced the relative calcium permeability, but a mutation (V619I) to the residue present in NR2C did not. Asterisks indicate significance relative to NR2A. E, the relative calcium permeability was not correlated with the degree of inactivation for these mutants (linear regression, $P = 0.42$).

NR2 affect inactivation, including the threonine and serine residues at position 625–627. These residues could in principle be phosphorylated. We did find reduced inactivation in some of the relevant mutants after inhibition of staurosporine-sensitive kinases or casein kinase II. Whereas the two threonines at positions 625 and 626, as well as serine 627, are within good recognition sequences for several staurosporine-sensitive kinases (Kemp and Pearson, 1990), the M2–3 loop of NR2 does not contain a casein kinase II recognition sequence (Guerra et al., 1999). It is also not clear whether phosphorylation of any of the three residues is indeed involved in the effects seen with the kinase inhibitors. However, it is certainly plausible that phosphorylation could affect inactivation. For example, casein kinase II affects the open probability of NMDA channels (Lieberman and Mody, 1999), and protein kinase C can increase inactivation in hippocampal neurons, possibly by regulating the efficiency of receptor interactions with intracellular proteins like calcium/calmodulin or α-actinin (Lu et al., 2000). Because we used the NR1–4a subunit, phosphorylation of the C1-exon of NR1 (Tingley et al., 1997) was not a factor in our experiments.

Some of the chimeras and point mutants, including the

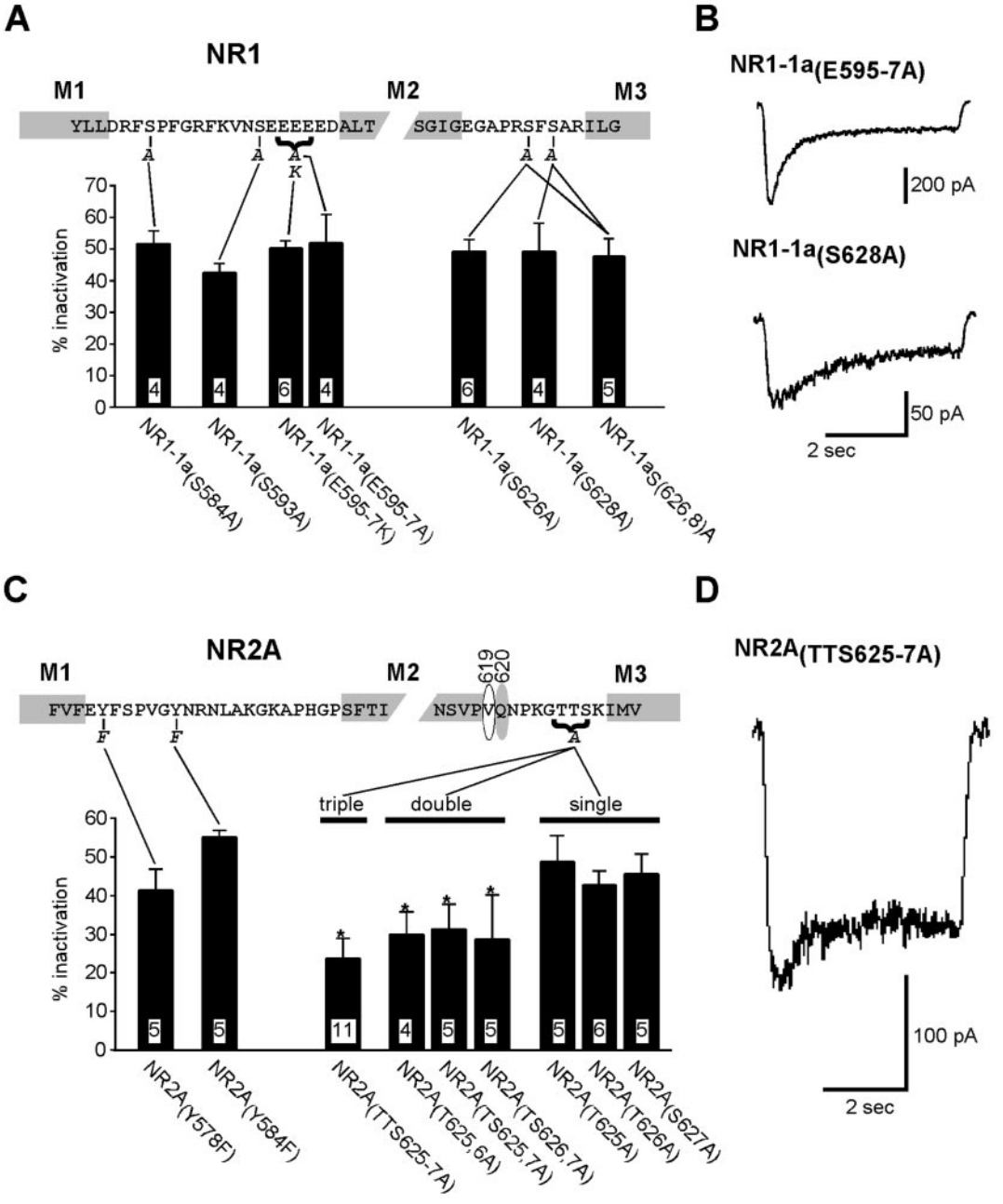


Fig. 4. Mutations of putative phosphorylation-sites in the M2–3 loop of NR2A affect calcium-dependent inactivation. A and B, in NR1–1a, mutations in the intracellular M1–2 and M2–3 loops did not alter inactivation. The amino acid sequence of these loops (one-letter code) and the mutations studied are indicated. Cells were cotransfected with NR2A. C and D, mutations in the intracellular M2–3 loop, but not the M1–2 loop, of NR2A reduced inactivation. The amino acid sequence of these loops (one-letter code) and the mutations studied are indicated. Double and triple mutations of three putative phosphorylation sites in the M2–3 loop of NR2A significantly reduced inactivation as shown in the example in D. Because the point mutations in the M2–3 loop of NR2A were cotransfected with NR1–4a, significance, as indicated by asterisks, was tested against NR1–4a/2A (see Krupp et al., 1999). $NR2A_{(Y578F)}$ and $NR2A_{(Y584F)}$ were cotransfected with NR1–1a.

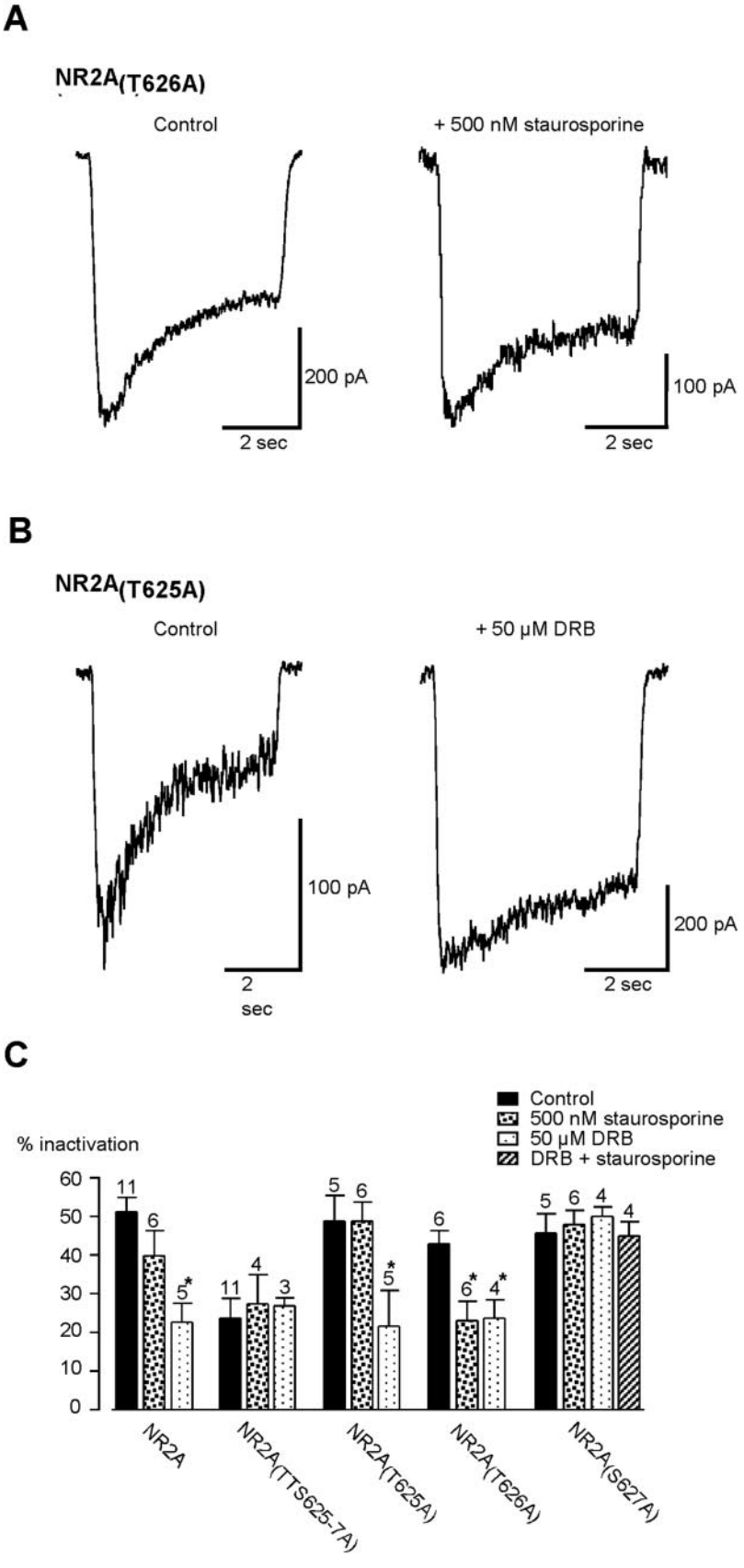


Fig. 5. The 625–627 residues in NR2A are involved in modulation of inactivation by protein kinase inhibitors. A, incubation with the membrane-permeable broad-spectrum kinase inhibitor staurosporine (500 nM) reduced inactivation in NR1–4a/2A$_{(T626A)}$. B, incubation with DRB (50 μM), a membrane-permeable casein kinase II-specific inhibitor, reduced inactivation in NR1–4a/2A$_{(T625A)}$. C, pooled data from experiments as shown in A and B indicate that TTS625–7A and S627A were no longer sensitive to DRB suggesting that residue 627 is involved in a casein kinase-mediated effect. The staurosporine results cannot be explained by effects on a single residue. Asterisks indicate significance relative to their respective control.

double or triple-alanine mutations in the M2–3 loop, reduced inactivation with 5-s applications, but full (~ 50%) inactivation was apparent with longer agonist applications, suggesting that the mutations reduced the likelihood of inactivation rather than eliminating it. In addition to this variability in the degree of inactivation, such mutations often resulted in a biphasic onset of inactivation. This is in contrast to the situation at native NMDA receptors and NR1–1a/2A heteromers, where inactivation can be described by monoexponential functions, largely because of the accumulation of calcium at the intracellular mouth of the channel (Legendre et al., 1993; Krupp et al., 1996). It is plausible that the structural alterations caused by the mutations interfere with protein rearrangements that occur during inactivation, resulting in a biphasic process. Because it is unlikely that conformational movements are responsible for such slow kinetics, the mutations may lower the frequency of interactions between subunits. Such interactions must occur, because inactivation is

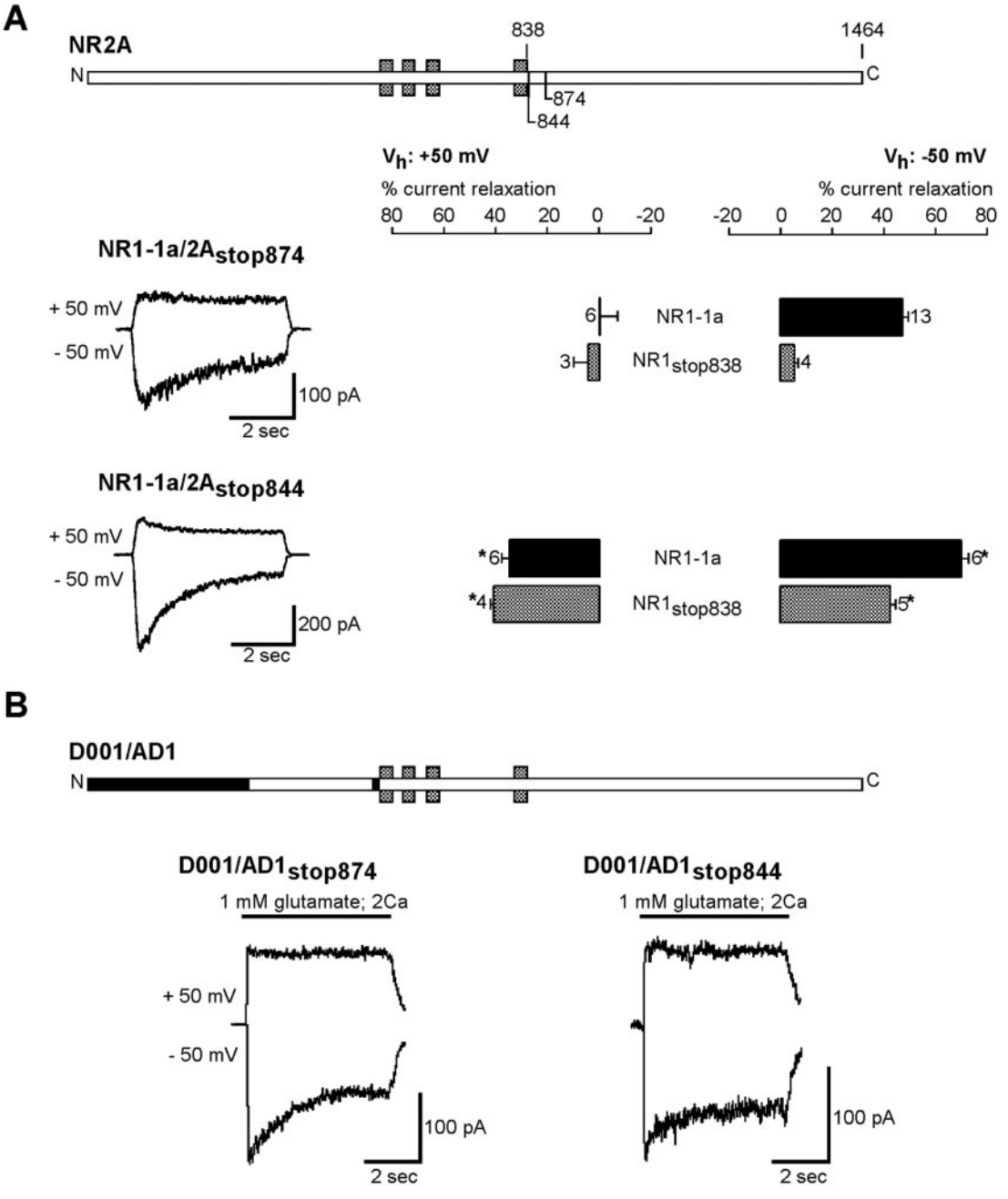


Fig. 6. The C terminus of the NR2 subunit modulates calcium-dependent inactivation. A, the scheme at top illustrates the site of the C-terminal truncations shown. Inactivation in NR1–1a/NR2A$_{stop874}$ was similar to full-length NR2A. Coexpression of NR2A$_{stop874}$ with NR1$_{stop838}$ confirmed that the relaxation was caused by inactivation (▨, upper row). However, complete truncation of the C terminus (NR2A$_{stop844}$) resulted in a calcium- and voltage-independent current relaxation as well as a calcium-dependent inactivation. The calcium-independent relaxation was not abolished in NR1$_{stop838}$/NR2A$_{stop844}$heteromers (▨, lower row). Asterisks indicate significance relative to the respective control with full-length NR2A. B, the calcium-independent relaxation in the C-terminal truncation was abolished by re-engineering the truncation into the nondesensitizing chimera D001/AD1. Inactivation was significantly smaller (31.9%) in heteromers containing D001/AD1$_{stop844}$ compared with D001/AD1 or D001/AD1$_{stop874}$. This suggests that the C-terminal region between residues 844 and 874 modulates inactivation.

NR2-subunit specific (Krupp et al., 1996) yet requires the C terminus of the NR1-subunit as an essential determinant (Krupp et al., 1998, 1999; Zhang et al., 1998). Such a scenario could also provide a basis for the critical role of the M2–3 loop in inactivation. We have shown previously that residues 834 to 843 in NR1 have an intrinsic effect on NMDA channel gating (Krupp et al., 1999). Among the characteristics of these residues (EIA*YKRHKD*A) is a short string of polar residues (underlined) that could provide a basis for electrostatic interactions. In fact, the polarity profile of this sequence $(-+++++-)$ is nearly a negative mirror $(+n---+)$ of the C-terminal part (underlined) of the M2–3 loop of NR2A (VQNP*KGTTSK*), raising the possibility of an interaction of both regions.

Implications for Synaptic Transmission. Calcium-dependent inactivation provides a mechanism for activity-dependent regulation of synaptic NMDA receptors. The slow kinetics of inactivation in whole-cell recordings might seem to indicate that it does not play a role during fast excitatory transmission. However, inactivation is present at synapses, presumably because local rapid accumulations of calcium are sufficient (Rosenmund et al., 1995; Umemiya et al., 2001). Although most synapses contain both NMDA and AMPA receptors, inactivation may be particularly important under conditions in which the NMDA receptors dominate. The present results add to the existing evidence that not only calcium influx, but also interactions between subunits and with regulatory proteins, such as calmodulin and α-actinin, are involved in the inactivation process.

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