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ACCELERATED COMMUNICATION

Calcium-Dependent Inactivation of Recombinant *N*-Methyl-D-aspartate receptors Is NR2 Subunit Specific

JOHANNES J. KRUPP, BRYCE VISSEL, STEPHEN F. HEINEMANN, and GARY L. WESTBROOK

Vollum Institute, Oregon Health Sciences University, Portland, Oregon 97201 (J.J.K., G.L.W.), and Salk Institute, La Jolla, California 92037 (B.V., S.F.H.)

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SUMMARY

Intracellular Ca^{2+} can reversibly reduce the activity of native *N*-methyl-D-aspartate (NMDA) receptors in hippocampal neurons, a phenomenon termed Ca^{2+}-dependent inactivation. We examined inactivation in heteromeric NMDA receptors expressed in human embryonic kidney (HEK) 293 cells using whole-cell recording. NR1-1a/2A heteromers showed reversible inactivation that was very similar to native NMDA receptors in cultured hippocampal neurons. Inactivation was dependent on the extracellular Ca^{2+} concentration and the degree of intracellular Ca^{2+} buffering. In 2 mM extracellular Ca^{2+}, inactivation resulted in a 46.1 ± 12.6% reduction in the whole-cell current during a 5-sec agonist application. Inactivation of NR1-1a/2A heteromers was unaffected by calcineurin inhibitors, staurosporine, or phalloidin. NR1-1a/2D heteromers also showed a similar degree of inactivation. In contrast, NR1-1a/2B and NR1-1a/2C heteromers showed no significant inactivation. At saturating concentrations of NMDA (1 mM), NR1-1a/2A heteromers also showed Ca- and glycine-independent desensitization, as seen in native hippocampal neurons. Ca^{2+}- and glycine-independent desensitization was less pronounced in NR1-1a/2B heteromers and absent in NR1-1a/2C heteromers. Activation of NR1-1a/2C heteromers triggered intracellular Ca^{2+} transients similar to NR1-1a/2A heteromers as verified by combined Ca^{2+} imaging and whole-cell recording. Thus differences in Ca^{2+} permeability were not responsible for the lack of inactivation in NR1-1a/2C heteromers. Our results show that inactivation of recombinant NMDA receptors requires either the NR2A or NR2D subunit, whereas both inactivation and desensitization were absent in NR2C-containing receptors. The gating of inactivating NMDA receptors is more likely to be influenced by ongoing NMDA receptor activity and Ca^{2+} transients, perhaps consistent with the prominent expression of NR2A in hippocampus and cerebral cortex.

The activity of NMDA receptors is highly regulated, including both allosteric modulation by extracellular substances and intracellular regulation by Ca^{2+}, kinases, and phosphatases (1). Transient increases in intracellular Ca^{2+} downregulate NMDA channel activity in cultured hippocampal neurons (2–5). Thus, intracellular Ca^{2+} may be important in dynamically influencing NMDA receptor activity at synapses (6, 7). In whole-cell recording, increases in intracellular Ca^{2+} evoked by NMDA applications induce a slow relaxation of agonist-induced current that has been termed Ca^{2+}-dependent inactivation (8) to distinguish it from either glycine-dependent or -independent receptor desensitization (1). Inactivation is a form of desensitization in that the current is reduced during agonist application (i.e., as Ca^{2+} increases in the cell). However, inactivation of NMDA receptors does not require ligand binding or channel opening (8), thus distinguishing it from NMDA receptor desensitization. Although inactivation does not seem to directly involve kinases or phosphatases (3), its molecular mechanism or mechanisms are not understood.

The cloning of NMDA receptor subunits has revealed significant functional heterogeneity among different subunit combinations (1, 9, 10). Subunit-specific differences in receptor function have also provided clues to the domains that

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; HEK, human embryonic kidney; GFP, green fluorescent protein; DMSO, dimethylsulfoxide; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CaN, calcineurin; CaM, calmodulin; EPSC, excitatory postsynaptic current; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; pCa, log[Ca^{2+}].

interact with regulatory molecules (1). NMDA receptors are heteromers of two classes of subunits that in the rat are termed NMDAR1 (or NR1) and NMDAR2 (NR2). NR1 subunits arise from one gene, with eight functional splice variants (11, 12), whereas the NR2 subunits (2A–2D) are coded by four different genes (9, 13, 14). The NR1 subunit is ubiquitously expressed in neurons, whereas NR2 subunits vary in their spatial and developmental expression patterns (15, 16). In cortical and hippocampal neurons, NR2A and NR2B are the predominant NR2 subunits (15–17), although mRNA encoding NR2C and NR2D subunits, presumably located in interneurons, can also be found (15, 16).

Whether Ca^{2+}-dependent inactivation of NMDA receptors is subunit specific has not been examined, although Ca^{2+} seems to regulate recombinant NMDA receptors (18). We report the effect of intracellular Ca^{2+} on recombinant NMDA receptors expressed in HEK 293 cells. We compared heteromers composed of NR1-1a plus NR2A, NR2B, NR2C, and NR2D, respectively. All four heteromers expressed functional channels, but only NR1-1a/2A and NR1-1a/2D heteromers showed significant Ca^{2+}-dependent inactivation. NR1-1a/2C heteromers also lacked Ca- and glycine-independent desensitization. The subunit-specific differences in Ca^{2+} regulation provides one mechanism by which neurons can differentially regulate synaptic-mediated Ca^{2+} influx.

Materials and Methods

Preparation of DNA and transfection and identification of transfected cells. NR1-1a, NR2B, and NR2D were cloned at the Salk Institute; NR2A and NR2C were a gift from S. Nakanishi (Dept. of Biological Science, Kyoto University, Kyoto, Japan). Bluescript cDNAs encoding NR1-1a, NR2A, NR2B, NR2C, and NR2D were inserted into the pcDNA1/Amp vector (InVitrogen, San Diego, CA). The terminology for the NMDA subunits and splice variants follows that of Hollmann *et al.* (12). All clones were confirmed by restriction and partial sequence analyses before use. To identify cells expressing NMDA receptors, HEK 293 cells were cotransfected with cDNA coding for GFP (19) or with cDNA for the lymphocyte CD4 receptor. Both constructs were inserted into the JPA vector kindly provided by John Adelman (Vollum Institute). GFP fluorescence was visualized on a Nikon Diaphot microscope with standard fluorescein filter sets. The expression of the CD4 receptor was confirmed with CD4 receptor antibody-coated beads. For bead coating, 1 μl of Dynabeads M-450 CD4 (Dynal, Oslo Norway) was added with 1 ml of medium to each 35-mm dish; then, the dish was gently swirled for 15–20 min before recording.

HEK 293 cells were plated 6–12 hr before transfection in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 1% glutamine (GIBCO, Grand Island, NY), and 1% penicillin/streptomycin (GIBCO; 37° at 5% CO_2). Cells were plated onto 31-mm glass coverslips placed in 35-mm dishes. The cDNAs for NR1/NR2x/GFP-CD4 were mixed in a 4:4:1 ratio and added to HEK 293 cells as a Ca^{2+}-phosphate complex (20). Kynurenic acid (3 mM, Sigma Chemical, St. Louis, MO) and DL-AP5 (1 mM; Tocris, Essex, UK) were routinely added to prevent NMDA receptor-mediated excitotoxic cell death (21). The transfection mixture was removed after 8–16 hr by exchange with fresh culture medium containing kynurenic acid and DL-AP5. FUDR (0.2 mg/ml 5′-fluoro-2-deoxyuridine 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 12–48 hr after the end of the transfection. The recording chamber was continuously superfused at room temperature (~20°) with an extracellular solution of 162 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, and 1 mM $CaCl_2$, pH 7.25 with NaOH, 325 mOsM. High performance liquid chromatography-grade water was used for all solutions 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 of 2–5 MΩ. The intracellular solution included an ATP-regenerating system (3, 22) composed of 115.5 mM $CsCH_4SO_3$, 10 mM HEPES, 6 mM $MgCl_2$, 4 mM Na_2ATP, 20 mM phosphocreatine, 500 units/ml creatine phosphokinase, 0.1 mM leupeptin, and 0.1 mM EGTA, pH 7.2 with CsOH, 320 mOsM (sucrose). In some experiments, the EGTA or BAPTA concentration was varied to test the influence of intracellular Ca^{2+} on inactivation. The pCa was calculated for no-added Ca^{2+} solution assuming a 50 μM Ca^{2+} contamination. 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, Burlingame, CA). The membrane voltage was clamped at −50 mV unless otherwise indicated. Currents were filtered at 5 kHz, low-pass filtered at 0.2 kHz, and digitized at 1 kHz. All recordings were made from isolated cells. Series resistance was routinely compensated (60–90%), and cell input resistance (range, 400-3000 MΩ) was continuously monitored with a short −10-mV voltage step preceding each agonist application.

NMDA (10 μM to 1 mM; Tocris) and L-glutamate (100 μM to 1 mM; Sigma) were applied with a fast microperfusion system described previously (3). Glycine (10 μM) was added to the control and drug solutions to prevent glycine-dependent desensitization (23). Unless otherwise noted, agonist was applied for 5 sec at 30-sec intervals. The extent of inactivation was measured as the percentage reduction in current amplitude at the end of the 5-sec application compared with the initial peak. Due to decreases in current amplitude during prolonged whole-cell recording, responses were analyzed during the first 5 min unless otherwise noted (see below). In experiments using FK506 (Fujisawa, Melrose Park, IL; DMSO), cyclosporine A (a gift from J. D. Scott, Vollum Institute; ethanol), or staurosporine (Sigma; DMSO); cells were incubated for ≥15 min before recording in extracellular solution containing the respective reagent. These reagents (plus solvent) were also present in control and agonist-containing solutions after establishment of the whole-cell configuration. Calmidazolium (Calbiochem, San Diego, CA; DMSO), CaM binding domain peptide (amino acids 290–309 of CaM II kinase; Calbiochem; water), and phalloidin (Molecular Probes, Eugene, OR; water) were added to the intracellular pipette solution. To allow time for diffusion of these low-molecular-weight substances into the cell (3, 24), measurements were made after 5 min of whole-cell recording. The activities of cyclosporine, staurosporine, calmidazolium, and the CaM binding domain peptide was verified using *in vitro* kinase assays (courtesy of Brian Perrino and Debra Brickey, Vollum Institute).

Combined Ca^{2+}-imaging and patch-clamp experiments. Combined whole-cell and Ca^{2+} imaging was performed using a inverted microscope (Axiovert 100, Zeiss) in combination with an Odyssey XL confocal scanning laser system (Noran Instruments, Middleton, WI). Cells were dye loaded through the whole-cell pipette with 0.2 mM fluo-3-tetrapotassium salt (Molecular Probes; water) added to the intracellular solution. Filter wavelengths were 488 nm for excitation and 515 nm for long-pass emission with a slit aperture of 100 μm. The fluorescence signal was obtained from a rectangular box placed over the cell soma. Video frames were digitized and eight frames averaged (0.267 Hz) using Intervision 1.4.1. software (Noran Instruments) running on an Indy workstation (Silicon Graphics). Changes in intracellular Ca^{2+} concentration are expressed as $\Delta F/F$, where F is the resting fluorescence preceding agonist application (mean of a 10-sec interval immediately preceding agonist application), and ΔF is the fluorescence at a given time. Values are given as peak $\Delta F/F$, where peak ΔF is the average value of a 2-sec stretch during the peak of the Ca^{2+} response on agonist application. Whole-cell currents were measured as above using a separate computer. To

avoid background fluorescence due to GFP, all imaging experiments were done with cells coexpressing the CD4 receptor.

Data and statistical analyses. Data are expressed as mean ± standard deviation. For statistical comparisons, Student's *t* test and analysis of variance were used as appropriate. Statistical significance was set at $p < 0.05$.

Results

Ca^{2+}-dependent inactivation in NR1-1a/2A heteromers expressed in HEK 293 cells. Native NMDA receptors in hippocampal neurons show glycine-dependent (23) and glycine-independent (25) desensitization, as well as Ca^{2+}-dependent inactivation. To examine inactivation in isolation from other forms of desensitization, a low concentration of NMDA (10 μM) was applied to HEK 293 cells in the presence of a saturating concentration of glycine (10 μM). This protocol elicits no macroscopic desensitization in native NMDA receptors (8). In HEK 293 cells expressing NR1-1a/2A heteromers, currents evoked by 10 μM NMDA in low extracellular Ca^{2+} (0.2 mM) also showed little or no desensitization (amplitude, 10–1070 pA; seven cells; Fig. 1A, *bottom right*). In contrast, currents evoked in 2 mM $[Ca^{2+}]_o$ inactivated slowly during the agonist application, reaching a steady state level of ~50% at the end of the 5-sec application (Fig. 1A, *bottom left*).

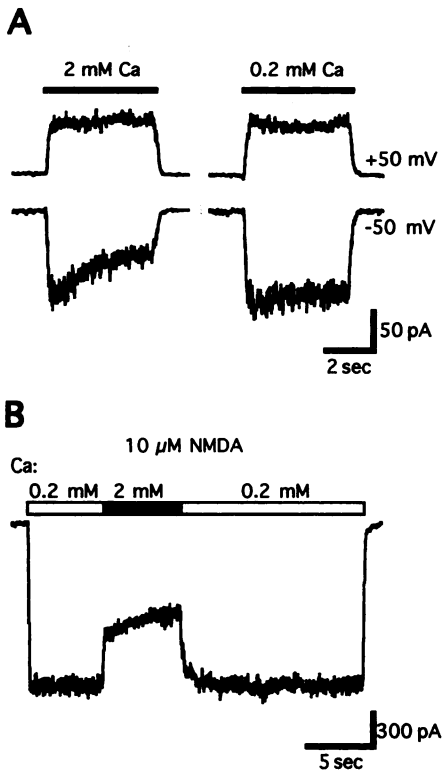

Fig. 1. NR1-1a/2A heteromers show reversible Ca^{2+}-dependent inactivation. A, 10 μM NMDA (5 sec, 0.033 Hz) was applied to a HEK 293 cell expressing NR1-1a/2A heteromers in high (2 mM) and low (0.2 mM) extracellular Ca^{2+}. At −50 mV, application of NMDA in high Ca^{2+} induced a slow relaxation (τ_{inact} = 2.1 sec) that was absent in low Ca^{2+} and at +50 mV. Inactivation reached 49.7% by the end of the 5-sec application in this cell. B, In another cell, Ca^{2+} was increased from 0.2 to 2 mM during a long (22 sec) application of NMDA (10 μM). On exposure to high Ca^{2+}, there was an instantaneous drop in the current amplitude (due to the lower unitary channel conductance), followed by the onset of inactivation. Inactivation was completely reversible on return to the low Ca^{2+} solution.

Longer agonist applications in high $[Ca^{2+}]_o$ resulted in no further reduction of the whole-cell current (not shown). No significant inactivation was present at +50 mV (Fig. 1A, *top*). Thus, NR1-1a/2A heteromers show inactivation similar to hippocampal NMDA receptors.

To further test the similarity of inactivation between NR1-1a/2A heteromers and native NMDA receptors, we tested the reversibility of inactivation (Fig. 1B). During a long (22 sec) application of NMDA (10 μM), an increase in $[Ca^{2+}]_o$ from 0.2 to 2 mM for 5 sec resulted in an instantaneous reduction in the whole-cell current due to the lower unitary conductance of NMDA channels in high $[Ca^{2+}]_o$ (26, 27). The subsequent development of inactivation is easily apparent due to its much slower onset. On return to low $[Ca^{2+}]_o$, a rapid increase in the whole-cell current, reflecting the increased unitary conductance, was followed by full recovery from inactivation in 4.8 ± 3.1 sec (seven cells). In some cells, the time course of recovery was multiexponential, suggesting that several steps were involved. Recovery did not require open NMDA channels because currents evoked by test pulses in low $[Ca^{2+}]_o$ 30 sec before and after an agonist application in high $[Ca^{2+}]_o$ had the same amplitude (not shown). The onset of inactivation and the degree of inactivation at steady state were similar with the use of the protocol in either Fig. 1A or 1B (not shown).

Inactivation in NR1-1a/2A heteromers was due to increases in intracellular Ca^{2+}. Ca^{2+}-dependent inactivation in NR1-1a/2A heteromers was affected by the $[Ca^{2+}]_o$ as well as by the extent of intracellular Ca^{2+} buffering. We first examined the effect of extracellular Ca^{2+} in HEK 293 cells buffered with 0.1 mM EGTA in the whole-cell pipette. The increases in inactivation for one cell in the presence of 0.2, 0.6, and 1.0 mM $[Ca^{2+}]_o$ are shown in Fig. 2A. No inactivation was present in 0.2 mM $[Ca^{2+}]_o$ (−2.1 ± 19.1%, seven cells) compared with 46.1 ± 12.6% inactivation in 2 mM $[Ca^{2+}]_o$ (23 cells; Fig. 2B). No further inactivation was observed by increasing $[Ca^{2+}]_o$ to 6 mM (two cells). The half-maximal inactivation was 747 μM $[Ca^{2+}]_o$. The inactivation time constant (τ_{inact}) in 2 mM $[Ca^{2+}]_o$ was 2.7 ± 1.8 sec (range, 0.4 to 7.5 sec; 20 cells). The τ_{inact} decreased with increasing $[Ca^{2+}]_o$ for individual cells, but the time constants between cells were more variable than observed in hippocampal neurons (8), perhaps reflecting differences in Ca^{2+} handling in the HEK 293 cells.

The degree of inactivation was dependent on the amount of EGTA in the whole-cell pipette. With 10 mM EGTA and 1 mM $CaCl_2$ (pCa = 7.8), inactivation was significantly less (29.2 ± 18.7%, 17 cells) than with 0.1 mM EGTA with no added Ca^{2+} (pCa = 6.8, Fig. 2C). Although the degree of inactivation was very consistent in 0.1 mM EGTA, the cell-to-cell variability in the degree of inactivation was more variable in the presence of higher concentrations of EGTA. In contrast, the fast Ca^{2+}-chelator BAPTA [either 2.4 mM (pCa = 7.6) or 10 mM (pCa = 7.9)] prevented inactivation in HEK 293 cells expressing NR1-1a/2A heteromers. Thus, inactivation of NR1-1a/2A heteromers seems to require increases in intracellular Ca^{2+} as for native NMDA receptors. However, higher pipette concentrations of EGTA or BAPTA were necessary to prevent inactivation in hippocampal neurons compared with HEK 293 cells. We suspect that this difference is due to more effective dialysis of HEK 293 cells compared with neurons rather than to intrinsic differences in the Ca^{2+} sensitivity of inactivation.

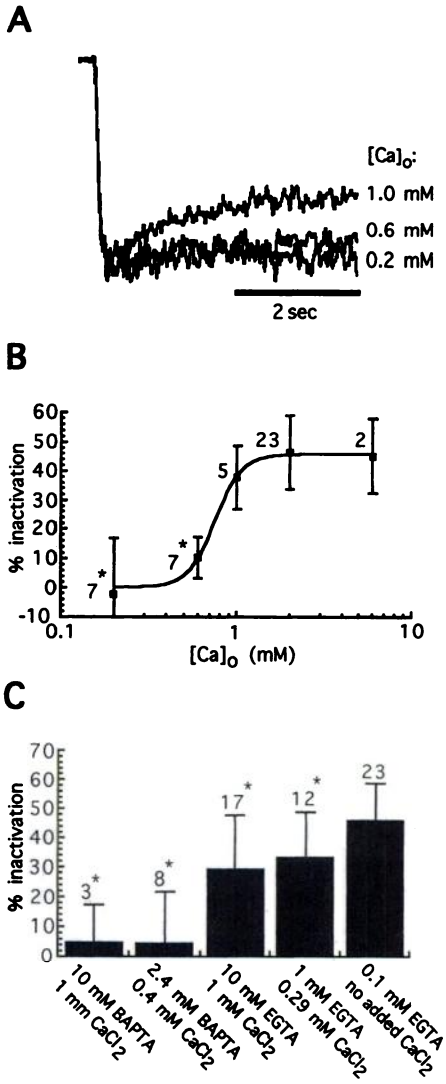

Fig. 2. The degree of Ca^{2+}-induced inactivation is influenced by $[Ca^{2+}]_o$ and the degree of intracellular Ca^{2+} buffering. A, NMDA (10 μM, 5 sec, 0.033 Hz) was applied at three different extracellular Ca^{2+} concentrations in an HEK 293 cell expressing NR1-1a/2A heteromers. Currents were normalized to the initial peak and superimposed. Inactivation was 5.5% (0.2 mM Ca^{2+}), 10.7% (0.6 mM Ca^{2+}), and 29.0% (1 mM Ca^{2+}) in this cell. B, The percent inactivation was plotted as a function of $[Ca^{2+}]_o$ for the NR1-1a/2A heteromers. Data were fitted with the logistic equation $I = I_{max} \times 1/(1 + (IC_{50}/[Ca^{2+}]_o)^n)$, where I is the percentage of inactivation, I_{max} is maximal inactivation (45.6%), IC_{50} is half-maximal inactivation (746.9 μM), and n is the slope factor (5.6). *Numbers*, number of cells tested under each condition. *, Statistically significant differences from 2 mM $[Ca^{2+}]_o$. C, Inactivation was compared with $[Ca^{2+}]_o$ of 2 mM for different intracellular Ca^{2+} buffering conditions. BAPTA almost completely prevented inactivation. However, inactivation was not prevented by several different EGTA/Ca^{2+} solutions. Inactivation was most consistent when using 0.1 mM EGTA. *, Significance compared with 0.1 mM EGTA/no-added $CaCl_2$.

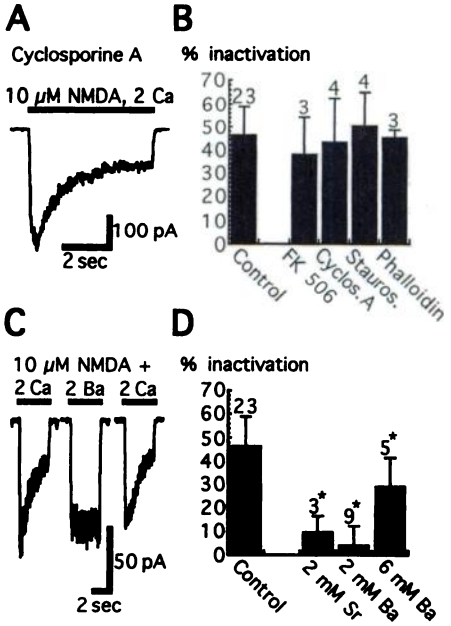

Fig. 3. Effect of CaN and kinase inhibitors as well as divalent cations on inactivation of NR1-1a/2A heteromers. A, Incubation of a HEK 293 cell expressing NR1-1a/2A heteromers with cyclosporine A (500 nM) did not prevent inactivation. Current was evoked by NMDA (10 μM) in 2 mM $[Ca^{2+}]_o$. The cell was incubated for >15 min in extracellular solution containing cyclosporine A (500 nM) before and during the whole-cell recording. B, Two different CaN inhibitors [100 nM FK506 and 500 nM cyclosporine A (*Cyclos. A*)] as well as the nonspecific kinase inhibitor staurosporine (*Stauros.*; 1 μM) had no significant effect on inactivation. The actin-stabilizing reagent phalloidin (1 μM) also did not prevent inactivation. The number of cells tested for each drug are indicated. C and D, Substitution of extracellular Ca^{2+} with equimolar concentrations of Ba^{2+} reversibly prevented inactivation in this cell, although higher concentrations of Ba^{2+} caused partial inactivation. The effects of 2 mM $[Sr^{2+}]_o$ as well as 2 and 6 mM $[Ba^{2+}]_o$ were significantly different from the control (2 mM $[Ca^{2+}]_o$). The control data shown in B and D are the same data shown in Fig. 2. All statistical comparisons were made with analysis of variance with post hoc unpaired Student's *t* tests.

Effect of Ca^{2+}-dependent enzymes on inactivation. Phosphatase 2B (CaN) can affect NMDA receptor activity (7, 28, 29), but our previous results gave no indication that dephosphorylation via CaN was responsible for NMDA receptor inactivation in cultured hippocampal neurons (3). We reinvestigated this with the use of NR1-1a/2A heteromers. Inactivation was examined in HEK 293 cells after preincubation (>15 min) with the CaN inhibitors FK506 (100 nM) or cyclosporine A (500 nM). Inactivation was 38.1 ± 16.1% (three cells) for FK506-treated cells and 43.0 ± 19.5% (four cells) for cyclosporine A-treated cells, which was not significantly different from untreated controls (Fig. 3, A and B). Preincubation with the nonspecific kinase inhibitor staurosporine (500 nM) also did not affect the degree of inactivation (50.2 ± 14.9%; four cells; Fig. 3B). Similarly, intracellular dialysis with 1 μM phalloidin, an actin-depolymerization inhibitor that has previously been shown to prevent Ca^{2+}-dependent rundown of NMDA responses (3), had no effect on inactivation (45.0 ± 3.4%; three cells; Fig. 3B). Thus, as for native NMDA receptors, inactivation in NR1-1a/2A heteromers does not seem to directly depend on the state of receptor phosphorylation. However, we examined a relatively small number of cells, so we cannot exclude that phosphorylation could have a small modulatory effect on inactivation.

Ehlers *et al.* (30) reported that CaM can bind to the cytoplasmic domain of NR1 and reduce recombinant NMDA channel activity in excised patches. We tested the effects of two CaM inhibitors, calmidazolium and CaM binding domain peptide, on inactivation in NR1-1a/2A heteromers. The CaM inhibitors were added to the whole-cell pipette and allowed to diffuse into the cell for 5 min before the first agonist application. If CaM is required for inactivation, treated cells would not be expected to show inactivation. However, the

NMDA current induced by the first agonist application showed prominent inactivation when 10 μM calmidazolium was present in the pipette (51.6 ± 18.0%; five cells). In five cells, inactivation was not significantly reduced by the CaM binding domain peptide (500 nM; 27.7 ± 15.6%). These results do not exclude a role for CaM in NMDA receptor regulation but suggest that CaM is not required for inactivation.

Effects of other divalents on inactivation in NR1-1a/2A heteromers. Ca^{2+}-dependent inactivation in cultured hippocampal neurons can be induced by other divalent cations, such as Ba^{2+} or Sr^{2+} (8). In NR1-1a/2A heteromers, no inactivation was apparent when extracellular Ca^{2+} was replaced with 2 mM Ba^{2+} (inactivation, 4.1 ± 7.9%; nine cells) or Sr^{2+} (9.5 ± 6.9%; three cells). Inactivation could be induced on reapplication of NMDA in a Ca^{2+}-containing solution (Fig. 3C). However, partial inactivation was present when extracellular Ba^{2+} was increased from 2 to 6 mM (29.1 ± 12.3%; five cells; Fig. 3D). Like in hippocampal neurons (8), the inactivation time constant was faster with 6 mM Ba^{2+} (1.5 ± 0.4 sec; four cells) than with 2 mM Ca^{2+} (2.7 ± 1.8 sec; 20 cells).

Ca^{2+}-dependent inactivation is NR2-subunit specific. To examine the role of the NR2 subunit on inactivation, we expressed NR1-1a with NR2A, NR2B, NR2C, and NR2D. However, the protocol used for examining inactivation in NR1-1a/2A heteromers was not sufficient for NR2B- and NR2C-containing receptors because of smaller amplitude currents. As shown in Fig. 4 (*left*), a low concentration of NMDA (10 μM) produced the expected inactivation in NR1-1a/2A, but the NMDA currents were much smaller in NR1-1a/2B heteromers (6–104 pA; six cells) and unmeasurable in NR1-1a/2C heteromers (10 cells). Thus, we compared currents evoked by a saturating concentration of NMDA (1 mM) in 2 or 0.2 mM $[Ca^{2+}]_o$. Glycine (10 μM) was added to all solutions. In hippocampal neurons, this protocol reveals two forms of NMDA receptor desensitization: fast glycine-independent desensitization (25) and the slower Ca^{2+}-dependent inactivation.

NMDA (1 mM) evoked large currents in NR1-1a/2A heteromers (108–1512 pA). In low Ca^{2+}, a fast relaxation was present at −50 mV as well as at positive holding potentials, which is consistent with glycine-independent desensitization. However, in 2 mM $[Ca^{2+}]_o$, a slow relaxation due to inactivation became apparent (Fig. 4A, *right*). The current at the end of the 5-sec application was reduced by 42.6 ± 12.0% in 2 mM $[Ca^{2+}]_o$, which was significant greater than the reduction for the same cells in low Ca^{2+} (27.4 ± 11.3%, eight cells).

The current amplitudes for NR1-1a/2B heteromers were smaller (90–570 pA) than for NR1-1a/2A. Desensitization was present for currents evoked by 1 mM NMDA for NR1-1a/2B heteromers, but the relaxations were not significantly different between 0.2 and 2 mM $[Ca^{2+}]_o$ (Fig. 4B, *right*). For five cells, the current at the end of the agonist application was reduced by 24.2 ± 10.3% in 2 mM $[Ca^{2+}]_o$ and 13.9 ± 3.6% for 0.2 mM $[Ca^{2+}]_o$. NR1-1a/2C heteromers showed only small currents (10–32 pA) on application of 1 mM NMDA (Fig. 4C, *right*). More interestingly, NR1-1a/2C heteromers did not show glycine-independent desensitization or Ca^{2+}-dependent inactivation. The currents at the end of the agonist application were 107.9 ± 9.8% of the initial peak for 2 mM $[Ca^{2+}]_o$ and 108.8 ± 10.3% for 0.2 mM $[Ca^{2+}]_o$ (five cells).

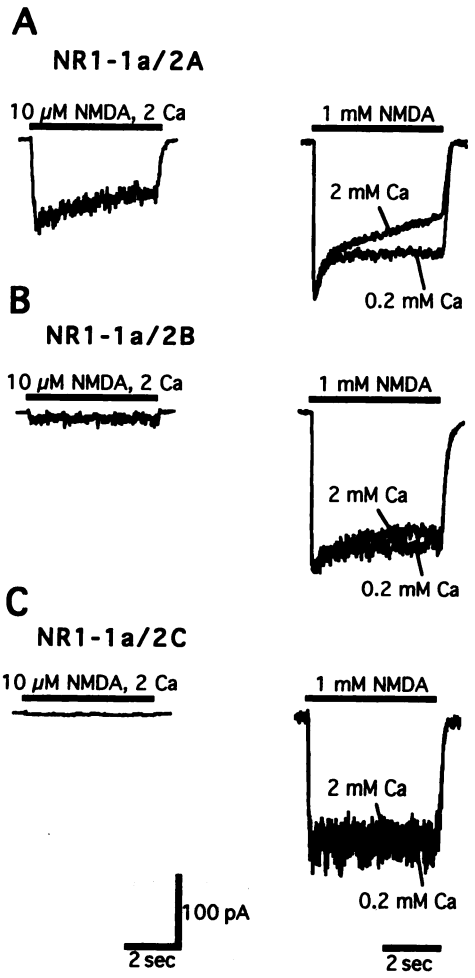

Fig. 4. Inactivation was present only in NR1-1a/2A heteromers. *Traces on the left*, responses to 10 μM NMDA in 2 mM $[Ca^{2+}]_o$ for HEK 293 cells expressing NR1-1a with NR2A, NR2B, or NR2C. *Traces on the right*, superimposed and normalized responses at a saturating concentration of NMDA (1 mM) in high (2 mM) and low (0.2 mM) $[Ca^{2+}]_o$. A, For a cell expressing NR1-1a/2A heteromers, currents evoked by 10 μM NMDA showed prominent inactivation (*left*). Responses evoked by 1 mM NMDA (*right*) showed fast glycine-independent desensitization in low Ca^{2+} solutions. However, in high $[Ca^{2+}]_o$, a slower component was apparent, which is consistent with Ca^{2+}-dependent inactivation. B, In a cell expressing NR1-1a/2B heteromers, 10 μM NMDA (*left*) induced only a small current without apparent inactivation. At 1 mM NMDA (*right*), glycine-independent desensitization was present in low Ca^{2+}, but no significant inactivation was apparent in 2 mM Ca^{2+}. C, In a cell expressing NR1-1a/2C heteromers, 10 μM NMDA induced very small currents that were difficult to measure (*right*). However, responses to 1 mM NMDA (*left*) showed no relaxation of the macroscopic current in 0.2 or 2 mM $[Ca^{2+}]_o$. Thus, NR1-1a/2C heteromers lack both desensitization and inactivation.

NR1-1a/2D heteromers also showed inactivation on application of 10 μM NMDA in 2 mM $[Ca^{2+}]_o$ (54.8 ± 14.8%; three cells). The inactivation time constant was similar to NR1-1a/2A heteromers (3.3 ± 1.6 sec; three cells). Consistent with the presence of inactivation, responses evoked by 1 mM glutamate in the presence of 2 mM $[Ca^{2+}]_o$ showed a relaxation at −50 mV of 48.7 ± 26.9% (five cells), whereas there was no significant relaxation at +50 mV (4.5 ± 9.1%). Likewise, currents evoked by 1 mM glutamate in low extracellular Ca^{2+} showed no significant relaxation (6.4 ± 20.3%, four cells). These results suggest that NR1-1a/2D heteromers have inactivation but lack glycine-independent desensitization.

The lack of inactivation in NR1-1a/2C heteromers is not due to insufficient Ca^{2+} influx. The lack of inactivation in NR1-1a/2C heteromers possibly could be explained by subunit differences in Ca^{2+} influx, although the fractional Ca^{2+} fluxes through NR1-1a/2A and NR1-1a/2C heteromers are very similar (31). Current amplitudes were smaller for NR1-1a/2C heteromers, but the charge transfer (in nanocoulombs) evoked by NMDA applications provides a measure of Ca^{2+} influx through the respective heteromeric receptors. As shown in Fig. 5A, inactivation was present in cells expressing NR1-1a/2A heteromers (■) even when the charge transfer during agonist application was very small. Therefore, no lower limit for the induction of inactivation was apparent. This result is not surprising because the effective site of Ca^{2+} is likely to be close to the membrane. In contrast, cells expressing NR1-1a/2C heteromers showed no inactivation (□ and ○) even when the charge transfer was equal to that of NR1-1a/2A-expressing cells that showed inactivation.

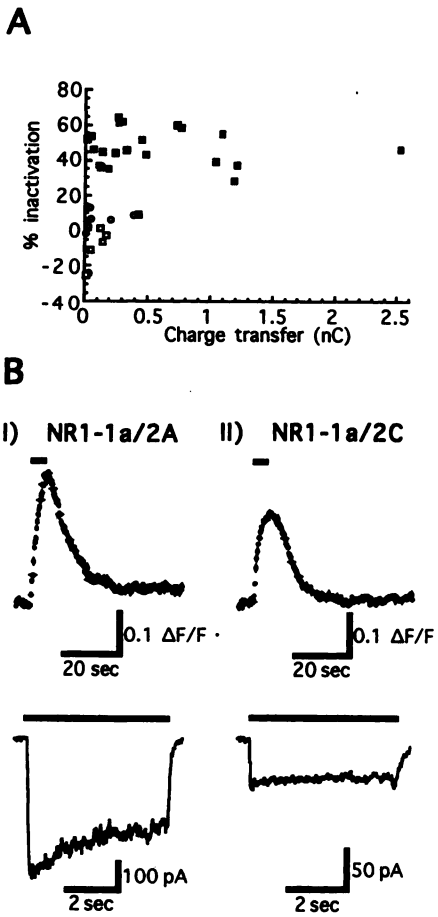

Fig. 5. NMDA evoked Ca^{2+} transients in cells expressing NR1-1a/2A or NR1-1a/2C, but only NR1-1a/2A heteromers showed inactivation. A, The percentage of inactivation was plotted as a function of the charge transfer for responses of NR1-1a/2A heteromers (10 μM NMDA, ■) and NR1-1a/2C heteromers (1 mM NMDA (□) or 1 mM glutamate (○). NR1-1a/2C heteromers showed no inactivation even when the charge transfer was sufficient to induce inactivation in NR1-1a/2A heteromers. B, Simultaneous Ca^{2+} imaging (*top traces*) and whole-cell recording (*bottom traces*) in two cells expressing either NR1-1a/2A (*left*) or NR1-1a/2C (*right*) heteromers. Five-second agonist applications (10 μM NMDA for NR1-1a/2A, 1 mM L-glutamate for NR1-1a/2C, *thick bar*) induced a comparable rise in intracellular Ca^{2+}. However, only the cell expressing NR1-1a/2A heteromers showed Ca^{2+}-dependent inactivation. Agonist solutions contained 2 mM Ca^{2+} in A and B. Note the different time base for the Ca^{2+}-imaging and whole-cell data.

To directly test whether NMDA evoked Ca^{2+} transients in NR1-1a/2C-expressing cells, we performed simultaneous Ca^{2+} imaging/whole-cell clamp experiments. In four cells expressing NR1-1a/2A heteromers, the application of 10 μM NMDA in 2 mM $[Ca^{2+}]_o$ induced Ca^{2+}-dependent inactivation (43.8 ± 8.2%) concomitant with a rise in intracellular Ca^{2+} (peak $\Delta F/F$, 1.3–5.2; mean, 2.4 ± 1.9; Fig. 5B, *left*). In cells expressing NR1-1a/2C heteromers, the application of 1 mM NMDA or L-glutamate in 2 mM $[Ca^{2+}]_o$ also produced increases in intracellular Ca^{2+} concentration with peak $\Delta F/F$ ranging from 1.1 to 2.0 (mean, 1.3 ± 0.4; five cells). The transient increases in fluo-3 fluorescence ($(\Delta F\text{-}F)/F$) ratios were not significantly different for the two heteromers when normalized to the charge transfer for each cell. The simultaneously recorded whole-cell currents in NR1-1a/2C heteromers also did not show significant inactivation (6.7 ± 10.8%; five cells), even for comparable increases in fluo-3 fluorescence (Fig. 5B, *right*).

Discussion

Our results demonstrate that Ca^{2+}-dependent inactivation in recombinant NR1-1a/2A receptors closely resembles that of native NMDA receptors in hippocampal neurons. More interestingly, our data show that inactivation is dependent on the presence of NR2A (or NR2D) because NR1-1a/2B and NR1-1a/2C heteromers showed no significant inactivation. In addition, NR1-1a/2C heteromers lacked glycine-independent desensitization, suggesting a critical role for the NR2 subunit in both inactivation and desensitization. Consistent with separate determinants for inactivation and desensitization, the NR1-1a/2D heteromers showed inactivation but apparently lack glycine-independent desensitization.

Ca^{2+}-dependent inactivation in recombinant and native receptors. The characteristics of inactivation in recombinant NR1-1a/2A heteromers closely resemble those of native NMDA receptors in cultured hippocampal neurons (3–5, 8) and other central nervous system neurons (32). In contrast, the behavior of NR1-1a/2B and especially NR1-1a/2C heteromers is clearly different. Because NR2A can combine with either NR2B or NR2C (33–35), at least two NR2 subunits (along with two NR1s; see Ref. 36) are likely to contribute to functional NMDA receptors. In the hippocampus, NR2A and NR2B are both highly expressed (15, 16). Thus, if NR2A and NR2B coassemble in a significant fraction of hippocampal NMDA receptors, it is plausible that a single copy of NR2A is sufficient to produce an inactivating receptor.

Inactivation of NR1-1a/2A heteromers did show some differences compared with native hippocampal receptors, but these were more quantitative than qualitative. For example, higher concentrations of Ba^{2+} were necessary to induce even partial inactivation in NR1-1a/2A heteromers. However, like for neurons, inactivation induced by 6 mM Ba^{2+} was faster than inactivation in 2 mM Ca^{2+}. This could indicate that Ba^{2+} reaches higher intracellular levels than Ca^{2+} due to less-effective buffering and sequestering mechanisms (37, 38) but that Ca^{2+} has a higher intrinsic activity in inducing inactivation. Inactivation of NR1-1a/2A heteromers was more sensitive to intracellular Ca^{2+} chelators, perhaps due

to the compact cell geometry of HEK 293 cells, allowing easier access of buffer to the submembrane region. In addition, ~80% of neuronal NMDA receptors are at synapses (39), where diffusion to the postsynaptic membrane may be limited by the postsynaptic density.

Our results differ from those of Medina *et al.* (18), who reported "inactivation" of both NR1/2A and NR1/2B heteromers. However, their protocols did not permit clear separation of Ca^{2+}-dependent inactivation from other forms of desensitization, both of which were included in their definition of "inactivation." This is not simply a semantic issue because both NR1-1a/2A and NR1-1a/2B heteromers showed glycine-independent desensitization in our experiments. For NR1/2B, Medina *et al.* (18) found inhibition of 12–32% in the presence of 2 mM $[Ca^{2+}]_o$; the comparable values for our results were 14% (0.2 mM $[Ca^{2+}]_o$) and 24% (2 mM $[Ca^{2+}]_o$), a difference that was not significant. Medina *et al.* also reported little "inactivation" for small responses (<0.2 nC), but Ca^{2+}-dependent inactivation was clearly present for responses of this size in our experiments (Fig. 5A). Because we found that inactivation was much more reliably observed with low intracellular EGTA (0.1 mM), the use of higher EGTA in their experiments may explain this latter discrepancy. Regardless of the presence or absence of inactivation in NR1-1a/2B heteromers, NR1-1a/2C heteromers showed no inactivation in our experiments. Because NR2C has a much shorter carboxyl-terminal cytoplasmic domain than NR2A and NR2B, it is possible that this difference is critical to inactivation.

Multiple actions of Ca^{2+} on NMDA channels. Several recent reports have described direct or indirect effects of Ca^{2+} on NMDA channel activity. For example, NMDA receptor function is linked to the cytoskeleton in a Ca^{2+}-dependent manner (4). By enhancing the depolymerization of actin filaments, Ca^{2+} induces NMDA-channel rundown. In neurons, this effect can be overcome with ATP or through inhibition of depolymerization of actin filaments by phalloidin (4). However, phalloidin had no effect on inactivation in hippocampal neurons (4) and did not affect inactivation in NR1-1a/2A heteromers. The degree of inactivation and the peak amplitude of currents in HEK 293 cells expressing NR1-1a/2A heteromers decreased with time of whole-cell recording (not shown). However, ATP regenerating solution was not able to prevent this gradual loss of the current; thus, it is likely that additional mechanisms contributed to NMDA channel "rundown" in HEK 293 cells.

A second Ca^{2+}-dependent modulation occurs through CaN, although whether CaN acts directly on the NMDA receptor has not been determined. In cell-attached patches from acutely isolated dentate granule cells, the duration of openings of NMDA channels was increased by CaN inhibitors (28). CaN inhibitors also decreased glycine-insensitive desensitization of NMDA receptors in outside-out patches (29) and reduced desensitization of synaptic NMDA currents in paired pulse experiments (7). The latter effect of CaN can be counterbalanced by phosphorylation through cAMP-dependent protein kinase (40). In contrast, we found no effect of CaN inhibitors on inactivation of NR1-1a/2A heteromers, which is consistent with previous studies on hippocampal neurons (3). Nevertheless, our results do not contradict the evidence implicating CaN in the regulation of NMDA receptor activity but merely indicate that CaN-mediated dephosphorylation is not responsible for inactivation.

CaM has recently been reported to bind to two sites in the carboxyl terminus of NR1 and to reduce NMDA channel activity (30). One of these binding sites is common to all NR1-splice variants; it is located immediately distal to the fourth transmembrane region. The second, higher affinity site is located on the C1 exon cassette, common to only NR1-1a/b and NR1-3a/b splice variants. The binding of CaM is Ca^{2+} dependent and reduces the open probability of channels in inside-out patches from NR1/2A-expressing HEK 293 cells. However, calmidazolium had no effect on inactivation of recombinant NR1-1a/2A channels in the present whole-cell recordings or in previous experiments on hippocampal neurons (3). We confirmed this prior result by demonstrating that whole-cell dialysis with CaM binding domain peptide has no affect on inactivation in cultured hippocampal neurons.[1]

These findings argue against the idea that Ca-CaM binding to the NR1 subunit is solely responsible for inactivation. In this respect, it is interesting that Ba^{2+} can induce inactivation in hippocampal neurons (8) as well as in NR1-1a/2A heteromers. Even at millimolar concentrations, Ba^{2+} does not activate CaM *in vitro* (37, 38, 41, 42). Patch excision is known to affect NMDA channel gating and regulation (39, 43). Thus, it is possible that patch excision disrupts protein interactions with the NR1 carboxyl terminus, allowing CaM to more easily access the receptor in excised patches. One candidate molecule in this respect is the actin-binding protein α_2-actinin, which can also bind to the carboxyl terminus of NR1 and may link NMDA receptors to the actin cytoskeleton (44).

Does Ca^{2+} bind directly to the NMDA receptor? The presence of inactivation in recombinant receptors expressed in HEK 293 cells makes it unlikely that a neuron-specific regulatory protein is required for inactivation. However, it remains unclear whether Ca^{2+} binds direct to the receptor or to another Ca^{2+}-binding protein. The facts that even small NR1-1a/2A currents showed inactivation and that the fast Ca^{2+} chelator BAPTA abolished inactivation support the hypothesis that Ca^{2+} acts close to the inner mouth of the channel (8). Because we were unable to demonstrate a role for several common Ca-dependent proteins, it remains plausible that inactivation requires Ca^{2+} binding to cytoplasmic domains of the receptor. NMDA receptor subunits do not possess a clear EF-hand motif or any other commonly known Ca^{2+}-binding motif (45). However, multiple regions of the NMDA receptor may contribute to a Ca^{2+}-binding site. For example, the Mg^{2+} block of NMDA channels is affected not only by the asparagine site in the P loop but also by three other regions of NR2 (46). Thus, it is conceivable that a Ca^{2+}-binding site could involve multiple domains.

The best evidence against the direct Ca^{2+} binding hypothesis is that application of Ca^{2+} to inside-out patches does not reduce the channel open probability (4). However, as mentioned above, patch excision alters NMDA channel kinetics. Thus, this result is consistent with a role for a soluble Ca^{2+}-binding protein such as CaM but could equally reflect disruption of Ca-dependent protein/protein interactions required for inactivation.

[1] J. J. Krupp, unpublished observations.

Functional significance of NR2 subunit specificity. Because inactivation also occurs at synaptic receptors (6), the NMDA receptor-mediated EPSC and the associated Ca^{2+} influx may be controlled by this mechanism. The mRNAs encoding different NR2 subunits have distinct regional and developmental expression patterns (15, 16). Our results predict that inactivation should occur primarily at synaptic receptors containing either NR2A or NR2D subunits. In the hippocampus, the expression of NR2B mRNA is higher during embryonic and early postnatal phases than NR2A (15, 16), suggesting that NR1/2B receptors may be the predominant combination during early development. The lack of this down-regulatory mechanism in a putative NR1/2B receptor might provide a relative constant Ca^{2+} signal at developing synapses, perhaps relevant to either synapse formation or receptor clustering. For example, clustering of NR1 subunits in fibroblasts has been reported to depend on the first carboxyl-terminal C1 exon cassette of NR1-1a (47). The CaM-binding site in the C1 exon cassette has a high degree of homology with MARCKS, a protein kinase C- and Ca/CaM-regulated protein capable of organizing and modulating the cytoskeleton by actin filament cross-linking (48). Thus, Ca^{2+}-dependent interactions involving the NR1 carboxyl terminus may provide a link between actin or actin-binding proteins and NMDA receptors.

The increased expression of NR2A subunits with development predicts that inactivation should become more prominent, providing a mechanism for dynamic gating of NMDA receptors. Thus, Ca^{2+} entry during ongoing high-frequency stimulation could progressively decrease the NMDA receptor-mediated EPSC similar to the effects of desensitization on AMPA receptor-mediated EPSCs (49). Such regulation may be specific, however, to only some neurons within a circuit. For example, NR2C and NR2D are apparently expressed in different sets of hippocampal interneurons (15). Thus, cells expressing NR2C might be expected to show less inactivation and therefore less accommodation to high-frequency stimulation.

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Send reprint requests to: Dr. Gary L. Westbrook, Vollum Institute L474, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, OR 97201. E-mail: westbroo@ohsu.edu