α-Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid Subtype Glutamate Receptor (AMPAR) Endocytosis Is Essential for N-Methyl-D-aspartate-induced Neuronal Apoptosis*

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Excessive activation of the N-methyl-D-aspartate subtype glutamate receptor (NMDAR) is thought to be involved in mediating programmed cell death (apoptosis) in numerous central nervous diseases. However, the underlying mechanisms remain unknown. We report here that stimulation of NMDARs activates intracellular signaling cascades leading to apoptosis and facilitates clathrin-dependent endocytosis of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid subtype glutamate receptors (AMPARs). Both broad spectrum inhibitors of clathrin-dependent endocytotic processes and a specific inhibitor of AMPAR endocytosis selectively inhibit NMDA-induced apoptosis without affecting apoptosis produced by staurosporine. These results demonstrate that clathrin-dependent endocytosis of AMPARs is an essential step in NMDAR-mediated neuronal apoptosis. Our study not only identifies a previously unsuspected step in NMDA-induced apoptosis but also demonstrates that AMPAR endocytosis, in addition to attenuating synaptic strength as previously demonstrated in models of synaptic plasticity, may play a critical role in mediating other important intracellular pathways.

Neuronal apoptosis induced by activation of NMDARs has been postulated to underlie the loss of neurons and neuronal function in many central nervous system disorders (1–5). However, the mechanisms linking NMDAR activation to neuronal apoptosis remain unclear, although excessive calcium influx has been proposed as a primary underlying processes (1, 3, 4). Interestingly, several recent studies have indicated that NMDAR activation can also lead to significant facilitation of clathrin-mediated endocytosis of AMPARs, leading to long term depression of AMPAR-mediated synaptic transmission (6, 7). The evidence that endocytosis of AMPARs, in addition to attenuating receptor activation on the plasma membrane, is essential to some signaling pathways mediated by AMPAR (8), we set out to investigate whether the stimulated endocytosis of AMPARs constitutes an essential step in the signaling pathway leading to NMDA-induced neuronal apoptosis.

**EXPERIMENTAL PROCEDURES**

Primary Culture of Hippocampal Neurons—Hippocampal cultures were prepared from embryonic E18 Sprague-Dawley rats and grown in culture medium (Neurobasal medium containing B-27 supplement and 0.5 mM glutamine (Invitrogen)). The medium from mature 14-day in vitro neurons was removed and replaced with 100 µM NMDA plus 10 µM glycine in extracellular solution (10 mM HEPES, pH 7.35, 140 mM NaCl, 2.5 mM KCl, 25 mM glucose, 5.4 mM KCl, 1.3 mM CaCl2, osmolarity: 310–320 mosm) for 1 h at 37 °C prior to restoring neurons to the culture medium. Twenty-four hours after NMDA/glycine application, neurons were processed using cell death assays. NMDA-induced (Ca2+) responses were evoked and measured using methods described previously (9).

**Apoptosis Assays**—NMDA-induced apoptosis was quantified using either a Cell Death Detection Eliasa Plus Kit (Roche Applied Sciences) or using TdT-mediated addition of biotinylated 11-dUTP to the free 3′-OH ends of DNA (Chemicon). Absorbance readings for both assays were determined using a spectrophotometric microplate reader. Data analysis and expression relative to appropriate controls were performed according to the manufacturer’s instructions for each kit.

**Propidium Iodide (PI) Staining of Nuclei**—After the induction of apoptosis, cells were fixed with 4% paraformaldehyde, 4% sucrose for 10 min followed by ice-cold acetone for 1 min, and then stained with 20 mg/ml PI in Dulbecco’s phosphate-buffered saline for 30 min and viewed with a Leica fluorescence microscope to identify condensed nuclei. Cells with condensed nuclei were counted as apoptotic, and the percentage of apoptotic cells to the total number of cells was calculated to give a semi-quantitative analysis, expressed as percentage of apoptosis.

**Peptide Treatments**—Synthetic peptides, GluR2Δα or GluR2Δα, were incubated with a carrier protein (Pep-1) (10) at a ratio of 1:20 in Dulbecco’s modified Eagle’s medium (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO2 for 30 min to allow the formation of GluR2/Pep-1 complexes. Hippocampal neurons (12–14 days in vitro) were then overlaid with the preformed complex to reach a final GluR2 peptide concentration of 1 µM and further incubated for 1 h before experiments commenced.

**ELISA Cell-surface Receptor Assay**—Quantification of cell-surface AMPA or NMDARs was performed by a colorimetric cell-ELISA assay essentially as described previously (11), using monoclonal antibodies.

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Fig. 1. NMDA induces apoptosis in primary cultures of rat hippocampal neurons. Neurons were treated without (Control) or with NMDA (100 μM plus 10 μM glycine, 1 h) in extracellular solution and then returned to culture medium for various times as indicated for caspase-3 activity assay in A and for 24 h for all other apoptosis assays in B–F. In this and the following figures all data are expressed as mean ± S.E. and analyzed using a non-paired Student’s t test. A, NMDA treatment increases caspase-3 activity. Top panel, Western blot of cell lysates using anti-cleaved caspase-3 antibody; bottom panel, ELISA assay detecting DEVD-p-nitroanilide cleavage (n = 4). * p < 0.5, compared with control. B, agarose gel electrophoresis shows significant DNA laddering 24 h after NMDA treatment. C, PI staining of cells after fixation shows condensed nuclei 24 h after NMDA treatment. Fluorescent (PI staining) and differential interference contrast (DIC) images were overlaid (Overlay). D, cell death ELISA assay for apoptosis shows that NMDA-induced apoptosis is blocked by competitive NMDAR antagonist, APV (50 μM), but not the non-NMDAR antagonist, DNQX (10 μM). E and F, cell death ELISA assays for apoptosis show that endocytosis inhibitors sucrose (Suc; 400 mM) or membrane-permeant dynamin peptide (myr-Dyn; 10 μM), but not non-membrane permeant Dyn (10 μM) or the scrambled myr-Dyn (s-myr-Dyn; 10 μM), specifically block NMDA-induced, but not staurosporine-induced (STS; 100 nM, 1 h), apoptosis. ** p < 0.01 compared with control; n = 36–72 wells from three separate experiments for each group.

RESULTS

NMDA treatment (100 μM plus 10 μM glycine, 1 h) induced a time-dependent increase in caspase-3 activity, a biochemical indicator of neuronal apoptosis (12), as detected by ELISA assay of DEVD-p-nitroanilide cleavage (Fig. 1A). The increase peaked 12–24 h after the treatment, at which time the majority of neurons were either dying or dead, exhibiting the hallmarks of apoptotic cell death, including DNA laddering as demonstrated by gel electrophoresis of extracted DNA (Fig. 1B), and nuclear condensation with disintegrating processes shown by nucleus staining with PI (Fig. 1C) or the intercalating DNA dye, Hoechst 33258 (bissbenzimide, data not shown). The degree of neuronal apoptosis was also quantified by measuring intracellular DNA cleavage with both 11-dUTP (Fig. 1D) and histone biotinylation assays (Fig. 1E). Furthermore, the NMDA-induced apoptosis was a result of specific activation of NMDARs, as it was fully blocked by the NMDAR antagonist, APV (50 μM; Fig. 1D), but not inhibited by blocking AMPARs with DNQX (10 μM; Fig. 1D). Therefore, in accordance with previous reports (12), the NMDA treatment paradigm used in the present study produced neuronal apoptosis.

To investigate the role of NMDA-induced endocytosis in mediating neuronal apoptosis, we first examined the effect of hypertonic sucrose, a well characterized inhibitor of clathrin-dependent endocytosis that prevents the assembly of clathrin-coated pits (11, 13). As shown in Fig. 1E, sucrose treatment (400 mM) dramatically reduced NMDA-induced apoptosis. To more specifically target clathrin-dependent endocytosis, we employed a dynamin-derived, myristoylated peptide (myr-Dyn). This peptide is membrane-permeable and effectively inhibits clathrin-mediated endocytosis (14, 15) by blocking the recruitment of dynamin to clathrin-coated pits by amphiphysin (16). We found that myr-Dyn (10 μM) was as effective as hypertonic sucrose in reducing NMDA-induced apoptosis (Fig. 1, E and F). In contrast, control Dyn peptides, both non-myristoylated (membrane-impermeable) Dyn (Dyn; Fig. 1E) and scrambled myr-Dyn (s-myr-Dyn; Fig. 1F), had little effect. To determine whether endocytosis is a general requirement for neuronal apoptosis, we next tested the effect of these inhibitors on a well characterized model of neuronal apoptosis induced by treating neurons with the kinase inhibitor, staurosporine (STS; 100 nM, 1 h) (12). As shown in Fig. 1E, both endocytosis inhibitors failed to significantly alter STS-induced neuronal apoptosis. These results demonstrate that a clathrin-dependent endocytic process is necessary for NMDAR-mediated, but not STS-mediated, apoptosis.

Ca2+ overload following NMDAR activation has been suspected as a primary causal factor leading to neuronal apoptosis (1, 3, 4). However, as summarized in Fig. 2A, sucrose at concentrations that inhibited endocytosis and apoptosis did not significantly alter NMDA-evoked transient [Ca2+]i responses measured with the Ca2+ dye, Fura-2. The fact that inhibition of endocytosis blocked NMDA-induced apoptosis without affecting its [Ca2+]i responses strongly suggests that intracellular increases in Ca2+ concentrations, although necessary (3, 4), may not be sufficient to produce NMDA-induced apoptosis.

Activation of certain forms of caspases, such as caspase-3 and caspase-7 (17) (also see Fig. 1A) has been implicated in NMDA-induced neuronal apoptosis. We therefore investigated the effects of inhibiting endocytosis on NMDA dependent activation of caspase-3. NMDA treatment dramatically increased the level of the activated form of caspase-3, as demonstrated by Western blots using an antibody that specifically recognizes
activated/cleaved caspase-3, and this effect was efficiently inhibited by the membrane-permeable myr-Dyn (Fig. 2B). The serine/threonine kinase Akt/PKB has previously been shown to be critically involved in protecting neurons from apoptotic cell death (18), and inhibition of this kinase activity has previously been suspected of being involved in NMDA-mediated apoptosis (19). We therefore investigated whether the endocytosis process plays a critical role in the inhibition of Akt activity by determining the level of Akt phosphorylation at serine 473, a residue whose phosphorylation is required for full activation of Akt (20).

As shown in Fig. 2C, treatment of neurons with NMDA resulted in a significant reduction in Ser^{473}-phosphorylated Akt and hence Akt activity without altering the levels of total Akt. This reduction in Akt activity was largely prevented by the inhibition of endocytosis with hypertonic sucrose. In striking contrast, sucrose treatment had no effect on the reduction of Akt phosphorylation following STS treatment (Fig. 2C). Thus, stimulated endocytosis appears to be an obligatory step that is downstream of rising \([Ca^{2+}]_i\), and upstream of caspase activation and Akt inhibition in NMDA-induced neuronal apoptosis.

Several recent studies in the synaptic plasticity field have demonstrated that NMDAR activation can selectively stimulate the clathrin-mediated endocytosis of AMPARs (6, 7, 21–23). Consistent with these previous studies, we observed a significant reduction of cell-surface AMPA, but not NMDA, receptors following the NMDA paradigm used here. Since the reduction in AMPARs was observed by measuring cell-surface expression of both the GluR2 subunit (Fig. 3A, A and B) and the GluR1 subunit (64 ± 2 and 51 ± 1% of the total receptors on the cell surface for control and NMDA-treated neurons, respectively; n = 5, p < 0.001), it appears to be a decrease in total cell-surface AMPARs and not a replacement of GluR2-containing with GluR2-lacking receptors as a result of GluR2-dependent endocytosis. The AMPAR reduction was blocked by the endocytosis inhibitor myr-Dyn but not the control peptide, Dyn (Fig. 3A), indicating the involvement of facilitated clathrin-dependent endocytosis. We have demonstrated previously that stimulated AMPAR endocytosis requires the GluR2 subunit (11), more specifically the short amino acid sequence between residues tyrosine 869 and glutamic acid 879 within the carboxyl-terminal region (21, 24). We have therefore reasoned that a clathrin-dependent endocytosis inhibitor hypertonic sucrose but not by Pep-1 + GluR2_{3Y} (Fig. 3A), indicating the involvement of facilitated clathrin-dependent endocytosis. We have demonstrated previously that stimulated AMPAR endocytosis requires the GluR2 subunit (11), more specifically the short amino acid sequence between residues tyrosine 869 and glutamic acid 879 within the carboxyl-terminal region (21, 24). We have therefore reasoned that a clathrin-dependent endocytosis inhibitor hypertonic sucrose but not by Pep-1 + GluR2_{3Y} (Fig. 3A), indicating the involvement of facilitated clathrin-dependent endocytosis. We have demonstrated previously that stimulated AMPAR endocytosis requires the GluR2 subunit (11), more specifically the short amino acid sequence between residues tyrosine 869 and glutamic acid 879 within the carboxyl-terminal region (21, 24). We have therefore reasoned that a clathrin-dependent endocytosis inhibitor hypertonic sucrose but not by Pep-1 + GluR2_{3Y} (Fig. 3A), indicating the involvement of facilitated clathrin-dependent endocytosis. We have demonstrated previously that stimulated AMPAR endocytosis requires the GluR2 subunit (11), more specifically the short amino acid sequence between residues tyrosine 869 and glutamic acid 879 within the carboxyl-terminal region (21, 24). We have therefore reasoned that a clathrin-dependent endocytosis inhibitor hypertonic sucrose but not by Pep-1 + GluR2_{3Y} (Fig. 3A), indicating the involvement of facilitated clathrin-dependent endocytosis.
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In the present study we have found that clathrin-dependent AMPAR endocytosis is specifically required for NMDA-induced apoptosis, as quantified by the histone bixinilution assay (Fig. 4A), and by PI nuclear staining (Fig. 4, B and C). Neither GluR2-3Y nor Pep-1 alone nor Pep-1 plus the control peptide GluR2-3A (in which the three tyrosine residues were replaced with alanines (24)) had any detectable effect on NMDA-induced apoptosis (Fig. 4A). Similar to the general blockade of the clathrin-mediated endocytic process with either sucrose or myr-Dyn, interference with AMPAR endocytosis by GluR2-3Y did not alter STS-induced neuronal apoptosis (Fig. 4B). Taken together, our results provide strong evidence for an obligatory requirement for AMPAR endocytosis in mediating NMDA-induced neuronal apoptosis.

DISCUSSION

In the present study we have found that clathrin-dependent AMPAR endocytosis is specifically required for NMDA-induced apoptosis, as quantified by the histone bixinilution assay (Fig. 4A), and by PI nuclear staining (Fig. 4, B and C). Neither GluR2-3Y nor Pep-1 alone nor Pep-1 plus the control peptide GluR2-3A (in which the three tyrosine residues were replaced with alanines (24)) had any detectable effect on NMDA-induced apoptosis (Fig. 4A). Similar to the general blockade of the clathrin-mediated endocytic process with either sucrose or myr-Dyn, interference with AMPAR endocytosis by GluR2-3Y did not alter STS-induced neuronal apoptosis (Fig. 4B). Taken together, our results provide strong evidence for an obligatory requirement for AMPAR endocytosis in mediating NMDA-induced neuronal apoptosis.

A large body of evidence accumulated recently has established a central role for stimulated clathrin-dependent AMPAR endocytosis in reducing receptor-mediated synaptic transmission, contributing to the expression of certain forms of well characterized synaptic plasticity including long term depression in both hippocampus (11, 25) and cerebellum (26). The requirement for AMPAR endocytosis in NMDA-induced apoptosis demonstrated in the present study further suggests that, in addition to the decrease in receptor-mediated synaptic transmission, endocytosed AMPARs also play previously unsuspected roles in intracellular signaling pathways. The mediation of NMDA-induced apoptosis is one example. The detailed mechanisms by which endocytosed AMPARs contribute to the initiation of downstream apoptotic cascades remain to be determined. However, analogous to the recently reported retinal cell apoptosis induced following stimulated endocytosis of rhodopsin (27–29) and activation of EGFR receptor-mediated mitogen-activated protein kinase signaling by endocytosed EGFR receptors (8), it is possible that endocytosed AMPARs, in response to NMDA stimulation, may form, with some of the clathrin-coat protein components, a signaling complex that initiates the apoptotic cascade. Nevertheless, the present study provides the first evidence for the involvement of stimulated AMPAR endocytosis in NMDA-induced neuronal apoptosis, thereby unveiling a novel pathway in mediating cell apoptosis. NMDAR-mediated apoptosis is one of the primary causes leading to the selective loss of neurons in both neurodegenerative diseases and acute brain insults, and clathrin-dependent AMPAR endocytosis is a fundamental process in the control of efficacy of synaptic transmission at glutamatergic synapses. By linking the endocytosis of AMPARs to neuronal apoptosis, our study suggests the components of the AMPAR endocytotic pathway may be novel therapeutic targets for the development of a new generation of neuroprotective agents.

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