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N-Methyl-D-aspartate Receptors Activate Transcription of c-*fos* and *NGFI-A* by Distinct Phospholipase A_2-Requiring Intracellular Signaling Pathways

LESLIE S. LEREA, NOEL G. CARLSON, and JAMES O. McNAMARA

Department of Medicine, Division of Neurology (L.S.L., N.G.C., J.O.M.), Epilepsy Research Laboratory, Veterans Affairs Medical Center (J.O.M.), and Departments of Pharmacology and Neurobiology (J.O.M.), Duke University, Durham, North Carolina 27710

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SUMMARY

Activation of *N*-methyl-D-aspartate (NMDA) receptors is required for induction of some lasting changes in nervous system structure and function. The cellular mechanisms involved in transducing receptor stimulation into long-lasting changes in cellular activity are unknown. Immediate-early genes (IEGs) have been implicated in the conversion of short term stimuli to long term changes in cellular phenotype, by regulation of gene expression. Activation of NMDA receptors on dentate gyrus neurons triggers the transcriptional activation of several IEGs. To determine whether the same intracellular pathways transduce the signal from this ligand-gated ion channel to the nucleus, we compared NMDA induction of two IEGs. NMDA was sufficient to produce a striking increase in both c-*fos* and *NGFI-A* mRNAs in dentate granule neurons, in a calcium-dependent manner. The induction of both IEGs was blocked by structurally distinct inhibitors of phospholipase A_2, an enzyme that catalyzes phospholipid degradation and formation of arachidonic acid. Arachidonic acid itself is catalyzed to biologically active metabolites by multiple enzymes, including cyclooxygenase and lipoxygenase. Selective inhibitors of cyclooxygenase attenuated NMDA induction of c-*fos* but not *NGFI-A*. Conversely, structurally distinct inhibitors of lipoxygenase blocked NMDA induction of *NGFI-A* but not c-*fos*. The signaling pathways linking NMDA receptors to the transcriptional activation of c-*fos* and *NGFI-A* are related but distinct. We suggest that phospholipase A_2 and the arachidonic acid cascade play a pivotal role in NMDA receptor regulation of gene expression.

Signals generated at the cell membrane by hydrophilic ligands evoke diverse, cell-specific responses, including growth, differentiation, and survival. Modification of gene expression through regulation of transcription is one determinant of the specificity of these diverse cellular responses. In the mature CNS, fleeting changes of activity produce lasting modifications of structure and function in postmitotic neurons (1–4). These activity-dependent modifications often require synaptic activation of glutamate receptors, in particular those of the NMDA subtype. The cellular and molecular mechanisms by which brief episodes of synaptic activity produce permanent modifications of neuronal phenotype are unknown. IEGs provide an attractive mechanism by which brief activation of NMDA receptors produces lifelong changes in neuronal structure and function (neuronal plasticity) through regulation of expression of late-response genes (5, 6). Many IEGs are induced rapidly and transiently after a stimulus and encode DNA-binding proteins that regulate the transcription of target or late-response genes (7–9). The precise late-response genes transcribed and the specificity of the long term consequences undoubtedly depend, at least in part, on the unique array of IEGs initially transcribed and the intracellular signaling mechanisms regulating IEG transcription.

High frequency stimulation of afferents in the CNS induces both lasting changes in synaptic efficacy and striking increases in mRNA content of the IEGs c-*fos* and *NGFI-A* in DG granule cells of the hippocampal formation (10–12). Activation of the NMDA subtype of glutamate receptors is necessary for this induction of c-*fos* (13) and *NGFI-A* (10) in the DG granule neurons. Activation of NMDA receptors is sufficient to induce the expression of c-*fos* and *NGFI-A* in DG granule cells *in vitro* (14). The signaling pathways involved in the NMDA receptor-mediated induction of one of these genes, c-*fos*, have been partially defined (14–18). The in-

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ABBREVIATIONS: CNS, central nervous system; NMDA, *N*-methyl-D-aspartate; IEG, immediate-early gene; DG, dentate gyrus; MEM, modified Eagle's medium; AA, arachidonic acid; NDGA, nordihydroguaiaretic acid; LTP, long term potentiation; PLA_2, phospholipase A_2; SSC, sodium chloride/sodium citrate; HBSS, Hanks' balanced salt solution; $[Ca^{2+}]_i$, intracellular calcium concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

duction of c-*fos* mRNA in DG granule neurons by NMDA requires increases of $[Ca^{2+}]_i$ and activation of PLA_2 and cyclooxygenase (18). Here we ask whether the same intracellular signaling pathways mediate the NMDA receptor-induced transcriptional activation of both c-*fos* and *NGFI-A*. By defining the intracellular mechanisms involved in the induction of transcription factors such as the IEGs, we may begin to understand the cellular and molecular mechanisms underlying the specificity of long term responses in a particular population of cells after brief synaptic activation.

NMDA receptor activation *in vitro* results in an increase in $[Ca^{2+}]_i$ in several neuronal populations (14, 19–21). Numerous calcium-dependent intracellular signaling pathways may be activated after NMDA receptor activation; these include protein kinase C, calcium/calmodulin-dependent kinase, tyrosine kinase, protein phosphatases, proteases, and phospholipases. We have tested the role of many of these calcium-dependent, intracellular signaling enzymes in IEG induction; here we use pharmacological tools to examine the effects of several enzyme inhibitors targeted against the PLA_2 signaling pathway on c-*fos* and *NGFI-A* mRNA expression after NMDA stimulation of isolated neurons derived from the DG. To detect changes in IEG expression, we use *in situ* hybridization with radiolabeled riboprobes prepared to specific sequences of c-*fos* or *NGFI-A* cDNA, and we report that closely related but distinct intracellular signal transduction mechanisms are required for the induction of these two distinct IEGs after NMDA receptor activation.

Experimental Procedures

Cell preparation. DG cells were prepared from 4-day-old rat pups as described by Lerea *et al.* (14). Briefly, each hippocampus was dissected and sectioned into 600–800-μm-thick transverse slices, and the DG was separated from the hippocampal gyrus by microdissection. Efforts were made to dissect as close to the hilar border of the granule cell layer as possible, thereby excluding the hilus. All tissue was enzymatically dissociated, rinsed, and dispersed into a single-cell suspension. The cell suspension was centrifuged and the pellet was resuspended in MEM supplemented to contain 33 mM glucose, 1 mM pyruvic acid, 2 mM $CaCl_2$, 20 mM KCl, and 10% fetal calf serum (MEM-C). An estimate of viable cells was obtained using trypan blue dye exclusion; cells were plated in a small volume, at a density of 4–6 $\times 10^3$ cells/cm^2, on poly-D-lysine-coated glass chamberslides or 22-mm glass coverslips. Cells were allowed to settle and adhere for several hours before addition of more MEM-C. Cells were maintained *in vitro* at 37°, in a humidified incubator with 5% CO_2/95% O_2, for 7–9 days before use.

Cell treatment for IEG induction. MEM-C was removed from each culture well and replaced with Ca^{2+}/Mg^{2+}-free HBSS supplemented to contain 2.3 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM HEPES, and 5 μM glycine (HBSS$^+$). Cells were returned to the 37° incubator for 3–4 hr before stimulation. Cells were incubated with the specified inhibitors and/or receptor agonists at 37° for the designated times. All drugs were added to the cellular environment as 10× stock solutions. After treatment, cells were fixed with 4% paraformaldehyde at 4° for 5 min, rinsed with HBSS containing 10 mM HEPES, and dehydrated through a series of ethanol concentrations (50%, 70%, 95% ethanol). Cells were stored at −70° until used for *in situ* hybridization. The concentrations chosen for each inhibitor have previously been shown to be selective and efficacious for the desired target (22–27). Aspirin, indomethacin, piroxicam, and quinacrine were purchased from Sigma Chemical Co. Aristolochic acid, esculetin, and NDGA were purchased from Biomol Research Laboratories. Ibuprofen was purchased from Aldrich Chemical Co. NMDA was purchased from Tocris Neuramin.

Riboprobe preparation. A full length c-*fos* cDNA insert (generously supplied by J. Morgan and T. Curran, Depts. of Neuroscience, and Molecular Oncology and Virology, respectively, Roch Institute of Molecular Biology, Nutley, NJ) was used to generate antisense and sense c-*fos* riboprobes. The full length c-*fos* cDNA insert is cloned in a pSP65 plasmid containing the SP6 promoter and contains a single *Xho*I restriction site at base 1353 from the 5′ end. *In vitro* translation of the transcript derived from this plasmid yields the complete c-Fos protein (28). The plasmid was linearized with *Xho*I and riboprobes were generated using SP6 polymerase and an *in vitro* transcription system, in the presence of ^{35}S-UTP. Riboprobes were hydrolyzed to approximately 200 nucleotides with sodium carbonate at 60°. Hybridization of brain sections prepared from rats sacrificed 30 min after an electrographic seizure, using antisense riboprobe generated from this plasmid, results in a discrete pattern of c-*fos* mRNA induction (Fig. 1B); this pattern exactly mimics the specific anatomic pattern described previously using oligonucleotide probes prepared to base pairs 270–319 of c-*fos* mRNA (11, 28). Sense riboprobe generated from this plasmid does not detect any signal (Fig. 1A). Northern blot analyses of RNA isolated from NMDA-stimulated cerebral cortical neurons maintained *in vitro* or from whole cerebral cortex after a pilocarpine (350 mg/kg)-induced seizure, when probed with a ^{32}P-labeled c-*fos* antisense riboprobe, reveal a single band of approximately 2.2 kilobases (data not shown), a size consistent with that of c-*fos* transcripts (28). Riboprobe used for the Northern blot analyses was prepared exactly as described above for the *in situ* experiments, except that [^{32}P]UTP was used instead of ^{35}S-UTP. Similar results were obtained with Northern blot analyses using a random-primed DNA probe generated from this plasmid (data not shown).

A plasmid containing nucleotides 414–184 of the *NGFI-A* cDNA (generously supplied by J. Milbrandt, Dept. of Pathology, Washington University School of Medicine, St. Louis, MO) was used to generate *NGFI-A* riboprobes. The 230-base pair *NGFI-A* sequence was cloned into Bluescript KS containing the T7 and T3 promoters. The plasmid was linearized with the restriction enzyme *Eco*RI and transcribed in the presence of ^{35}S-UTP with T7 polymerase, to generate *NGFI-A* antisense riboprobe. Northern blot analysis of RNA isolated from NMDA-stimulated cerebral cortical neurons maintained *in vitro* or from whole cerebral cortex after a pilocarpine-induced seizure yields a single band of approximately 3.3 kilobases (data not shown), a size consistent with that of *NGFI-A* transcripts (29), when probed with a riboprobe prepared exactly as described above for the *in situ* experiments, except that [^{32}P]UTP was used instead of ^{35}S-UTP. Similar results were obtained with Northern blot analyses using random-primed DNA probes generated from this plasmid (data not shown). Hybridization of brain sections prepared from rats sacrificed 30 min after an electrographic seizure, using an antisense riboprobe generated from this plasmid, results in a discrete pattern of *NGFI-A* mRNA induction (Fig. 1C); this pattern exactly mimics the specific anatomic pattern described previously with oligonucleotide probes prepared to base pairs 890–939 of *NGFI-A* mRNA (11).

***In situ* hybridization.** *In situ* hybridization was done as described by Lerea and McNamara (18). Briefly, cells were incubated for 3–4 hr at 55° with prehybridization buffer [50% formamide, 10% dextran sulfate, 3× SSC (0.45 M NaCl, 0.045 M citric acid), 5× Denhardt's solution, 500 mg/ml yeast tRNA (Sigma), 500 mg/ml salmon sperm DNA (Sigma), 10 mM dithiothreitol]. Cells were hybridized overnight (approximately 16 hr) at 55° in the aforementioned buffer containing 60 ng/ml ^{35}S-labeled riboprobe. Nonspecific hybridization was determined by using ^{35}S-labeled c-*fos* or *NGFI-A* sense riboprobe in adjacent wells. After hybridization, cells were rinsed with 4× SSC (three times, 15 min each) and treated with RNase A at 37° for 30 min. Cells were rinsed with 2×, 1×, and 0.5× SSC (15 min each) and with 0.1× SSC (30 min) at 55°. All slides were dipped in NTB-3 liquid emulsion and stored at 4° for 5–7 days. Emulsion-coated slides were developed in Kodak D-19 developer,

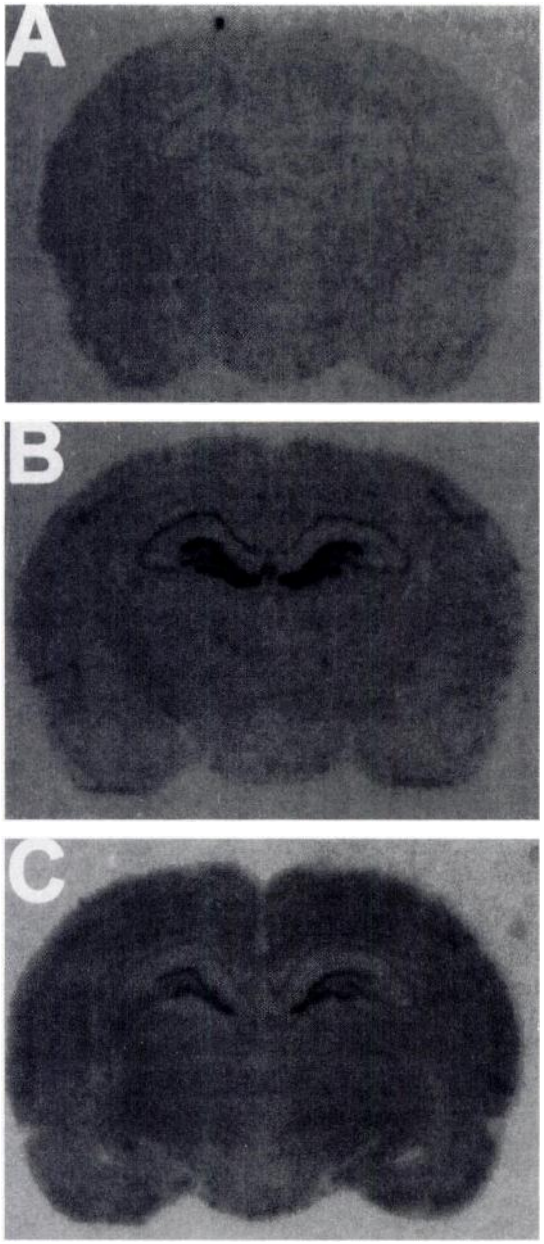

Fig. 1. Anatomic distribution of c-*fos* and *NGFI-A* mRNA expression in rat brain sections. Representative autoradiograms taken at the level of the dorsal hippocampus from rats sacrificed 30 min after an afterdischarge delivered to the right angular bundle are shown. A, Section hybridized with sense riboprobe prepared from a plasmid containing the c-*fos* cDNA insert. Note the lack of signal over the hippocampus and DG. B, Section hybridized with antisense riboprobe prepared from the plasmid containing the c-*fos* cDNA insert. Note the robust signal over the DG granule cells and, to a lesser extent, over CA3 and CA1 pyramidal cells. C, Section hybridized with antisense riboprobe prepared from the plasmid containing the *NGFI-A* cDNA insert. Note the signal over the DG granule cells. Sections hybridized with sense riboprobe prepared from this plasmid are similar to A.

rinsed in water, and fixed in Kodak fixer. Cells were stained for Nissl substance, and silver grains were visualized and counted, using bright-field optics, on a Zeiss Axiovert microscope interfaced with an Image 1 analysis system (Universal Imaging Corp.). Data were collected from all morphologically distinct neurons obtained from random fields across each chamberwell. At least three independent chamberslides were used for each experimental condition. Greater than 95% of DG neurons respond to glutamate receptor agonists. Data are presented as the mean ± standard error of silver grains/single cells.

Animal preparation. Adult male rats were implanted and stimulated according to the protocol of Simonato *et al.* (11). Animals were sacrificed 30 min after stimulation, and brains were removed, frozen in isopentane cooled in a dry ice/methanol bath, and stored at −70°. Brains were cut into 10-μm-thick sections through the hippocampal region and hybridized according to the protocol described above.

Results

NMDA receptor activation in DG neurons causes a robust and rapid increase of $[Ca^{2+}]_i$ (14). This increase of $[Ca^{2+}]_i$ is dependent on extracellular calcium and may activate multiple, calcium-dependent, intracellular enzymes. NMDA receptor activation in DG neurons *in vitro* also causes a robust increase in c-*fos* mRNA in >90% of the neurons, as detected with *in situ* hybridization and single-cell analysis (Fig. 2). This increase in c-*fos* mRNA is a calcium-dependent process (14). The calcium-dependent enzymes involved in NMDA induction of c-*fos* mRNA are not fully understood. The calcium-sensitive enzyme PLA_2 primarily hydrolyzes AA from the *sn*-2 position of membrane-bound phospholipids. NMDA stimulates PLA_2 activity by a calcium-requiring mechanism in a variety of neuronal cell populations (30, 31). We previously demonstrated that two structurally distinct inhibitors of PLA_2 inhibited the NMDA-mediated induction of c-*fos* mRNA in DG neurons without inhibiting NMDA-mediated increases of $[Ca^{2+}]_i$ (18). In the present studies, we tested the role of PLA_2 inhibitors on NMDA-mediated induction of a second IEG, *NGFI-A*. NMDA receptor activation in DG neurons also causes an increase in *NGFI-A* mRNA, as detected with *in situ* hybridization and single-cell analysis. The PLA_2 inhibitor quinacrine reduced NMDA-mediated increases in *NGFI-A* mRNA in a concentration-dependent manner, with 30 μM abolishing induction (Fig. 3A). The structurally distinct, PLA_2 inhibitor aristolochic acid (8-methoxy-6-nitrophenanthro[3,4-*d*]-1,3-dioxole-5-carboxylic acid) binds directly to the enzyme at domains other than the active site, resulting in changes in the secondary structure of PLA_2. Aristolochic acid has been shown to act selectively on PLA_2 in

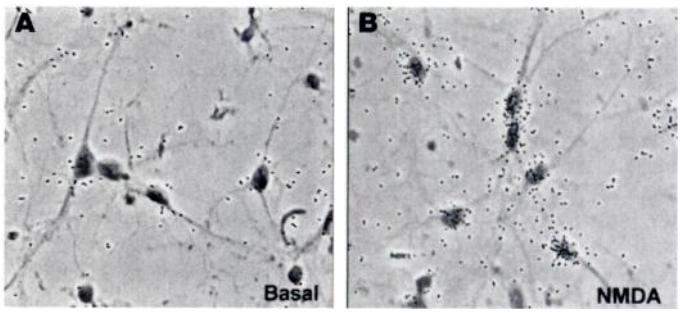

Fig. 2. Expression of c-*fos* in DG neurons after NMDA receptor activation. Representative phase-contrast photomicrographs of DG cells obtained from 4-day-old rat pups and maintained *in vitro* for 9 days are shown. c-*fos* mRNA was detected using an antisense riboprobe generated to the full length clone of c-*fos* DNA and hydrolyzed to 200 nucleotides. Cells were hybridized under basal conditions (A) or after treatment with 50 μM NMDA (B). DG neurons respond to NMDA with a robust increase in c-*fos* mRNA, as evidenced by the increased density of silver grains localized over neurons in B, compared with the neurons in A. Silver grains correspond to radiolabeled riboprobe bound to c-*fos* mRNA. NMDA-stimulated cells hybridized with a sense riboprobe to c-*fos* mRNA exhibited the same density of silver grains as seen in A.

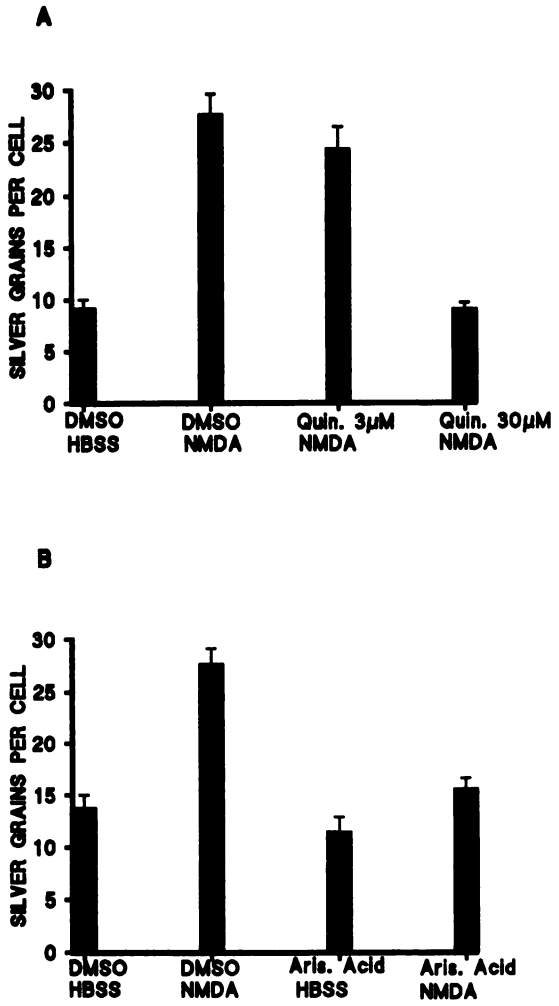

Fig. 3. Effects of PLA_2 inhibitors on *NGFI-A* mRNA induction after NMDA treatment. NMDA was added in the presence of 10 μM 6-cyano-2,3-dihydroxy-7-nitroquinoxaline. All receptor stimulations were done in the presence of inhibitors. A, Cells were pretreated with vehicle or 3 or 30 μM quinacrine (*Quin*) for 30 min before NMDA (50 μM) stimulation. Quinacrine was dissolved in dimethylsulfoxide (DMSO) and diluted into $HBSS^+$. Cells were hybridized with antisense riboprobe to *NGFI-A* mRNA. Each data point was determined by counting silver grains over at least 30 individual neurons. Quinacrine blocked NMDA-induced *NGFI-A* mRNA in a dose-dependent manner. B, Cells were pretreated with vehicle or aristolochic acid (*Aris. Acid*) (50 μM) for 30 min before NMDA (50 μM) or vehicle ($HBSS^+$) stimulation. Aristolochic acid was dissolved in dimethylsulfoxide and diluted into $HBSS^+$. Each data point was determined by counting silver grains over 30–60 individual neurons. Aristolochic acid blocked NMDA-induced *NGFI-A* mRNA.

inhibiting AA and platelet-activating factor release (25, 26). Similarly to quinacrine, aristolochic acid markedly reduced NMDA-mediated increases in *NGFI-A* mRNA, inhibiting its expression by 74% (Fig. 3B; Table 1). Quinacrine and aristolochic acid inhibited NMDA induction of c-*fos* and *NGFI-A* to equivalent extents (Table 1).

AA is rapidly metabolized by distinct enzymes, each giving rise to potentially biologically active metabolites. We previously reported that two distinct inhibitors of cyclooxygenase, the metabolizing enzyme giving rise to prostaglandins and thromboxanes, inhibited NMDA-mediated increases of c-*fos* mRNA (18). To our surprise, cyclooxygenase inhibitors did not block NMDA induction of *NGFI-A* mRNA. The cyclooxygenase inhibitor aspirin (2-acetyloxybenzoic acid) had no effect on NMDA induction of *NGFI-A* mRNA when used at its IC_{50} concentration (22, 24) (Fig. 4A). This same concentration attenuated NMDA-mediated increases in c-*fos* mRNA by 58% (Fig. 4C; Table 1). Ibuprofen [2-(4-isobutylphenyl)propionic acid], a structurally distinct and reversible inhibitor of cyclooxygenase (23), did not inhibit NMDA induction of *NGFI-A* mRNA when used at a concentration of 100 μM (Fig. 4B); this same concentration inhibited the induction of c-*fos* mRNA by 38% (Fig. 4D). Ibuprofen used at 300 μM produced no inhibition of *NGFI-A* mRNA but a 58% inhibition of c-*fos* mRNA (Table 1). Other cyclooxygenase inhibitors exhibited similar selective inhibition of NMDA-mediated increases of c-*fos* mRNA (Table 1).

TABLE 1

Inhibition by various inhibitors of the PLA_2/AA cascade of c-*fos* and *NGFI-A* mRNAs after NMDA treatment

Each value represents the mean ± standard error of the number of experiments indicated in parentheses. The percentage inhibitions were calculated by dividing the average number of silver grains/cell in a treatment group (e.g., inhibitor plus NMDA) by the average number of silver grains/cell in a vehicle group (e.g., vehicle plus NMDA), after their respective basal values had been subtracted. Indomethacin alone often resulted in an unexplained increase in basal *NGFI-A* mRNA expression. An increase in basal expression leads to an artificially high value when percentage inhibition is calculated. This precluded determination of meaningful data on the effect of indomethacin on *NGFI-A* mRNA induction. Such an effect on the basal levels of *NGFI-A* mRNA was not seen with aspirin, ibuprofen, or piroxicam, nor did indomethacin affect the basal expression of c-*fos* mRNA.

	Inhibition	
	c-*fos*	*NGFI-A*
	%	
Quinacrine, 30 μM	100 (3)	100 (3)
Aristolochic acid, 50 μM	78 ± 5.5 (4)	74 ± 2.3 (3)
Indomethacin, 1 μM	85 ± 3.6 (5)	NC[a]
Aspirin, 100 μM	56 ± 2.4 (5)	0 (3)
Ibuprofen, 100 μM	38 ± 3.0 (3)	0 (3)
Ibuprofen, 300 μM	58 (2)	2 (2)
Piroxicam, 100 μM	58 (2)	0 (2)
NDGA, 1 μM	10 ± 2.8 (4)	82 ± 1.3 (3)
Esculetin, 5 μM	6.7 ± 4.8 (3)	88 ± 8.7 (3)

[a] NC, not calculated.

The reduction by PLA_2, but not by cyclooxygenase, inhibitors suggests that either platelet-activating factor, AA itself, or an alternate route of AA metabolism mediates NMDA induction of *NGFI-A* mRNA expression. To test this last possibility, we examined the effects of structurally distinct inhibitors of lipoxygenase, the enzyme that metabolizes AA to leukotrienes and hydroxyeicosatetraenoic acids, on NMDA induction of *NGFI-A* and c-*fos* mRNA. NDGA, used at a concentration (1 μM) reported to inhibit lipoxygenase (32), reduced NMDA-mediated increases of *NGFI-A* mRNA by 82% (Fig. 5A). This concentration inhibited NMDA-induced increases in c-*fos* mRNA by only 10% (Fig. 5C; Table 1). Esculetin (6,7-dihydroxycoumarin), a noncompetitive lipoxygenase inhibitor (33, 34), reduced NMDA-induced increases in *NGFI-A* mRNA by 88% but c-*fos* mRNA increases by only 7% (Fig. 5, B and D; Table 1).

Discussion

Our principal conclusion is that the transcription of two distinct IEGs, c-*fos* and *NGFI-A*, after NMDA receptor activation is mediated by related, but divergent, intracellular pathways. The following findings support this conclusion. NMDA induces c-*fos* and *NGFI-A* mRNAs in DG neurons in

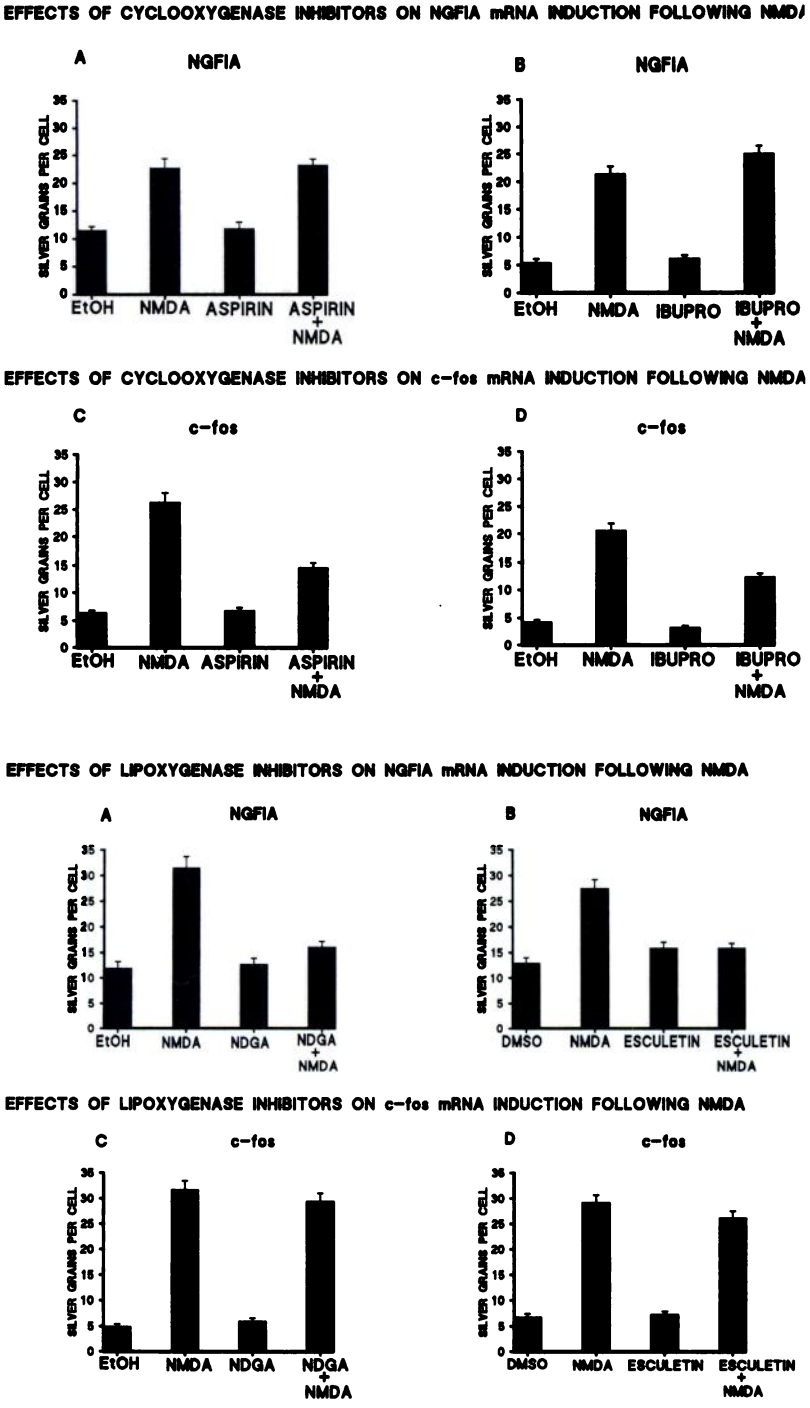

Fig. 4. Effects of cyclooxygenase inhibitors on c-*fos* or *NGFI-A* mRNA induction after NMDA treatment. Cells were pretreated with vehicle (*EtOH*), 100 μM aspirin (A and C), or 1 μM ibuprofen (*IBUPRO*) (B and D) for 15 min before NMDA. Aspirin and ibuprofen were dissolved in ethanol and diluted into $HBSS^+$. Each data point was determined by counting silver grains over at least 30 individual neurons. A and B, Data from cells hybridized with antisense riboprobe to *NGFI-A* mRNA. Neither aspirin nor ibuprofen inhibited NMDA-mediated induction of *NGFI-A* mRNA. B and D, Data from cells hybridized with antisense riboprobe to c-*fos* mRNA. Both aspirin and ibuprofen inhibited NMDA-mediated induction of c-*fos* mRNA.

Fig. 5. Effects of lipoxygenase inhibitors on c-*fos* or *NGFI-A* mRNA induction after NMDA treatment. Cells were pretreated with vehicle, 1 μM NDGA (A and C), or 5 μM esculetin (B and D) for 15 min before NMDA. NDGA was dissolved in ethanol (*EtOH*) and diluted into $HBSS^+$; esculetin was dissolved in dimethylsulfoxide (*DMSO*) and diluted into $HBSS^+$. Each data point was determined by counting silver grains over at least 30 individual neurons. A and B, Data from cells hybridized with antisense riboprobe to *NGFI-A* mRNA. Both NDGA and esculetin inhibited NMDA-mediated induction of *NGFI-A* mRNA. B and D, Data from cells hybridized with antisense riboprobe to c-*fos* mRNA. Neither NDGA nor esculetin inhibited NMDA-mediated induction of c-*fos* mRNA.

a calcium-requiring manner (data not shown). Two structurally distinct inhibitors of PLA_2, a calcium-dependent enzyme, abolish NMDA-mediated increases in both c-*fos* and *NGFI-A* mRNA. Structurally distinct inhibitors of cyclooxygenase reduce the induction of c-*fos* mRNA while having little effect on the induction of *NGFI-A* mRNA. Conversely, distinct inhibitors of lipoxygenase markedly reduce NMDA-induced increases in *NGFI-A* without attenuating the increases in c-*fos* mRNA. The selective effects exhibited by the cyclooxygenase and lipoxygenase inhibitors on the induction of either c-*fos* or *NGFI-A* mRNA demonstrate that these inhibitors act at sites distal to the NMDA receptor and do not nonspecifically block NMDA receptor activation. Additionally, many of these compounds have been shown not to interfere with NMDA-mediated increases in $[Ca^{2+}]_i$ (18). These data demonstrate that PLA_2 and the AA cascade play a pivotal role in NMDA regulation of gene expression.

Numerous compounds have been described to inhibit AA release and eicosanoid production by interfering with the action of PLA_2. The mechanism of action of PLA_2 inhibitors can be either direct (action on the enzyme itself) or indirect (action at nonenzymatic sites). Quinacrine is a cationic amphiphilic compound that complexes with phospholipids, thereby blocking the ability of PLA_2 to interact with its

substrate (27). Several laboratories have demonstrated that quinacrine blocks NMDA-mediated AA release from various neuronal preparations (30, 31). In separate experiments, we have also demonstrated that quinacrine blocks NMDA-mediated release of AA from cortical neurons *in vitro*.[1] Aristolochic acid is an alternate PLA_2 inhibitor that directly binds to the enzyme, interfering with its mechanism of action (26). Rosenthal *et al.* (26) demonstrated that aristolochic acid effectively blocks A23187-stimulated PLA_2 activity *in vitro*, in a substrate-independent manner. Therefore, we have used two distinct compounds that block PLA_2 activity via differing mechanisms; both compounds markedly reduce the induction of both c-*fos* and *NGFI-A* mRNA after NMDA receptor activation on DG neurons.

Stimulation of NMDA receptors activates several different enzymes in a calcium-requiring manner. These include mitogen-activated protein kinase (35), calcium/calmodulin-dependent kinase (36), nitric oxide synthase (37), and PLA_2 (18, 30). Little information is available, however, on whether any or all of these enzymes are required for NMDA receptor regulation of gene expression. We previously proposed an intracellular sequence involving NMDA receptor-mediated activation of PLA_2, generation of AA, and activation of the cyclooxygenase metabolic pathway, leading to induction of c-*fos* mRNA (18). The robust inhibition of NMDA receptor-mediated *NGFI-A* mRNA expression by structurally distinct PLA_2 inhibitors strongly implicates this enzyme in the induction of a second IEG. However, the phospholipid products resulting from AA turnover and the subsequently formed, biologically active metabolites responsible for *NGFI-A*, in contrast to c-*fos*, mRNA induction involve the lipoxygenase metabolic pathway. Two distinct metabolic branches of the AA signaling cascade are involved in mediating NMDA receptor-induced expression of two different IEGs (Fig. 6).

AA and its metabolites are gaining increasing support as diffusible signaling mediators. The levels and turnover rates of the various eicosanoids formed after AA metabolism may be crucially involved in regulating the array of IEGs expressed after NMDA receptor activation. To date, it has been difficult to isolate and reliably identify the various AA metabolites that may be released from neurons after NMDA receptor activation. The amount of neuronal material required to obtain a detectable signal has hampered such studies. Given the size of the DG in 4-day-old rat pups and the limited number of cells obtained from this region, such studies are not feasible in the cellular preparation used in the present study. Once accomplished, identification of these specific eicosanoids will greatly enhance our understanding of NMDA-mediated changes in gene expression. AA and its metabolites are thought to modulate numerous cellular responses. Our results emphasize the potential importance of the PLA_2 pathway for the long term consequences of NMDA receptor activation. AA is increased *in vivo* after synaptic stimulation (38), as well as in a variety of neuronal cell populations *in vitro* after NMDA receptor activation (30, 31). AA has been shown to affect synaptic transmission and NMDA receptor function in the CNS (39), be involved in the induction of LTP (40, 41), and generate free radicals that are potentially involved in neuronal cell death (42). The permanent changes in neuronal function observed after brief epi-

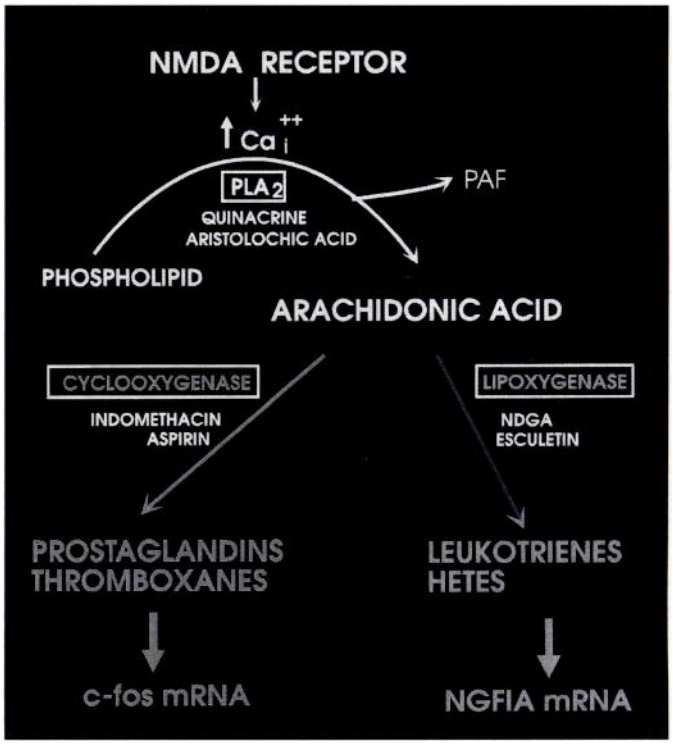

Fig. 6. Schematic diagram of the intracellular signaling pathways involved in NMDA-mediated induction of c-*fos* and *NGFI-A* mRNAs. Stimulation of DG neurons with NMDA causes an increase in $[Ca^{2+}]_i$ and an increase in the expression of c-*fos* and *NGFI-A* mRNAs. The induction of both of these IEGs is reduced by PLA_2 inhibitors (quinacrine and aristolochic acid). When activated, the calcium-sensitive enzyme PLA_2 increases the intracellular pool of AA. The NMDA-mediated increase in $[Ca^{2+}]_i$, activation of PLA_2, and generation of AA are common to the induction of both IEGs. The specific AA-metabolizing pathway required for the full induction of each gene is distinct. Inhibitors of cyclooxygenase (aspirin and indomethacin) reduce NMDA-mediated increases in c-*fos* mRNA, whereas inhibitors of lipoxygenase (NDGA and esculetin) reduce NMDA-mediated induction of *NGFI-A* mRNA. *PAF*, platelet-activating factor; *HETES*, hydroxyeicosatetraenoic acids.

sodes of neuronal activity, such as the changes in neuronal excitability seen in seizure models or the increased synaptic efficacy observed in LTP (a cellular model of learning and memory), may involve the induction of IEGs and specific target gene transcription. Both c-*fos* and *NGFI-A* encode transcription factors that are able to regulate the expression of target genes through DNA-binding proteins (7, 43). The long term consequences of the induction of c-*fos* and/or *NGFI-A* transcription are not well understood. The transcription factor c-Fos plays a crucial role in normal developmental processes (44, 45), as well as in activity-dependent plasticity.[2] Other IEGs, such as *NGFI-A*, may also be involved in developmental and nondevelopmental changes (46). Many examples of activity-induced neuronal plasticity mimic changes in neuronal function and morphology observed during postnatal development. Increases in IEGs such as c-*fos* and *NGFI-A* have been temporally correlated with the induction of LTP (6, 10), seizure activity (11, 13, 47), axonal sprouting,[2] cell death (48), and other forms of activity-induced neuronal plasticity in the mature nervous system. We hy-

[1] N. G. Carlson, unpublished observations.

[2] Y. Watanabe, R. S. Johnson, L. S. Butler, B. M. Spiegelman, V. E. Papaioannou, and J. O. McNamara. Null mutation of c-*fos* impairs functional and structural plasticities in the kindling model of epilepsy. Submitted for publication.

pothesize that, to gain specificity of long term consequences of glutamate receptor activation, distinct intracellular signaling pathways are necessary to regulate the initial induction of varying arrays of IEGs. Once induced, these distinct arrays may trigger a sequence of genomic events resulting in specific long-lasting changes.

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Send reprint requests to: Leslie S. Lerea, 401 Bryan Research Building, Box 3676, Department of Medicine, Duke University, Durham, NC 27710.