Hypoxia modulates nitric oxide-induced regulation of NMDA receptor currents and neuronal cell death

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1Interdisciplinary Program in Neuroscience and 2Department of Physiology and Biophysics, Georgetown University Medical Center, Washington, District of Columbia 20007; 3Department of Pharmacology, University of Connecticut Health Center, Farmington, Connecticut 06030; and 4Radiation Biology, National Cancer Institute, and 5Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

Gbadegesin, Muyiwa, Stefano Vicini, Sandra J. Hewett, David A. Wink, Michael Espey, Ryszard M. Pluta, and Carol A. Colton. Hypoxia modulates nitric oxide-induced regulation of NMDA receptor currents and neuronal cell death. Am. J. Physiol. 277 (Cell Physiol. 46): C673–C683, 1999.—Nitric oxide (NO) released from a new chemical class of donors enhances N-methyl-d-aspartate (NMDA) channel activity. Using whole cell and single-channel patch-clamp techniques, we have shown that (Z)-1-[(3-ammoniopropyl)-N-(n-propyl)amino]-NO (PAPA-NO) and diethylamine NO, commonly termed NONOates, potentiate the glutamate-mediated response of recombinant rat NMDA receptors (NR1/NR2A) expressed in HEK-293 cells. The overall effect is an increase in both peak and steady-state whole cell currents induced by glutamate. Single-channel studies demonstrate a significant increase in open probability but no change in the mean single-channel open time or mean channel conductance. Reduction in oxygen levels increased and prolonged the PAPA-NO-induced change in both peak and steady-state glutamate currents in transfected HEK cells. PAPA-NO also enhanced cell death in primary cultures of rodent cortical neurons deprived of oxygen and glucose. This potentiation of neuronal injury was blocked by MK-801, indicating a critical involvement of NMDA receptor activation. The NO-induced increase in NMDA channel activity as well as NMDA receptor-mediated cell death provide firm evidence that NO modulates the NMDA channel in a manner consistent with both a physiological role under normoxic conditions and a pathophysiological role under hypoxic conditions.

N-methyl-d-aspartate; oxygen-glucose deprivation; PAPA-NO

The N-METHYL-D-ASPARTATE (NMDA) receptor is one of the subtypes of excitatory amino acid receptors found on neurons in the central nervous system and plays important physiological roles in both the fetal and adult central nervous systems. Activation of the NMDA receptor affects processes ranging from migration of neurons in the developing brain to learning and memory (8, 15, 43, 53, 63). The NMDA receptor also mediates pathophysiological states such as glutamate-mediated excitotoxicity, which is commonly associated with ischemia-reperfusion injury, persistent seizures, and chronic neurodegenerative disease (7, 11, 13, 27, 37, 67).

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agents results in a reduction in NMDA receptor responses (20, 31, 33, 35, 61). However, NO may not be the primary species produced by these donors, since other reactive nitrogen species that act as nitrosonium ion (NO+·) donors have been implicated in their actions (33). In support of this, the actual level of NO generated by SIN-1, SNOC, or SNAP is very low (62). Recently, a new generation of pure NO donors, the NONOates, has been developed, which produce up to 15 µM NO with variable half-lives ranging from seconds to hours (36, 62). NONOates, therefore, can serve as a reliable, long-lasting source of NO. The aim of the present study was to characterize the effects of the NO generated by this new class of agents on NMDA receptor activity and neuronal cell death under normoxic and hypoxic conditions.

METHODS

Cell cultures. Murine mixed cortical cell cultures were prepared as described in Refs. 22 and 61. First, cerebral cortices of 1- to 3-day-old mouse pups (CD-1; Charles River) were removed under sterile conditions, minced, and incubated for 20–30 min (37°C) in 0.025% trypsin diluted in Hanks’ balanced salt solution supplemented with glucose (15 mM) and sucrose (20 mM). Cells were pelleted by light centrifugation (1,850 rpm, 3 min), the supernatant was discarded, and the cell pellet was resuspended in plating medium of modified Eagle’s medium (Earle’s salts; Mediatech) supplemented with 2 mM glutamine, 20 mM glucose [media stock (MS)], 10% fetal bovine serum (FBS), 10% horse serum (HS), and epidermal growth factor (10 ng/ml). The cells were plated in 24-well dishes (Primaria; Falcon) at a density of 1 hemisphere·plate-1·10 ml-1. Once confluent (9–11 days), the cells, primarily astrocytes, were shifted into a maintenance medium (MS + 10% HS).

Cortical neurons were obtained in a similar manner from embryonic day 15 (E15) fetuses and then plated at a density of 3 hemispheres per plate per 10 ml on an established bed of astrocytes (12–24 days in vitro) in MS supplemented with 5% HS and 5% FBS. After 5–7 days in vitro, cultures were exposed to 0.5 µg/ml mitomycin C for 2 days to eliminate any mitotic cells (microglia, oligodendrocytes). Cells were then shifted into maintenance medium, and the medium was changed twice weekly. Experiments were performed on mixed cultures between 14 and 16 days in vitro. All cultures were kept at 37°C in a humidified 5% CO2-containing atmosphere.

Human embryonic kidney-293 (HEK-293) cells (ATCC No. CRL1573; American Type Culture Collection, Manassas, VA), were grown at 37°C in a 6% CO2-94% air atmosphere in MEM (GIBCO BRL, Gaithersburg, MD), supplemented with 10% FBS and 100 U/ml penicillin-streptomycin (GIBCO BRL). Transfection of NMDA receptor subunits. Exponentially growing HEK-293 cells were dispersed with trypsin 15–20 h in advance of transfection, resuspended at 2 × 106 cells in 1.5 ml of culture medium, and plated on poly-L-lysine (Sigma, St. Louis, MO)-coated coverslips. HEK-293 cells were transfected with rat NMDA receptor cDNAs using the calcium phosphate precipitation method as described in Ref. 60. Mixed plasmids (3 µg total) coding for NR1a, NR1b, and NR2A subunits of the NMDA receptor were added to the dish containing the 1.5-ml culture medium. cDNA for pGreenLantern-1 protein (GIBCO BRL) was cotransfected with the NMDA receptor cDNAs to allow for visualization of the transfected cells. Greater than 90% of the cells that expressed GreenLantern protein also expressed NMDA receptors. The transfection mixture was removed after 8–10 h, the cells were washed twice with PBS, and then the PBS was replaced with fresh culture media containing 500 µM ketamine to reduce glutamate-mediated cell toxicity. Transfected cells were allowed to equilibrate overnight before use in the experimental protocols. Experiments were performed on a minimum of three different transfected cell cultures (n = the number of cells studied). Statistical significance was determined using an unpaired or paired Student’s t-test according to the experimental protocols.

Electrophysiology. With the use of the patch-clamp technique, transfected HEK-293 cells were voltage clamped at −60 mV in the whole cell or outside-out patch configuration. Electrodes were pulled in two stages on a vertical pipette puller from borosilicate glass capillaries (Wiretrol II; Drummond, Broomall, PA). Typical pipette resistance was 5–7 MΩ. Intracellular (patch pipette) solutions contained (in mM) 145 potassium gluconate, 5 EGTA, 2 MgCl2, 10 HEPES, 2 NaATP, and 0.2 NaGTP, pH to 7.2 with KOH. The cells were bathed in extracellular solution containing (in mM) 145 NaCl, 5 KCl, 1 CaCl2, 5 HEPES, and 5 glucose. The extracellular solution contained saturating concentrations of glycine (10 µM).

All drugs were applied to the bath using a gravity-fed Y-tube delivery system placed within 500 µm of the cell under study. The tip of the Y-tube had a diameter of ~100 µm and allowed solution exchange in <20 ms. In addition, the chamber (0.5 ml total volume) was continually perfused at a rate of ~4–5 ml/min. All drugs were dissolved in the same extracellular solution used to bathe the cells.

For experiments in low oxygen (hypoxia), control solution or solutions containing glutamate or glutamate plus the test drugs were vigorously aerated with 100% N2 at ambient pressure in separate enclosed and vented chambers. These solutions were then used to perfuse the transfected HEK cells. Exact oxygen tensions were not determined under these conditions.

Data acquisition and analysis. Whole cell currents were monitored with an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt/Eberstadt, Germany), filtered at 1.5 kHz with an eight-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA), and digitized with Axotape 2 software (Axon Instruments, Foster City, CA). Clampfit (Axon Instruments) was used for off-line analysis, and Origin (MicroCal Software, Northampton, MA) was used for figure preparation and statistical analysis. Single-channel recordings were filtered at 1.5–2.5 kHz and digitized at 10–20 kHz. Analysis and curve fitting for the single-channel recordings were performed using Fetchan and pStat analysis programs (Axon Instruments). The threshold for measuring open and shut intervals was set at one-half the maximal amplitude of the main conductance level of the channel. Log-binned interval histograms for shut and open duration as well as burst duration were plotted with a square root vertical axis and fitted using maximum likelihood fitting (52). For display purposes, traces were filtered at 50 Hz.

NO donors. (Z)-1-[N-(3-ammoniopropyl)-N-(propyl)-amino]-NO (PAPA-NO) and diethylamine-NO (DEA-NO) were gifts from Dr. David Wink (National Institutes of Health) or were obtained from Alexis Biochemical (San Diego, CA). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PITO; potassium salt) and SIN-1 were obtained from Alexis Biochemicals. All NONOates were prepared as stock solutions of 10 or 100 mM in alkaline solutions and were diluted into the desired media immediately before use. Exchanged solutions were prepared by allowing the NO donor solutions to sit for 24 h at room temperature.
Combined oxygen-glucose deprivation. Cultures were placed in an anaerobic chamber (Forma Scientific, Marietta, OH) that contained a gas mixture of 5% CO₂-10% H₂-85% N₂ (0.2% O₂). Culture media were replaced by thorough exchange with a deoxygenated, glucose-free balanced salt solution alone or a balanced salt solution containing PAPA-NO (0.3–10 μM) and then were placed in a 37°C humidified incubator within the chamber for 40–45 min. Cultures were subsequently removed from the incubator, and 100 μl of cell culture supernatant were collected to assess early cell death using the lactate dehydrogenase (LDH) assay (see below). Exposure medium was then exchanged with oxygenated MEM, and the cells were returned to a normoxic (21% O₂) incubator at 37°C containing 5% CO₂. Delayed neuronal death was assessed 20–24 h later. The balanced salt solution contained (in mM) 116 NaCl, 5.4 KCl, 0.8 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, 1.8 CaCl₂, and 0.01 glycine.

For exposure under normoxic conditions, cultures were exposed for 45 min to PAPA-NO (0.3–10 μM) diluted into MS alone (or in the presence of 10 μM NMDA) at 37°C in a 5% CO₂-containing incubator. Cultures were subsequently removed from the incubator, and 100 μl of cell supernatants were collected for assessment of early neuronal injury. The exposure medium was then exchanged with fresh MS and the cells returned for an additional 20–24 h, whereupon delayed neuronal death was determined.

Assessment of neuronal cell death. Neuronal cell death was estimated by examination of cultures under phase-contrast microscopy and quantitatively assessed by the measurement of LDH released by damaged or destroyed cells into the bathing medium following treatment. LDH activity was quantified by the rate of oxidation of NADH, which was followed spectrophotometrically at 340 nm (29). The small amount of LDH present in the medium of sister cultures subjected to sham wash (generally <15% of total) was subtracted from the levels in experimental conditions, to yield the LDH signal specific to experimental injury. Data are expressed as the percentage of total neuronal LDH (=100%) that was determined for each experiment by assaying the supernatant of sister cultures after 24 h of exposure to 300 μM NMDA.

NO measurement. NO release from the NO donors was measured continuously for 12 h with an electrochemical probe (Diamond General) placed in a 3-ml sample of the perfusion media. After stabilization, the probe was calibrated with a probe (Diamond General) placed in a 3-ml sample of the perfusion media. After stabilization, the probe was calibrated with a 1 M NaOH solution containing NO (0.3–10 μM) and then were placed in a 37°C humidified incubator within the chamber for 40–45 min. Cultures were subsequently removed from the incubator, and 100 μl of cell culture supernatant were collected to assess early cell death using the lactate dehydrogenase (LDH) assay (see below). Exposure medium was then exchanged with oxygenated MEM, and the cells were returned to a normoxic (21% O₂) incubator at 37°C containing 5% CO₂. Delayed neuronal death was assessed 20–24 h later. The balanced salt solution contained (in mM) 116 NaCl, 5.4 KCl, 0.8 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, 1.8 CaCl₂, and 0.01 glycine.

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NO measurement. NO release from the NO donors was measured continuously for 12 h with an electrochemical probe (Diamond General) placed in a 3-ml sample of the perfusion media. After stabilization, the probe was calibrated against known concentrations of NO with the use of in vitro methods previously described (38). NO stock solutions used for calibration of the probe were prepared by bubbling NO into a solution of 1 M NaOH to eliminate other nitrogen oxide species and then through a sealed container with deoxygenated normal saline. The concentration of NO in the stock solution was confirmed using colorimetric methods.

RESULTS

Whole cell response to PAPA-NO. The effects of the NO donors PAPA-NO and DEA-NO on NMDA receptor activity were studied in HEK-293 cells transiently transfected with NMDA NR1a, NR1b, and NR2A subunit cDNAs. Glutamate (30 μM) was applied in 10-s pulses to the cells and whole cell currents were recorded. As shown in Fig. 1 for HEK cells transfected with NR1a and NR2A subunits, the evoked currents demonstrated slow onset and offset kinetics similar to those recorded using native NMDA receptors (40). On average, the peak responses of NR1a/NR2A transfected cells to 30 μM glutamate were 300 ± 74 pA (SE; n = 8 cells), whereas average steady-state currents, determined after 10 s of application of agonist, were 217 ± 48 pA (n = 8). The response to glutamate was reexamined in the presence of the NO donors by adding the donor directly to the glutamate-containing perfusion media and applying this mixture in 10-s pulses onto the cell using the Y-tubing system. Because NO production by PAPA-NO rises quickly then falls over 20 min (Fig. 1B), PAPA-NO was added to the glutamate solution <1 min before application to the cell. This insured maximal NO generation. The presence of glutamate and PAPA-NO (200 μM) significantly increased both the peak current and the steady-state current compared with glutamate alone. The effect of PAPA-NO was reversible by removing the NO donor from the perfusion media or by the simultaneous addition of C-PTIO, an NO scavenger that rapidly and irreversibly inactivates NO (Fig. 1A) (4). DEA-NO, a similar NO donor but with a shorter half-life than PAPA-NO (~7 min) also produced an increase in glutamate-mediated whole cell currents (Table 1).

The putative NO donor SIN-1 did not generate NO under the same conditions as PAPA-NO and did not increase the glutamate-mediated response in transfected HEK cells (Fig. 1, C and D). In contrast, the presence of 1 mM SIN-1 decreased the whole cell currents produced by application of 30 μM glutamate to the bath.

To rule out the possibility that the amine backbone of PAPA-NO mimicked the glycine-independent potentiation of the NMDA receptor by spermine, we tested the effect of PAPA-NO on whole cell currents in HEK-293 cells transfected with the NR1b and NR2A subunit cDNAs. The NR1b splice variant contains a 21-amino acid NH₂-terminal insert generated by exon 5 and shows no potentiation by spermine at saturating concentrations of glycine (66). If PAPA-NO or the other NONOates mimics the effect of spermine, potentiation should not be observed in recombinant receptors containing the NR1b subunit splice variant. As shown in Table 1, recombinant NMDA receptors containing the splice variant demonstrated increased whole cell currents in response to PAPA-NO. The average control peak current value for the NR1b/NR2A variant was 296 ± 141 pA (n = 5), and the average control steady-state current value was 207 ± 84 pA (n = 5). In the presence of 200 μM PAPA-NO, these values were increased by 33 ± 15% and 33 ± 14%, respectively, compared with glutamate alone (Table 1). The NR1b splice variant was chosen for all subsequent experiments, thereby eliminating both the spermine and pH sites involved in NMDA channel regulation (57) and allowing a more direct analysis of the effect of NO.

Control studies were done to examine the effect of expended PAPA-NO on the response to pulses of 30 μM glutamate (Fig. 2). PAPA-NO solutions equilibrated at 25°C for 24 h induced a depression of 27 ± 5% (n = 10) in glutamate-mediated whole cell currents in cells transfected with the NR1a/NR2A and NR1b/NR2A subtypes, indicating that the increased response of the
NMDA receptor induced by PAPA-NO was not due to the parent molecule.

To examine the NO-mediated effect on NMDA receptor function in more detail, we determined the effect of PAPA-NO on NMDA channel activity in outside-out patches excised from transfected HEK-293 cells. A representative recording is shown in Fig. 3 and demonstrates that, under control conditions (5 µM glutamate, 10 µM glycine), the open probability was 0.023 and, in the presence of 100 µM PAPA-NO plus glutamate, the open probability increased to 0.041. The increased open probability returned to the untreated value when the NO scavenger, C-PTIO, was added to the perfusion media. The average open probability in the presence of glutamate alone was 0.029 \( \pm \) 0.008 (n = 11 patches) and was 0.072 \( \pm \) 0.02 (n = 11) for PAPA-NO plus glutamate.

Fig. 1. Modulation of whole cell N-methyl-D-aspartate (NMDA) receptor responses by (Z)-1-(N-(3-ammoniopropyl)-N-(n-propyl)amino)-nitric oxide (PAPA-NO). NMDA whole cell responses were elicited in HEK-293 cells transiently transfected with the NMDA receptor subunits NR1a and NR2A. Traces indicate responses from a typical transfected HEK cell to 30 µM glutamate applied via a Y-tube. A: responses to sequential application of 30 µM glutamate (GLUT) and 30 µM glutamate + 200 µM PAPA-NO. Responses to glutamate + PAPA-NO were elicited 40 s after addition of PAPA-NO to glutamate solution. A 2-min wash period was interposed between each application of drugs. Right trace removal of effect of PAPA-NO by addition of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO), an NO scavenger. Bars, time of drug application. B: electrochemical detection of NO production by PAPA-NO over time. NO production by PAPA-NO was measured in separate control experiments using an NO-sensitive electrode in same media as that used for electrophysiological recordings. C: whole cell current recordings obtained from a representative HEK cell transiently transfected with NMDA receptor subunits NR1a and NR2A in response to 30 µM glutamate in presence and absence of 1 mM 3-morpholinosydnonimine (SIN-1). Cells were washed for 2 min between each drug application and were voltage clamped at −60 mV. D: electrochemical detection of NO production from SIN-1 using an NO-sensitive electrode. Measurement of NO production from SIN-1 was carried out in same media used for electrophysiological recordings. Final concentration of SIN-1 was 1 mM.

Distributions of channel open time and burst duration were also determined and are shown for a representative patch in Fig. 4, A-D. PAPA-NO did not alter the distribution of channel open times (Fig. 4, A and B). Average values of the mean open times for 11 patches were 3.7 \( \pm \) 0.4 and 4.1 \( \pm \) 0.4 ms in the presence of glutamate alone and glutamate plus PAPA-NO, respectively. Determination of the distribution of burst durations revealed an increase in burst duration (Fig. 4, C and D). The average burst duration changed from 8 \( \pm \) 1 ms (n = 8 patches) in glutamate alone to 15 \( \pm \) 3 ms (n = 8 patches) in the presence of PAPA-NO. Current-voltage relationships were also obtained for the main conductance state over a range of membrane potentials from −80 to +80 mV (Fig. 4F). Conductance and reversal potential values for the main conductance state of the channels under control conditions were 51.6 pS and 3.8 mV, respectively, whereas, for PAPA-NO-
treated channels, conductance and reversal potential of the main conductance state were 50.8 pS and 3.4 mV, respectively. Thus no change in single-channel conductance was observed.

Effect of low oxygen tensions on the changes in glutamate-mediated whole cell currents by PAPA-NO. To determine whether hypoxic conditions altered the response of the NMDA receptor to NO, ambient oxygen tension in the solutions was decreased. For these experiments, the glutamate or glutamate plus PAPA-NO solutions perfusing the cells was vigorously and continuously agitated with 100% N₂ immediately before application to the cells. In contrast to the previous experiments where 30 µM glutamate was used, PAPA-NO (200 µM) was added in the presence of saturating concentrations of glutamate (200 µM). For that reason, the potentiation of the peak current induced by PAPA-NO was not pronounced. However, use of saturating concentrations of glutamate allowed discernment of the effect of PAPA-NO in normoxic and hypoxic conditions. In addition, use of saturating concentrations of glutamate allowed the study of the effect of PAPA-NO on desensitization, which appeared to be a possible mechanism for the action of PAPA-NO. This mechanism was suggested by the effect of PAPA-NO on burst duration in the single-channel studies (see DISCUSSION). The relative change in the response to PAPA-NO was followed over time, and average values were obtained by determining the ratio of the sequential responses to glutamate plus PAPA-NO to the average response to glutamate alone. Only cells with stable control responses to repetitive glutamate pulses were used in these experiments. As shown in Fig. 5, the effect of PAPA-NO on both the peak and steady-state current in response to 5-s pulses of 200 µM glutamate is enhanced in a low-oxygen environment. A comparison of the normalized values for peak and steady-state currents in normoxic and hypoxic conditions is shown in Fig. 5, C and D. A significant ($P < 0.01$) increase in both the peak and steady-state current was seen initially but decayed with time.

NMDA-mediated neuronal injury in normoxic and hypoxic conditions with PAPA-NO. The relationship between the increase in NMDA channel response induced by exposure to PAPA-NO and a downstream functional end point, i.e., neuronal injury, was also investigated. First, cortical neurons in primary culture were exposed to PAPA-NO (0.3–10 µM) for 45 min under normoxic conditions to determine whether it was directly cytotoxic. Under these control conditions, exposure to PAPA-NO alone produced no early (data not shown) or late cortical neuronal cell death over the concentration range studied (Fig. 6A). Furthermore, PAPA-NO did not enhance cell death induced by low doses of NMDA (10 µM; Fig. 6B). However, when the cortical cultures were deprived of oxygen and glucose in the presence of PAPA-NO, cell killing was significantly increased over that produced by oxygen-glucose depri-

<table>
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<th>Channel Type</th>
<th>%Control Peak Current 200 µM PAPA-NO</th>
<th>%Control Steady-State Current 200 µM PAPA-NO</th>
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<tr>
<td>NR1a/NR2A</td>
<td>132 ± 4 (8)</td>
<td>159 ± 6 (8)</td>
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<tr>
<td>NR1b/NR2A</td>
<td>133 ± 15 (5)</td>
<td>133 ± 14 (5)</td>
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<tr>
<td>1 mM DEA-NO</td>
<td>134 ± 14 (4)</td>
<td>136 ± 22 (4)</td>
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Values are means ± SE of responses of cells from several independent cell transfections; no. of cells recorded for each channel type in parentheses. Values are whole cell current activated by 30 µM glutamate (in presence of 200 µM PAPA-NO) expressed as a percentage of control response to 30 µM glutamate (in absence of PAPA-NO). PAPA-NO was added to glutamate solution 40 s before application to the cell, to ensure near-maximal levels of NO production. NR1a, −exon 5; NR1b, +exon 5; PAPA-NO, (Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)amino]-nitric oxide; DEA-NO, diethylamine-nitric oxide; NMDA, N-methyl-D-aspartate. $P < 0.05$, paired t-test.
vation (OGD) alone (Fig. 7A). The expended NONOate had no effect on OGD-mediated killing (data not shown), indicating that the PAPA-NO-mediated enhancement of cell death was not due to a nonspecific effect of a metabolic product produced by PAPA-NO. To determine whether cell death in the low-oxygen, low-glucose condition was dependent on a functional NMDA receptor response, the irreversible NMDA antagonist MK-801 was utilized. As shown in Fig. 7B, the PAPA-NO-mediated enhancement of OGD-induced cell death was prevented by the addition of MK-801 (10 µM) to the exposure medium.

**DISCUSSION**

NO released from well-characterized, time-dependent, pure NO donors such as the NONOates enhances NMDA channel activity. Using whole cell and single-channel patch-clamp techniques, we have shown that the two NONOates studied, i.e., PAPA-NO and DEA-NO, potentiate glutamate-mediated responses of recombinant rat NMDA receptors expressed in HEK-293 cells. The overall effect is an increase in both the peak and steady-state currents induced by glutamate. Single-channel kinetics demonstrate a significant increase in open probability and an increase in burst duration but no change in channel open time or single-channel conductance. The effect of NO could be readily reversed by NO scavengers, and the addition of expended NONOate (both PAPA-NO and DEA-NO) produced a slight depression in whole cell currents rather than the enhancement seen in the presence of NO. The action of PAPA-NO was highly time dependent and could be visualized most clearly within 10 min of addition to the cells. The falloff in activity most likely represents the time-dependent release of NO from the NONOates. The polyamine-like backbone that remains after release of NO could be responsible for the inhibition of the NMDA response seen with expended NONOate solutions, since polyamines induce a voltage-dependent inhibition of the NMDA channel (40).

The enhancement of NMDA channel whole cell and single cell responses is in contrast to the effect on the NMDA channel of other NO donors reported in the literature. Previous studies with donors such as SNAP (25), SIN-1 (35), SNOC (31), and SNP (25, 31, 47) have demonstrated that the NMDA receptor is inhibited by NO. One potential explanation for the discrepancy is that reactive species other than NO are produced downstream of NO, which inhibit rather than potentiate receptor activity (33). NO is well known to undergo a number of rapid interactions with oxygen, forming reactive nitrogen-oxygen species (RNOs) that may themselves have effects on a variety of molecules and reactions within the cell (64, 65). RNOs can react with glutathione or with sulfhydryl groups on proteins and lipids, producing independent effects on the NMDA receptor. In our experiments, the action of SIN-1 on NMDA channel activity is consistent with the action of these other NO species. Using an NO electrode in separate control studies, we showed that SIN-1 does not release measurable amounts of NO over 12 h. SIN-1 has been reported, however, to produce superoxide anion. Thus any NO generated by SIN-1 would rapidly combine with superoxide anion to form the powerful oxidizing agent peroxynitrite (59). Colton et al. (14), Tang and Alizeman (55, 56), and others (30, 54) have
demonstrated that oxidation depresses NMDA channel function and that both whole cell currents and single-channel properties are reduced under oxidative conditions. SIN-1 in our study inhibits whole cell glutamate-mediated currents, a result that is consistent with oxidation of the NMDA channel.

Both the whole cell and single-channel studies show a direct effect of NO released from PAPA-NO on the NMDA channel. This is apparent from the potentiation of the peak and steady-state current at the whole cell level and the increase in open probability and burst duration at the single-channel level. The exact site of NO action in our study is not clear. Aizenman and Potthoff (3) have indicated that NO does not appear to act on Cys-744 and Cys-798, the redox sites commonly associated with the inhibition of NMDA channel func-
tion by oxidation. These authors suggest that inhibition by NO utilizes an alternative site. Our data also indicate alternative sites of action for NO. In this case, NO-induced potentiation of the NMDA response may be due to a decrease in desensitization, since burst duration is increased. Lester and Jahr (32) have demonstrated that an increase in burst duration represents entry of the NMDA receptor into a relatively nondesensitizing state.

NO-mediated enhancement of NMDA receptor activity under aerobic conditions is likely to be a normal physiological function of NO. In our experiments, the increase in whole cell currents in response to PAPA-NO is generated using rapid and short-duration application pulses of the NO donor. Although the exact concentration of NO is not known, the cell is exposed in a transient fashion to low levels of NO. This exposure pattern to NO is reminiscent of the action of nNOS in the hippocampus and is consistent with a physiological role for NO. For example, NO has been implicated in the regulation of synaptic transmission and is released from postsynaptic neurons in a calcium/calmodulin-dependent fashion (8, 12, 51). One potential role, as proposed by Bredt et al. (8) and others (43, 51), is that NO serves as a retrograde messenger, strengthening synaptic connections during long-term potentiation. Localization of nNOS to the synaptic region, as indicated by Brenman et al. (9) and Aoki et al. (5), would provide easy access of NO to the NMDA receptor. Because the overall level of NO is low, direct effects of

Fig. 5. Changes in effect of NO in a low-oxygen environment. A: effect of PAPA-NO on glutamate-induced whole cell currents in an ambient oxygen (normoxic) environment. Traces represent typical recordings from NR1b/NR2A transfected cells. Glutamate (200 µM) applications were for 5 s followed by a 5-s wash period in presence and absence of 200 µM PAPA-NO. PAPA-NO was added to glutamate-containing solution and was thus also applied in 5-s intervals with a wash between each application. B: typical glutamate responses (5 s, 200 µM) from a different cell in presence and absence of 200 µM PAPA-NO in a low-oxygen (hypoxic) environment. C and D: relative change in response to glutamate induced by exposure to PAPA-NO was followed over time. Values were obtained by determining an average control (untreated) response to glutamate and then determining the ratio of individual responses to the average control response for a sequential series of responses to glutamate in presence of PAPA-NO. Data points represent average ± SE relative change for 5 cells under normoxic condition and for 4 cells under hypoxic conditions. C: average relative peak currents (I_{peak}). D: average relative steady-state currents (I_{steady state}). *P < 0.05.
NO would dominate (19, 65). Consistent with a physiological modulatory role, our data demonstrate that neither NO-mediated cell death nor an enhancement of NMDA-induced neuronal injury occurs under these conditions. However, during ischemia, the fall in oxygen level may significantly alter the response to NO, allowing a pathophysiological state to ensue.

Hypoxic conditions alter the effect of PAPA-NO on the NMDA receptor. Reduction in oxygen levels increased and prolonged the PAPA-NO-induced change in both peak and steady-state glutamate currents in the transfected HEK cells. Although the mechanism of this effect is unclear, separate studies using mixed cortical cultures indicate that the action of PAPA-NO in a low-oxygen environment may have functional consequences. Cell death mediated by oxygen and glucose deprivation was increased by exposure to low concentrations of PAPA-NO. MK-801-induced blockade of the enhanced toxicity further suggests that the effect of PAPA-NO in OGD-treated cultures is due to activation of the NMDA channel. An increased NMDA receptor activity would also provide an explanation for the association between increased astrocytic inducible NOS activity and increased neuronal death seen during OGD (23). Alternatively, one might argue that NO-mediated enhancement of OGD-induced neuronal injury could result from alterations in astrocytic glutamate uptake. However, NO derived from DEA-NO at concentrations up to 10 mM had no effect on glutamate uptake by purified or recombinant high-affinity glutamate transporters (58). By contrast, peroxynitrite and oxygen radicals effectively prevented glutamate uptake (58). Under hypoxic conditions, the formation of peroxynitrite or other oxidizing species is low. Because our data clearly demonstrate that neuronal cell death is enhanced in the presence of low oxygen, i.e., under those conditions where formation of RNOs is minimal, a direct effect on the NMDA receptor is favored.

The source of NO in hypoxic episodes is not clear, but direct measurement of NO using NO electrodes during rat carotid occlusion has shown a burst of NO at the onset of the occlusion, followed by a slow decline (34). A variety of studies using different techniques have also indirectly implicated that NO is generated during the early phase of ischemia (6, 18, 44, 45). Although continued lack of oxygen, as would occur in the core of an ischemic infarct, would preclude the generation of NO by NOS (1), the low oxygen tension that is main-

Fig. 7. PAPA-NO potentiates oxygen and glucose deprivation-induced neuronal injury. A: primary cortical cultures were deprived of oxygen and glucose in absence and presence of varying concentrations of PAPA-NO (0.3–10 µM). At 20–24 h after 45 min of oxygen-glucose deprivation (OGD), LDH release into the bathing medium was measured. Data are expressed relative to LDH signal corresponding to neuronal death in sister cultures (taken as = 100; measured after exposure to 300 µM NMDA for 24 h). *P < 0.01 as assessed by ANOVA followed by Dunnett’s t-test (n = 12–20 wells assayed for 5 separate culture groups). B: cell cultures were deprived of oxygen and glucose alone or in presence of NMDA receptor antagonist MK-801 (10 µM) alone, PAPA-NO (10 µM) alone, or MK-801 (10 µM) + PAPA-NO (10 µM). At 20–24 h after 40–45 min of oxygen-glucose deprivation, LDH release into the bathing medium was measured. Data are expressed relative to the LDH signal corresponding to neuronal death in sister cultures (taken as = 100; measured after exposure to 300 µM NMDA for 24 h). *P < 0.001 as assessed by ANOVA followed by Student-Newman-Keuls test (n = 7–8 wells assayed for 3 separate culture groups).
tained in the penumbral region could be enough to sustain NO formation. Further, the return of oxygen with reperfusion would promote a different chemical profile of NO species, including the generation of oxidizing RNO compounds that might be expected to decrease NMDA channel function via its redox modulatory site.

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REFERENCES


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