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Mechanism for Nitric Oxide's Enhancement of NMDA-stimulated [^{3}H]Norepinephrine Release from Rat Hippocampal Slices

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Summary—Previous studies in our laboratory have shown that nitric oxide (NO) gas enhances NMDA-stimulated release of preloaded tritiated norepinephrine ([^{3}H]NA) from rat brain slices in a dose-dependent, oxygen-sensitive, and cyclic GMP-independent manner. In this study we have attempted to determine the mechanism for the enhancement of neurotransmitter release seen with NO. NO-enhanced transmitter release was not due to buffer acidification or generation of NO degradation products, since reducing buffer pH below 7.3 inhibited NMDA-stimulated [^{3}H]NA release and nitrite or nitrate ions (3–100 μM) had no significant effect on release. Carbon monoxide (CO, 10–300 μM), another diatomic gas with properties similar to NO including heme binding and guanylate cyclase activation, had no significant effect on depolarization-induced [^{3}H]NA release. The NO effect was probably not due to mono-ADP-ribosylation of cellular proteins, since the ADP-ribosyltransferase (ADPRT) inhibitors nicotinamide (10 μM–10 μM) and luminol (1 μM–1 mM) did not diminish the enhancement of transmitter release seen with NO. The NA reuptake inhibitor desmesthylimipramine (DMI, 10 nM–10 μM) neither mimicked nor blocked the effect of NO, suggesting that NO was not acting via inhibition or reversal of the NA transporter. Similar to NO, the metabolic inhibitors sodium azide (NaN_3, 0.1–3 mM), potassium cyanide (KCN, 0.1–3 mM), and 2,4-dinitrophenol (2,4-DNP, 10–300 μM) also dose-dependently enhanced NMDA-stimulated [^{3}H]NA release. These results suggest that NO may enhance neurotransmitter release by inhibiting cellular respiration and perhaps ultimately via altering calcium homeostasis.

Keywords—Nitric oxide, N-methyl-D-aspartate (NMDA), carbon monoxide, ADP-ribosyltransferase, azide, cyanide, norepinephrine.

Nitric oxide is a diatomic, gaseous free radical molecule synthesized from the amino acid L-arginine by the enzyme NO synthase. In neurons, NO synthesis is calcium/calmodulin-dependent (Bredt and Synder, 1990) and is stimulated by NMDA receptor activation (Garthwaite *et al.*, 1988). Once NO is made, it can act in a variety of ways. NO is known to activate soluble guanylate cyclase (Murad *et al.*, 1978). NO can enhance the activity of mono-ADP-ribosyltransferase (Brune and Lapetina, 1989; Dunman *et al.*, 1991) and activate poly-ADP-ribosylation via DNA damage (Zhang *et al.*, 1994). NO is also believed to react with iron-containing proteins of the electron transport chain to inhibit cellular respiration (Erecinska and Wilson, 1981; Nathan and Hibbs, 1991; Bolanos *et al.*, 1994). Nitric oxide is rapidly degraded to nitrite and nitrate ions in the presence of oxygen, and the peroxynitrite radical formed by the reaction of NO with superoxide may also be an important cellular effector molecule (Beckman *et al.*, 1990).

The phenomenon of long-term potentiation (LTP), which is a long-lasting enhancement in synaptic strength following repetitive stimulation of a neuronal pathway, is thought to represent a cellular model of learning and memory. There is some disagreement as to whether this phenomenon of enhanced synaptic transmission involves primarily enhanced neurotransmitter release from presynaptic neurons (Bekkers and Stevens, 1990; Malinow and Tsien, 1990) or is due mainly to enhanced postsynaptic sensitivity (Kauer *et al.*, 1988; Muller and Lynch, 1988). Nitric oxide has been reported to play a critical role in LTP (Bohme *et al.*, 1991; O'Dell *et al.*, 1991; Schuman and Madison, 1991; Haley *et al.*, 1992). Consistent with these findings, we have previously demonstrated that authentic nitric oxide gas can enhance the depolarization-induced release of preloaded tritiated neurotransmitter from rat brain slices (Stout and Woodward, 1994). This enhancement of release by NO was only apparent under helium-degassed conditions and

appeared to be independent of the brain region used (hippocampus, cortex, striata, or cerebellum), the depolarization stimulus employed (NMDA, KCl, or 4-aminopyridine), or the neurotransmitter measured ([^{3}N]NA or [^{3}H]dopamine). In this study we have attempted to determine the mechanism by which NO enhances neurotransmitter release.

METHODS

[^{3}H]NA release from rat brain slices

Male Sprague–Dawley rats (200–250 g) were killed by rapid decapitation, and the appropriate brain regions were removed and cross-chopped (350 × 350 μm) using a McIlwain tissue chopper. The slices were gently separated by repeated pipetting in 37°C bubbled (95% O_2/5% CO_2) Krebs–Ringer bicarbonate buffer. The composition of the buffer was (in mM): NaCl (118), KCl (4.7), $NaHCO_3$ (24.8), KH_2PO_4 (1.18), $CaCl_2$ (1.0), glucose (10), HEPES (25), pH 7.4. Magnesium was omitted from the buffer because it has been shown to completely block NMDA-stimulated release of neurotransmitter (Woodward and Gonzales, 1990). The buffer was removed every 15 min, and the slices were resuspended in 45 ml of fresh buffer. After 1 hr of washing, slices were incubated for 30 min in the presence of 100 nM [^{3}H]NA (42 Ci/mmol, Amersham, Arlington Heights, IL). Pargyline (a monoamine oxidase inhibitor, 20 μM) and ascorbic acid (an antioxidant, 1 mM) were present during the loading period to protect the labelled catecholamine from degradation and metabolism. Following loading, slices were washed three times with fresh buffer to remove unincorporated radioactive neurotransmitter. Aliquots (20 μl) of gravity-packed slices ($\sim$10 mg tissue wet weight) were then loaded into individual nylon mesh-bottomed baskets (210 μm nylon mesh, Tetko Inc., Elmsford, NY) and suspended in mini-vials each containing 3 ml of buffer. Baskets were transferred at 2-min intervals with continuous bubbling with 95% O_2/5% CO_2 (except as noted below) through a series of vials which contained the various modulators to be tested. NMDA (100 or 500 μM) or KCl (15 mM) was present in the last row of vials. In most experiments the buffer in the stimulated row was degassed with helium and not bubbled with 95% O_2/5% CO_2 in order to prevent degradation of NO or mimic the experimental conditions of experiments with NO. Following the 2-min stimulation with NMDA or KCl, baskets were transferred to another row of vials which contained 3 ml of 0.1 M perchloric acid in order to lyse the tissue and release all the neurotransmitter remaining in the slices. Three mls of scintillation cocktail (Budget-Solve, Research Products Inc., Mount Prospect, IL) was added to each vial, and the radioactivity contained in the vial was quantitated by liquid scintillation spectrometry. The fractional release neurotransmitter in each vial was calculated as a percent of the total neurotransmitter present at that time. The total amount of radioactivity taken up by an aliquot of slices ranged between 200,000 and 600,000 dpm. HPLC analysis of the tritium recovered in the vials following stimulation verified that >90% of it was NA. Data were analyzed by one-way ANOVA with repeated measures. *Post-hoc* testing was performed using the paired t-test with a significance level of 0.05.

Chemicals and animals

Nitric oxide gas (chemically pure) was from MG Industries (Malvern, PA). Carbon monoxide gas (99.3%) was from Airco (Murray Hill, NJ). NO and CO gas solutions were made by diluting saturated stocks (2 and 1 mM, respectively) prepared by bubbling each gas through room temperature, helium-degassed Krebs buffer. 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA AM) was from Molecular Probes Inc. (Eugene, OR). BAPTA AM was dissolved in anhydrous dimethyl sulfoxide (10 mM) and stored at −20°C until used. Thapsigargin was from Research Biochemicals Inc. (Natick, MA). A thapsigargin stock solution (1 mM) was also prepared in dimethyl sulfoxide. Sodium nitrite, sodium nitrate, and potassium cyanide were from Fisher Scientific Co. (Fair Lawn, NJ). All other chemicals were from Sigma Chemical Co. (St Louis, MO). Male Sprague–Dawley rats (200–500 g) were obtained from Zivic Miller (Allison Park, PA). All animals use procedures were in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Virginia Commonwealth University's Institutional Animal Care and Use Committee.

RESULTS

NMDA (25–500 μM) concentration-dependently stimulated the release of preloaded [^{3}H]NA from rat hippocampal slices under helium-degassed conditions ($EC_{50} = 43.4 \pm 4.8$ μM, Fig. 1). Non-stimulated basal release averaged <1% per 2-min period, while a 2-min stimulation with 500 μM NMDA caused approx. 12% release. A 2-min exposure to helium-degassed buffer alone did not alter basal or stimulated release by itself (not shown). Nitric oxide gas (20 μM) had no significant effect or non-stimulated basal release, but caused approximately a doubling of NMDA-stimulated release regardless of the NMDA concentration used (Fig. 1). The EC_{50} value for stimulation of [^{3}H]NA release by NMDA in the presence of 20 μM NO was 42.4 ± 1.5 μM.

Effect of pH

Concentrated aqueous solutions of NO are acidic, and the pH of a 200 μM solution was approx. 5. In order to determine whether the enhancement of transmitter release seen with NO was due to acidification of the experimental buffer, slices were stimulated in buffers of varying pH. Reducing the pH of the experimental

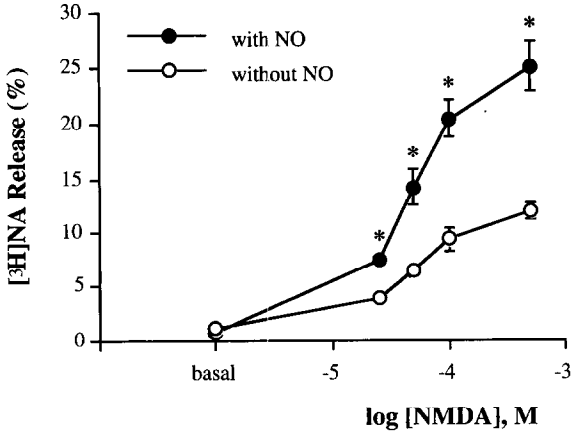

Fig. 1. Dose–response curves for NMDA-stimulated [^{3}H]NA release from rat hippocampal slices in the presence and absence of NO. Slices were preloaded with [^{3}H]NA, dispensed into mesh-bottomed baskets, and transferred through a series of vials containing Krebs buffer before a two-minute stimulation with NMDA. [^{3}H]NA release was quantitated using liquid scintillation spectrometry, and the fractional release of neurotransmitter in each vial was calculated as a percent of the total neurotransmitter present at that time (see Methods). NO (20 μM) was only present during the 2-min stimulation under helium-degassed conditions. Each point represents the mean $\pm$ SEM of four experiments. *$P < 0.05$ compared to paired control (NO = 0 μM) release.

buffer from 7.3 to 7.0 or lower (for 2 min prior to stimulation and during the 2-min stimulation) significantly inhibited the NMDA-induced release of preloaded [^{3}H]NA from rat hippocampal slices (Fig. 2). Raising the pH above 7.3 had no significant effect on NMDA-stimulated transmitter release. None of the alternative pH buffers tested had any significant effect on non-stimulated basal release.

Effects of nitrite and nitrate

Slices were treated with nitrite or nitrate ions during the 2-min stimulation with NMDA to see if the effect of NO could be attributed to the production of one of its degradation products. Neither sodium nitrite (3–100 μM) nor sodium nitrate (3–100 μM) had any significant effect on NMDA-stimulated [^{3}H]NA release under helium-degassed conditions (not shown). In the presence of 100 μM sodium nitrite, NMDA-stimulated [^{3}H]NA release was 104% of control (13.5 $\pm$ 2.0% release vs 13.0 $\pm$ 2.3% release, $n = 4$). Similarly, in the presence of 100 μM sodium nitrate, NMDA-stimulated [^{3}H]NA release was 99% of control (13.2 $\pm$ 2.2% release vs 13.2 $\pm$ 2.0% release, $n = 4$).

Effect of carbon monoxide

Slices were exposed to carbon monoxide gas to determine if the effect of NO could be mimicked by another diatomic gaseous species known to bind heme proteins and activate guanylate cyclase (Brune and Ullrich, 1987; Graser *et al.*, 1990). CO gas (10–300 μM) present during the two-minute stimulation had no significant effect on either NMDA- or KCl-stimulated [^{3}H]NA release from rat hippocampal slices under helium-degassed conditions (Fig. 3).

Effects of ADPRT and NADase inhibitors

Since nitric oxide can enhance endogenous ADP-ribosylation in rat brain homogenates (Duman *et al.*, 1991), we wanted to determine whether the effect of NO on NMDA-stimulated [^{3}H]NA release was due to enhanced mono-ADP-ribosylation of cellular proteins. ADPRT enzymes attach ADP-ribose groups onto cellular proteins, while ADP-ribose groups are removed by the enzyme NAD glycohydrolase (NADase); and the NADase inhibitors isoniazid and 3-acetylpyridine adenine dinucleotide (APAD) have been shown to enhance endogenous ADP-ribosylation by blocking the enzymatic removal of ADP-ribose units (Duman *et al.*, 1991). We tested the effects of both ADPRT and NADase inhibitors in the hippocampal slices assay.

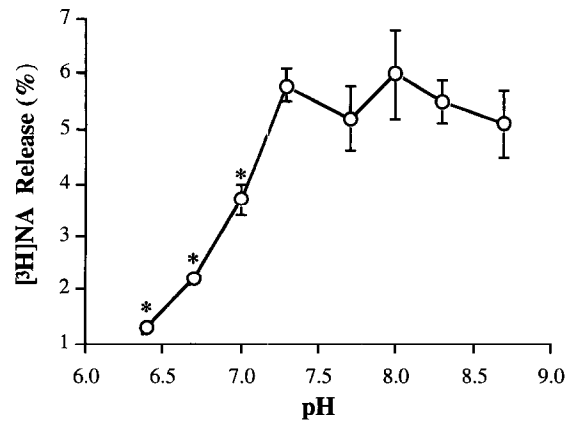

Fig. 2. Effect of pH on NMDA-stimulated [^{3}H]NA release from rat hippocampal slices. Acidic or basic buffer was present for 2 min prior to stimulation and present during the 2-min stimulation with 100 μM NMDA. Each point represents the mean $\pm$ SEM of four or more experiments. *$P < 0.05$ compared to control (pH = 7.3) release.

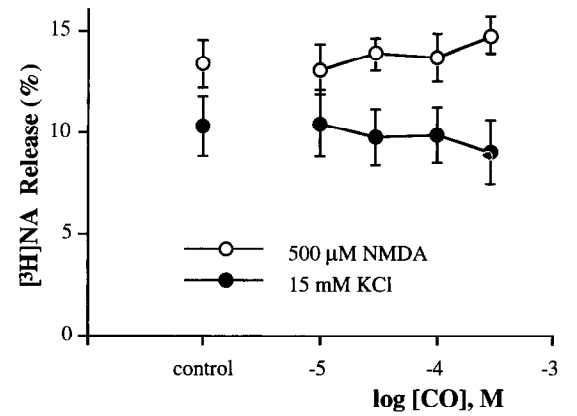

Fig. 3. Effect of CO gas on NMDA- and KCl-stimulated [^{3}H]NA release from rat hippocampal slices under helium-degassed conditions. CO was only present during the two-minute stimulation with 500 μM NMDA or 15 mM KCl. Each point represents the mean $\pm$ SEM of four experiments. *$P < 0.05$ compared to control release.

Pretreatment of slices with the mono-ADPRT inhibitors nicotinamide (10 μM–10 mM) or luminol (1 μM–1 mM) for 30 min did not significantly diminish the enhancement of NMDA-stimulated [^{3}H]NA release caused by a 2-min exposure to 20 μM NO (not shown). NMDA-stimulated [^{3}H]NA release in the presence of 20 μM NO after pretreatment with 10 mM nicotinamide was 92% of control (21.8 $\pm$ 1.5% release vs 23.8 $\pm$ 1.2% release, $n = 4$). Similarly, NMDA-stimulated [^{3}H]NA release in the presence of 20 μM NO after pretreatment with 1 mM luminol was 85% of control (19.3 $\pm$ 0.5% release vs 22.8 $\pm$ 1.4% release, $n = 4$). Neither ADPRT inhibitor had any significant effect on non-stimulated basal release.

Pretreatment of slices with isoniazid (10 μM–10 mM) or APAD (1 μM–1 mM) for 30 min did not significantly augment the enhancement of NMDA-stimulated [^{3}H]NA release caused by a 2-min exposure to 20 μM NO (not shown). NMDA-stimulated [^{3}H]NA release in the presence of 20 μM NO after pretreatment with 10 mM isoniazid was 90% of control (23.1 $\pm$ 1.5% release vs 25.6 $\pm$ 1.4% release, $n = 4$). Similarly, NMDA-stimulated [^{3}H]NA release in the presence of 20 μM NO after pretreatment with 1 mM APAD was 97% of control (18.3 $\pm$ 1.4% release vs 18.9 $\pm$ 1.4% release, $n = 4$). Neither NADase inhibitor had any significant effect on non-stimulated basal release.

Effects of desipramine

Slices were exposed to the NA reuptake inhibitor DMI in the absence and presence of NO to determine if the effect of NO could be mimicked or blocked by transporter inhibition. The concentrations of DMI used here (10 nM–10 μM) were 10–10,000-fold greater than the IC_{50} for inhibition of NA reuptake (1.2 nM, Baldessarini, 1985), so the NA transporter should have been completely blocked. DMI (10 nM–1 μM, present for 6 min prior to stimulation and during the 2-min stimulation) caused small but statistically significant increases in NMDA-stimulated [^{3}H]NA release but had no significant effect on non-stimulated basal release (Fig. 4). Higher concentrations of DMI (10 μM) significantly inhibited NMDA-stimulated [^{3}H]NA release, consistent with previous reports that micromolar concentrations of DMI act as NMDA antagonists (Reynolds and Miller, 1988; Sernagor *et al.*, 1989). A 2-min exposure to nitric oxide (20 μM) in the absence of DMI significantly enhanced NMDA-stimulated [^{3}H]NA release (176% of non-NO-treated control), and NO significantly enhanced NMDA-stimulated release at every concentration of DMI tested. The enhancement of release by NO was only significantly diminished by the highest concentration of DMI (10 μM). However, even in the presence of 10 μM DMI, nitric oxide still significantly enhanced NMDA-stimulated [^{3}H]NA release (148% of non-NO-treated control).

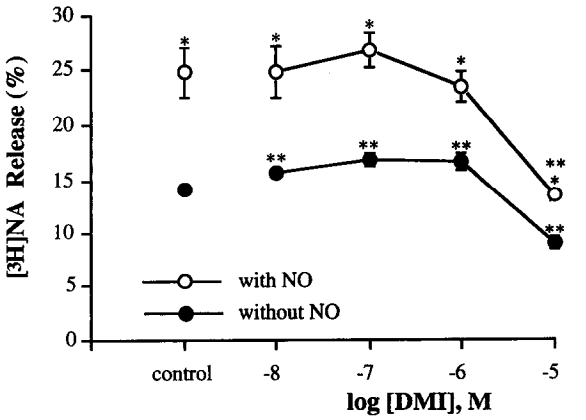

Fig. 4. Effects of desmethylimipramine or NMDA-stimulated [^{3}H]NA release from rat hippocampal slices in the presence and absence of nitric oxide under helium-degassed conditions. DMI was present for 6 min prior to stimulation and present during the 2-min stimulation with 500 μM NMDA. NO (20 μM) was only present during the 2-min stimulation. Each point represents the mean $\pm$ SEM of three experiments. $*P < 0.05$ compared to paired control (NO = 0 μM) release. $**P < 0.05$ compared to control (DMI = 0 μM) release.

Effects of metabolic inhibitors

NO is thought to react with iron-containing proteins of the mitochondrial electron transport chain to

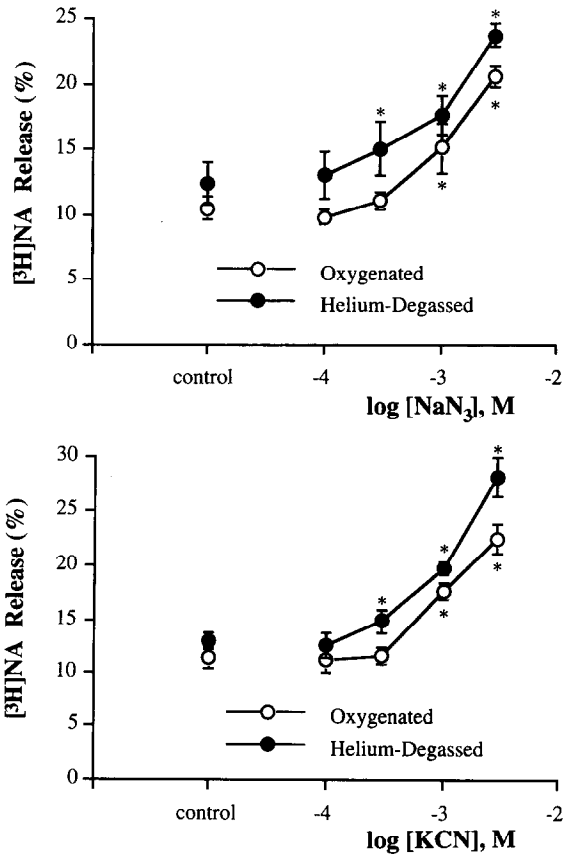

Fig. 5. Effects of sodium azide and potassium cyanide on NMDA-stimulated [^{3}H]NA release from rat hippocampal slices under oxygenated and helium-degassed conditions. NaN_3 or KCN was only present during the two-minute stimulation with 100 μM NMDA. Each point represents the mean $\pm$ SEM of three experiments. $*P < 0.05$ compared to control release.

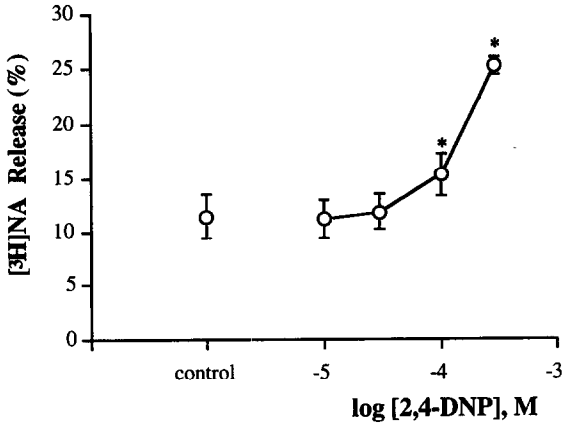

Fig. 6. Effect of 2,4-dinitrophenol on NMDA-stimulated [^{3}H]NA release from rat hippocampal slices under helium-degassed conditions. 2,4-DNP was only present during the two-minute stimulation with 100 μM NMDA. Each point represents the mean $\pm$ SEM of three experiments. $^*P < 0.05$ compared to control release.

inhibit cellular respiration (Erecinska and Wilson, 1981; Nathan and Hibbs, 1991; Bolanos *et al.*, 1994). Therefore, we tested other known inhibitors of cellular respiration to see if they mimicked NO under identical experimental conditions. Slices were exposed to NaN_3, KCN, or 2,4-DNP for 2 min concurrent with stimulation with 100 μM NMDA. Low millimolar concentrations of NaN_3 significantly enhanced the NMDA-stimulated release of [^{3}H]NA from hippocampal slices under both oxygenated and helium-degassed conditions [Fig. 5(A)]. The dose response curves for NaN_3 under oxygenated and helium-degassed conditions were not significantly different. Low millimolar concentrations of KCN also significantly enhanced NMDA-stimulated [^{3}H]NA release under both oxygenated and helium-degassed conditions [Fig. 5(B)]. 2,4-DNP also significantly enhanced NMDA-stimulated [^{3}H]NA release under helium-degassed conditions at concentrations of 100 μM and above (Fig. 6). The concentrations of NaN_3, KCN, or 2,4-DNP which caused a two-fold increase in NMDA-stimulated release were 3 mM, 3 mM, and 300 μM, respectively. Although preliminary experiments reveal that NaN_3, KCN, or 2,4-DNP treatment alone can cause small but reversible increases in non-stimulated basal release (from $\sim$0.4% to $\sim$1.9%), these increases cannot account for the doubling of [^{3}H]NA release (from $\sim$12% to $\sim$25%) that occurs when these compounds are present during NMDA stimulation.

DISCUSSION

Previous studies in this laboratory have demonstrated that authentic nitric oxide gas can enhance depolarization-induced release of preloaded tritiated neurotransmitter from rat brain slices (Stout and Woodward, 1994). In this study we have attempted to determine the mechanism by which nitric oxide enhances NMDA-stimulated [^{3}H]NA release from hippocampal slices, and we have eliminated several possibilities. Since NO did not alter the EC_{50} value for stimulation of [^{3}H]NA release by NMDA (Fig. 1) and since NO enhanced both KCl- and 4-aminopyridine-stimulated release of [^{3}H]NA from rat hippocampal slices (Stout and Woodward, 1994; unpublished observations), these results suggest that the effect of NO is not due to selective modification of NMDA receptors. In addition, the effect of NO is not due to its ability to acidify aqueous solutions, since reducing extracellular pH significantly inhibited rather than enhanced NMDA-stimulated neurotransmitter release (Fig. 2). Similarly, the effect of NO is probably not mediated by a breakdown product, since sodium nitrite and sodium nitrate did not alter NMDA-stimulated [^{3}H]NA release (not shown) and NO gas did not enhance release in the presence of high oxygen tension (Stout and Woodward, 1994). The enhancement of [^{3}H]NA release by NO in our studies was not mimicked by cyclic-GMP analogs (Stout and Woodward, 1994) nor by the guanylate cyclase activator CO (Fig. 3), suggesting a cyclic GMP-independent mechanism. Likewise, ADPRT inhibitors (nicotinamide and luminol) did not block the enhancement of transmitter release caused by NO, and NADase inhibitors (isoniazid and APAD) did not amplify the NO-induced increases in NMDA-stimulated [^{3}H]NA release, suggesting that the effect of NO is not via ADP-ribosylation of cellular proteins. In contrast to other studies which suggest that nitric oxide may inhibit or reverse the action of neurotransmitter transporters (Lonart and Johnson, 1993; Kuhar and Pogun, 1993; Pogun and Kuhar, 1993), the results of the present study suggest that the enhancement of NMDA-stimulated [^{3}H]NA release by NO was not mediated by inhibition or reversal of NA reuptake; and these results are consistent with a report by Hanbauer *et al.* (1993) that authentic NO does not alter the ability of cultured neurons to take up [^{3}H]dopamine.

The results of the present study suggest that the enhancement of NMDA-stimulated [^{3}H]NA release by NO gas may be related to NO's ability to inhibit cellular respiration (Erecinska and Wilson, 1981; Nathan and Hibbs, 1991; Bolanos *et al.*, 1994). Azide, cyanide, and carbon monoxide are known to block electron transport at the cytochrome oxidase level (Erecinska and Wilson, 1981). Although azide and cyanide enhanced NMDA-stimulated [^{3}H]NA release from rat hippocampal slices similar to NO (Fig. 5), it is unclear why CO had no effect (Fig. 3). Perhaps the concentrations of CO used were not high enough to inhibit mitochondrial function. Alternatively, there is one report that cytochrome oxidase from brain, unlike cytochrome oxidase from heart and liver, is not inhibited by CO (Shigezane *et al.*, 1989). The enhancement seen with azide may be an indirect effect, since azide has been shown to be metabolized to NO by catalases found *in vivo* (Keilin and Hartree, 1954; Murad *et al.*, 1978). Interestingly, azide and cyanide have both been shown to inhibit evoked transmitter release in the

squid giant synapse (Adams and Thomas, 1989; Adams *et al.*, 1985). In contrast, 2,4-DNP does not block electron transport but does inhibit ATP synthesis by dissipating the proton gradient across the inner mitochondrial membrane (Heytler, 1981). Despite the difference in its mechanism of action, 2,4-DNP also enhanced NMDA-stimulated release similar to NO (Fig. 6).

As a consequence of inhibiting cellular respiration, NO might significantly reduce ATP levels, which could disrupt calcium homeostasis and ultimately enhance exocytotic release. The increased potency of NO compared to the other metabolic inhibitors is consistent with this hypothesis; because, in addition to its effects on electron transport, NO can also inhibit glycolysis (via ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase; Dimmeler *et al.*, 1992; Zhang and Snyder, 1992) to further reduce ATP levels. Although ATP levels in the hippocampal slices after treatment with NaN_3, KCN, 2,4-DNP, CO, or NO were not measured in this study, there is evidence that metabolic inhibitors can increase free intracellular calcium. Cyanide has been shown to increase resting free intracellular calcium levels in dorsal root ganglia within two minutes (Duchen *et al.*, 1990), and the putative NO-generating compound sodium nitroprusside has also been shown to cause rapid and reversible increases in free intracellular free calcium levels in a murine marcophage cell line (Kong *et al.*, 1994). Authentic NO has been shown to release calcium from isolated mitochondria (Schweizer and Richter, 1994), and in preliminary experiments we have detected rapid and sustained increases in free intracellular calcium levels in FURA 2-loaded cultured cortical neurons upon exposure to NO gas under helium-degassed conditions (unpublished observations).

To further investigate the possibility that enhanced calcium underlies NO's effect, we have also attempted to mimic or block the effect of NO by stimulating or blocking the release of calcium from intracellular pools. Attempts to mimic or prevent the NO effect by concurrent calcium release or prior pool depletion using thapsigargin, an inhibitor or the calcium-ATPase of the inositol 1,4,5-triphosphate-sensitive pool (Thastrup *et al.*, 1990), have been unsuccessful (unpublished observations). However, previous studies in our laboratory using carbachol to stimulate inositol 1,4,5-triphosphate production have demonstrated an enhancement of NMDA-stimulated release (Woodward and Harms, 1992). Attempts to block the NO effect by blocking calcium-induced calcium release with dantrolene were also ineffective (unpublished observations). Finally, attempts to block the effects of NO, azide, cyanide, and 2,4-DNP by loading slices with the calcium chelator BAPTA AM were also unsuccessful (unpublished observations). Thus, the involvement of increased free intracellular calcium in the NO-induced potentiation of NMDA-stimulated neurotransmitter release is largely speculative at this point. Interestingly, there is one recent report that nitric oxide stimulates calcium-independent vesicle release (Meffert *et al.*, 1994).

It is unclear whether the results of the present study are physiologically relevant. Even though the slices were stimulated with NMDA in helium-degassed buffer in these experiments, these conditions did not appear to place the slices in a state of extraordinary metabolic stress. It is possible that there is enough oxygen inside the slices to support normal functioning during the two-minute exposure to helium-degassed buffer. The fact that neither basal or NMDA-stimulated release was altered in helium-degassed buffer supports this hypothesis. In addition, if the slices were severely compromised by exposure to deoxygenated buffer, one might expect that the metabolic inhibitors would have been more effective under these conditions. However, both azide and cyanide had very similar effects in both oxygenated and helium-degassed conditions (Fig. 5). The conditions of low O_2 and high NO found in our experiments might be similar to those found in some parts of the brain during ischemia.

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