Lack of interaction between nitric oxide and the redox modulatory site of the NMDA receptor

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1 The inhibitory effects of nitric oxide (NO) on N-methyl-D-aspartate (NMDA) receptor function have been proposed to be mediated *via* the interaction of this gas with a redox-sensitive thiol moiety on the receptor. Here, we evaluated this suggested mechanism by examining the actions of various NO donors on native neuronal receptors as well as in wild-type and cysteine-mutated recombinant NMDA receptors expressed in Chinese hamster ovary (CHO) cells.

2 The NO donor N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine (NOC-12; 100 μ M) produced a rapid and readily reversible inhibition of whole-cell currents induced by NMDA (30 μ M) in cultured cortical neurons. The inhibition was apparent at all holding potentials, though a more pronounced block was observed at negative voltages. The effects of NOC-12 disappeared when the donor was allowed to expire. A similar receptor block was observed with another NO-releasing agent, S-nitroso-N-acetylpenicillamine (SNAP; 1 mM).

3 The blocking effects of NO released by SNAP, 3-morpholinosydnonimine (SIN-1; 1 mM), and 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine (NOC-5; 100 μ M) on currents mediated by recombinant NR1/NR2B receptors were virtually indistinguishable from those observed on native receptors. Furthermore, mutating cysteines 744 and 798 of NR1, which constitute the principal redox modulatory site of the NR1/NR2B receptor configuration, did not affect the inhibition produced by NO.

4 The NR2A subunit may contribute its own redox-sensitive site. However, the effects of NO on NR1/NR2A receptors were very similar to those seen for all other receptor configurations evaluated. Hence, we conclude that NO does not exert its inhibition of NMDA-induced responses *via* a modification of any of the previously described redox-sensitive sites on the receptor.

Keywords: NMDA receptor; nitric oxide; redox modulatory site; cortical neurons; recombinant expression; patch-clamping

Abbreviations: CHO, Chinese hamster ovary; cys, cysteine; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOC-5, 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine; NOC-12, N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine; NR, NMDA receptor; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetylpenicillamine

Introduction

Disulfide reducing agents can potentiate physiological responses mediated by the N-methyl-D-aspartate (NMDA) receptor while thiol oxidants can reverse the effects of reductants and, in some instances, depress native responses (Tang & Aizenman, 1993a). Several redox-active substances of endogenous origin can modulate NMDA receptor function (Aizenman, 1994), and further, it has been observed that the redox state of the receptor can change without the addition of exogenous reductants or oxidants *in vitro* (Aizenman *et al.*, 1989; Gozlan & Ben-Ari, 1995; Sinor *et al.*, 1997). Hence, NMDA receptor function in the brain may be modulated by redox substances, thereby affecting the physiological and pathophysiological consequences of receptor activation. Indeed, substances known to oxidize the redox modulatory site *in vitro* have neuroprotective and anticonvulsive activity *in vivo* (Jensen *et al.*, 1994; Quesada *et al.*, 1997). It has been suggested that more than one redox-sensitive site may be present in a functional NMDA receptor (Sullivan *et al.*, 1994; Köhr *et al.*, 1994; Brimecombe *et al.*, 1997; Arden *et al.*, 1998; but see Paoletti *et al.*, 1997). However, only one site has been unequivocally demonstrated to be formed by a pair of cysteines, cys-744 and cys-798, which are localized in an

extracellular region between two transmembrane domains in the NR1 subunit (Sullivan *et al.*, 1994).

Nitric oxide (NO), or a closely related derivative (Butler *et al.*, 1995), has been shown to inhibit NMDA receptor-mediated physiological responses in a number of preparations (Hoyt *et al.*, 1992; Manzoni *et al.*, 1992; Lei *et al.*, 1992; Fagni *et al.*, 1995). Several mechanisms have been proposed to account for this inhibition, including voltage-dependent channel block (Fagni *et al.*, 1995) and interference with down-stream calcium homeostatic processes (Hoyt *et al.*, 1992). However, a widely-held view is that the gas can directly interact with the receptor's redox modulatory site (Lei *et al.*, 1992), and specifically, with the one formed by cys-744 and cys-798 of NR1 (Stamler *et al.*, 1997). This hypothesis is based partly on sequence homology to proposed targets for NO in other proteins (Stamler *et al.*, 1997). The putative ensuing cysteine nitrosylation has been suggested to be responsible for the inhibition of the receptor-mediated responses (Lipton *et al.*, 1993; Stamler *et al.*, 1997). However, our group (Hoyt *et al.*, 1992), as well as others (Fagni *et al.*, 1995), previously suggested that the redox modulatory site does not, in fact, mediate any of the actions of NO at the NMDA receptor. In the present study, we re-examine the effects of NO in native, as well as in wild-type and cysteine mutated recombinant NMDA receptors in order to test directly the degree to which NO can

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modulate receptor function in the presence or absence of a functional redox site.

Methods

Tissue culture

All tissue culture reagents were purchased from Sigma (St. Louis, MO, U.S.A.), with the exception of iron-supplemented bovine calf serum (Hyclone Laboratories, Logan, UT, U.S.A.), and minimal essential medium, (Gibco BRL, Gaithersburg, MD, U.S.A.). Chinese hamster ovary cells (CHO-K1; ATTC CCL61) were grown in Ham's F-12 nutrient medium with 10% foetal bovine serum and glutamine (1 mM) at 37°C in 5% CO₂. These cells were passaged every 2 days at a 1 : 10 dilution for a maximum of 30 times. Cerebral cortices were dissociated from E-16 Sprague Dawley rats according to methods described previously (Hartnett *et al.*, 1997). After dissociation, cells were plated at a density of $3-5 \times 10^5$ cells ml⁻¹ in growth medium (v/v 80% Dulbecco's modified Eagle's minimum, 10% Ham's F12 nutrient mixture, 10% heat-inactivated iron-supplemented bovine calf serum, HEPES 25 mM, penicillin 24 U ml⁻¹, streptomycin 24 mg ml⁻¹ and L-glutamine 2 mM) into 35-mm tissue culture dishes containing 12-mm poly-L-lysine-coated glass coverslips. The cells were fed three times a week with growth medium. Non-neuronal cell proliferation was inhibited with cytosine arabinoside (2 mM) at 15 days *in vitro* after which the growth medium was changed to 2% serum and no F-12. Cells were maintained at 37°C in 5% CO₂. Neurons were utilized for electrophysiological measurements between 25–29 days *in vitro*.

Transfection protocol

Generation of mammalian expression vectors for the NMDA receptor (NR) subunits (Boeckman & Aizenman, 1994, 1996) and green fluorescent protein (Brimecombe *et al.*, 1997) have been previously described. The double mutant NR1 (C744A, C798A) plasmid was a kind gift from Dr S. Traynelis (Emory University, Atlanta, GA, U.S.A.). CHO cells were seeded at 3×10^5 cells well⁻¹ in 6-well plates (35-mm wells) containing CHO media approximately 24 h prior to transfection. Serum-free CHO media (1 ml) containing 1.3 mg of total DNA and 6 ml of LipofectAMINE (Gibco-BRL) was added to each well. A ratio of 1 : 4.3 was used for GFP to total NR plasmid DNA, and a ratio of 1 : 3 was used for NR1 to NR2 subunits (Cik *et al.*, 1993). After a 4 h incubation at 37°C in 5% CO₂ with the transfection solution, cells were re-fed with serum-containing CHO medium plus 300 µM ketamine (Boeckman & Aizenman, 1996). Recordings were performed approximately 2 days after transfection.

Whole-cell voltage-clamp recordings

Electrophysiological measurements were obtained from cortical neurons and transiently transfected CHO cells using the whole-cell configuration of the patch-clamp technique. Methods for data acquisition and analysis have been previously described (Tang & Aizenman, 1993a). The extracellular recording solution consisted of (in mM) NaCl 150, KCl 2.8, CaCl₂ 1.0, HEPES 10 and 10 µM glycine (pH 7.2). For cortical neuron recordings, 0.25 µM tetrodotoxin (CalBiochem, La Jolla, CA, U.S.A.) was also added to this solution. Patch electrodes (2 MΩ) were filled with (in mM) CsF 140, EGTA 10, CaCl₂ 1, and HEPES 10 (pH 7.2). Drugs were

applied onto cells by a multi-barrel fast perfusion system (Warner Instrument Corp., Hamden, CT, U.S.A.). Freshly-prepared stock solutions of 3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine (NOC-5; CalBiochem) and N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine (NOC-12; CalBiochem) were made in NaOH (10 mM), diluted in external recording solution and used immediately. 3-Morpholinohydronimine (SIN-1; BIOMOL, Plymouth Meeting, PA, U.S.A.) and S-nitroso-N-acetylpenicillamine (SNAP; BIOMOL) were dissolved directly in external recording solution and used immediately. When necessary, NO was allowed to expire by allowing the NO donor-containing solution to be in direct contact with air and exposed to light overnight.

Results

Whole-cell currents were obtained from cortical neurons during the application of NMDA (30 µM) alone or in the presence of freshly prepared NOC-12 (100 µM) at several holding potentials. The half-life of NO release by this compound is 327 min at 22°C in physiological solutions, according to the supplier (CalBiochem). We observed that inclusion of the NO donor produced a readily reversible inhibition of the NMDA-induced responses at all holding voltages tested (Figure 1A); Current-voltage relationships were generated by measuring the steady state current amplitude in the absence or presence of NOC-12 (Figure 1B); a more pronounced block was apparent at negative membrane potentials when compared to positive voltages. The block at -60 mV for this drug was near 25% ($n=5$; Table 1). In a separate group of neurons ($n=6$), we tested for the ability of expired NOC-12 to block the NMDA receptor-mediated responses. As is evident from Figure 1C, the spent donor did not influence the currents to any measurable extent. These results corroborate the previously noted actions of other NO donors such as SIN-1, SNAP and isosorbide dinitrate which demonstrate that the gas can reversibly block the NMDA receptor in both a voltage-dependent and voltage-independent manner, and that the use of NO-expired donors, or NO scavengers such as haemoglobin, can prevent the actions of the gas on the responses mediated by this receptor (Fagni *et al.*, 1995). Similar NMDA receptor inhibition was observed in the present investigation with the use of another NO donor, SNAP (1 mM; $n=5$), on a separate group of cortical neurons (Table 1).

Table 1 Comparison of the NO-mediated block of whole-cell NMDA currents (-60 mV)*

Receptor subtype	SIN-1 (1 mM)	SNAP (1 mM)	NOC-12 (0.1 mM)	NOC-5 (0.1 mM)
Neuronal		78.6 ± 5.1 (5)	77.0 ± 5.3 (5)	
NR1/NR2B	77.7 ± 7.7 (6)	67.0 ± 4.7 (4)		59.0 ± 9.5 (3)
NR1(C744A, C798A)/NR2B	66.7 ± 8.9 (5)	66.6 ± 5.5 (4)		
NR1/NR2A	70.5 ± 8.6 (8)	70.8 ± 10.2 (4)		

*Values shown represent the mean ± s.d. per cent of control current remaining in the presence of the NO donor indicated. The number of cells tested for each conditions is in parentheses.

Figure 1 Inhibition of NMDA-activated currents by NO in neurons. (A) Representative whole-cell current traces obtained at -60 , -30 , 0 , $+15$ and $+30$ mV in rat cortical neurons in culture during activation by NMDA ($30 \mu\text{M}$) in the absence and presence of freshly-prepared NOC-12 ($100 \mu\text{M}$). (B) Current-voltage relationship of steady-state NMDA responses in the absence and presence of NOC-12 for the same cell shown in A. (C) Whole-cell current trace during activation by NMDA at -60 mV in the absence and presence of NOC-12 ($100 \mu\text{M}$) which had been allowed to expire overnight.

We next turned our attention to the effects of NO on NMDA-induced responses mediated by recombinant receptors. We chose first to utilize the NR1/NR2B receptor configuration as cys-744 and cys-798 of NR1 constitute the principal redox-sensitive site of this subunit combination (Sullivan *et al.*, 1994). As seen with native receptors in neurons, NO, released by the donors NOC-5 ($100 \mu\text{M}$; $n=3$), SIN-1 (1 mM ; $n=6$), and SNAP (1 mM ; $n=4$), produced similar block of the whole cell currents mediated by this receptor configuration (Table 1). The half-life of NO release by NOC-5 is only 93 min (CalBiochem) and thus may transiently produce a higher concentration of the gas than a comparable amount of another donor. This likely accounts for the slightly greater degree block observed with this donor (Table 1). Figure 2A shows the typical current block observed with the NO donor SIN-1. The current-voltage relationship obtained from NR1/NR2B receptor-mediated responses (Figure 2B) demonstrate that the voltage-dependence of the inhibition is similar to what was observed with native neuronal receptors. Although SIN-1 also produces superoxide anions, a previous investigation reported that the effects of this donor on NMDA receptor-mediated currents are not affected by the addition of superoxide dismutase and catalase (Fagni *et al.*, 1995). A separate set of CHO cells were then transfected with the double mutant NR1(C744A, C798A) together with NR2B. In these, the pattern NMDA response inhibition produced by the NO donors SIN-1 (Figure 2B,C; $n=5$) and SNAP ($n=4$) was

virtually indistinguishable to that seen in the wild-type receptors and native channels (Table 1). Thus, the presence or absence of the redox site on NR1 had no influence on the actions of NO on NR2B-containing receptors.

The redox sensitivity of NR1/NR2A receptors is not critically dependent on cys-744 and cys-798 of NR1 (Sullivan *et al.*, 1994; Arden *et al.*, 1998). Although individual thiol-reactive residues in NR2A have not yet been localized (Köhr *et al.*, 1994), results of a recent study from our laboratory suggest that this subunit does indeed have unique redox attributes (Arden *et al.*, 1998). However, the effects of both SIN-1 ($n=8$) and SNAP ($n=4$) on this subunit configuration were almost identical to those seen in neuronal receptors and in wild-type and cysteine mutated NR1/NR2B receptors (Table 1). This suggests that the NO inhibition of NMDA receptor currents is not subunit dependent.

Discussion

Cysteine residues 744 and 798 on NR1 appear to constitute the principal redox modulatory site of receptors composed of this subunit when it is co-expressed with either NR2B, NR2C or NR2D (Sullivan *et al.*, 1994). Receptors which are assembled with NR1 and NR2A contain a second redox sensitive site which has yet to be localized at the molecular level (Köhr *et al.*, 1994; Arden *et al.*, 1998). Our present results suggest that NO

Figure 2 Inhibition of NMDA-activated currents by NO in transfected CHO cells does not require the presence of a functional redox site. (A) Representative whole-cell currents obtained at -60 and $+30$ mV in a CHO cell expressing NR1/NR2B receptors during application of NMDA ($30 \mu\text{M}$) in the absence and presence of SIN-1 (1 mM). (B) Current-voltage relationship of steady-state NMDA responses in the absence and presence of NOC-12 for the same cell shown in A. (C) Representative whole-cell currents obtained in a CHO cell expressing NR1 (C744A, C798A)/NR2B receptors, which lack a functional redox site, during application of NMDA with or without SIN-1. (D) Current-voltage relationship of steady-state NMDA responses in the absence and presence of NOC-12 for the same cell shown in C.

does not interact with the redox modulatory site formed by cys-744 and cys-798 of NR1. In fact, given the almost identical effects of NO on native receptors and on recombinant receptors composed of either NR1/NR2A or NR1/NR2B, it is unlikely that the gas interacts with any of the previously investigated sites on the NMDA receptor which are sensitive to redox reagents. This is in direct contrast to the widely accepted mechanism of NO inhibition of NMDA receptors that has been suggested by other investigators (Lei *et al.*, 1992; Lipton *et al.*, 1993; Stamler *et al.*, 1997).

Our conclusion, however, is not altogether surprising as the actions of NO on the NMDA receptor do not seem to fit the pattern of effects seen for other agents known to interact with the redox site. For instance, the inhibitory effects of thiol oxidants on the NMDA receptor do not spontaneously reverse (Aizenman *et al.*, 1989; Tang & Aizenman, 1993a). That is, a subsequent treatment with a disulfide reducing agent is necessary to reverse the effects of oxidants. In contrast, as seen in this and prior studies (Hoyt *et al.*, 1992; Manzoni *et al.*, 1992; Lei *et al.*, 1992; Fagni *et al.*, 1995), the effects of NO appear to be readily and spontaneously reversible. Inhibition by NO is also present even after prior oxidation of the receptor with a thiol agent (Hoyt *et al.*, 1992). Additionally, previous

studies have shown that after chemical alkylation and subsequent functional elimination of the redox site in neurons (Tang & Aizenman, 1993a), the inhibitory effects of NO remain virtually intact (Hoyt *et al.*, 1992; Fagni *et al.*, 1995). Finally, redox agents do not alter the conductance of native or recombinant single NMDA receptor channels (Tang & Aizenman, 1993a; Brimecombe *et al.*, 1997), while one of the principal reported actions of NO on unitary currents is to decrease their amplitude (Fagni *et al.*, 1995).

Lei *et al.* (1992) first suggested that NO could interact with the redox modulatory site of the NMDA receptor. This group reported that NO donors could depress NMDA receptor-mediated responses in neurons in a partially reversible manner. This reversibility, inconsistent with an effect at the redox site, was attributed to a rapid decomposition of putative S-nitrosothiol groups formed by the interaction of NO with the cysteine moieties that formed this site. However, these investigators also noted that NO could reversibly depress responses even after exposure of the receptor to thiol oxidants, which would eliminate any available free thiols for NO to nitrosylate. Lei and co-workers argued that NO may have reacted with thiols spared by the oxidants. However, this argument is not consistent with the fact that (i) oxidants

depress NMDA-induced responses by interacting with the redox site, and (ii) this inhibitory actions of thiol oxidants have been shown to be completely abolished following alkylation of the receptor (Tang & Aizenman, 1993a,b; Aizenman *et al.*, 1994). As mentioned earlier, in our previous studies (Hoyt *et al.*, 1992) as well as in those of others (Fagni *et al.*, 1995), it was observed that alkylation did not alter, to any extent, the inhibitory effects of NO. Indeed, a close look at the data presented by Lei *et al.* (1992) reveal that the effects of NO in depressing whole-cell currents before (38%) and after (26%) treatment with an alkylating agent are not dramatically different. Hence, we must conclude that the inhibitory effects

of NO on NMDA receptor function appear not to be determined by the subunit composition of the receptor, nor by the presence or absence of the redox site on NR1. The present findings refute the hypothesis that cys-744 and cys-798 on the NR1 subunit are the target of NO (Stamler *et al.*, 1997), at least with respect to the functional inhibition of the channel.

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