Nitric Oxide-Induced Blockade of NMDA Receptors

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Summary

West studied two effects of nitric oxide (NO)-producing agents on N-methyl-D-aspartate (NMDA) receptor activation in cultured neurons. 3-Morpholino-sydnonimine (SIN-I) blocked both NMDA-induced currents and the associated increase in intracellular Ca$^{2+}$. The actions of SIN-I were reversible and suppressed by hemoglobin. A degraded SIN-1 solution that did not release NO was unable to block NMDA receptors. This showed that the SIN-I effects were due to NO and not to another breakdown product. Similar results were obtained with 1-nitrosopyrrolidine (an NO-containing drug) and with NO released from NaN02. Pretreatment with hemoglobin potentiated NMDA receptor activation. This result suggests a feedback inhibition of NMDA receptors by endogenous NO modulates NMDA receptors. Since NMDA receptor activation induces NO synthesis, these results suggest a feedback inhibition of NMDA receptors by NO under physiological condition.

Introduction

Excitatory amino acids and glutamate are thought to play a critical role in important phenomena such as memory and neurotoxicity. Several glutamate receptors have been described and named according to their specific agonist (Monaghan et al., 1989). The receptor sensitive to N-methyl-D-aspartate (NMDA) is a channel permeant to monovalent cations and Ca$^{2+}$. These receptors are thought to play a key role in a model of elementary learning, long-term potentiation (for reviews see Kennedy, 1989; Collingridge and Singer, 1990).

In the cerebellum and hippocampus, NMDA receptor stimulation has been shown to induce nitric oxide (NO) synthesis, which then activates soluble guanylate cyclase (Garthwaite, 1991; Garthwaite et al., 1996) and leads to formation of cGMP. NO has been implicated in neuron-glial interactions (Garthwaite, 1991), synaptic plasticity (Gally et al., 1990), and, especially, long-term depression (Shibuki and Okada, 1991), as well as in AMPA ((RS)-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor desensitization in the cerebellum (Ito and Karachot, 1990). Because of its ability to diffuse across cell membranes rapidly, NO has been proposed as a transynaptic messenger in the development of long-term potentiation and depression (Gally et al., 1990; Böhmme et al., 1991; Schuman and Madison, 1991; Errington et al., 1991, Soc. Neurosci., abstract; O'Dell et al., 1992).

Thus, glutamate receptors and NO are both involved in synaptic plasticity, in which complex phenomena are likely to occur. This report, addresses the question of a possible feedback action of NO on NMDA receptors. In preliminary studies, we demonstrated the existence of a site sensitive to ferrocyanide and responsible for the blockade of NMDA receptor activation when neurons are pretreated with sodium nitroprusside, a classic NO donor (Manzoni et al., 1992). Thus, to study the effects of NO itself on NMDA receptors, we found drugs that produced NO without generating ferrocyanide ions. These drugs were two NO-producing compounds, 3-morpholino-sydnonimine (SIN-I) and NaN02, and one NO-containing compound, 1-nitrosopyrrolidine.

SIN-1 is spontaneously transformed by photolysis into SIN-IA, an unstable molecule containing one NO group. SIN-IA then releases NO and SIN-IC (Böhmme et al, 1984). Around pH 7, NaN02 is a very stable molecule, but when the pH solution drops to acidic values, NaN02 releases NO. At physiological pH, NO is rapidly transformed into NO2 and NO3. The 1-nitrosopyrrolidine molecule contains an NO group, but does not decompose to give free NO molecules.

These drugs all blocked NMDA receptor-induced increases in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) and current. These effects were reversed by hemoglobin, which strongly binds NO, confirming the idea that the blockade is due to NO itself and not to a side product of the drugs used. In addition, we tested the possible blockade of NMDA receptors by NO produced by neurons themselves (i.e., without the addition of exogenous NO). We found that hemoglobin potentiated NMDA receptor activation. This result suggests that endogenous production of NO is responsible for a sustained blockade of NMDA receptor activity.

Results

Effects of NO-Producing or NO-Containing Compounds on cGMP Formation

NO is known to activate the soluble guanylate cyclase (Knowles et al., 1989) in the central nervous system and to stimulate cGMP formation in cerebellar granule cells (Garthwaite et al., 1988) and striatal neurons (Marin et al., 1992). We therefore used cGMP formation as a test to measure the ability of several compounds either to act as NO donors or to mimic the NO effect.

SIN-1 at 1 mM stimulated the production of cGMP in a dose-dependent manner in striatal cultures (EC50 = 3.2 μM; maximal induced cGMP formation, 8.4 ± 0.4 pmol per well; n = 3). 1-Nitrosopyrrolidine also induced cGMP formation (maximal induced cGMP formation, 20 ± 0.8 pmol per well; n = 3). We also formed NO from an NaN02 solution. An acidic (pH
Effects of NO-Producing or NO-Containing Compounds on NMDA Receptor Activation
In primary cultures of striatal neurons, the NMDA-induced increase in \([\text{Ca}^{2+}]\) was monitored by quantitative ratio imaging of the fluorescent \(\text{Ca}^{2+}\) chelator fura-2. SIN-1 up to 1 mM was without significant effect on basal \([\text{Ca}^{2+}]\) (Figure 1C). The NMDA-induced \([\text{Ca}^{2+}]\) increase (Figure 1B) was blocked by a 5 min pretreatment with 1 mM SIN-1 (Figure ID), and this effect was reversible after a 10 min wash. We examined the time dependency of this phenomenon and found that a 2 min preincubation with SIN-1 was sufficient to induce a maximal inhibition of NMDA receptors. SIN-1 had no inhibitory effect on the increase in \([\text{Ca}^{2+}]\) produced by 100 \(\mu\text{M}\) AMPA (data not shown), suggesting that NO specifically blocks NMDA receptor activation in striatal neurons. SIN-1 inhibited NMDA-induced \([\text{Ca}^{2+}]\) increase in a dose-dependent manner with a relatively low affinity (1-10 \(\mu\text{M}\)). The maximal effect was always obtained at 1 mM (Figure 2A).

To exclude the possibility that these inhibitory effects were due to an interaction between NO and the \(\text{Ca}^{2+}\) measurement procedure, we tested the effects of SIN-1 on NMDA receptor-mediated current in the whole-cell recording configuration of the patch-clamp technique. Cerebellar granule cells were used since they are known to possess a high density of NMDA receptors and to contain the highest level of NO synthase in the brain (Garthwaite, 1991). As shown in Figure 2, solution of NaNO\(_2\) (10 mM) was prepared in the presence of glutathione (100 \(\mu\text{M}\)) and diluted (1:100 or 1:200) in normal incubation medium just before application to the cells. We verified that at such a dilution, the acidic solution of NaNO\(_2\) did not modify the pH (7.4) of our incubation medium. The final solution still contained a sufficient amount of NO to stimulate cGMP formation in striatal neurons (maximal induced cGMP formation, 15 ± 0.4 pmol per well; \(n = 9\)).
Figure 2. SIN-1 Specifically Inhibits NMDA Receptor Action

(A) Dose-dependent inhibition of the NMDA response by SIN-1. Neurons were preincubated for 10 min in the presence of the indicated doses of SIN-1 prior application of 100 μM NMDA. [Ca²⁺]i measured in the absence of NMDA (open circles) or after a 90 s treatment with NMDA (closed circles). IC₅₀ = 84 ± 46 μM (mean ± SEM, n = 34). Similar results were observed in two other preparations from different cultures, corresponding to a total of 85 cells.

(B and C) Specific NMDA-induced current blockade by SIN-1. In this and the following figures, NMDA currents were recorded under the whole-cell configuration at -60 mV, and horizontal bars represent NMDA applications. (B) Trace a, control NMDA response (100 μM); trace b, NMDA response following a 5 min incubation with 1 mM SIN-1; trace c, NMDA response following washout. (C) Similar legend as in (B), except that here horizontal bars represent AMPA (10 μM) applications. Recordings in (B) and (C) were obtained from the same cell.

(D) Evidence that SIN-1 produces its blocking effects through NO formation. NMDA (100 μM) responses obtained under control conditions (trace a), after a 2 min incubation with 1 mM SIN-1 alone (trace b), following a 3 min wash (trace c), after a 3 min incubation with 100 μM hemoglobin (trace d), and after a 3 min incubation with SIN-1 and hemoglobin (trace e). Comparing trace b with e, one can see that hemoglobin protected NMDA receptors from SIN-1 inhibition. This protective effect of hemoglobin was reversible after a 5 min wash of the protein. Similar results were obtained in 6 other cells.

Figure 2B (compare trace a with b), a 5 min preincubation of the recorded neurons with 1 mM SIN-1 inhibited the NMDA-induced current by 47% ± 9% (mean ± SD, n = 15). Indeed, a 10 s preincubation with SIN-1 was sufficient to reach the maximal effect as a function of time. Inhibition induced by this drug was easily reversible, since 87% ± 12% of the control NMDA currents was restored after a 2 min wash (Figure 2B, trace c) and could be reproduced several times and to a similar extent in the same cell. The effect was apparently dose dependent, since at 10 mM, SIN-1 inhibited 68%-86% of the NMDA current (3 cells) and this effect was also reversible (71%-83%) after a 2 min wash. SIN-1 (1 mM) did not significantly change the AMPA current (Figure 2C, compare trace a with b). Hence, an inhibition only of 10%-8% (n = 15) of the AMPA response was found using 1 mM SIN-1, which was within the normal range of fluctuation of the AMPA current.

The question of whether or not the final breakdown product of SIN-1, SIN-1C, mediates the observed inhibitory effects on NMDA responses was raised. We therefore tested a solution of 1 mM SIN-1 left in contact with normal air and light at room temperature for 28 hr. By measuring the kinetics of the NO release from SIN-1 under these conditions (see Experimental
effects (Fig- increases (basal, 92 in a hemoglobin-reversible manner. or patch-clamp techniques, we verified that an I-nitrosopyrrolidine, compare traces a and b with e), confirming that Similar were obtained from the same (data not shown). Note that (basal, 80 at physiological inhib- after a 90 s application of 100 i-nitrosopyrrolidine, respectively, n = 43) were affected by procedures). one can predict that there was no more NO produced in the solution. Such a solution was without effect on NMDA currents (data not shown). Moreover, coincidence of hemoglobin (100 µM) with SIN-1 abolished the inhibitory effect of SIN-1 (Figure 2D, compare traces a and b with e), confirming that NO-and not another breakdown product of SIN-1—was responsible of the inhibition.

Our hypothesis was that the NO group blocked the NMDA receptor. To test whether the effects of NO were due to free NO molecules, bound NO groups, or both, we tested the effects of I-nitrosopyrrolidine, a molecule containing but not producing free NO molecules as shown by our spectrophotometric measurements of free NO and NO₂ (see Experimental Procedures).

Treatment with I-nitrosopyrrolidine (1 mM) inhibited NMDA receptor-induced currents by 44% ± 3% (n = 5) (Figure 3A). It also inhibited the NMDA-induced increase in [Ca²⁺] (basal, 80 ± 9 and 76 ± 8 mM; after a 90 s application of 100 µM NMDA, 546 ± 52 and 183 ± 16 nM without and with a 5 min pretreatment with 1 mM I-nitrosopyrrolidine, respectively, n = 43). A 3 min incubation was sufficient to observe maximum inhibition, and the effects were reversed (87% ± 9%) after a 3 min wash. As with SIN-1, neither AMPA currents recorded in the same cells (Figure 3B), nor AMPA-induced [Ca²⁺] increases (basal, 92 ± 10 and 89 ± 8 mM; after a 90 s application of 100 µM AMPA, 541 ± 66 and 483 ± 52 nM without or with a 5 min pretreatment with 1 mM I-nitrosopyrrolidine, respectively, n = 43) were affected by I-nitrosopyrrolidine. The inhibitory effects of I-nitrosopyrrolidine were reversible after washing (Figure 3A, trace c) and were suppressed by coinubation of the drug with 100 µM hemoglobin, suggesting that these effects were due to its NO group.

Figure 4 shows that an acidic solution of NaNO₂ (prepared as described above), applied 2 min before NMDA (100 µM), blocked the NMDA-induced increase in [Ca²⁺], in a hemoglobin-reversible manner. This NaNO₂ solution was without effect on the AMPA-induced increase in [Ca²⁺] (data not shown). Note that the same solution, prepared without NaNO₂, had no effect on the NMDA-induced Ca²⁺ response (data not shown). In additional experiments performed with fura-2 or patch-clamp techniques, we verified that an NaNO₂ solution (10 mM) at physiological pH (a solution that did not contain NO) did not alter the NMDA responses.

Evidence for a Blockade of NMDA Receptors by Endogenous NO
Since exogenously applied NO could block NMDA receptors and since cerebellar (Garthwaite, 1991) and striatal (Marín et al., 1992) neurons have been shown to produce NO, we wondered whether endogenous NO production could interfere with NMDA receptor activity. To test this hypothesis, we examined whether basal endogenous NO production could be responsible for a sustained inhibition of NMDA receptors. In this case, incubation with an NO scavenger should increase NMDA action. Such a possibility was suggested by our observation that NMDA currents in cerebellar granule cells were always of slightly larger amplitude when recorded in the presence than in the absence of hemoglobin (Figure 2D, compare trace a with d). However, because of the difficulty in discriminating between the actions of hemoglobin and the washout due to liquid perfusion per se in removing NO from the environment of the recorded cell, we preferred to test the above hypothesis using fura-2 measurements. In [Ca²⁺] ratio imaging, we found that treatment of neurons for 2 min with hemoglobin (100 µM) clearly potentiated NMDA (100 µM) effects (Figure 5).
Mechanisms of the NO Blockade
Could cGMP mediate the inhibitory effects of SIN-1? Bath application of 1 mM cGMP had no effect on the increase in [Ca\(^{2+}\)]\(_i\) induced by 100 \(\mu\)M NMDA (data not shown), excluding a possible direct effect of cGMP on the NMDA recognition site (Baron et al., 1989). Preincubation (10 min) of striatal neurons with 300 \(\mu\)M dibutyryl-cGMP, a cGMP analog that diffuses into cells, in the presence of isobutyl-methylxanthine (IBMX; 750 \(\mu\)M), an inhibitor of phosphodiesterases, induced a partial but significant inhibition of the NMDA-induced rise in [Ca\(^{2+}\)]\(_i\). (after a 90 s application of 100 \(\mu\)M NMDA, 831 \pm 70 and 429 \pm 52 nM Ca\(^{2+}\) without or with a 10 min pretreatment with 300 \(\mu\)M dibutyryl-cGMP, respectively, \(n = 42\)). This effect was reversible after a 10 min wash. Since this inhibition was only partial, we wondered whether cGMP was really the messenger of NO-induced inhibition of NMDA receptor activation.

Addition of 1 mM cGMP and 750 \(\mu\)M IBMX in the whole-cell recording pipette solution did not block NMDA-induced currents (Figure 6A, trace a). Since we could not have direct control in the same cell because cGMP could not be introduced into the pipette during the course of the whole-cell recording, it was difficult to exclude the possibility that cGMP partially reduced NMDA current. However, application of 10 mM SIN-1 to the same cell (recorded in the whole-cell configuration with a pipette already containing 1 mM cGMP plus 750 \(\mu\)M IBMX) inhibited NMDA currents up to 80% (Figure 6A trace b), and this effect was reversible (trace c). Such an inhibitory effect was similar to that obtained with a normal recording pipette solution. This clearly indicated that the inhibitory effects of SIN-1 did not require cGMP production. Similar results were observed in 5 cells.

We examined whether SIN-1 required any other soluble second messenger to block NMDA receptors in excised outside-out patch of membranes (Figure 6B). In normal medium and in the presence of 100 \(\mu\)M NMDA, the integrated activity (\(\Delta P_0\), \(P_0\) is the putative number of NMDA channels in the patch; \(\Delta P_0\) is the cumulative open probability for all channels in the patch) of NMDA channels was 0.68 \pm 0.23 (mean \pm SD, \(n = 5\); Figure 6B, trace a). In the presence of SIN-1 (10 mM), \(\Delta P_0\) dropped to a mean value of 0.09 \pm 0.08 (Figure 6B, trace b). This change was reversible after washout of the drug (Figure 6B, trace c). Given that the presence of any soluble messenger in the patch was very unlikely, these data indicated that SIN-1 blocks NMDA channels by acting directly on the receptor-channel complex and alters the channel gating process. Other actions of SIN-1 on unitary NMDA currents were not tested in further details.

To test the hypothesis of a direct action of NO at NMDA-binding site, we performed binding displacement studies on rat forebrain membranes, using a specific ligand of the NMDA binding site, \(\text{[H]}\text{CGS} 19755\) (which acts as a competitive antagonist [Murphy et al., 1988; Manzoni et al., 1992]) and SIN-I. We found that the binding of \(\text{[H]}\text{CGS} 19755\) (30 \(\mu\)M) was not displaced by pretreatment of the membranes with SIN-1 (100% \pm 6.4% of control and 132\% \pm 10.3% of control, \(n = 6\), in the absence and presence of 1 mM SIN-I, respectively).

Discussion
The present results show that NO-producing or NO-containing compounds block NMDA receptor activity. Indeed, SIN-1, l-nitrosoeryprylidine, and NaNO\(_2\) specifically suppress the NMDA-induced increase in [Ca\(^{2+}\)] and NMDA-induced currents in striatal and cerebellar neurons, respectively. AMPA responses were not affected, showing the specificity of the drug effects. We also found that drug inhibitory effects were antagonized by hemoglobin. Moreover, a degraded solution of SIN-1 or an NaNO\(_2\) solution at pH 7 was ineffective in blocking NO-induced currents. Finally, we observed that NMDA-induced currents and the NMDA-induced increase in [Ca\(^{2+}\)] could both be potentiated by hemoglobin, a potent NO scavenger.

NO inhibits NMDA Receptor Function Independently of Any Second Messenger
First, our results show that NO is the active radical blocking NMDA receptors. Thus we found NaNO\(_2\) (NaNO\(_2\) solution at pH 7) to be ineffective in blocking NMDA receptors. In contrast, both NO released from either pH 2 NaNO\(_2\) (used after dilution and pH neutralization) or SIN-1 solutions, or contained in l-nitrosopropylidine solution, gave similar blockade of NMDA receptors. Moreover, these compounds became ineffective when stored and applied in the presence of hemoglobin.

Second, our data clearly indicate that second messenger production is not required for the NO blocking action. Since NMDA triggers cGMP production in granule cells via NO synthase stimulation (Garthwaite, 1991), it is conceivable that NO-producing drugs inhibit NMDA receptors via cGMP. However, high external concentrations of dibutyryl-cGMP and IBMX reduce, but do not suppress, the NMDA-induced increase in [Ca\(^{2+}\)]\(_i\) as does SIN-I. Furthermore, in the presence of 1 mM cGMP and IBMX in the patch pipette, the inhibitory effect of SIN-1 on NMDA currents is not significantly reduced. The observation that SIN-1 also blocks NMDA receptor activity in outside-out patches further strengthens the conclusion of a direct action of NO on the receptor-channel complex in the absence of any other cytosolic messenger.

Our results raise the question of whether NO free radical or a related compound is responsible for this inhibitory effect. The fact that l-nitrosoeryprylidine, which does not release NO radicals, still inhibited NMDA responses showed that bound NO groups could also block NMDA receptors. This suggests that...
Figure 4. Specific Blocking Effects of Acidic NaNO₂ Solution on the NMDA-Induced Increase in [Ca²⁺]

[Ca²⁺] measurements obtained in HEPES-buffered saline (A), after a 90 s treatment with 100 µM NMDA (B), after a 2 min incubation in a 1:100 dilution of acidic NaNO₂ (C), after the NaNO₂ treatment followed by a 90 s application of NMDA, in the presence of NaNO₂ (D), in the presence of NMDA alone after washout (E), and in the presence of NMDA (100 µM; 90 s application) after treatment with NaNO₂ and hemoglobin (100 µM) together (F). Comparison of (E) and (D) shows that hemoglobin blocked the inhibitory effect of the NaNO₂ solution on NMDA responses. The color scale also applies to Figure 5.
Figure 6. SIN-1-induced Inhibition of NMDA Current Does Not Require Any Soluble Intracellular Messenger

(A) Whole-cell recordings performed using a patch pipette containing 1 mM cGMP and 750 μM IBMX. NMDA (100 μM) responses obtained under control conditions (trace a), following a 2 min perfusion of 10 mM SIN-1 (trace b), and after a 3 min wash (trace c). Similar results were obtained in 4 other cells.

(B) Outside-out recordings of NMDA-mediated (100 μM) unitary currents obtained under control conditions (trace a), after a 2 min incubation with 10 mM SIN-1 (trace b), and after a 3 min wash (trace c). Horizontal bars represent NMDA applications. Recordings were performed at -60 mV holding potential. Similar results were obtained in 4 other outside-out patches.

Figure 5. NO Produced by Endogenous Metabolism Blocks NMDA Receptors

[Ca\(^{2+}\)] measurements obtained in the same population of cells as in Figure 4 in HEPES-buffered saline (A), after a 90 s treatment with 100 μM NMDA (B), after a 10 min wash (C), following a 10 min incubation with hemoglobin (100 μM) and then a 90 s application of NMDA and hemoglobin (D), after a 10 min wash of both NMDA and hemoglobin (E), and finally in the presence of NMDA (100 μM, 90 s application) alone (F). Comparison of (B) and (D) shows that hemoglobin potentiated the NMDA response. The similarity between (B) and (F) attests to the reversibility of the change induced by hemoglobin in (F). Similar results were observed in 53 different cells.
under physiological conditions, NO radicals may be stabilized by binding to chemical structures, which would prolong the action of NO. This is in agreement with the fact that NO is highly reactive with cysteine-rich proteins, forming nitrosocysteine compounds that could mimic NO free radical effects (Myers et al., 1990).

Possible Site of Actions of NO
A direct inactivation of the NMDA-binding sites by NO is unlikely in view of the following observations: the number of [1H]CGS19755-binding sites is not reduced upon incubation of brain membranes with SIN-1 or NO produced from NaN02. Whether the NO-sensitive site of the NMDA receptor is identical to the redox-sensitive site recently identified on NMDA receptors remains to be examined (Alizeman et al., 1989, 1990). Nevertheless, such an explanation is very unlikely, since we found that NO effects were not reversed by di-dithiothreitol (unpublished data), a compound interacting with the NMDA redox site (Alizeman et al., 1989). Specific experiments are needed to determine the exact site of action of NO on the NMDA receptor-channel complex.

Several authors have described rapid desensitizations of NMDA responses that are either reversed or not by glycine, depending on experimental recording conditions (Mayer et al., 1989; Sather et al., 1990). It is difficult to know whether the present blockade of NMDA responses by NO is somehow related to these desensitizations. Considering that our experiments were performed in the presence of glycine, it is tempting to propose the absence of any relationship between these two phenomena. However, one cannot exclude an acceleration of the previously described desensitization phenomena by NO in a way similar to the phosphorylation-mediated acceleration of nicotinic receptor desensitization (Huganir and Green, 1990). Indeed, NO has been shown to desensitize AMPA receptors in Purkinje cells (Ito and Karachot, 1990) via cGMP production. Under our experimental conditions, we found no desensitization of the AMPA receptor-induced increase in [Ca2+] by NO in striatal neurons. This apparent discrepancy could be due to a difference in cGMP kinase and/or AMPA receptor properties in Purkinje and striatal cells (Barnard and Henley, 1990). Specific patch-clamp studies are needed to answer the question of a possible involvement of NO in NMDA receptor desensitization.

Physiological Roles of NO Inhibitory Effects
Interestingly, we observed that neutralizing endogenous NO with hemoglobin potentiated NMDA receptor actions. This suggests that endogenous NO or a related compound could be physiological inhibitory factors used by neurons to modulate their own or neighboring cells’ NMDA receptor activities. The feedback inhibition hypothesis is particularly interesting, since it has been recently proposed that NO plays a major role in NMDA-induced neurotoxicity (Dawson et al., 1991). Hence, NO-induced NMDA receptor blockade could be a protective mechanism against an excess of NMDA receptor stimulation.

Experimental Procedures

Striatal Neuron and Granule Cell Cultures
Primary cultures of striatal neurons and granule cells were prepared as previously described (Weiss et al., 1986; Van-Vliet et al., 1989). Briefly, striata or cerebella were removed from 14 to X-day-old mouse embryos or mice at postnatal days 6-7, respectively (Ifla Credo, Lyon, France). The cells were gently dissociated in culture medium with a fire-narrowed Pasteur pipette and plated (1.5 × 105 cells per dish) in 35 mm diameter culture dishes and in 6 well clusters at 2 ml per well for biochemical experiments. Dishes, glass coverslips, and wells had been previously coated with poly-l-ornithine (15 μg/ml; molecular weight 40,000) and medium containing 10% fetal calf serum, successively. Culture medium was composed of a 1:1 mixture of Dulbecco's minimal essential medium and F-12 nutrient, supplemented with glucose (30 mM), glutamine (2 mM), sodium bicarbonate (3 mM), and HEPES (5 mM). For granule cell cultures, 5% calf serum, 5% horse serum, and 25 mM KCl were added to this culture medium. Under these conditions, the cultures have been shown to be highly enriched in neurons that form many mature and functional synapses (Weiss et al., 1986; Van-Vliet et al., 1989). Only 7% of the striatal cells have been identified as astrocytes (representing 5% of the surface area) after 12 days in vitro. Similar results were obtained in cerebellar cultures (unpublished data). Granule neurons were identified as 5-10 μm, round cells with thin, long neurites. This cell type represented 58% of the neurons in our cerebellar cultures.

Kinetics of NO Production
The kinetics of NO production by the tested drugs were studied using the method described by Ignarro et al. (1987). Briefly, the method is based on the diazotation of sulfanilic acid by NO, at acidic pH, followed by a coupling reaction with N-(1-naphthyl)-ethylenediamine. This leads to the formation of a colored compound, the optical density of which was measured by spectrophotometry. The diazotation reaction is specific to NO, but other NO derivatives, like NO2-, can also be detected by this method. This is important since NO is a very unstable compound that is rapidly transformed into NO2-.

We examined the kinetics of NO production during photolysis of the tested drugs under normal light, at pH 7.4, using the procedure described above. The accumulation of NO and NO2- in a solution of SIN-1 followed an exponential increase, reaching half-maximal saturation after 4 hr and a plateau after 16 hr. Considering the very short lifetime (a few seconds) of NO in water considering the very short lifetime (a few seconds) of NO in water, this result indicates that under our experimental conditions, there are no more NO molecules produced in the SIN-1 solution after 16 hr. We therefore prepared fresh SIN-1 solution every day and used it within 30 min for fura-2 experiments or within 5 hr for patch-clamp recordings.

Similar measurements performed with 1 mM N-(1-naphthyl)-ethylenediamine showed no detectable release of NO by this drug over 24 hr. Because the method used to detect the presence of NO in our solutions was also sensitiveloto the presence of NO2-, we could not use it to measure NO produced by our acidic NaN02 solution.

Fura-2 Ratio Imaging
Intracellular Ca2+ measurements were performed as previously described (Manzoni et al., 1991). Briefly, 7-13 days in vitro mouse...
striatal neurons were loaded with the fluorescent Ca²⁺ chelator fura-2-AM, by incubation in 3.3 μM fura-2-AM for 1 hr at 37°C in HEPES-buffered saline (146 mM NaCl, 4.2 mM KCl, 1.1 mM CaCl₂, 5 mM glucose, 1 μM glycine, 20 mM HEPES [pH 7.2]). After the incubation, the cells were washed 3 times in the same buffer. The coverslips containing the loaded and washed cells were mounted for microscopic analysis. The cells were alternately excited with 340 and 380 nm light, and the emission was measured at 500 nm (Gryniewicz et al., 1985) on a Zeiss Axiovert 10 inverted microscope coupled to an MFP 21 microprocessor responsible for the filter changes. The images were taken by a low light level STILH-4036 camera from Lhesa (Paris) and digitized on-line by the Quantel Crystal Imaging System. During the 2 s excitation period at each wavelength, we averaged 8 video frames per digitized image. Each image was coded on 512 x 576 x 8 bits. The camera dark noise was subtracted from the recorded images. The values were estimated from the images using the procedure described by Grynkiewicz et al. (1985). The constants necessary for this procedure were obtained with fura-2 free acid standard solutions. The whole-image treatments were done by homemade software. For experiments on cell somata, [Ca²⁺] levels were determined on the final image individually for each cell soma using the Crystal particle analysis package together with homemade software.

Patch Clamp Techniques
For patch-clamp recording, the culture medium was replaced by a control solution containing 140 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 10 mM HEPES, 1 μM glycine, and 0.3 μM tetrodotoxin (pH 7.4). Drug solutions were prepared in this same medium, and pH was adjusted to 7.4. Control and drugs solutions were applied using a rapid perfusion system similar to the one described by Johnson and Ascher (1987). The recording pipette solution contained 140 mM CaCl₂, 0.5 mM CaCl₂, 5 mM EGTA-Tris, and 10 mM HEPES (pH 7.2). Whole-cell currents were recorded using a List-EPC7 amplifier and stored on a videotape recorder. Recordings were then filtered at 500 Hz and digitized at 3 kHz for analysis. Macroscopic NMDA currents were measured at their initial peak amplitude (before desensitization of the response). Single-channel recordings were filtered at 1 kHz and digitized at 5 kHz. Analyses were performed using the patch-clamp 5.5 program of Axon Instruments. For a given level of channel opening, the integrated time during which the channels stayed open was divided by the duration of the NMDA application and gave the value ΔPo.

cGMP Formation
Striatal neurons grown in 12 well dishes were loaded with cGMP indicator with greatly improved fluorescence properties. This work was supported by grants from CNRS (Centre National de la Recherche Scientifique), INSERM (Institut National de la Sante et de la Recherche Medicale), Bayer France/Tropenwerke (Germany), and DRET (Direction des Recherches Etudes et Techniques grant 88/163).

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