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Nitric Oxide-Induced Blockade of NMDA Receptors

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Summary

We studied the 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-1 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 $NaNO_2$. Pretreatment with hemoglobin potentiated NMDA-induced effects, demonstrating that 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., 1988) and leads to formation of cGMP. NO has been implicated in neuron-glia 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 transsynaptic messenger in the development of lon-term potentiation and depression (Gally et al., 1990; Böhme 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, adresses 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 $NaNO_2$, and one NO-containing compound, I-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 (Böhme et al., 1984). Around pH 7, $NaNO_2$ is a very stable molecule, but when the pH solution drops to acidic values, $NaNO_2$ releases NO. At physiological pH, NO is rapidly transformed into NO_2 and NO_3. 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 ($[Ca^{2+}]_i$) 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 (EC_{50} = 3.2 μM,; maximal induced cGMP formation, 8.4 ± 0.4 pmol per well; n = 3). I-Nitrosopyrrolidine also induced cGMP formation (maximal induced cGMP formation, 20 ± 0.8 pmol per well; n = 3). We also formed NO from an $NaNO_2$ solution. An acidic (pH

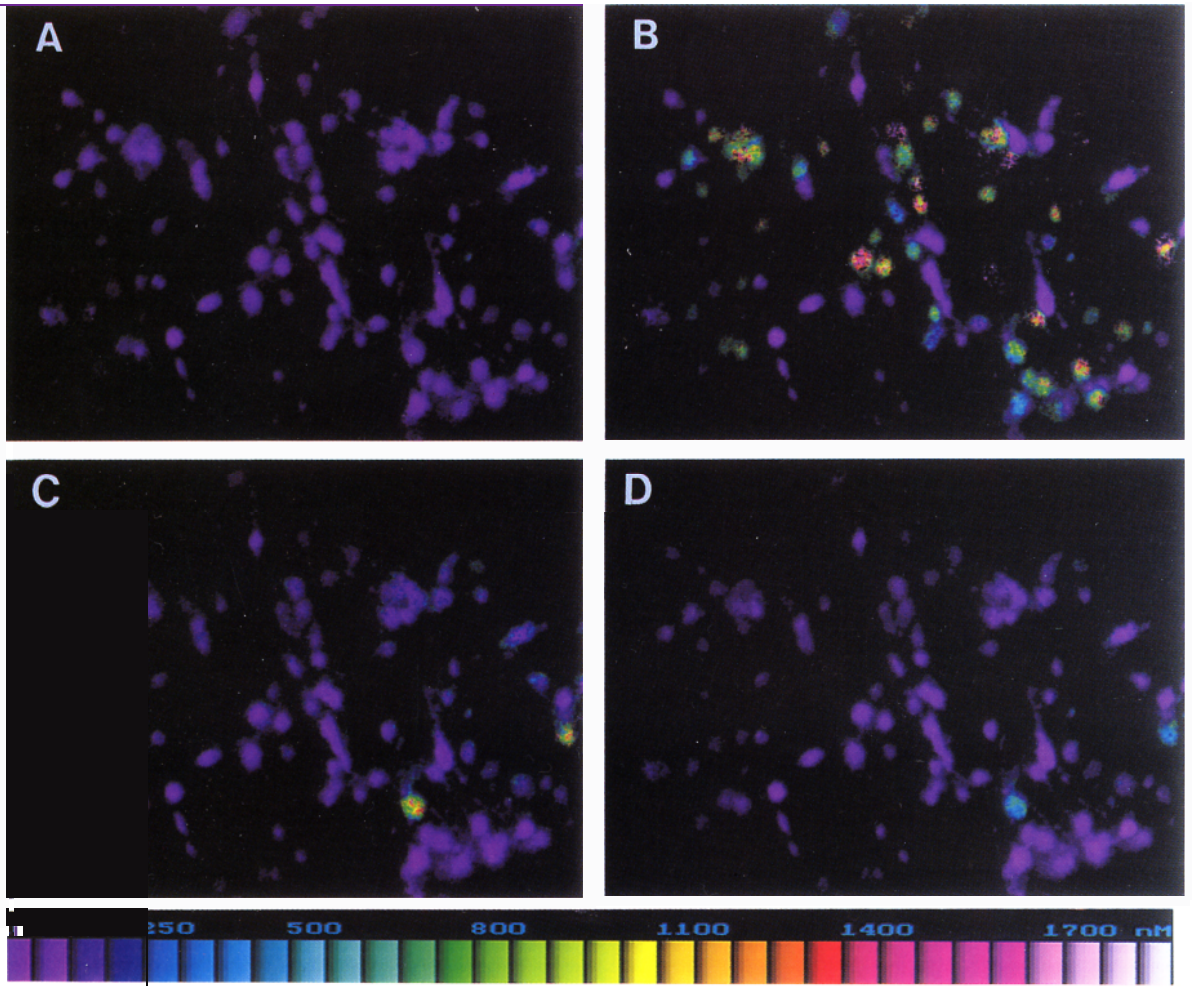


Figure 1. Specific Blocking Effects of SIN-1 on NMDA-Induced Increase in $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ measurements in HEPES-buffered saline (A), after a 90 s treatment wrth 100 μM NMDA (B), after a 5 min incubation with 1 mM SIN-I, in the absence of NMDA (C), and after a 5 min preincubation with SIN-1 and then a 90 s application of NMDA, in the presence of SIN-1 (D). The inhibitory effect of SIN-I on the NMDA response was completely reversible after a 10 min wash of the drug (data not shown). All pictures were obtained from the same population of cells. Similar results were obtained in four other preparations from different cultures, corresponding to a total of 206 cells.

2) solution of $NaNO_2$ (IO mM) was prepared in the presence of glutathione (100 μM) 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 = 3).

Effects of NO-Producing or NO-Containing Compounds on NMDA Receptor Activation

In primary cultures of striatal neurons, the NMDA-induced increase in $[Ca^{2+}]_i$ was monitored by quantitative ratio imaging of the fluorescent Ca^{2+} chelator fura-2. SIN-1 up to 1 mM was without significant effect on basal $[Ca^{2+}]_i$ (Figure 1C). The NMDA-induced $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ produced by 100 μM AMPA (data not shown), suggesting that NO specifically blocks NMDA receptor activation in striatal neurons. SIN-1 inhibited NMDA-induced $[Ca^{2+}]_i$ increase in a dose-dependent manner with a relatively low affinity (I-IO μM). 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 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 Fig-

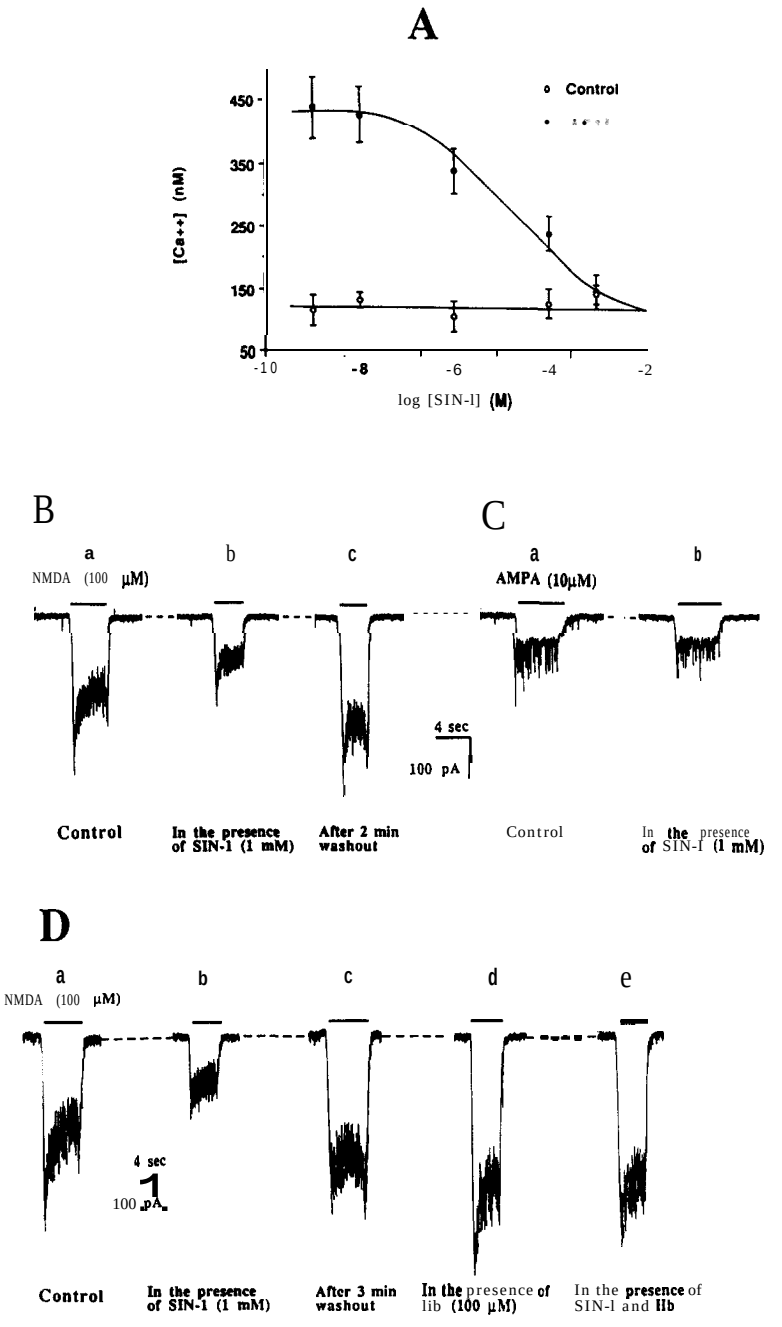


Figure 2. SIN-I Specifically Inhibits NMDA Receptor Action

(A) Dose-dependent inhibition of the NMDA response by SIN-I. Neurons were preincubated for 10 min in the presence of the indicated doses of SIN-1 prior application of 100 μM NMDA. $[Ca^{2+}]_i$ measured in the absence of NMDA (open circles) or after a 90 s treatment with NMDA (closed circles). IC_{50} = 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 thewhole-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-I; 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-I 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 (traced), 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.

ure 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 ceils) 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-I, which was within the normal range of fluctuation of the AMPA current.

The question of whether or not the final breakdown product of SIN-I, SIN-IC, 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

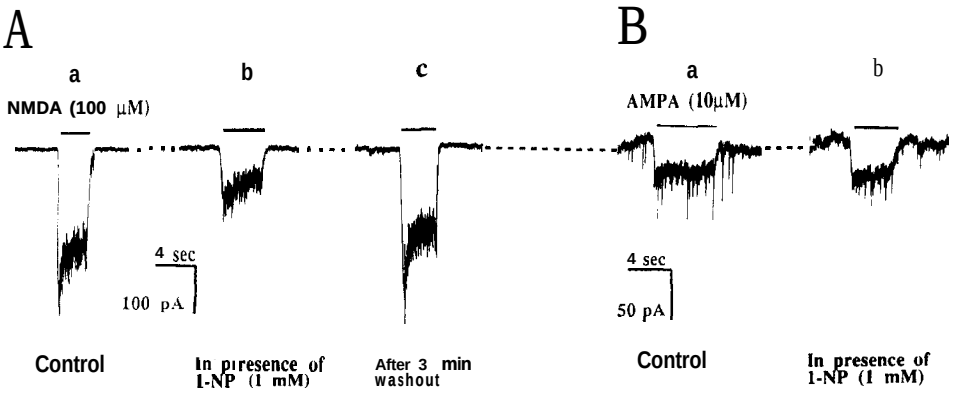


Figure 3. I-Nitrosopyrrolidine-Induced Inhibition of NMDA Receptors

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

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, coincubation 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 NO_2^- (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 $[Ca^{2+}]_i$ (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 maximal inhibition, and the effects were reversed (87% ± 9%) after a 3 min wash. As with SIN-I, neither AMPA currents recorded in the same cells (Figure 3B), nor AMPA-induced $[Ca^{2+}]_i$ increases (basal, 92 ± IO 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 1-nitrosopyrrolidine. The inhibitory effects of I-nitrosopyrrolidine were reversible after washing (Figure 3A, trace c) and were suppressed bycoincubation 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 $NaNO_2$ (prepared as described above), applied 2 min before NMDA (100 μM), blocked the NMDA-induced increase in $[Ca^{2+}]_i$ in a hemoglobin-reversible manner. This $NaNO_2$ solution was without effect on the AMPA-induced increase in $[Ca^{2+}]_i$ (data not shown). Note that the same solution, prepared without $NaNO_2$, had no effect on the NMDA-induced Ca^{2+} response (data not shown). In additional experiments performed with fura-2 or patch-clamp techniques, we verified that an $NaNO_2$ solution (IO 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 (Marin et al., 1992) neurons have been shown to produce NO, we wondered whether this 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 amplitudewhen recorded in the presence than in the absence of hemoglobin (Figure 2D, compare trace a with d). However, because of thedifficulty 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 $[Ca^{2+}]_i$ 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-I? Bath application of 1 mM cGMP had no effect on the increase in $[Ca^{2+}]_i$ induced by 100 μM NMDA (data not shown), excluding a possible direct effect of cGMP on the NMDA recognition site (Baron et al., 1989). Preincubation (IO min) of striatal neurons with 300 μM dibutyryl-cGMP, a cGMP analog that diffuses into cells, in the presence of isobutyl-methylxanthine (IBMX; 750 μM), 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 μM NMDA, 831 ± 70 and 429 ± 52 nM Ca^{2+} without or with a 10 min pretreatment with 300 μM 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 μM 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 μM 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 μM NMDA, the integrated activity (NP_o, N is the putative number of NMDA channels in the patch; P_o is the cumulative open probability for all channels in the patch) of NMDA channels was 0.68 ± 0.23 (mean ± SD, n = 5; Figure 6B, trace a). In the presence of SIN-1 (IO mM), NP_o dropped to a mean value of 0.09 ± 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 indicate 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, [^{3}H]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 [^{3}H]CGS 19755 (30 μM) was not displaced by pretreatment of the membranes with SIN-1 (100.4% ± 6.4% of control and 132% ± 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-I, I-nitrosopyrrolidine, and $NaNO_2$ specifically suppress the NMDA-induced increase in $[Ca^{2+}]_i$ 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 NMDA responses. Finally, we observed that NMDA-induced currents and the NMDA-induced increase in $[Ca^{2+}]_i$ 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 1-nitrosopyrrolidine 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 I-nitrosopyrrolidine, which does not release NO radicals, still inhibited NMDA responses showed that bound NO groups could also block NMDA receptors. This suggests that

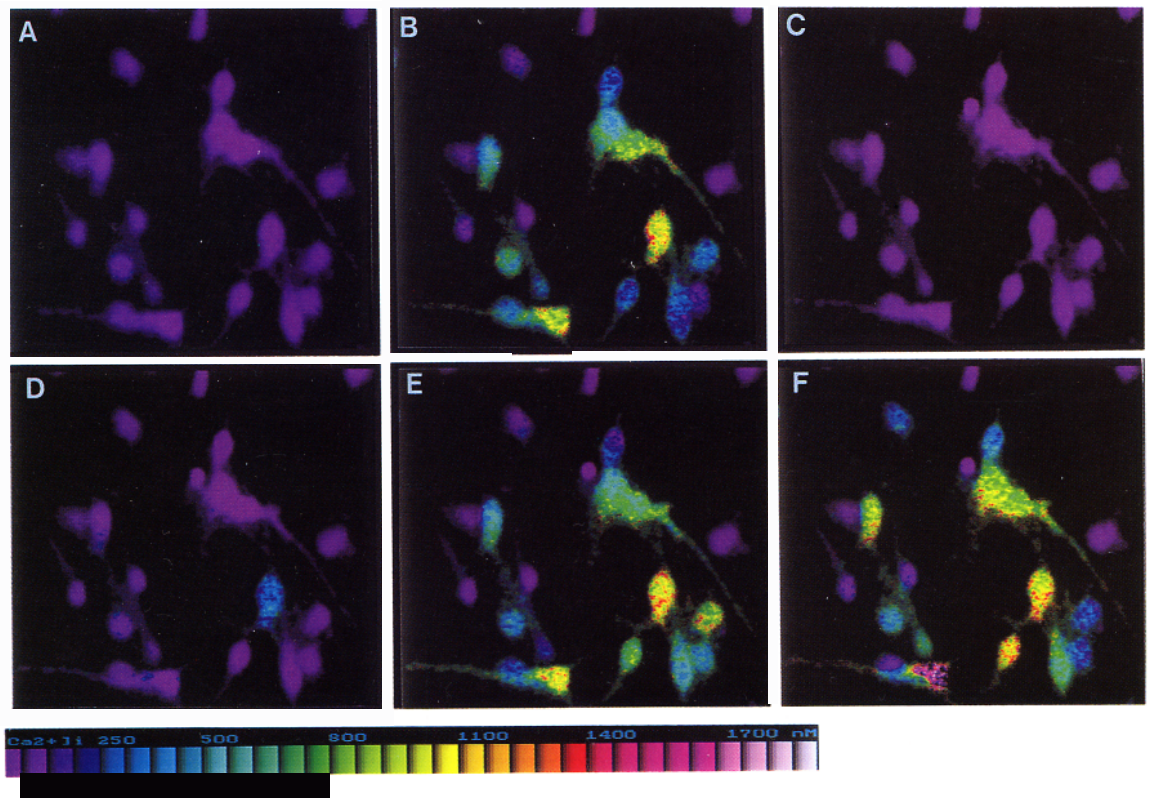
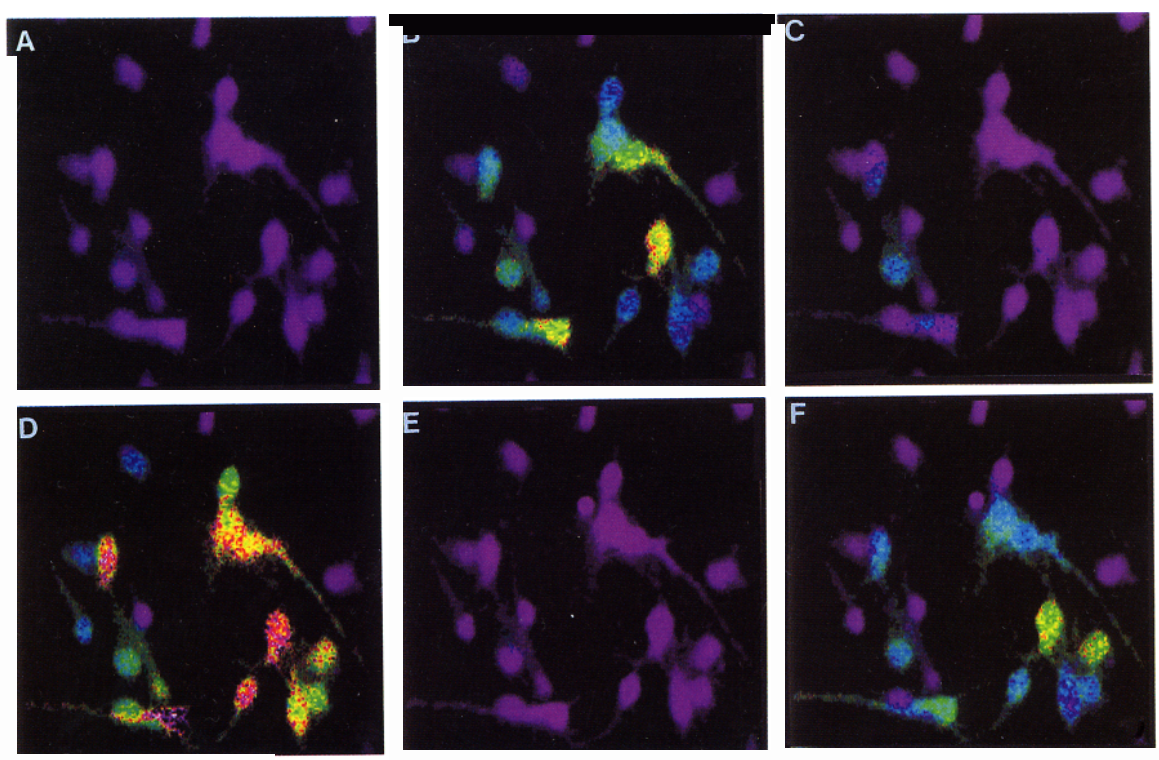


Figure 4. Specific Blocking Effects of Acidic $NaNO_2$ Solution on the NMDA-Induced Increase in $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ 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 $NaNO_2$ (C), after the $NaNO_2$ treatment followed by a 90 s application of NMDA, in the presence of $NaNO_2$ (D), in the presence of NMDA alone after washout (E), and in the presence of NMDA (100 μM; 90 s application) after treatment with $NaNO_2$ and hemoglobin (100 μM) together (F). Comparison of (E) and (D) shows that hemoglobin blocked the inhibitory effect of the $NaNO_2$ solution on NMDA responses. The color scale also applies to Figure 5.



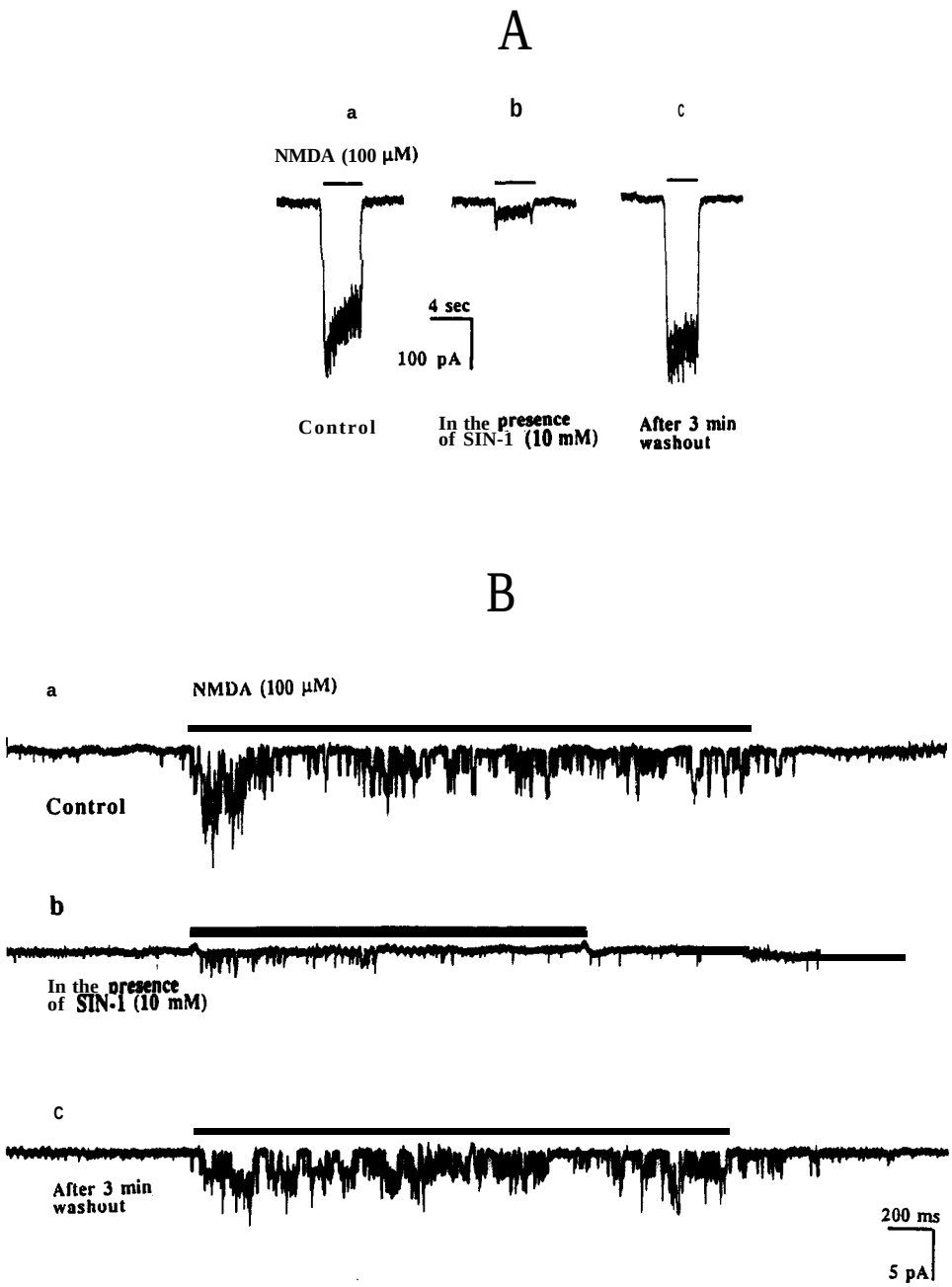


Figure 6. SIN-l-induced Inhibition of NMDA Current Does Not Require Any Soluble Intracellular Messenger

(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

$[Ca^{2+}]_i$ 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 [^{3}H]CGS 19755-binding sites is not reduced upon incubation of brain membranes with SIN-1 or NO produced from $NaNO_2$. 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 (Aizenman et al., 1989, 1990). Nevertheless, such an explanation is very unlikely, since we found that NO effects were not reversed by DL-dithiothreitol (unpublished data), a compound interacting with the NMDA redox site (Aizenman 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 Greengard, 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 $[Ca^{2+}]_i$ 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 cerebelli were removed from 14- to X-day-old mouse embryos or mice at postnatal days 6-7, respectively (Iffa Credo, Lyon, France). The cells were gently dissociated in culture medium with a fire-narrowed Pasteur pipette and plated (1.8 x 10^6 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 mixtureof 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 wereobtained in cerebellar cultures (unpublished data). Granule neurons were identified as 5-10 μm, round cells with thin, long neurites. This cell type represented 95% 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 diazotization of sulfanilic acid by NO, at acidic pH, followed by a coupling reaction with N-(1-naphtyl)-ethylene-diamine. This leads to the formation of a colored compound, the optic density of which was measured by spectrophotometry. The procedure was as follows: 100 μl of 4 M HCl was added to 1 ml of the tested solution. A 70 μl sample of this final solution was added to 100 μl of 2 M HCl and 100 μl of sulfanilic acid (2 mg/ml). After 10 min, 100 μl of N-(1-naphtyl)-ethylene-diamine (1 mg/ml) was added to the sample solution, and 30 min later, absorbance of the solution was measured at 548 nm wavelength by spectrophotometry. The diazotization reaction is specific to NO, but other NO derivatives, like NO_2, can also be detected by this method. This is important since NO is a very unstable compound that is rapidly transformed into NO_2.

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 NO, 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 at pH 7.4, 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 measurement performed with 1 mM 1-nitrosopyrrolidine showed no detectable release of NO by this drug over 24 hr. Because the method used to detect the presence of NO in oursolutionswasalsosensitivetothepresenceof NO_2, we could not use it to measure NO produced by our acidic $NaNO_2$ solution.

Fura-2 Ratio Imaging

Intracellular Ca^{2+} measurements were performed as previously described (Manzoni et al., 1991). Briefly, 7-13 days in vitro mouse

striatal neurons were loaded with the fluorescent Ca^{2+} 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 $CaCl_2$, 5 mM glucose, 1 μM glycine, 20 mM HEPES [pH 7.21). 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 alternatively excited with 340 and 380 nm light, and the emission was measured at 500 nm (Grynkiewicz et al., 1985) on a Zeiss Axiovert 10 inverted microscope coupled to an MSP 21 microprocessor responsible for the filter changes. The images were taken by a low light level SIT LH 4036 camera from Lhésa (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 × 576 × 8 bits. The camera dark noise was subtracted from the recorded crude images. $[Ca^{2+}]_i$ values were estimated from the images using the procedure described by Grynkiewicz et al. (1985). The constants necessary for this procedure wereobtained with fura-2 free acid standard solutions. The whole-image treatments were done by homemade software. For experiments on cell somata, $[Ca^{2+}]_i$ 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 $CaCl_2$, 10 mM HEPES, 1 μM glycine, and 0.3 μM tetrodotoxin (pH 7.4). Drug solutionswere 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 CsCl, 0.5 mM $CaCl_2$, 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 3 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 NPo.

cGMP Formation

Striatal neurons grown in 12 well dishes during 10–13 days were washed 3 times with 1 ml of Krebs bicarbonate buffer (3.5 mM KCl, 0.75 mM $CaCl_2$, 124 mM NaCl, 1.25 mM K_2HPO_4, 26.3 mM $NaHCO_3$ [pH 7.4]), preequilibrated with 95% O_2, 5% CO_2, and then incubated during 30 min in this medium. Neurons were stimulated for 5 min with the indicated concentrations of drugs for 10 min in the same medium containing 750 μM IBMX. The incubation was stopped by replacing the medium with 0.5 ml of 95% EtOH 5% HCOOH. The cellular content of cGMP was determined by radioimmunoassay.

Binding of [^{3}H]CGS 19755 to Rat Brain Membranes

Membrane preparation and radioligand binding were performed as previously described (Murphy et al., 1988; Manzoni et al., 1992)

Materials

Pharmacological agents used in this study were obtained from the following sources: NMDA, $NaNO_2$, and I-nitrosopyrrolidine were from Sigma (L'Isle d'Abeau, France); AMPA was from Tocris Neuramin (Essex, England); 35 mm diameter dishes for tissue cultures were from Costar (Cambridge, MA); Dulbecco's minimal essential medium and F-12 nutrient were from GIBCO Europe (Paris, France); fura2-AM was from Calbiochem. SIN-1 was generously donated by Dr. Kunstmann (Hoechst, Frankfurt). All other compounds were of the highest possiblegradefrom commercial sources.

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