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Sodium Nitroprusside Inhibits *N*-Methyl-D-aspartate-Evoked Calcium Influx via a Nitric Oxide- and cGMP-Independent Mechanism

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SUMMARY

In primary cultures of rat cerebellar granule cells, sodium nitroprusside (SNP), a vasodilator that generates nitric oxide (NO), potently inhibited *N*-methyl-D-aspartate (NMDA)-evoked $^{45}Ca^{2+}$ influx (IC_{50} = 6.6 μM). This inhibition was time dependent and was complete when SNP was applied 10 min before NMDA stimulation. The effect of SNP was transient and the ability of NMDA to stimulate $^{45}Ca^{2+}$ influx was restored after SNP withdrawal. The effect of SNP was selective for the NMDA-sensitive glutamate receptor, because SNP failed to antagonize kainate-stimulated $^{45}Ca^{2+}$ influx. The action of SNP was independent of the ability of this agent to generate NO; *S*-nitroso-*N*-acetylpenicillamine, an NO-containing compound that was 100 times more potent than SNP in stimulating cGMP accumulation, failed to inhibit NMDA-evoked $^{45}Ca^{2+}$ influx. In contrast, $K_4Fe(CN)_6$, a compound structuraly similar to SNP but devoid of NO, inhibited both $^{45}Ca^{2+}$ influx (IC_{50} = 27 μM) and cGMP accumulation evoked by NMDA; $K_3Fe(CN)_6$ was inactive. Thus, in cerebellar granule cells, SNP and $K_4Fe(CN)_6$ interfere with the function of NMDA receptors, possibly at the level of the receptor recognition site. The resulting blockade of Ca^{2+} influx through NMDA receptor channels accounts for the reported ability of these compounds to protect granule cells from NMDA-induced neurotoxicity. This protection is not mediated by an NO-dependent mechanism but depends on the action of the ferrocyanide portion of the SNP molecule.

NO (1) or its nitrosothiol derivative (2) accounts for the biological action of endothelium-derived relaxing factor, a potent activator of soluble guanylate cyclase (3, 4). NO synthase, the enzyme that catalyzes the conversion of arginine to NO and citrulline, was recently cloned (5); the enzyme is present in brain (6, 7) and is particularly abundant in the cerebellum (8). Activation of NO synthase mediated by the NMDA-sensitive glutamate receptor has been demonstrated in cerebellar granule neurons (9). SNP, which spontaneously generates NO (10), and nitroglycerin, which releases NO after metabolism in the body (11), are commonly used both as vasodilators in individuals with severe congestive heart failure (12) and as tools to study the biological activities of NO. Recently, we showed that SNP protects cerebellar granule neurons against NMDA-induced toxicity and that this property of SNP is independent from its ability to release NO (13). In order to elucidate the mechanism of neuroprotection mediated by SNP, we have studied the effects of SNP on NMDA-induced $^{45}Ca^{2+}$ influx and cGMP accumulation in primary cultures of neonatal rat cerebellar granule neurons.

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Materials and Methods

Granule cell cultures. Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rats (Zivic-Miller), as described previously (14, 15). Cells were plated on 35-mm plastic culture plates coated with poly-L-lysine (10 μg/ml), at a density of 2.5 × 10^6 cells/plate, in basal Eagle's medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, and 50 μg/ml gentamicin. Glial cell proliferation was prevented by the addition of 1 μM cytosine arabinofuranoside 24 hr after plating. Immunocytochemical studies show that these primary cultures comprise 97% neurons and 3% glial and other contaminating cells (15).

Measurement of $^{45}Ca^{2+}$ influx and cGMP formation. Granule cell cultures were washed three times with 1 ml of buffered CSS, which contained 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 1.3 mM $CaCl_2$, and 10 mM HEPES (pH 7.35), and were incubated for 5 min in the same solution; the medium was then replaced with fresh CSS supplemented with the drugs under study and the cells were incubated for the indicated time periods (usually 10 min). The cells

ABBREVIATIONS: NO, nitric oxide; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzocyclohepten-5,10-imine maleate; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; NMDA, *N*-methyl-D-aspartate; CSS, control salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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were then stimulated with the indicated agonist; $^{45}Ca^{2+}$ (3 μCi/plate) was added simultaneously with the agonist. Incubations were terminated after 5 min by rapid aspiration of the medium, three washes with 2.5 ml of ice-cold salt solution containing 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2 mM EGTA, and 10 mM HEPES (pH 7.35), and the addition of 1 ml of 0.3 M $HClO_4$. The $HClO_4$ cell extract was neutralized with K_2CO_3. Aliquots of the neutralized extract were used to measure $^{45}Ca^{2+}$ influx and cGMP accumulation, as described previously (9). Precipitated cellular proteins were dissolved in 0.5 M NaOH and measured according to the method of Lowry *et al.* (16).

Measurement of $^{45}Ca^{2+}$ efflux. Cells were loaded with $^{45}Ca^{2+}$ during a 5-min incubation with 1 ml of CSS supplemented with 10 μCi of $^{45}Ca^{2+}$ and 20 μM glutamate. Cells were then washed three times with 1 ml of CSS or CSS containing 100 μM SNP and were incubated for the indicated times with the same solution. The medium was aspirated and the cells were washed three times with 2.5 ml of ice-cold CSS. The experiment was terminated by dissolving the cells in 1 ml of 0.5 M NaOH, and aliquots of this solution were used for measurement of $^{45}Ca^{2+}$ by liquid scintillation counting and for measurement of protein.

Materials. $^{45}CaCl_2$ (13 Ci/g) was from Du Pont/NEN; ^{125}I-labeled cGMP Amerlex-M radioimmunoassay kits were from Amersham. SNAP was synthesized from $NaNO_2$ and *N*-acetylpenicillamine according to the method of Field *et al.* (17). Reduced hemoglobin was prepared as described by Martin *et al.* (18). Stock solutions of SNP (10 mM) were prepared in deionized water 30–60 min before the experiment and were protected from light. The photolysis of SNP was achieved by a continuous exposure of a 10 mM solution to white fluorescent light for 7 days. The color of the resulting solution was dark blue due to Prussian blue formation. Cell culture media were purchased from GIBCO. A23187 was from Boehringer Mannheim, $K_4Fe(CN)_6$, KCN, $FeCl_2$, and $FeCl_3$ from Aldrich, and MK-801 from Research Biochemicals Inc. All other chemicals were obtained from Sigma.

Results

Treatment of cerebellar granule neurons with SNP (100 μM) led to a time-dependent inhibition of NMDA-evoked $^{45}Ca^{2+}$ influx but did not significantly affect basal $^{45}Ca^{2+}$ influx. SNP inhibited NMDA-evoked $^{45}Ca^{2+}$ influx when added simultaneously with NMDA; however, complete inhibition could be observed when the cells were incubated with SNP for 5–10 min before NMDA application (Fig. 1). This inhibitory effect of SNP was transient; cells from which SNP was withdrawn after 15 min of treatment showed NMDA-induced $^{45}Ca^{2+}$ influx 10 min after SNP removal (Fig. 1).

The possibility that SNP increases $^{45}Ca^{2+}$ extrusion rather than inhibits $^{45}Ca^{2+}$ influx was investigated by loading cells with $^{45}Ca^{2+}$ in the presence of 20 μM glutamate. This procedure allowed the monitoring of $^{45}Ca^{2+}$ extrusion for at least 20 min in the absence or presence of SNP. We found that 100 μM SNP failed to change the rate of $^{45}Ca^{2+}$ efflux from granule cells (Fig. 2).

To determine whether SNP inhibition of NMDA-evoked $^{45}Ca^{2+}$ influx is mediated by NO, we investigated the effect of SNAP, which stimulates guanylate cyclase via an NO-related mechanism (19), on NMDA-induced $^{45}Ca^{2+}$ influx. SNAP stimulated cGMP with a potency (EC_{50} = 0.35 ± 0.013 μM; three experiments) that was >100 times that of SNP (EC_{50} = 60 ± 3.7 μM; three experiments) (Fig. 3A) but failed to inhibit NMDA-evoked $^{45}Ca^{2+}$ influx (Fig. 3B). The products of SNP photolysis (7-day exposure to light) showed a very low potency in enhancing cGMP accumulation, indicating a low NO content. Nevertheless, the photolyzed SNP inhibited NMDA-evoked $^{45}Ca^{2+}$ influx with a potency similar to that of the freshly

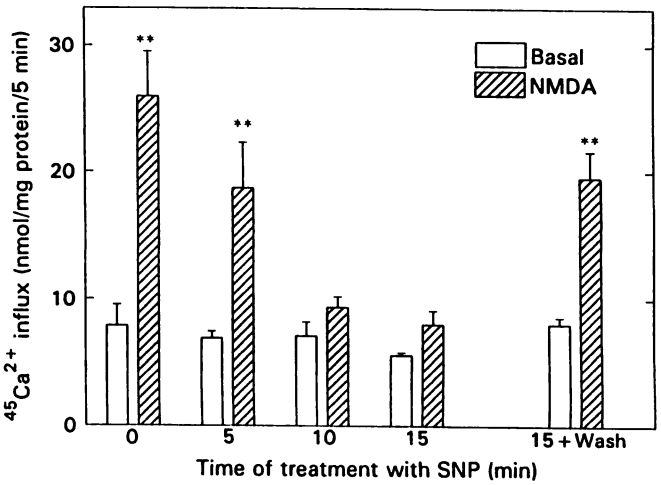

Fig. 1. Time course and transient nature of SNP effect on NMDA-evoked $^{45}Ca^{2+}$ influx in cerebellar granule neurons. Cells were incubated for 5 min with $^{45}Ca^{2+}$ (3 μCi) in the absence and presence of NMDA (100 μM). SNP (100 μM) was added together with NMDA (5 min of incubation with SNP), 5 min before NMDA (10 min of incubation with SNP), or 10 min before NMDA (15 min of incubation with SNP). To illustrate the transient nature of the SNP effect, SNP was washed out and replaced with CSS after 15 min of incubation. Cells were stimulated with NMDA 10 min after removal of SNP. Data are means ± standard errors from three separate experiments. **, $p < 0.01$ versus basal $^{45}Ca^{2+}$ influx measured in respective CSS-treated control (Student's *t* test).

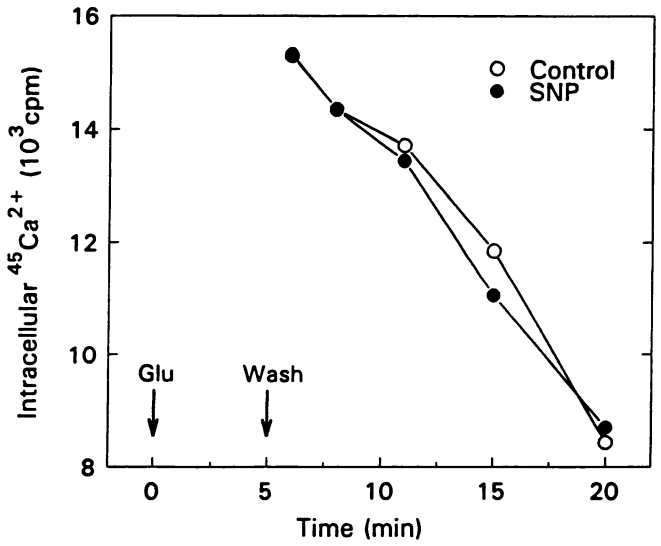

Fig. 2. Effect of SNP on $^{45}Ca^{2+}$ efflux from cerebellar granule neurons. Cells were incubated with $^{45}Ca^{2+}$ (10 μCi/ml) in the presence of 20 μM glutamate for 5 min. The medium was then replaced with CSS or CSS supplemented with SNP (100 μM). The efflux of $^{45}Ca^{2+}$ was terminated at the indicated times, as described in Materials and Methods. The data represent $^{45}Ca^{2+}$ associated with the cells and are means from duplicates obtained in a single experiment that was repeated with the same result.

dissolved SNP (data not shown). In control experiments, we observed that both SNP and SNAP that were incubated for 15 min with granule cells and then transferred to a new batch of cells were able to stimulate cGMP accumulation to the same extent as freshly prepared solutions, indicating that NO was present throughout the whole incubation period. Preincubation of granule cells for 10 min with dibutyryl-cGMP or 8-bromo-cGMP (both 100 μM) failed to inhibit NMDA-evoked $^{45}Ca^{2+}$ influx (data not shown), indicating that the ability of SNP to increase cGMP concentration is not related to its inhibitory

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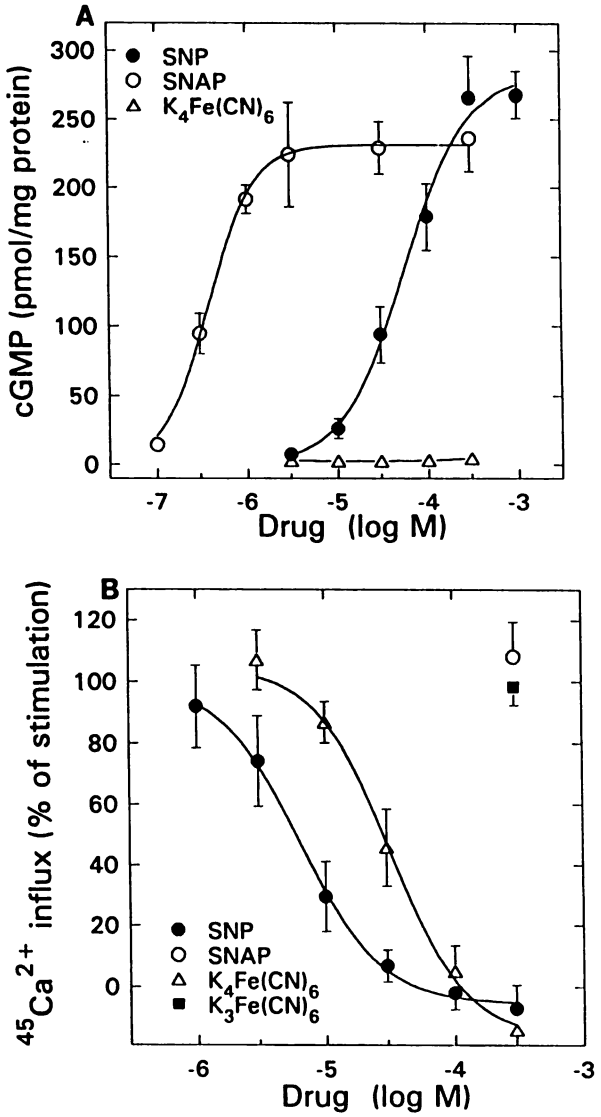

Fig. 3. cGMP accumulation (A) and NMDA-evoked $^{45}Ca^{2+}$ influx (B) in cerebellar granule neurons. cGMP was measured after 5-min exposure of the cells to the indicated concentrations of SNP, SNAP, or $K_4Fe(CN)_6$. For $^{45}Ca^{2+}$ influx experiments, cells were treated for 10 min with CSS or the indicated concentrations of SNP, SNAP, $K_4Fe(CN)_6$, or $K_3Fe(CN)_6$ and then stimulated for 5 min with NMDA (100 μM). Results are expressed as a percentage of the basal $^{45}Ca^{2+}$ influx (CSS-treated control that was not stimulated with NMDA). Data are means ± standard errors from three to six separate experiments.

effect. Moreover, the preincubation (10 min) of granule cells with an inhibitor of NO synthase, N^G-nitro-L-arginine (3 μM), failed to affect NMDA-evoked $^{45}Ca^{2+}$ influx, although it completely blocked NMDA-stimulated cGMP formation (data not shown). These findings suggest that neither NO nor cGMP can be considered a mediator of the SNP inhibition of NMDA-evoked $^{45}Ca^{2+}$ influx.

We then determined whether the effect of SNP on $^{45}Ca^{2+}$ influx could be mimicked by the oxidation products of NO or by the ferrocyanide moiety of the SNP molecule. NO_2^- ions (100 μM) did not affect NMDA-evoked $^{45}Ca^{2+}$ influx (data not shown), whereas CN^- ions (100 μM), which are degradation products of SNP (20), actually increased NMDA-induced $^{45}Ca^{2+}$ influx (data not shown). However, $K_4Fe(CN)_6$, which did not stimulate cGMP accumulation (Fig. 3A), inhibited NMDA-evoked $^{45}Ca^{2+}$ influx (Fig. 3B). The inhibitory effect of $K_4Fe(CN)_6$ on NMDA-mediated $^{45}Ca^{2+}$ influx, similarly to that of SNP, was transient. It could be observed when $K_4Fe(CN)_6$ and NMDA were applied simultaneously, but a complete inhibition was achieved when the cells were preincubated with $K_4Fe(CN)_6$ (data not shown). SNP was more potent (IC_{50} = 6.6 ± 2.4 μM; six experiments) than $K_4Fe(CN)_6$ (IC_{50} = 28 ± 8.0 μM; three experiments) in inhibiting NMDA-induced $^{45}Ca^{2+}$ influx. In contrast to $K_4Fe(CN)_6$, $K_3Fe(CN)_6$ was inactive (Fig. 3B). $FeCl_2$ and $FeCl_3$ (both at 100 μM) failed to affect NMDA-evoked $^{45}Ca^{2+}$ influx (data not shown).

Because hemoglobin is known to complex NO and inhibit NO-dependent processes (21), we investigated the effect of this protein on NMDA-evoked $^{45}Ca^{2+}$ influx. Hemoglobin alone (10 μM) potentiated the NMDA-induced $^{45}Ca^{2+}$ influx. Moreover, hemoglobin counteracted the inhibitory effects of both SNP and $K_4Fe(CN)_6$ and shifted to the right the dose-response curves obtained with each of these compounds for the inhibition of NMDA-evoked $^{45}Ca^{2+}$ influx (Fig. 4).

Stimulation of cerebellar granule neurons for 5 min with NMDA evoked a 17-fold increase in the cellular cGMP concen-

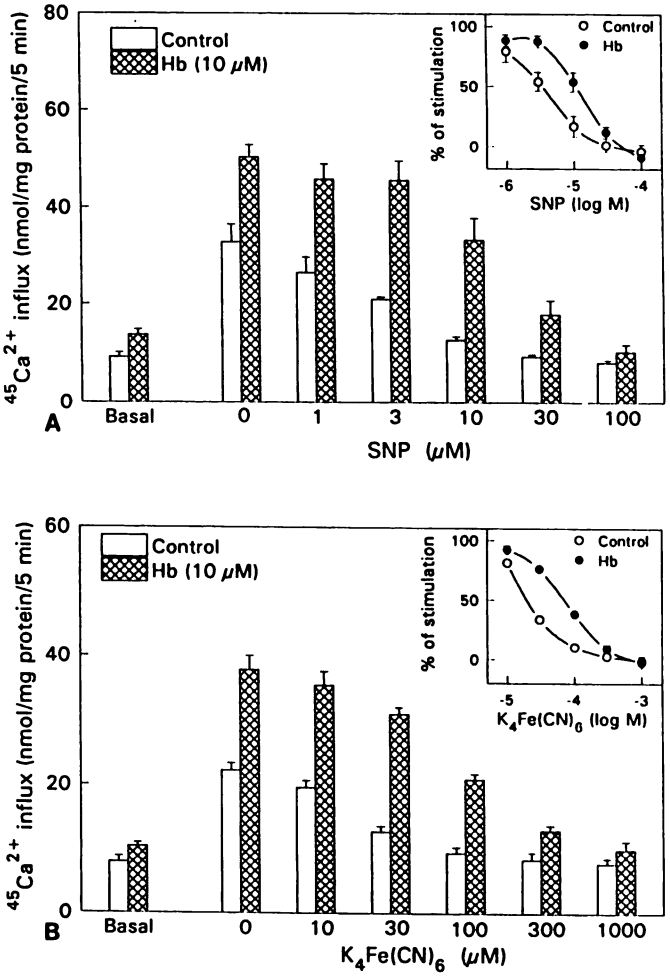

Fig. 4. Effect of reduced hemoglobin (*Hb*) on the inhibition of NMDA-evoked $^{45}Ca^{2+}$ influx by SNP (A) and $K_4Fe(CN)_6$ (B). Cells were treated for 10 min with hemoglobin (10 μM) and either SNP or $K_4Fe(CN)_6$ and were then stimulated with NMDA (100 μM) for an additional 5 min. *Insets*, standardized inhibition curves from the same results expressed as a percentage of maximal stimulation. Basal represents cells not stimulated with NMDA; control represents cells not treated with hemoglobin. Data are means ± standard errors from three separate experiments.

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tration; this effect was inhibited by prior treatment for 10 min with $K_4Fe(CN)_6$ but not with $K_3Fe(CN)_6$ (Fig. 5).

To determine whether the effects of SNP and $K_4Fe(CN)_6$ were selective for NMDA receptor-mediated Ca^{2+} influx, we compared their actions on $^{45}Ca^{2+}$ influx elicited by other glutamate receptor agonists and by veratidine. Treatment of cells with SNP or $K_4Fe(CN)_6$, but not with SNAP or $K_3Fe(CN)_6$, inhibited NMDA- and glutamate-induced $^{45}Ca^{2+}$ influx. However, SNP and $K_4Fe(CN)_6$ failed to affect $^{45}Ca^{2+}$ influx elicited by 100 μM kainate (Fig. 6) or 10 μM veratridine (data not shown). The effects of kainate and veratridine were studied in the presence of the NMDA receptor antagonist MK-801 (22), in order to avoid the indirect stimulation of NMDA receptors by glutamate released as a result of cell depolarization. SNP, SNAP, $K_4Fe(CN)_6$, and $K_3Fe(CN)_6$ did not affect basal $^{45}Ca^{2+}$ influx (Fig. 6) or $^{45}Ca^{2+}$ influx in the presence of MK-801 (data not shown).

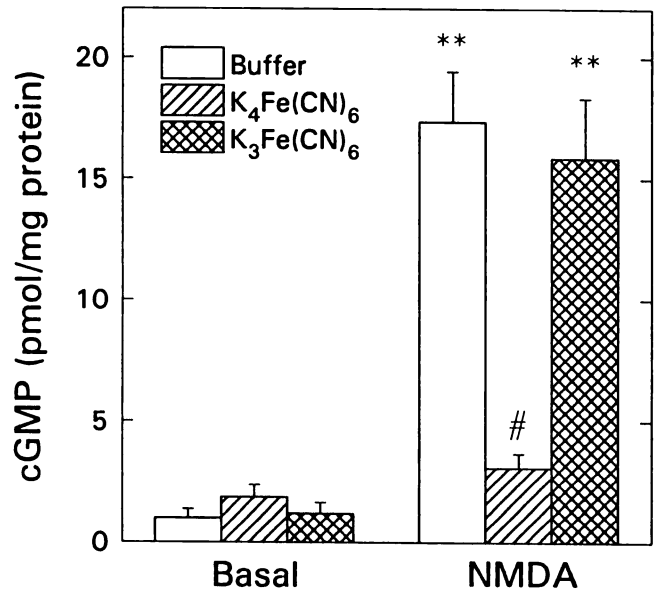

Fig. 5. Effect of NMDA on cGMP accumulation in cerebellar granule neurons that had been treated with $K_4Fe(CN)_6$ or $K_3Fe(CN)_6$. Cells were incubated with $K_4Fe(CN)_6$ or $K_3Fe(CN)_6$ (both at 300 μM) for 10 min and then in the absence or presence of NMDA (100 μM) for an additional 5 min. Data are means ± standard errors from three separate experiments. **, $p < 0.01$ versus respective basal value (Dunnett's test); #, $p < 0.01$ versus NMDA alone (Newman-Kuels test).

Discussion

Our results show that SNP inhibits NMDA-induced $^{45}Ca^{2+}$ influx in a dose-dependent manner. This effect is selective for NMDA-sensitive glutamate receptors, because SNP fails to inhibit Ca^{2+} influx evoked by kainate. Three lines of evidence indicate that this effect of SNP is independent of its ability to generate NO. First, SNAP, which, like SNP, potently stimulates cGMP accumulation via an NO-dependent mechanism, fails to inhibit $^{45}Ca^{2+}$ influx evoked by NMDA. Second, $K_4Fe(CN)_6$, which structurally resembles SNP but is devoid of the ability to generate NO and to activate guanylate cyclase, also inhibits NMDA-evoked $^{45}Ca^{2+}$ influx. Third, if NO inhibits NMDA receptors, then the NO synthase inhibitor N^G-nitro-L-arginine would be expected to potentiate NMDA-evoked $^{45}Ca^{2+}$ influx; however, this is not observed.

Because both SNP and $K_4Fe(CN)_6$ contain Fe(II) (20), one might argue that the oxidation state of iron in the prusside ion is critical for these agents to exert their inhibitory effects on NMDA-induced $^{45}Ca^{2+}$ influx. Indeed, $K_3Fe(CN)_6$ [which contains Fe(III)] failed to inhibit NMDA-evoked $^{45}Ca^{2+}$ influx. However, ferrocyanide ions exert their effect as an entity, because Fe^{2+} ions alone, when present at the same concentration as the effective doses of SNP or $K_4Fe(CN)_6$, failed to inhibit NMDA-evoked $^{45}Ca^{2+}$ influx.

We have shown that SNP and $K_4Fe(CN)_6$ inhibit NMDA receptor-mediated $^{45}Ca^{2+}$ influx specifically and that the iron oxidation state is important for their inhibitory action. These results might suggest that the mechanism of action of SNP and $K_4Fe(CN)_6$ is related to a modification in the redox state of the NMDA receptor. The redox state of the NMDA receptor has been proposed as a possible factor that modulates NMDA receptor activity (23, 24). In this regard, reducing agents, such as dithiothreitol, potently increase NMDA-evoked $^{45}Ca^{2+}$ influx (23). Our results show, however, that $K_4Fe(CN)_6$, which contains a reduced iron atom, inhibits, rather than increases, the NMDA-induced Ca^{2+} influx.

Hemoglobin counteracted the inhibition of NMDA-induced $^{45}Ca^{2+}$ influx not only by SNP but also by $K_4Fe(CN)_6$, which does not generate NO. However, 10 μM hemoglobin failed to

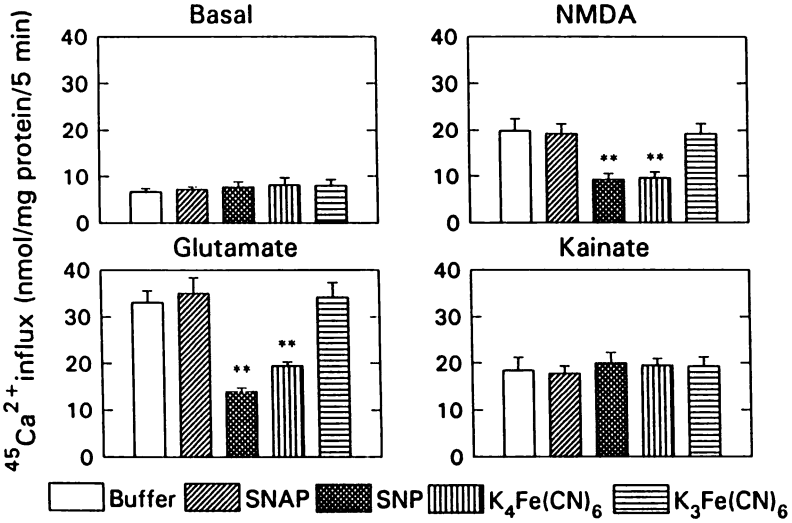

Fig. 6. Effects of SNP, SNAP, $K_4Fe(CN)_6$, and $K_3Fe(CN)_6$ on $^{45}Ca^{2+}$ influx induced by NMDA, glutamate, and kainate in cerebellar granule neurons. Cells were treated with SNP, SNAP, $K_4Fe(CN)_6$, or $K_3Fe(CN)_6$ (all at 300 μM) for 10 min and then stimulated with NMDA (100 μM), glutamate (50 μM), or kainate (100 μM) for an additional 5 min. The effect of kainate was studied in the presence of MK-801 (1 μM) to block indirect activation of NMDA receptors. MK-801 did not affect basal $^{45}Ca^{2+}$ influx. Data are means ± standard errors from three to five separate experiments. **, $p <$ 0.01 versus CSS-treated control (Dunnett's test).

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counteract the action of 100 μM SNP (Fig. 4A), whereas at concentrations as low as 1 μM hemoglobin strongly inhibits the NO-mediated actions of 100 μM SNP (21). Thus, it seems that the effect of hemoglobin reported here cannot be attributed to the complexing of NO released from SNP. It has been reported recently that serum albumin greatly potentiates calcium-dependent NMDA-evoked toxicity (25). Hemoglobin might potentiate NMDA-evoked calcium influx in a similar way as serum albumin. The inhibition of NMDA receptors by SNP and $K_4Fe(CN)_6$ and the permissive action of hemoglobin may reflect the importance of the redox state of the NMDA receptor. The understanding of the functional significance of these effects requires further studies.

We have shown that, in intact granule cells, $K_4Fe(CN)_6$, but not $K_3Fe(CN)_6$ inhibited NMDA-induced cGMP accumulation. In contrast, cGMP production by guanylate cyclase in cell homogenates has been shown to be inhibited by $K_3Fe(CN)_6$ but not by $K_4Fe(CN)_6$ (26). Together, these data suggest that in our experiments $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ did not penetrate into the cells, but instead $K_4Fe(CN)_6$ exerted its effect extracellularly. The cGMP accumulation elicited by excitatory amino acids in cerebellar granule cells depends on the presence of extracellular Ca^{2+} (27), which reflects the fact that NO synthase is Ca^{2+}/calmodulin dependent (28). It appears that $K_4Fe(CN)_6$ inhibits NMDA-evoked Ca^{2+} influx and thereby prevents the activation of NO synthase and the NO-mediated stimulation of soluble guanylate cyclase. In fact, we have observed that NMDA fails to stimulate NO synthase in cerebellar granule cells that have been treated for 10 min with SNP or $K_4Fe(CN)_6$ (29).

The possibility that SNP and $K_4Fe(CN)_6$ inhibit NMDA-evoked Ca^{2+} influx at the level of the receptor recognition site is further supported by the recent observation that SNP inhibits glutamate binding to NMDA receptors (30). The reported potency of SNP in inhibiting NMDA-specific [^{3}H]glutamate binding was similar to that determined for the inhibition of NMDA-induced $^{45}Ca^{2+}$ influx. Moreover, the inhibition of [^{3}H] glutamate binding by SNP was counteracted by hemoglobin, as was the inhibition of NMDA-induced $^{45}Ca^{2+}$ influx in our study.

We have recently shown that treatment of cerebellar granule cells with SNP and $K_4Fe(CN)_6$ protects these neurons against NMDA- and glutamate-evoked neurotoxicity (13). The present study illustrates the NO-independent mechanism by which this protection is achieved. In contrast, SNP was shown to be neurotoxic in primary cultures from cerebral cortex of fetal rats, and this toxicity appears to be mediated by NO (31). However, in these cortical cultures a very small number of neurons (1%) contain NO synthase, and these neurons are resistant to NO toxicity (32). It appears that cells that are highly specialized for NO production, such as cerebellar granule cells, have also developed a resistance to NO toxicity. Moreover, the ability of NO to produce toxicity could be determined developmentally. Thus, earlier maturating cortical neurons may be more sensitive to NO than cerebellar granule neurons. Neuronal death occurs physiologically during ontogeny of the nervous system (33), and it may be speculated that selective sensitivity of certain neurons to NO may be an important factor in determining cell survival and death during development.

In conclusion, SNP blocks NMDA-evoked Ca^{2+} influx by an NO-independent mechanism that is mediated by the ferrocyanide portion of the molecule. SNP can thus no longer be considered as a specific tool for mimicking the action of endogenously produced NO. Furthermore, blockade of the effects of SNP by hemoglobin cannot automatically be interpreted as meaning that the phenomenon under study is mediated by NO. The properties of SNP reported here should also be taken into consideration with regard to the use of SNP in human therapy.

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