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Zinc protoporphyrin IX suppresses nitric oxide production through a loss of L-arginine in rat cerebellar slices

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Abstract

Zinc protoporphyrin IX (ZnPP) inhibits production of carbon monoxide (CO) but not nitric oxide (NO) synthase activity in vitro, therefore, it has been used to discriminate the effects of these activators of cyclic GMP production. However, intracellular enzyme activity is supported by endogenous substrates and cofactors, therefore, in vitro experiments with an optimal supply of these substances do not necessarily represent the intracellular circumstances. The present study was conducted to examine whether ZnPP affects NO production in rat cerebellar slices. Incubation with 1-aminocyclopentane-trans-1,3-dicarboxylic acid (ACPD) increased the conversion of [^{3}H]-L-arginine into [^{3}H]-citrulline in the slices. ZnPP at 1 μM, not only suppressed the ACPD-dependent production of [^{3}H]-citrulline but also reduced the basal [^{3}H]-citrulline level, accumulation of [^{3}H]-L-arginine and ATP content in the slices, but ZnPP at 1 μM did not affect the catalytic activity of partially purified NO synthases in vitro. In contrast, a selective inhibitor of NO synthase, N$^\omega$-nitro-L-arginine, inhibited ACPD-dependent [^{3}H]-citrulline production without affecting the basal [^{3}H]-citrulline level or [^{3}H]-L-arginine accumulation. These results indicate that ZnPP suppresses NO production through a loss of its precursor, even at concentrations exhibiting no inhibition of the catalytic activity of NO synthase. It is concluded that ACPD activates NO synthase in the slice and that ZnPP cannot discriminate the roles of CO from NO in cyclic GMP production in brain slices.

Keywords: Zinc protoporphyrin IX; Nitric oxide synthase; L-arginine; Carbon monoxide; Intracellular ATP; N$^\omega$-nitro-L-arginin

1. Introduction

Both nitric oxide (NO) and carbon monoxide (CO) are endogenous activators of soluble guanylyl cyclase (sGC), a major enzyme catalyzing the production of cyclic GMP (Murad et al., 1978; Verma et al., 1993). NO synthase (NOS) produces citrulline and NO, the most potent sGC activator, by oxidation of L-arginine (for a review, see Marletta, 1993), while CO is 50-fold less potent than NO as a sGC activator (Kharitonov et al., 1995) and believed to be a coproduct of heme degradation by hemeoxygenase-2 (HO2) in the brain (Verma et al., 1993). Since the cerebellar cortex contains both NOS (Bredt et al., 1990) and HO2 (Verma et al., 1993), it is necessary to determine which of the two sGC activators mediates an agonist-induced cyclic GMP production in the cerebellum. For this aim, specific inhibitors of NOS and HO2 are often used. N$^\omega$-nitro-L-arginine (NNA) is known to inhibit constitutive NOS selectively through competition with L-arginine (Furfine et al., 1993). Indeed, Okada (1995) observed that NNA inhibited NOS activity in cerebellar homogenates with an IC_{50} concentration about 0.5 μM. NNA also blocked cyclic GMP production following incubation of rat cerebellar slices with a specific agonist

Abbreviations: ACPD, 1-aminocyclopentane-trans-1,3-dicarboxylic acid; CO, carbon monoxide; DMSO, dimethylsulfoxide; HO2, hemeoxygenase-2; NNA, N$^\omega$-nitro-L-arginine; NOS, nitric oxide synthase; sGC, soluble guanylyl cyclase; ZnPP, zinc protoporphyrin IX.

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of the metabotropic glutamate receptor, (±)1-aminocyclopentane-trans-1,3-dicarboxylic acid (ACPD), showing the involvement of NOS in ACPD-dependent cyclic GMP production (Okada, 1992). On the other hand, zinc protoporphyrin IX (ZnPP) has been used as a pharmacological reagent which selectively blocks HO2 but not NOS, since ZnPP at 1–10 μM inhibited HO2 activity in nasal epithelium homogenates (Verma et al., 1993) but not NOS in the cytosolic fraction prepared from hippocampal homogenates (Meffert et al., 1994).

Previous studies, however, have cast doubt on the specificity of ZnPP. ZnPP can interact with heme proteins such as NOS (Klatt et al., 1992) and sGC (Gerzer et al., 1981) by displacing the protein-bound heme group (Luo and Vincent, 1994). Indeed, ZnPP inhibited purified sGC in vitro with K_i at 50 nM (Ignarro et al., 1984), and in rat cerebellum in vivo (Luo and Vincent, 1994). ZnPP at high concentrations only, (over 50 μM) inhibited NOS in cytosolic fraction in vitro (Meffert et al., 1994), however, it does not necessarily ensure the ineffectiveness of ZnPP at lower concentrations (1–10 μM) on NO production in tissue. Intracellular enzyme activity is supported by endogenous substrates and cofactors. The results obtained from in vitro experiments with optimal conditions of substrates and cofactors available do not necessarily represent the intracellular circumstances. Nathanson et al. (1995) found activation of the Na^+-K^+ pump by CO in cerebellar Purkinje cells, suggesting the possibility that ZnPP through HO2 inhibition affects cellular membrane potentials, which may largely affect intracellular biochemical homeostasis.

The present study was conducted to examine whether ZnPP affects NO production in rat cerebellar slices. The results revealed that ZnPP at 1 μM considerably reduced L-arginine, citrulline and ATP contents in the slices, but the same concentration of ZnPP did not significantly inhibit the activity of partially purified NOS in vitro. Thus, ZnPP appears to interfere with NO synthesis through components other than NOS.

2. Methods

2.1. NOS assay in slice

Parasagittal slices of cerebellar vermis, 400 μm in thickness, were prepared from adult (7–10 week old) male Wistar-ST rats as described previously (Okada, 1995). A slice weighed 13 mg (wet weight) and contained 1.4 mg of total protein, on average. After a 90-min recovery period, a slice was incubated with 111 kBq of [^{3}H]-L-arginine (Amersham) for 45 min in a test tube containing 1 ml of Krebs-bicarbonate buffer composed of 120 mM, NaCl; 2 mM, KCl; 1.2 mM, $MgCl_2$; 2 mM, $CaCl_2$; 1.2 mM, KH_2PO_4; 26 mM, $NaHCO_3$; and 11 mM, glucose, and supplied with 5% CO_2 + 95% O_2 at 34°C. The slice was then incubated with 1 mM ACPD (Tocris Cookson, UK) for 5 min. NNA (Sigma) at 30 μM, 0.1% (v/v) dimethylsulfoxide (DMSO) (Sigma) or 1 μM ZnPP (Aldrich) in 0.1% DMSO was added 15–20 min prior to the addition of ACPD. DMSO concentration was 0.1% in all experiments unless otherwise stated. Slices were washed and homogenized in cold 20 mM HEPES-NaOH buffer at pH 5.5 containing 2 mM EDTA. To measure the total tritium, radioactivity in aliquots of the homogenate was counted. For the NOS assay, [^{3}H]-citrulline in the homogenate was separated from [^{3}H]-L-arginine by Dowex AG50W-X8 (Na^+) and counted as described previously (Okada, 1996).

2.2. In vitro NOS assay

NOS was partially purified with 2′5′-ADP-agarose (Sigma) and assayed in vitro. A cerebellum was homogenized with a polytron-type homogenizer (Ika, Japan) in 1 ml of 50 mM Tris-acetate buffer at pH 7.4 containing 1 mM EDTA, 1 mM EGTA, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 0.1 mg/ml of phenylmethanesulfonylfluoride and 1.4 μM pepstatin A. The homogenate was centrifuged at $10^5 \times g$ for 1 h at 4°C. The supernatant was agitated in the presence of 5 mg 2′5′-ADP-agarose for 15 min. The resin was packed onto a glass filter cartridge (Whatman) and washed with 5 ml of buffer A (50 mM Tris-acetate buffer at pH 7.4 containing 1 mM EDTA and 1 mM EGTA) containing 0.5 M NaCl, then with 3 ml of buffer A. NOS was eluted with 0.6 ml of buffer A containing 10 mM NADPH (Sigma). NOS assay was initiated by the addition of 10 μl of 50 mM HEPES-NaOH buffer at pH 7.4 containing 3 mM $CaCl_2$, 5 μM L-arginine, 10 kBq of [^{3}H]-L-arginine, 1 mM NADPH, 3 μM FAD (Sigma), 300 nM calmodulin (bovine, Seikagaku Kogyo, Japan) and 3 μM (6R)-5,6,7,8-tetrahydrobiopterin (RBI, USA) and the mixture (20 μl) was incubated at 25°C for 5 min. The reaction was terminated by the addition of 0.8 ml of 20 mM HEPES-NaOH buffer at pH 5.5 containing 2 mM EDTA and analyzed as described above.

2.3. Arginine accumulation

Slices were incubated in Krebs-bicarbonate buffer containing 1 μM ZnPP or 0.1% DMSO at 34°C for 15 min, then 111 kBq of [^{3}H]-L-arginine was added. After various incubation periods, slices were washed in cold Krebs-bicarbonate buffer three times, then homogenized in 1 ml of 0.1 M NaOH. After 30 min, the homogenate was centrifuged and the radioactivity in the supernatant was counted as described by Schmidt et al. (1995).

2.4. ATP measurement

A slice incubated in Krebs-bicarbonate buffer containing 1 μM ZnPP or 0.1% DMSO at 34°C for 30 min was homogenized in 0.4 ml of cold 50 mM Tris-acetate buffer at pH 7.4 containing 5 mM EDTA and the homogenate was centrifuged at $10^4 \times g$ for 1 min. ATP in the supernatant was analyzed by HPLC after filtration with a cellulose acetate filter as described previously (Okada, 1995). Briefly, ATP was separated by a TSK-DEAE2SW (4 × 250 mm: Toso, Japan) column and eluted isocratically with 70 mM potassium phosphate buffer at pH 7.0 containing 0.1 mM EDTA and 20% acetonitrile with a flow rate of 0.8 ml/min at 22°C, and detected by absorption at 259 nm. Complete separation of ATP was confirmed by digestion with hexokinase (Sigma). A standard curve for ATP quantification was constructed using known concentrations of authentic ATP (Sigma). Protein concentration was determined using a BCA protein assay kit (Pierce). Data were statistically analyzed using the unpaired t-test.

3. Results

Fig. 1 shows that incubation of rat cerebellar slices with ACPD increased the conversion of the preloaded [^{3}H]-L-arginine into [^{3}H]-citrulline to 136% of the control level. Incubation with 30 μM NNA, added 15 min prior to the agonist, inhibited the increase without affecting the basal citrulline level. These results confirm the previous observations that metabotropic glutamate receptor mediates NOS activation in cerebellar slices (Okada, 1992, 1995). Fig. 1 also shows the effects of ZnPP on [^{3}H]-citrulline counts. First, ZnPP at 1 μM considerably reduced the basal [^{3}H]-citrulline counts. The average [^{3}H]-citrulline count in the slices incubated with 0.1% (v/v) DMSO, the ZnPP vehicle, was 23814 ± 2132 cpm (mean ± S.E.M.), whereas that incubated with ZnPP was 10038 ± 1689 cpm. DMSO at 0.1% did not affect the basal [^{3}H]-citrulline counts. Second, ACPD no longer increased [^{3}H]-citrulline counts in the slices incubated with 1 μM ZnPP. DMSO at 0.1% did not affect the ACPD-dependent increase in [^{3}H]-citrulline (Okada, 1995). Inhibition of the ACPD-dependent [^{3}H]-citrulline increase may suggest NOS inhibition by ZnPP. However, the reduction in the basal [^{3}H]-citrulline suggested loss of intracellular [^{3}H]-compounds. These possibilities were further studied.

To evaluate the possible involvement of NOS inhibition by ZnPP in the reduction in [^{3}H]-citrulline, the catalytic activity of partially purified NOS was assayed in the presence of ZnPP in vitro. As shown in Fig. 2, ZnPP at concentrations higher than 5 μM significantly inhibited NOS activity, but the inhibition at 1 μM was not significant and too small to explain the reduction in the citrulline counts shown in Fig. 1. Activity of the partially purified NOS was not affected by DMSO at 0.1% (v/v) (104% of the control level), however, it was reduced to 82 and 73% of the control level by 1 and 5% DMSO, respectively. These results showed that ZnPP at 1 μM did not inhibit the catalytic activity of the partially purified NOS in vitro, and it seemed unlikely that NOS inhibition by ZnPP could account for the [^{3}H]-citrulline reduction.

A possible loss of intracellular [^{3}H]-L-arginine by ZnPP was examined by measuring the total tritium counts in the slices. Fig. 3A shows that NNA, ACPD and DMSO did not significantly affect the total tritium counts in the slices, whereas incubation with ZnPP at 1 μM for 15 min markedly reduced the total tritium counts from 140453 ± 17312 cpm to 69030 ± 10684 cpm. Fig. 3B shows the time-dependent accumulation of the tritium counts carried by [^{3}H]-L-arginine in the slices. Preincubation with 1 μM ZnPP for 15 min did not affect the [^{3}H]-L-arginine accumulation during the initial 5 min, however thereafter, it considerably reduced the tritium accumulation. These results suggest that the reduction in the basal [^{3}H]-citrulline shown in

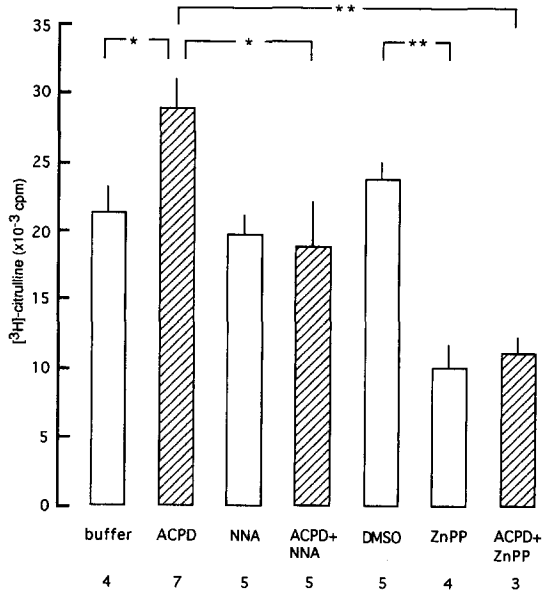

Fig. 1. Effects of NNA and ZnPP on NOS activation by ACPD in cerebellar slices. Cerebellar slices were incubated with [^{3}H]-L-arginine for 45 min before addition of ACPD. NNA, ZnPP or DMSO was added 15 min prior to the addition of ACPD. ACPD significantly activated NOS in the slice as indicated by increase in [^{3}H]-citrulline production (comparison of buffer and ACPD, significant at $P < 5\%$ as indicated by *). NNA blocked the effect of ACPD (comparison of ACPD + NNA and ACPD, $P < 5\%$) without affecting the basal level (comparison of buffer and NNA). ZnPP at 1 μM significantly reduced the basal level of citrulline (comparison of DMSO and ZnPP, significant at $P < 0.1\%$ as indicated by **) and inhibited the ACPD-induced increase (comparison of ACPD and ACPD + ZnPP, $P < 0.1\%$). The numbers below columns indicate the number of slices tested. The vertical bars are S.E.M.

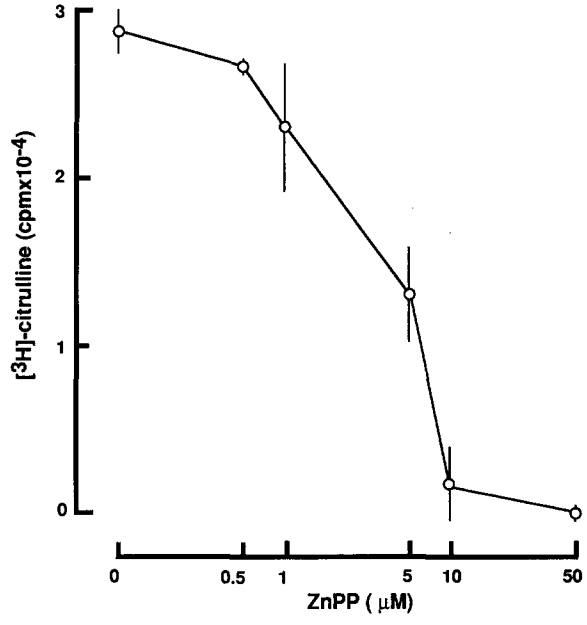

Fig. 2. Dose dependency of NOS inhibition by ZnPP. Partially purified NOS was assayed in vitro in the presence of the various concentrations of ZnPP. The concentration of DMSO was 0.1% (v/v). Data points are the mean of three experiments. Vertical bars indicates S.E.M.

Fig. 1 seems to be due to loss of intracellular [^{3}H]-L-arginine.

HPLC analysis revealed that the intracellular ATP content was also reduced by 1 μM ZnPP. Slices incubated with 1 μM ZnPP for 30 min contained only 10.5 ± 1.4 nmol ATP/mg protein (mean $\pm$ S.E.M., $n = 5$), whereas the control slices incubated with DMSO contained 20.5 ± 2.8 nmol ATP/mg protein ($n = 8$). According to Lipton and Whittingham (1984) this value found in the control slices is about 2/3 of the ATP content found in the guinea pig hippocampus in vivo. Considering the fact that cells on the cut surfaces of slices are ruptured, this value observed in slices with 400 μm thickness seems to match the in vivo data. These data suggest that 1 μM ZnPP significantly ($P <$ 1%) reduces the ATP content of the slice and causes a general loss of intracellular compounds.

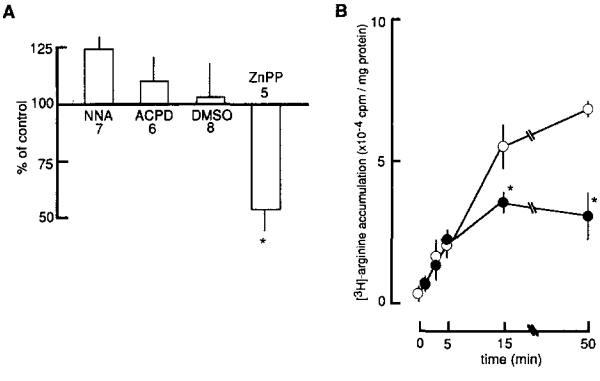

Fig. 3. (A) ZnPP reduced total tritiated compounds in the slice. Cerebellar slices were incubated with [^{3}H]-L-arginine for 45 min. NNA (30 μM), DMSO (0.1% v/v) or ZnPP (1 μM) in 0.1% DMSO was added 15 min before withdrawal. ACPD was added 5 min before withdrawal. Then, the total tritium in the homogenate was counted. The tritium count in control slices (incubated with buffer alone) was 125720 ± 6094 cpm/slice (mean $\pm$ S.E.M.; $n = 5$) and expressed as 100% in the ordinate. ZnPP greatly reduced the tritium counts. The numerals below the compound names represent the number of slices tested. The asterisk represents significant reduction at $P < 0.1\%$. (B) Time course of [^{3}H]-L-arginine accumulation. Slices were incubated with 0.1% (v/v) DMSO (open circles) or 1 μM ZnPP (closed circles) for 15 min, then [^{3}H]-L-arginine was added at time 0 on the abscissa. Slices were withdrawn at the indicated time, and total tritium was counted. The means of three separate experiments are shown with vertical bars representing S.E.M. The asterisks show significant reduction at $P < 1\%$.

4. Discussion

The present study revealed that although ZnPP at 1 μM did not inhibit partially purified NOS activity directly (Fig. 2), it abolished ACPD-dependent, NOS-dependent [^{3}H]-citrulline production (Fig. 1). ZnPP at 1 μM also greatly reduced the total [^{3}H]-compounds (Fig. 3) and ATP content in the slices. These results suggested that ZnPP suppress NO production through depletion of intracellular L-arginine. Inhibition of cyclic GMP production in cerebellar slices by 1 μM ZnPP, reported by Nathanson et al. (1995), can be explained by one or a combination of the following three possible causes: (1) HO2 inhibition that suppresses the CO-mediated sGC activation (Verma et al., 1993); (2) direct inhibition of sGC (Ignarro et al., 1984); or (3) depletion of intracellular L-arginine (this work). Thus, ZnPP does not allow discrimination of HO2 from NOS, even if its concentration is below those directly inhibiting NOS.

Since the radioactivity is supplied originally as [^{3}H]-L-arginine, all [^{3}H]-citrulline should be derived from L-arginine. Existence of the basal [^{3}H]-citrulline in the slice and its insensitiveness to NNA (Fig. 1) suggested basal activities converting L-arginine to citrulline, such as the urea cycle (Nakamura et al., 1990). Comparison between data in Figs. 1 and 3 indicates that the basal [^{3}H]-citrulline counts accounted for only 17 and 15% of the total tritium counts in the DMSO-treated and the ZnPP-treated slices, respectively, suggesting that the citrulline to arginine ratio in slices without stimulation by the agonist was not affected by ZnPP. These considerations suggest that although reduction in [^{3}H]-citrulline, the indicator of the assay, directly affects the NOS assay, it might be a secondary effect. In other words, although NOS activity in the slice incubated with ZnPP cannot be measured by [^{3}H]-citrulline, the conversion is likely to be suppressed due to a substantial reduction in the precursor. The loss of intracellular L-arginine indicates that NO production can no longer be expected in the slices incubated with 1 μM ZnPP.

Although actual intracellular concentration of ZnPP in the slice is not known, it is likely that hydrophobic compounds, such as ZnPP, are retained in cell membranes when applied to brain slices (Alger et al., 1984). Therefore, when a slice is incubated with 1 μM ZnPP, the actual intracellular concentration of ZnPP is likely to be lower than 1 μM, suggesting that NOS in the slice can catalyze conversion of L-arginine into citrulline, if the substrates and activators are supplied, as in in vitro experiments (Fig. 2). Meffert et al. (1994) reported that direct inhibition of NOS activity in the hippocampal cytosol required higher concentrations of ZnPP (50 μM) than shown in Fig. 2 and, Luo and Vincent (1994) failed to observe direct inhibition by ZnPP up to 100 μM. The discrepancy between these and the present reports may be attributed to the difference in NOS stability between crude and purified preparations and to endogenous L-arginine present in crude preparations.

The mechanisms underlying the depletion of intracellular compounds by ZnPP remain unclear. Two mechanisms can cause depletion of intracellular L-arginine, one is inhibition of L-arginine uptake and the other is enhancement of leakage. If ZnPP suppresses [^{3}H]-L-arginine uptake without altering the large endogenous L-arginine pool itself, the [^{3}H]-labelled fraction of any intracellular compounds will be reduced. However, this is unlikely because a substantial reduction in ATP content was also observed. It is likely that ZnPP caused a loss of these intracellular components. Therefore, cytotoxicity implied by the following mechanism may be a possible explanation for this effect of ZnPP. Due to the difference in stability and reactivity of NO and CO, it is expected that NO exerts temporally and spatially more limited effects than CO. Thus, it is likely that NO contributes to transient, stimulus-dependent production of cyclic GMP, whereas CO works tonically. Nathanson et al. (1995) found that CO activated the Na^+-K^+ pump. Considering the expected tonic nature of CO, the activity of the Na^+-K^+ pump might be tonically maintained by endogenous CO. According to this hypothesis, ZnPP would suppress the activity of the pump by reduction of the endogenous CO level, causing tonic depolarization and cell death. Thus, a cytotoxic effect of ZnPP can be expected from the specific inhibition of HO2. However, to confirm this hypothesis, it is necessary to estimate endogenous CO concentration.

In contrast to ZnPP, NNA did not affect levels of basal [^{3}H]-citrulline and total [^{3}H]-compounds in the slices. NNA inhibited NOS activity in the homogenates of cerebellar slices (Okada, 1995) and the inhibition by NNA of the ACPD-induced cyclic GMP production in the slice is reversed by excess L-arginine (Okada, 1992). These observations are consistent with the idea that NNA is a competitive inhibitor of NOS (Furfine et al., 1993). In the present study, ACPD increased [^{3}H]-citrulline production in cerebellar slices and this increase was blocked by NNA (Fig. 1), confirming the previous observation that ACPD activates NOS in cerebellar slices (Okada, 1992, 1995). NNA is known to be incorporated through L- and T-systems of amino acid transporters (Schmidt et al., 1995). To inhibit NOS in 400 μm thick cerebellar slices, NNA incubation for at least 15 min prior to agonist addition was necessary. When NNA and ACPD were added simultaneously, no inhibitory effect was observed (data not shown). This lagtime suggests that intracellular NNA accumulation is required for competitive inhibition. In other words, NNA inhibition depends on intracellular L-arginine concentration. This may account for the failure of NOS inhibition by NNA in the slices shown in some reports (Nathanson et al., 1995). Although the possible involvement of CO is not excluded, the present report further supports the notion that NO is required for ACPD-dependent cyclic GMP production in cerebellar slices.

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