Barium Activates Rat Cerebellar Nitric Oxide Synthase

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ABSTRACT— Ba^{2+} is known to pass through *N*-methyl-D-aspartate receptor channels and cause an increase in cGMP levels in rat cerebella. In the present study, we compared the activation of rat cerebellar nitric oxide synthase (NOS) by Ba^{2+} with that produced by Ca^{2+} . Both cations stimulated L-citrulline formation in the presence of calmodulin in identical fractions eluted from an anion exchange column with a salt gradient. EC_{50} values for Ca^{2+} and Ba^{2+} were 200 nM and 50 μM , respectively. The IC_{50} of *N*^G-monomethyl-L-arginine was the same (200 nM). These indicate a possible action of Ba^{2+} on NOS through the calmodulin-dependent pathway.

Keywords: Barium, Nitric oxide synthase, Calmodulin

Nitric oxide (NO) is thought to be a messenger molecule in cerebella, and the characteristics of cerebellar nitric oxide synthase (NOS) have been well-defined; activation of NOS is absolutely dependent on Ca^{2+} and calmodulin (1, 2), and this underlies the mechanism of the physiological response to *N*-methyl-D-aspartate (NMDA) in cerebella. Glutamate and NMDA release endothelium-derived relaxing factor (EDRF)-like messengers from cerebellar cells (3) increase cGMP levels (4–6) and enhance the conversion of L-arginine to L-citrulline (4, 6) through NMDA-receptor activation in cerebellar cells. This messenger is produced in a Ca^{2+} -dependent manner (3, 7).

Another alkaline earth cation, Ba^{2+} , causes a Ca^{2+} -mimetic action in NO-generating tissue. Recently, we reported that Ba^{2+} as well as Ca^{2+} relaxes canine coronary artery and increases cGMP levels (8). Electrophysiological studies with central nervous cells have demonstrated that Ba^{2+} can substitute for Ca^{2+} and pass through NMDA-receptor channels (9). Interestingly, Carter et al. (10) have shown that the NMDA-induced increase in cGMP in the cerebellum is not blocked by Ba^{2+} and persists when Ba^{2+} is substituted for Ca^{2+} . This raises the possibility that Ba^{2+} activates cerebellar NOS. To explain this phenomenon, direct evidence for Ba^{2+} -induced activation of NOS is needed, and also, it should be determined if calmodulin is required. Therefore, the present study was undertaken to investigate whether

Ba^{2+} can directly activate NOS in rat cerebella.

Cerebella of twelve male Wistar rats (150–200 g, Charles River Japan, Atsugi) were homogenized in 30 ml ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 100 mg/l phenylmethylsulfonyl fluoride, 10 mg/l leupeptin and 10 mg/l pepstatin A (Sigma, St. Louis, MO, USA). The homogenate was centrifuged at $20,000 \times g$ for 15 min at 4°C. The supernatant was loaded onto a 20-ml column of DEAE Sepharose Fast Flow (Pharmacia Biotech, Uppsala, Sweden). After the column had been washed with 50 ml buffer, a 100-ml linear gradient (0–300 mM) NaCl was applied at a speed of 1.5 ml/min. The eluate fraction was collected and stored at -80°C until use.

NOS activity was measured by monitoring the conversion of L-arginine to L-citrulline. Unless otherwise indicated, the assay mixture contained 50 mM Hepes (pH 7.4), 1 mM dithiothreitol, 1 mM NADPH, 10 μM (6R)-5,6,7,8-tetrahydro-L-biopterin, 100 nM calmodulin (from bovine brain, Sigma) and 1 mM EDTA. Ca^{2+} / Ba^{2+} -EDTA buffer was prepared according to the previously described method for computing metal ion concentrations (11). One hundred microliters of the assay mixture, 25 μl 100 nM [³H]L-arginine (NEN, Wilmington, DE, USA) and 25 μl enzyme protein were mixed and incubated for 6 min at 37°C. The enzyme reaction was terminated with a stop buffer containing 2 ml 20 mM Hepes (pH 5.5) and 6 mM EDTA. The reaction mixture was applied to a 1 ml column of Dowex AG50WX-8 (Na^{+} form; BioRad,

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Fig. 1. Partial purification of cerebellar NOS on DEAE sepharose. NOS activity was measured in the presence of Ca^{2+} and Ba^{2+} by monitoring the conversion of 100 nM [^3H]L-arginine (2.5 pmol) to [^3H]L-citrulline in the $20,000\times g$ supernatant (57.3 μg) (a) and DEAE eluate fractions (b). Data are from a representative purification. ○, Ca^{2+} (300 nM) plus calmodulin (CaM) (100 nM); ●, Ba^{2+} (100 μM) plus CaM (100 nM); □, Ca^{2+} (300 nM), CaM-free; ■, Ba^{2+} (100 μM), CaM-free. Western blot analysis is shown for the supernatant (S) and DEAE effluent (I, II and III) samples using rabbit anti-brain NOS antibody.

Hercules, CA, USA) before [^3H]citrulline (NEN) was eluted with 4 ml water. The eluate was collected and counted by a liquid scintillation spectrometer (Beckman, Palo Alto, CA, USA) after addition of a scintillation cocktail (Aquasol-2, NEN). The protein content was measured by the Bio-Rad assay with bovine serum albumin as the standard. Results are expressed as means $\pm$ S.E.M.

In crude rat cerebellar supernatant, Ca^{2+} (300 nM) and Ba^{2+} (100 μM) increased [^3H]citrulline formation (Fig. 1a). The activity of Ba^{2+} was approximately 65% that of Ca^{2+} . The crude cerebellum supernatant was loaded onto a DEAE-Sepharose column. When the column was washed with buffer, the effluent showed no L-citrulline forming activity during concomitant incubation with Ca^{2+} or Ba^{2+} . Thus, the enzyme appeared to have adhered to the DEAE column (data not shown). However, the eluate obtained by NaCl gradient (0–300 mM) elution could catalyze Ca^{2+} - and Ba^{2+} -induced citrulline formation in the presence of calmodulin (Fig. 1b). Both Ca^{2+} - and Ba^{2+} -induced activities eluted in the same fraction, which contained approximately 120 mM NaCl. The peak

activity of Ba^{2+} was approximately 65% that of Ca^{2+} . When calmodulin was omitted from the assay mixture, the activity in the DEAE eluate was lost (Fig. 1b), whereas activity still remained in the crude fraction because of the presence of endogenous calmodulin (Fig. 1a).

Samples of the crude supernatant fraction and fractions from the DEAE column were denatured with sodium dodecyl sulfate, electrophoresed on a 7.5% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane and immunoblotted with polyclonal (rabbit) anti-brain NOS antibody (Affinity BioReagents, Neshanic Station, NJ, USA) and HRP-conjugated donkey anti-rabbit IgG (Amersham, Arlington Heights, IL, USA). A single band (approximately 160 kDa) was recognized in the crude fraction (S) and the peak fraction (II) of the DEAE eluate, but not in the fractions that exhibited little L-citrulline formation (Fig. 1b). These findings indicate that the Ba^{2+} and Ca^{2+} can enhance the formation of L-citrulline from L-arginine using an identical NOS.

Ca^{2+} - and Ba^{2+} -induced L-citrulline formation (NOS

Fig. 2. Ca^{2+} , Ba^{2+} /calmodulin (CaM) dependency of partially purified cerebellar NOS. **a:** CaM dependency of the NOS activation by Ca^{2+} (300 nM) and Ba^{2+} (100 μM). Data are the means of three separate experiments with S.E.M. ▨, CaM-free; □, CaM (100 nM); ■, CaM (100 nM) plus calmidazolium (100 nM). **b:** Concentration-dependency on Ca^{2+} / Ba^{2+} . NOS activity was assayed with the DEAE eluate fraction ($17.9 \pm 2.0 \mu\text{g}$) containing the major activity peak and is expressed as a percentage of the maximal activity stimulated by Ca^{2+} (300 nM or 1 μM). CaM (100 nM) was present. Data are the means of four separate experiments with S.E.M. ○, Ca^{2+} ; ●, Ba^{2+} ; □, EDTA only.

activity) in the peak DEAE effluent fraction was dependent on calmodulin, and it was completely inhibited by the calmodulin inhibitor calmidazolium (100 nM, Sigma) (Fig. 2a). Ca^{2+} and Ba^{2+} increased NOS activity in a concentration-dependent manner (Fig. 2b). The EC_{50} value for Ca^{2+} was 200 nM, similar to those obtained in previous studies (1, 2). The EC_{50} for Ba^{2+} was 50 μM , which is 250 times higher than that for Ca^{2+} . The maximal activity of Ba^{2+} was approximately 70% that of Ca^{2+} . The NOS inhibitor N^G -monomethyl-L-arginine (L-NMMA; donated by Tanabe Seiyaku, Toda) inhibited the NOS-activating effect of both cations in a concentration-dependent manner in four separate experiments. More than 3 μM L-NMMA completely abolished the activity of both Ca^{2+} (300 nM) and Ba^{2+} (100 μM). The IC_{50} (200 nM) of L-NMMA for Ba^{2+} was the same as that for Ca^{2+} .

Stimulation of NMDA receptors has been shown to increase cGMP levels in the presence of Ba^{2+} as a replacement for Ca^{2+} (10). This raises the possibility that Ba^{2+} may enter into the cerebellar cells and activate NOS, leading to an increase in cGMP levels. The present study aimed to provide substantial evidence for Ba^{2+} -induced activation of NOS. To this end, we used the conversion of [^3H]L-arginine to [^3H]L-citrulline as a measure of the NOS

activity in rat cerebella. The present crude and partially purified NOS preparation requires the addition of Ca^{2+} to the reaction mixture for activity. This agrees well with the results of previous studies using constitutive NOS from the cerebellum (1, 2). The requirement of Ca^{2+} for NOS activation in the crude homogenate indicates that EDTA effectively chelates endogenous Ca^{2+} so that free Ca^{2+} present cannot increase NOS activity.

Ba^{2+} also increased NOS activity in the crude preparation. However, since much higher concentrations of Ba^{2+} are needed (see below), care should be taken to avoid the possibility of contamination by free Ca^{2+} since the Ca^{2+} bound to the chelator may be displaced by Ba^{2+} . In the partially purified NOS preparation, which excludes any interference by endogenous divalent cations, Ba^{2+} as well as Ca^{2+} increased L-citrulline formation in a concentration-dependent manner. The elution pattern of Ba^{2+} -induced L-citrulline formation was, however, similar to that of Ca^{2+} -induced formation. Immunoblotting analysis showed that the fraction exhibiting the highest activity with both cations included neuronal constitutive NOS, suggesting that L-citrulline formation may be due to the activation of this type of NOS. In addition, Ba^{2+} -activated NOS was blocked by a NO synthesis inhibitor, L-NMMA, at the same concentration required for inhibi-

tion of Ca^{2+} -activated NOS. These results afford evidence that Ba^{2+} activates an almost identical NOS to that which can be activated by Ca^{2+} .

The effective concentration range for Ba^{2+} was 250 times higher than that for Ca^{2+} . The maximal activation by Ba^{2+} was 50–70% of the maximal activation by Ca^{2+} . This may be due to the different affinities of these cations for calmodulin, which is known to play a crucial role in the activation of NOS (1). Previous studies have shown that Ba^{2+} , which had a lower affinity for calmodulin (12), was less effective than Ca^{2+} in eliciting phosphodiesterase activity (12) and phosphorylation of myosin light chain kinase (13). In the present study, the activation of NOS depended completely on the presence of calmodulin and was abolished by the calmodulin inhibitor calmidazolium. Therefore, the affinity of these divalent cations for calmodulin could markedly affect their NOS activation capabilities.

Our results do not directly address the issue of whether the extracellular Ba^{2+} concentration, 2.5 mM, used in the previous study (10) is sufficient to activate intracellular NOS during the stimulation of NMDA receptors. The correlation of a known extracellular concentration with the eventual intracellular one does not appear to be the same between Ca^{2+} and Ba^{2+} , because they are taken up into intracellular stores or removed to the extracellular spaces in different ways. The differences in handling these cations have been discussed in previous studies concerning the calmodulin-dependent contraction of smooth muscle (13) and Ca^{2+} -ATPase in human erythrocytes (14). In these experiments Ba^{2+} was shown to accumulate readily inside cells. To determine any quantitative relationship between the required concentration of Ba^{2+} to affect NOS activity and the extracellular concentration required for the physiological response, further studies of the accumulation of Ba^{2+} inside NOS-containing cells are needed. Although the physiological significance of the Ba^{2+} -induced NOS activation is yet unknown, the present results, at least in part, explain some interesting phenomena: the Ba^{2+} -induced increase in cGMP in rat cerebellum (10) and the Ba^{2+} -induced endothelium-dependent relaxation in canine coronary arteries (8).

In conclusion, Ba^{2+} can activate cerebellar NOS in a calmodulin-dependent manner. This may lead to an increase in cGMP in the cerebellum stimulated by NMDA in the presence of Ba^{2+} . The present study provides evidence of a possible action of Ba^{2+} on the calmodulin-dependent pathway.

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