Nitric oxide synthase in ferret brain: localization and characterization

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1 In the present study, we have investigated the distribution of nitric oxide synthase in the ferret brain. Nitric oxide synthase was determined biochemically and immunochemically.

2 In the rat brain, the highest nitric oxide synthase activity has been detected in the cerebellum. However, in the ferret brain, the highest activity was found in the striatum and the lowest in the cerebellum and cerebral cortex. The enzymatic activity was localized predominantly in the cytosolic fractions, it was dependent on NADPH and Ca^{2+} , and inhibited by N^G -nitro-L-arginine or N^G -methyl-L-arginine.

3 Western blot analysis revealed that all regions of the ferret brain contained a 160 kD protein crossreacting with an antibody to nitric oxide synthase purified from the rat cerebellum, and the levels of relative intensity of staining by the antibody correlated with the distribution of nitric oxide synthase activity.

4 These results indicate that the ferret brain contains a nitric oxide synthase similar to the rat brain, but the distribution of enzymatic activity in the ferret brain differs markedly from the rat brain.

Keywords: Nitric oxide; nitric oxide synthase; arginine; citrulline; guanylyl cyclase; cyclic GMP

Introduction

It has been recognized that nitric oxide (NO) is formed in the central nervous system (Garthwaite *et al.*, 1988; Knowles *et al.*, 1989) as well as in many other cell types (Ishii *et al.*, 1989; Förstermann *et al.*, 1991b) and may play an important role in neuronal signal transduction (Garthwaite, 1991; Bredt & Snyder, 1992). Recently, NO synthase has been purified from the rat cerebellum (Bredt & Snyder, 1990; Schmidt *et al.*, 1991), and the regional distribution of the enzymatic activity in the rat brain has been shown (Förstermann *et al.*, 1990). In the rat brain, the highest NO synthase activity was found in the cerebellum. Furthermore, immunohistochemical mapping of NO synthase in the rat brain has recently been reported (Bredt *et al.*, 1991). These facts suggest that NO synthase exists in neuronal cells in the brain and may be closely related to their physiological functions. Therefore, it seems important to know whether the distribution of NO synthase in the brain is the same in other mammals. Here we show that there is a different regional distribution of NO synthase activity in the ferret brain.

Methods

Dissection of ferret brain

Male ferrets (1.1–1.5 kg) were killed by intraperitoneal injection of an overdose of sodium pentobarbitone (more than 60 mg kg⁻¹). The brains were removed immediately and dissected on ice into seven different regions. The regions were as follows: olfactory bulb, medulla oblongata (including pons), cerebellum, hippocampus, midbrain (including thalamus and hypothalamus), striatum and cerebral cortex. The brain parts were frozen in liquid nitrogen and weighed. Subsequently, they were homogenized in five volumes (w/v) of homogenization buffer (Tris/HCl 50 mM, pH 7.4) containing EDTA (0.1 mM), EGTA (0.1 mM), β -mercaptoethanol

(12 mM), leupeptin (1 μM), pepstatin A (1 μM), phenylmethylsulphonyl fluoride (1 mM). Protein concentrations were determined according to Bradford (Bradford, 1976) with bovine serum albumin used as a standard.

Measurement of NO synthase activity

NO synthase activity was determined in crude homogenates of different regions by measuring the formation of [³H]-L-citrulline from [³H]-L-arginine as previously described (Bredt & Snyder, 1990; Schmidt *et al.*, 1991). Briefly, crude homogenates of each region (approximately 100 μg protein) were incubated in the presence of [³H]-L-arginine (10 μM , 5 GBq mmol⁻¹), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (BH_4 , 3 μM) and Ca^{2+} (2 mM) in a total volume of 100 μl . After 20 min incubation at 25°C, the reaction was stopped by the addition of 1 ml of HEPES buffer (20 mM, pH 5.5) containing EDTA (2 mM) and EGTA (2 mM). The incubations were then applied to 1 ml Dowex AG 50WX-8 columns (Na^+ form, Bio-Rad) and the eluted [³H]-L-citrulline was measured by liquid scintillation counting. In some experiments, crude homogenates were incubated in the absence of NADPH, in the presence of EGTA (1 mM; without Ca^{2+}) or in the presence of N^G -nitro-L-arginine (L-NNA, 100 μM) or N^G -methyl-L-arginine (L-NMA, 100 μM).

In further experiments, NO synthase activity in subcellular fractions was analyzed. Crude homogenates were centrifuged (150,000 g, 1 h, 4°C), and cytosolic and particulate fractions were collected. The particulate fractions were incubated with 1 M KCl in homogenization buffer for 5 min at 4°C to remove loosely bound cytosolic proteins, and after centrifugation (150,000 g, 30 min, 4°C), the particulate fractions were resuspended in the homogenization buffer, as described previously (Förstermann *et al.*, 1991a). NO synthase activity in the cytosolic and KCl-washed particulate fractions was then determined by the [³H]-L-citrulline formation assay.

NO synthase activity in the cytosolic fractions was also determined by its stimulating effect on soluble guanylyl cyclase of cultured rat foetal lung fibroblasts (RFL-6 cells). Because contamination of the cytosolic fractions with haemo-

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globin prevents the detection of NO synthase activity, the cytosolic fractions were treated with 10% (v/v) phosphocellulose (in potassium phosphate 2 mM, pH 6.7, containing 100 μ M EDTA and 10% glycerol) for 30 min at 4°C to remove haemoglobin, as previously described (Förstermann *et al.*, 1990). After the incubation, the cytosolic fractions were separated from phosphocellulose by centrifugation, and used in the subsequent assay. The accumulation of guanosine 3',5'-cyclic monophosphate (cyclic GMP) in RFL-6 cells was used to measure NO synthase activity, as described previously (Ishii *et al.*, 1989; 1991). Briefly, RFL-6 cells were preincubated for 20 min at 37°C with Locke solution (composition, mM: NaCl 154, KCl 5.6, CaCl₂ 2.0, MgCl₂ 1.0, NaHCO₃ 3.6, glucose 5.6 and HEPES 10, pH 7.4) containing 3-isobutyl-1-methylxanthine (300 μ M) and superoxide dismutase (SOD; 20 U ml⁻¹). The cytosolic fractions were then added to the RFL-6 cells together with L-arginine (100 μ M), NADPH (100 μ M), calmodulin (30 nM) and BH₄ (3 μ M). After incubation for 3 min at 37°C, the reaction was stopped by the removal of medium, the addition of ice cold sodium acetate (50 mM, pH 4.0) and the rapid freezing of the samples with liquid nitrogen. Cyclic GMP content in each sample was determined by radioimmunoassay (Ishii *et al.*, 1991). In some cases, incubations were performed in the absence of SOD or the presence of haemoglobin (1 μ M).

Western blot analysis

Western blot analysis of the different regions of ferret brain was performed as described previously (Schmidt *et al.*, 1992). Briefly, equal amounts of protein in each brain region were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% w/w gel) and transferred to a nitrocellulose membrane. All procedures were done in Tris buffer (40 mM, pH 7.55) containing 0.3 M NaCl and 0.3% Tween 20. The membrane was blocked with dried milk (6% w/v), and subsequently incubated with specific polyclonal rabbit antibody prepared against rat cerebellar NO synthase and a horseradish peroxidase-conjugate of affinity purified goat antibody to rabbit IgG. The immune-complexes were detected on a photographic film by H₂O₂/luminol chemiluminescence and the bands were scanned with a laser scanner.

Materials

L-[2,3,4,5-³H]-arginine monohydrochloride was purchased from Amersham (Arlington Heights, IL, U.S.A.). L-Arginine and N^G-nitro-L-arginine (L-NNA) were purchased from Sigma (St. Louis, MO, U.S.A.). N^G-methyl-L-arginine (L-NMA) was the kind gift of Dr J.F. Kerwin Jr. (Abbott Laboratories, IL, U.S.A.). Tetrahydrobiopterin (BH₄) was obtained from Dr B. Schircks Laboratories (Jona, Switzerland). All other reagents were of the highest grade available.

Statistics

All values represent means $\pm$ s.e.mean from *n* experiments. Statistical difference between groups was assessed by Student's *t*-test for unpaired data and a *P* value of less than 0.05 taken as significant.

Results

Distribution of NO synthase activity in different regions of ferret brain

Western blot analysis of crude homogenates from different brain regions revealed that all regions contained a 160 kD protein crossreacting with anti-rat NO synthase antiserum, but the levels of relative intensity of staining by the antibody differed among these regions. The highest levels were found

in the striatum and olfactory bulb and the lowest in the cerebellum (Figure 1a). When the specific NO synthase activity in crude homogenates of each region were assayed, marked differences among these regions were detected. As shown in Figure 1b, the highest specific activity of NO synthase was found in the striatum and the lowest in the cerebellum and cerebral cortex. A relatively high specific activity was found in the olfactory bulb of the ferret, which is similar to rat brain (Bredt *et al.*, 1991). The levels of relative intensity of staining by the antibody were nearly identical to the distribution of specific NO synthase activity determined by the [³H]-L-citrulline formation assay. The highest total activity was found in the cerebral cortex owing to the tissue mass (Figure 1c).

Characteristics of NO synthase in ferret brain

As shown in Figure 2, the specific activity of NO synthase in crude homogenates of each region was greatly reduced when NADPH or Ca²⁺ were omitted from the incubations converting L-arginine to L-citrulline. Figure 2 also shows the inhibition of NO synthase by L-NNA or L-NMA in all regions. The enzymatic activity was localized predominantly in the cytosolic fractions of all regions, while negligible enzymatic activity remained in KCl-washed particulate fractions (data not shown). In addition, when the cytosolic fractions of each region were added to RFL-6 cells, increases in the cyclic GMP content in these cells were detected (Figure 3). Similar to the L-citrulline formation assay, the highest increase in cyclic GMP was found in the striatal preparation followed by the midbrain and the hippocampus. Accumulation of cyclic GMP in RFL-6 cells was markedly reduced when SOD was omitted from the incubation buffer (Figure 3) or in the presence of haemoglobin (data not shown).

Figure 1 Distribution of NO synthase activity in the ferret brain. Representative Western blot analysis (a) of crude homogenates of each region (equal amounts of protein) with anti-rat NO synthase antiserum. Proteins were separated by SDS-PAGE (7.5%), transferred to a nitrocellulose membrane and probed with the antibody to rat NO synthase. Immune-complexes were visualized with a horseradish peroxidase-linked secondary antibody on a photographic film and scanned. Specific (b) and total (c) activity of NO synthase in crude homogenates of each region. The enzymatic activities were assayed by the formation of [³H]-L-citrulline from [³H]-L-arginine (10 μ M) in the presence of NADPH (1 mM), Ca²⁺ (2 mM), calmodulin (30 nM) and BH₄ (3 μ M). Data are means ($\pm$ s.e.mean, vertical bars) from 4 experiments (b and c). Abbreviations: OB, olfactory bulb; MO, medulla oblongata (including pons); CB, cerebellum; HC, hippocampus; MB, midbrain (including thalamus and hypothalamus); ST, striatum; CX, cerebral cortex.

Figure 2 Effect of removal of cofactors or addition of inhibitors on NO synthase activity in crude homogenates of different regions of the ferret brain. The enzymatic activity was determined by the [^3H]-L-citrulline formation assay. Control activity (solid column) was measured in the presence of L-arginine (10 μM), NADPH (1 mM), Ca^{2+} (2 mM), calmodulin (30 nM) and BH_4 (3 μM). In the absence of NADPH (hatched columns) or in the presence of EGTA (1 mM) without Ca^{2+} (stippled columns), the enzymatic activity was reduced. When 100 μM N^G -methyl-L-arginine (checked columns) or 100 μM N^G -nitro-L-arginine (open columns) was added to the preparation, the enzymatic activity was inhibited. Data are means ($\pm$ s.e.mean, vertical bars) from 4–6 experiments. Abbreviations are the same as in Figure 1.

Figure 3 Effect of the absence of superoxide dismutase (SOD) on NO synthase activity in the cytosolic fractions of different regions of the ferret brain (after removal of contaminating haemoglobin with phosphocellulose). Control activity (solid columns) was determined by the accumulation of cyclic GMP in RFL-6 detector cells in the presence of L-arginine (100 μM), NADPH (100 μM), calmodulin (30 nM) and BH_4 (3 μM) in Locke solution containing 3-isobutyl-1-methylxanthine (300 μM) and SOD (20 U ml^{-1}). In the absence of SOD (stippled columns), soluble guanylyl cyclase stimulation was reduced. Data are means ($\pm$ s.e.mean, vertical bars) from 3–4 experiments. Abbreviations are the same as in Figure 1.

Discussion

Several isoforms of NO synthase have been described and at least three isoforms have been purified and well characterized (Förstermann *et al.*, 1991b). The rat brain NO synthase has been described as cytosolic and Ca^{2+} -dependent, and localized with the highest enzymatic activity in rat cerebellum (Förstermann *et al.*, 1990). Our present results show that although the ferret brain has a similar type of NO synthase to the rat brain, the highest NO synthase activity is localized in the striatum and the activity is very low in the cerebellum and cerebral cortex of this species. The possibility that different concentrations of residual haemoglobin in crude homogenates of each region were responsible for the measured differences in NO synthase activity can be excluded by the fact that there was a good correlation between the formation of L-citrulline (the detection of which is not affected by haemoglobin) and NO production as determined by cyclic GMP accumulation in RFL-6 detector cells.

Several species of animals have been found to possess NO synthase in the brain (Schmidt *et al.*, 1989; Mayer *et al.*, 1990; Bredt *et al.*, 1991; Salter *et al.*, 1991), and in the monkey brain, NO synthase seems to exist in neuronal cells as in the rat brain (Bredt *et al.*, 1991). Human NO synthase has recently been purified from the cerebellum and shown to crossreact with anti-rat cerebellar NO synthase antiserum (Schmidt & Murad, 1991). Our present results suggest that

there are differences in the distribution of brain NO synthase among species. According to a recent report, the cerebellum of the bovine brain does not contain the highest NO synthase activity (Ohshima *et al.*, 1992).

Although the link of glutamatergic neurotransmission, especially via N-methyl-D-aspartic acid (NMDA) receptors, with NO synthase has been suggested, recent studies indicate that in the rat brain the regional distribution of NMDA receptors does not match that of NO synthase (Garthwaite, 1991). The regional distribution of glutamate has been reported in rat brain (Palkovits *et al.*, 1986). Although high glutamate levels were reported in the cerebellum of the rat, glutamate distribution in other brain areas does not correlate with that of NO synthase. There is no information thus far about the distribution of glutamate or NMDA receptors in the ferret brain.

Little is known about the function of NO in the brain, but the marked differences in distribution of brain NO synthase between species may be indicative of differences in its function.

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