Kinetic characteristics of nitric oxide synthase from rat brain

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The relationship between the rate of synthesis of nitric oxide (NO) and guanylate cyclase stimulation was used to characterize the kinetics of the NO synthase from rat forebrain and of some inhibitors of this enzyme. The NO synthase had an absolute requirement for L-arginine and NADPH and did not require any other cofactors. The enzyme had a $V_{\max}$ of 42 pmol of NO formed $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ and a K_m for L-arginine of 8.4 μM . Three analogues of L-arginine, namely N^G -monomethyl-L-arginine, N^G -nitro-L-arginine and N^G -iminoethyl-L-ornithine inhibited the brain NO synthase. All three compounds were competitive inhibitors of the enzyme with K_i values of 0.7, 0.4 and 1.2 μM respectively.

INTRODUCTION

An enzyme in vascular endothelial cells (Palmer *et al.*, 1988a) and macrophages (Marletta *et al.*, 1988) synthesizes nitric oxide (NO) from the terminal guanidino nitrogen atom(s) of L-arginine. The NO synthesized plays a role in the vasodilator responses of the vasculature and in the maintenance of blood pressure (Moncada *et al.*, 1989) and may also mediate in part the anti-tumour effects of cytotoxic activated macrophages (Hibbs *et al.*, 1987a,b) and the direct effects of interferon- γ on EMT-6 adenocarcinoma cells (Amber *et al.*, 1988; Lepoivre *et al.*, 1989). The formation of NO from L-arginine has also been demonstrated in neutrophils (McCall *et al.*, 1989; Salvemini *et al.*, 1989; Wright *et al.*, 1989), hepatocytes (Curran *et al.*, 1990) and adrenal glands (Palacios *et al.*, 1989).

A similar enzyme has been identified in the rat forebrain (Knowles *et al.*, 1989). Studies on cerebellar cells and slices show that, in the central nervous system, NO, synthesized from L-arginine, links activation of postsynaptic *N*-methyl-D-aspartate receptors to functional changes in neuronal and glial cells (Garthwaite *et al.*, 1988, 1989). The NO synthase in the brain is calcium-dependent and, like the endothelial and macrophage enzymes (Marletta *et al.*, 1988; Palmer & Moncada, 1989), forms citrulline as a co-product and is inhibited by N^G -monomethyl-L-arginine (L-NMMA) (Knowles *et al.*, 1989). However, the concentration-dependence of the brain NO synthase for its substrate L-arginine or for L-NMMA as an inhibitor could not be interpreted in that study in terms of K_m or K_i values for this enzyme, since the results were a combination of the activities of two enzymes, namely the NO synthase and the soluble guanylate cyclase (Knowles *et al.*, 1989).

In the present study we have characterized the stimulation by NO of the soluble guanylate cyclase in the cytosolic fraction of synaptosomes. We have used the observed relationship between the rate of NO synthesis and cyclic GMP formation to quantify NO production by the brain NO synthase, permitting the determination of the K_m for L-arginine, the demonstration of competitive inhibition by L-NMMA and determination of its K_i , none of which has previously been reported. It has also permitted the identification of two novel inhibitors of the brain NO synthase and the determination of their K_i values. Finally, we have been able to show that, unlike the macrophage NO synthase (Stuehr *et al.*, 1989; Kwon *et al.*, 1989; Tayeh & Marletta, 1989), the brain NO synthase requires only NADPH as a cofactor, suggesting that the macrophage and brain NO synthases are distinct enzymes.

MATERIALS AND METHODS

Materials

Cyclic GMP radioimmunoassay kits were obtained from Amersham. L-NMMA and L-NIO were synthesized as described (Scannell *et al.*, 1972; Patthy *et al.*, 1977). Other chemicals were obtained from Sigma or Aldrich.

Preparation of crude synaptosomal cytosol

This was prepared from the forebrains of male rats (200–300 g), essentially as described by Knowles *et al.* (1989). Briefly, a crude synaptosomal fraction was prepared and homogenized under hypo-osmotic conditions (1 mM-DL-dithiothreitol; included to stabilize the soluble guanylate cyclase) by 20 strokes of a Dounce homogenizer. The high-speed supernatant obtained after centrifugation at 150 000 g for 30 min was passed through Chelating Resin (Na^+ form; Sigma) to remove endogenous bivalent cations and arginine. 1 mM-DL-Dithiothreitol was used to make up a final volume of 50 ml per forebrain. This material contained approx. 40 μg of protein/ml.

Preparation of the high-molecular-mass fraction of synaptosomal cytosol

A high-molecular-mass fraction was obtained by passing 0.7 ml of the undiluted 150 000 g supernatant through a 2 ml column (6 mm $\times$ 40 mm) of Sephadex G-25 pre-equilibrated with 1 mM-DL-dithiothreitol and collecting 0.5 ml of the void-volume material. The efficiency of removal of low-molecular-mass material was determined by measuring the removal of [^{14}C]sucrose (0.02 $\mu\text{Ci}/\text{ml}$; 0.1 mM) from the starting material; in four separate experiments the efficiency was > 98 %. The high-molecular-mass fraction obtained was diluted and used at a protein concentration similar to that of the unfractionated synaptosomal cytosol (see above).

A low-molecular-mass fraction was obtained by centrifuging 2 ml of the undiluted 150 000 g supernatant in a Centriscart 1 ultrafiltration tube (molecular-mass cut-off 20 kDa; Sartorius, Göttingen, Germany) for 10 min at 4000 g . The ultrafiltrate was used undiluted.

Determination of NO and cyclic GMP formation

The rate of NO formation in the presence of sodium nitroprusside was determined spectrophotometrically by measuring the change in the absorption of oxyhaemoglobin when it is oxidized to methaemoglobin by NO (Feelisch &

Abbreviations used: L-NIO, *N*-iminoethyl-L-ornithine; L-NitroArg, N^G -nitro-L-arginine; L-NMMA, N^G -monomethyl-L-arginine.

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Noack, 1987). The assay was carried out in 25 mM-Tris/HCl buffer, pH 7.2 (at 37 °C), containing 5 mM-MgCl₂ and 1 μ M-oxyhaemoglobin; 0.5 ml of this solution was placed in the cuvette and prewarmed to 37 °C before addition of sodium nitroprusside. After rapid mixing, the A_{401} was monitored over 5 min against a blank incubation with no sodium nitroprusside. The initial rate of change in absorption was used to calculate the rate of NO formation, using the molar absorption coefficient of methaemoglobin ($\epsilon = 19700$; Feelisch & Noack, 1987).

The rate of cyclic GMP formation was determined as described by Knowles *et al.* (1989), the 10 min reactions being initiated by addition of 150 μ l of synaptosomal cytosol to prewarmed (37 °C) buffer (25 mM-Tris, 5 mM-GTP, 5 mM-MgCl₂, 1 mM-3-isobutyl-1-methylxanthine, 0.75 mM-DL-dithiothreitol, pH 7.2, at 37 °C). The 3-isobutyl-1-methylxanthine was included as a phosphodiesterase inhibitor. Cyclic GMP formed was then determined by radioimmunoassay. The synthesis of NO from L-arginine was measured in the presence of NADPH (1 mM) unless otherwise specified. The synaptosomal NO synthase is dependent on Ca²⁺, which is present in the buffers in sufficient quantities to allow full expression of its activity in the absence of any added Ca²⁺.

Other methods

The protein concentration of aliquots of synaptosomal cytosol, at a concentration 20-fold higher than that used for assays of cyclic GMP formation, was assayed using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Watford, Herts., U.K.), with BSA as the standard.

Linear and non-linear least-squares fittings of equations to data were carried out using the RS/1 programme (BBN Software Products Corp., Cambridge, MA, U.S.A.). Kinetic constants were obtained by non-linear least-squares regression of rate data to the Michaelis-Menten equation or to the equation for competitive inhibition.

RESULTS AND DISCUSSION

Relationship between NO synthesis and guanylate cyclase stimulation

The rate of NO formation from sodium nitroprusside increased linearly with the concentration of sodium nitroprusside present up to 400 μ M (Fig. 1a). This linear relationship was then used to characterize the effect of different rates of NO synthesis on guanylate cyclase activity.

Because the rate of NO breakdown is second-order at a constant oxygen concentration (von Temkin & Pyzhov, 1935), at the steady-state the NO concentration available to stimulate guanylate cyclase would be predicted to be linearly related to the square root of the rate of NO formation. If there exists a simple Michaelis-Menten relationship between NO concentration and guanylate cyclase stimulation, then this should be apparent if the square of the guanylate cyclase activity is plotted against the sodium nitroprusside concentration. The data are indeed consistent with simple saturable kinetics of stimulation of guanylate cyclase by NO (Fig. 1b), with half-maximal stimulation at 1.45 μ M-sodium nitroprusside. No significant cyclic GMP synthesis was observed in the absence of added sodium nitroprusside or L-arginine.

It is noteworthy that the stimulation of cytosolic ADP-ribosyltransferase by NO reported by Brune & Lapetina (1989) appears to need higher NO concentrations: approx. 30-fold higher concentrations of sodium nitroprusside (> 50 μ M) were required for half-maximal stimulation of the ADP-ribosyltransferase than were required for half-maximal effects on the guanylate cyclase.

Fig. 1. Relationship between NO formation, guanylate cyclase stimulation and sodium nitroprusside concentration

(a) Concentration-dependence of NO formation from sodium nitroprusside. The line drawn was obtained by linear regression:

$$y = 9.83 \times 10^{-4}x, r = 0.994$$

(b) Concentration-dependence of guanylate cyclase stimulation by NO formed from sodium nitroprusside. The guanylate cyclase activity is expressed as a percentage of its activity in the presence of 100 μ M-sodium nitroprusside. The line drawn was obtained by non-linear regression:

$$y = 10000x/(4.35 + x), r = 0.986$$

The hyperbolic relationship between NO synthesis and the square of the guanylate cyclase activity, demonstrated by the data in Fig. 1, was used to calculate rates of NO synthesis by the NO synthase, using the following equation derived from those obtained in Fig. 1:

$$\text{NO formation rate (nmol/min per mg of protein)} = \frac{4.35 (G_x/G_{\max})^2}{1 - (G_x/G_{\max})^2} \times 9.83 \times 10^{-4} \times \frac{1}{p}$$

where G_x is the activity of guanylate cyclase, $G_{\max}$ is the activity of guanylate cyclase in the presence of a maximally effective concentration of sodium nitroprusside and p is the concentration of synaptosomal cytosol protein in the incubation, in mg of protein/ml. The units of the three terms on the right-hand side of the equation are μ M-sodium nitroprusside, nmol of NO/ml per min per μ M-sodium nitroprusside and (mg of protein/ml)⁻¹ respectively.

Substrate kinetics of brain NO synthase

The rates of NO formation by the brain NO synthase in the presence of 1 mM-NADPH and in the presence of 0–500 μ M-L-arginine, calculated from the above equation, showed that the enzyme had a $V_{\max}$ of 43 ± 8.5 pmol/min per mg of protein and a K_m for L-arginine of 8.4 ± 2.7 μ M (both means $\pm$ S.E.M. from four independent determinations; Fig. 2); NO synthesis was not detectable in the absence of added L-arginine. This maximal activity of the rat brain NO synthase is very similar to the activity measured by determination of the formation of the NO breakdown product NO₂⁻ (40 pmol/min per mg of protein; Knowles *et al.*, 1989). The specific activity of the NO synthase in the synaptosomal cytosol is approx. 14-fold less than that in macrophage cytosol [approx. 600 pmol/min per mg of protein;

Fig. 2. L-Arginine concentration-dependence of brain NO synthase

The NO synthase activity of four synaptosomal cytosol preparations was determined in the presence of 0–500 μM-L-arginine. The data, shown as a double-reciprocal plot, are from one representative experiment. The data were fitted by non-linear regression to the untransformed data in order to obtain K_m and V_{max} values.

calculated from the results of Marletta *et al.* (1988) and Stuehr *et al.* (1989)].

The low K_m of the NO synthase for L-arginine (8.4 μM) suggests that the enzyme will normally be saturated with L-arginine, which is present in rat plasma and brain tissue at approx. 100 μM (Remesy *et al.*, 1971; Barbul, 1986). It may also explain why NO synthesis by vascular endothelial cells (Palmer *et al.*, 1988b) or neuroblastoma extracts (Deguchi & Yoshioka, 1982) is only significantly stimulated by addition of exogenous L-arginine when endogenous L-arginine has previously been depleted.

Inhibitor kinetics of NO synthase

The inhibition of the NO synthase by L-NMMA, L-NitroArg and L-NIO was studied. These L-arginine analogues were purely competitive inhibitors, with K_i values of 0.66 ± 0.13 μM, 0.44 ± 0.22 μM and 1.2 ± 0.49 μM for L-NMMA, L-NitroArg and L-NIO respectively (mean $\pm$ S.E.M., $n = 3$ or 4 for each compound; Fig. 3).

Cofactor-dependence of brain NO synthase

NADPH has been postulated as the cofactor of the NO synthase reaction (Marletta *et al.*, 1988; Knowles *et al.*, 1989; Palmer & Moncada, 1989). In a previous study of the brain NO synthase, an absolute requirement for NADPH could not be demonstrated in the unfractionated synaptosomal cytosol preparation, because of the variable activity measured in the absence of exogenous NADPH. This was presumably a consequence of the presence of low and variable concentrations of endogenous NADPH. However, in the present study, using a 20-fold lower concentration of the synaptosomal cytosol preparation than in the previous study (approx. 50 μg of protein/ml as against 1000 μg/ml), no significant synthesis of NO from L-arginine was observed in the absence of added NADPH (Table 1); NO synthase activity was restored by adding NADPH. Parallel experiments were carried out with cytosol which had been fractionated to remove endogenous low-molecular-mass cofactors. The high-molecular-mass fraction contained an NO synthase which was as active as the unfractionated preparation when assayed in the presence of NADPH and L-arginine, but was inactive in the absence of added NADPH (Table 1). The activity of the NO synthase in the high-molecular-mass fraction was not increased by addition of the concentrated low-molecular-mass fraction (results not shown). Thus the brain NO synthase requires only NADPH and L-arginine for activity, in apparent contrast

Fig. 3. Dixon plots of the inhibition of brain NO synthase by L-NMMA (a), L-NIO (b) and L-nitroArg (c)

The NO synthase activity of three or four synaptosomal cytosol preparations was determined in the presence of 0–10 μM-inhibitor and of the various concentrations of L-arginine shown, as described in the Materials and methods. The data shown are from individual experiments, representative of three or four carried out. The lines shown were obtained by non-linear least-squares regression of the data to the equation for competitive inhibition:

$$v = \frac{V_{max} \cdot s}{s + K_m [1 + (i/K_i)]}$$

in order to obtain the kinetic constants.

Table 1. Cofactor-dependence of brain NO synthase

Unfractionated or high-molecular-mass-fraction enzyme was incubated in the presence of 100 μM-L-arginine and in the presence or absence of 1 mM-NADPH, and the guanylate cyclase stimulation was measured and compared with the maximal guanylate cyclase activity measured in the presence of 100 μM-sodium nitroprusside. The data shown are means $\pm$ S.E.M. from four experiments; the value for (b)/(a) $\times 100$ was $91 \pm 13.5\%$

Enzyme	NADPH	Guanylate cyclase stimulation (% of maximal)
Unfractionated	–	0.1 $\pm$ 0.09
	+	29.8 $\pm$ 5.81 (a)
High-molecular-mass-fraction	–	–0.9 $\pm$ 1.05
	+	28.8 $\pm$ 8.86 (b)

with the macrophage enzyme, which requires an additional low-molecular-mass constituent of the cytosol (Stuehr *et al.*, 1989), recently shown to be tetrahydrobiopterin (Kwon *et al.*, 1989; Tayeh & Marletta, 1989).

Properties of NO synthases from different tissues

These results, together with previously published data (Knowles *et al.*, 1989), show that the rat brain NO synthase requires only the presence of L-arginine, NADPH and Ca^{2+} in

order to synthesize NO and citrulline. The brain NO synthase seems to differ from the macrophage enzyme in its cofactor requirement. Differences in the potency of L-homoarginine as a substrate and L-canavanine as an inhibitor also suggest that the brain and endothelial NO synthases may differ from the macrophage and neutrophil enzymes (reviewed by Moncada *et al.*, 1989).

The substrate and inhibitor kinetic constants obtained are consistent with the observed inhibition by L-NMMA of citrulline formation from L-arginine both by the endothelial (Palmer & Moncada, 1989) and brain (Knowles *et al.*, 1989) enzymes. In contrast, the degree of inhibition of NO₂⁻ (approx. 90%) and citrulline (approx. 75%) formation from 1.2 mM-L-arginine in macrophages by 0.1 mM-L-NMMA (Hibbs *et al.*, 1987a) is greater than would be predicted from the kinetics of the brain NO synthase (50%).

In addition, the NO synthase from the rat adrenal gland appears to have a different pattern of substrate and inhibitor potency from both the brain and the macrophage NO synthases (Palacios *et al.*, 1989): L-canavanine inhibits the macrophage and adrenal enzymes, but not that in brain, whereas L-homoarginine is a poor substrate for the brain and adrenal enzymes, but a good substrate for the macrophage NO synthase. These differences imply that distinct NO synthase enzymes may exist in different tissues.

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