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COMMENTARY

ISOFORMS OF NITRIC OXIDE SYNTHASE

CHARACTERIZATION AND PURIFICATION FROM DIFFERENT CELL TYPES

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Research which started more than a decade ago in such diverse fields as receptor-mediated increases in cyclic GMP levels in the central nervous system [1], the search for nitrate producing cells in mammals [2], endothelium-mediated relaxation of vascular smooth muscle [3], and autonomic non-adrenergic non-cholinergic neurotransmission [4] has been unified recently as it has become apparent that all these findings are based on the same phenomenon: the synthesis of nitric oxide radical or a related material (hereinafter abbreviated as NO) in mammalian cells. During the last two years, several isoenzymes responsible for NO formation have been characterized and purified from different cell types and tissues. The properties and mechanisms of regulation of these different isoforms are discussed in this commentary.

NO synthase in brain and neuronal cells

It has been known since the mid-seventies that neurotransmitters, especially excitatory amino acids, produce increases in cyclic GMP concentrations in many regions of the brain [1]. This cyclic GMP accumulation was abolished in the absence of extracellular Ca^{2+} [1]. As guanylyl cyclase activity is not directly regulated by Ca^{2+} [5] and the stimulators had no effect in broken cells, the mechanism underlying this cyclic GMP increase remained unclear. In 1988, Garthwaite *et al.* [6] demonstrated that stimulation of *N*-methyl-D-aspartate receptors led to the Ca^{2+}-dependent production of an NO-like material in brain slices which, in turn, stimulated soluble guanylyl cyclase in the tissue. Subsequent studies revealed that the cytosolic fraction of brain and neuroblastoma cells contained an enzyme that produces NO from L-arginine [7–10]. These findings also explained an earlier report by Deguchi and Yoshioka [11] that L-arginine activates soluble guanylyl cyclase in the crude cytosol of neuroblastoma cells and that this stimulation was lost after partial purification of the guanylyl cyclase. The presence of the NO signal transduction pathway in the brain focussed interest on the key enzyme of this pathway, the neuronal NO synthase. In our research we found that the enzyme in N1E-115 neuroblastoma cells was localized exclusively in the cytosol [10], used L-arginine as a substrate and required reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as a cofactor, was regulated by Ca^{2+} in the physiological intracellular range (Fig. 1a), and was dependent on calmodulin [12]. Also, in whole rat brain the enzyme was cytosolic and had the same characteristics as the neuroblastoma enzyme [14]. When the enzymatic activities in different areas of the brain were compared, the highest activity was found in the cerebellum [14], so enzyme purification was attempted from this brain region [15–17]. We purified the soluble enzyme to homogeneity by sequential affinity chromatographic steps on 2′,5′-ADP Sepharose and calmodulin agarose [16]. Enzyme activity during purification was bioassayed by the stimulation of soluble guanylyl cyclase in rat fetal lung fibroblasts (RFL-6 cells) following the L-arginine-, NADPH- and Ca^{2+}/calmodulin-dependent formation of NO. RFL-6 cells contain significant amounts of soluble and particulate guanylyl cyclase [18] and we have established them as a sensitive detector system for NO activity [19, 20]. In addition to synthesizing guanylyl cyclase-activating material, purified soluble NO synthase also produces L-citrulline from L-arginine (as measured by the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline [16]). Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) revealed a single protein band for the purified enzyme with a molecular mass of 155 kDa. A molecular mass of about 279 kDa was calculated for the native protein based on gel permeation chromatography (Superose 6) and velocity sedimentation [16]. These data suggest that soluble synthase purified from rat cerebellum is a dimer of 155 kDa subunits. Others described a similar molecular mass of the denatured protein (150 kDa [15] and 160 kDa [17] and similar kinetic properties of the enzymes plus the additional finding that 5,6,7,8-tetrahydrobiopterin was a cofactor for

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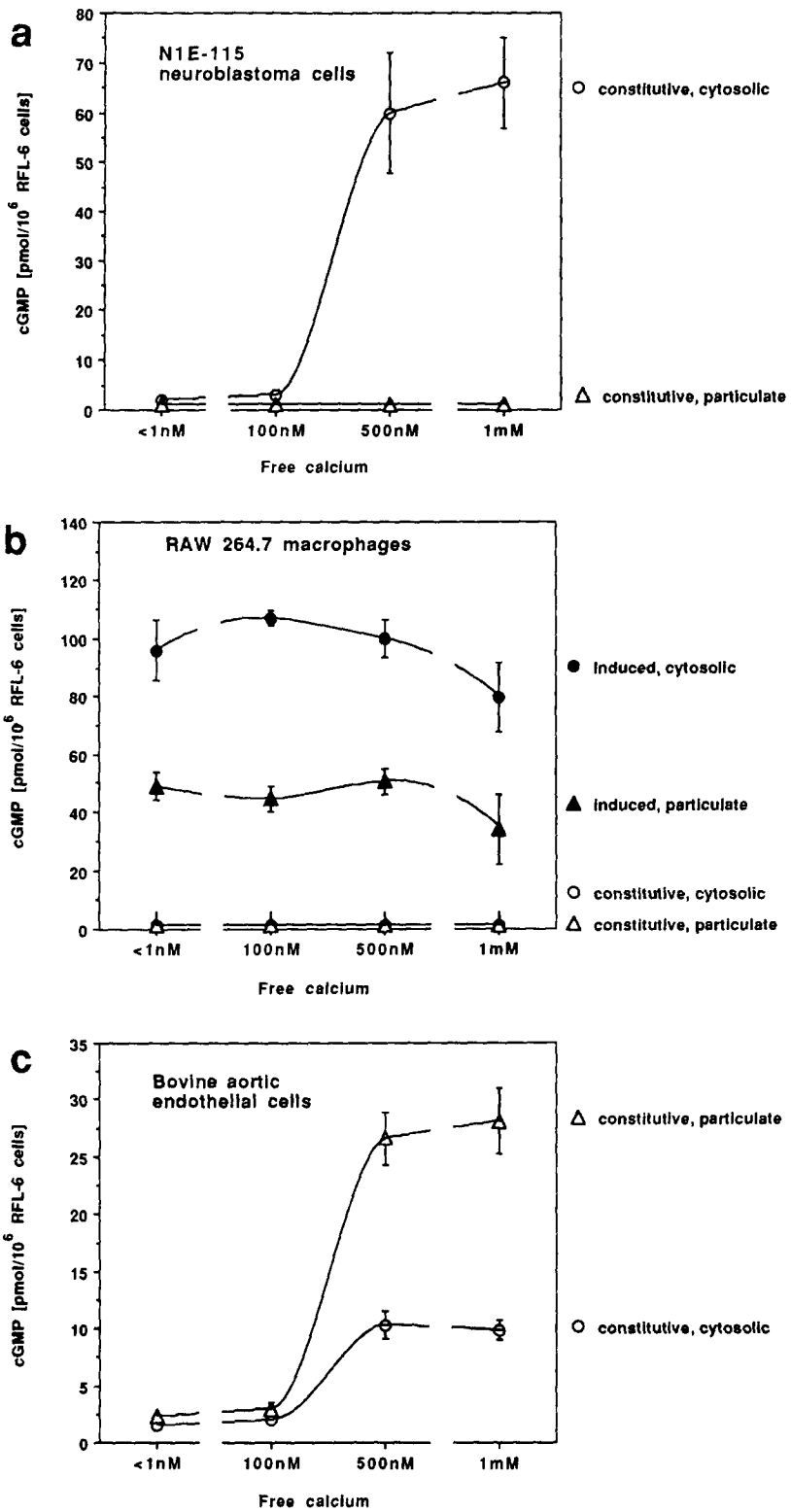

Fig. 1. Subcellular distribution and Ca^{2+} dependence of constitutive NO synthase activity in N1E-115 neuroblastoma cells (panel a), induced and constitutive activity in RAW 264.7 macrophages (panel b), and constitutive activity in bovine aortic endothelial cells (panel c). Specific activities (obtained with 100 μg of protein) are shown. Cytosolic and particulate fractions were obtained from homogenates of these cells as previously described [13]. All particulate fractions were washed with 1 M KCl to remove soluble proteins. L-Arginine (100 μM), NADPH (100 μM) and superoxide dismutase (20 units/mL) were present in the incubation mixture and the RFL-6 detector cells were treated with 3-isobutyl-1-methyl xanthine (300 μM). Some of the macrophages were induced for 16 hr with 1 μg/mL lipopolysaccharide and 100 units/mL interferon-γ prior to homogenization. Ca^{2+} concentrations in the RFL-6 assay were buffered with 1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) [13]. Basal cyclic GMP levels in the RFL-6 detector cells were 1–2 pmol/10^6 cells. The particulate fraction of N1E-115 neuroblastoma cells, as well as the particulate and cytosolic fractions of non-induced RAW 264.7 macrophages, produced no significant stimulation of cyclic GMP levels. The cytosolic fraction from rat brain showed the same characteristics as the cytosolic fraction from N1E-115 neuroblastoma cells. Values are means ± SEM, N = 4–6.

the enzyme [17]. As the first isoform purified, we termed the soluble NO synthase from rat cerebellum isoform Ia (Table 1). We subsequently found that enzymes from other brain regions had identical properties as the cerebellar enzyme. Also, platelets have been reported to contain small amounts of a soluble, Ca^{2+}-sensitive NO synthase [40], but this enzyme has not been characterized any further and it is not yet certain if the activity resulted from other contaminating cells in the preparation.

Induced NO synthase in macrophages and other cells

Mammals, including humans, excrete more nitrate in their urine than they ingest [2]. The original perception that this positive nitrate balance represented synthesis by the intestinal microflora [2] proved incorrect when it was determined that germfree rats synthesized the same amount of nitrate as control animals [41]. Subsequent reports demonstrated that injection of rats with *Escherichia coli* lipopolysaccharide markedly increases urinary nitrate excretion [42]. This triggered the search for mammalian cells capable of sythesizing nitrate and, in 1985, led to the discovery that macrophages could be induced with lipopolysaccharide to produce significant amounts of both nitrite and nitrate [43]. In 1987 further work demonstrated that L-arginine was the substrate of this pathway, that L-citrulline was formed as a co-product [44, 45], and that the pathway could also be expressed upon immunostimulation with the endogenous cytokine interferon-γ [46]. One year later NO was identified as the intermediate product [47]. The enzyme responsible for NO synthesis in lipopolysaccharide/interferon-γ-stimulated macrophages was reported to have been localized in the cytosolic fraction [47] and has since been characterized with respect to its kinetic properties and cofactor requirements. The enzyme requires NADPH, 5,6,7,8-tetrahydrobiopterin, flavin-adenine-dinucleotide and flavin-mononucleotide, and thiols for full activity [23–27] (Table 1). The induced enzyme has been purified recently from the cytosolic fraction of induced rat peritoneal macrophages [48] and from induced cells of the RAW 264.7 macrophage cell line [23]. On SDS–PAGE the peritoneal macrophage enzyme migrated as a single protein band corresponding to a molecular mass of 150 kDa; the RAW 264.7 enzyme migrated as a tight triplet of bands of estimated molecular mass between 125 and 135 kDa [23]. By gel filtration the apparent molecular masses of the native proteins were found to be 300 kDa for the peritoneal macrophage enzyme [48] and 250 kDa for the RAW 264.7 enzyme [23]. Both groups of authors concluded from their data that the catalytically competent enzyme is a dimer. We have no explanation at this time for the differences in molecular mass reported. When investigating the NO- and L-citrulline-forming properties of subcellular fractions of RAW 264.7 macrophages (using stimulation of soluble guanylyl cyclase in RFL-6 cells [20] and conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline [16]), we found that the majority (about 70%) of the total enzymatic activity of lipopolysaccharide/interferon-γ-induced cells was indeed associated with the cytosolic fraction, but about 30% of the activity was particulate [28,*]. Neither of the enzyme activities from induced RAW 264.7 cells was dependent on Ca^{2+}. We termed the endotoxin/cytokine-induced cytosolic enzyme isoform II and the induced particulate enzyme isoform IV (Table 1). Low passage numbers of *non-induced* RAW 264.7 macrophages showed small amounts of Ca^{2+}-dependent NO synthase activity [28, *] which disappeared with higher passage numbers (Fig. 1b). The specific enzyme activities in the cytosolic and particulate fractions of induced and non-induced RAW 264.7 cells are shown in Fig. 1b.

Several other cell types can also be induced with lipopolysaccharide and/or immune mediators such as tumor necrosis factor, interleukin-1 or interferon-γ to produce nitrogen oxides. These cells include hepatocytes [29] and Kupffer cells [30], endothelial cells [31, 32], vascular smooth muscle cells [33, 34], fibroblasts [35] and mesangial cells [36]. In some cells (e.g. RAW 264.7 macrophages) endotoxin or one cytokine is sufficient to produce maximal enzyme induction [28,*] whereas other cells (e.g. hepatocytes or endothelial cells) seem to require a specific combination of these mediators to maximally induce NO synthase [29, 31]. Some initial characterization has been performed recently on inducible enzymes from cells other than macrophages. Treatment of rats with endotoxin resulted in significant induction of NO synthase activity in the lung and the liver, but not in the brain [37]. The induced NO synthase activity in the cytosolic fractions of lung and liver was largely independent of Ca^{2+} (whereas the constitutive brain enzyme was Ca^{2+} sensitive [37]). Similarly, the cytosolic fraction from cytokine-induced smooth muscle cells contained Ca^{2+}-independent NO synthase activity [33]. Thus, endotoxin/cytokine-induced enzymes in different cells seem to be uniformly Ca^{2+}/calmodulin independent. However, further characterization and purification of these enzymes is required before a final conclusion can be reached.

Endothelium-derived relaxing factor (EDRF) synthase in endothelial cells

In 1980, Furchgott and Zawadzki [3] reported that several receptor agonists relax vascular preparations only if the endothelium of the preparation is intact. They termed the substance released from the endothelium as "endothelium-derived relaxing factor (EDRF)". Subsequently it became clear that the EDRF-mediated relaxations were associated with increased levels of cyclic GMP in the smooth muscle cells [49]. This mechanism of relaxation was similar to that of nitrovasodilators which also exert their effect through the formation of cyclic GMP [50, 51], but in an endothelium-independent fashion [3, 49]. The relaxing properties of nitrovasodilators were known to depend most probably on the formation of NO [51, 52]. Bioassay experiments revealed that EDRF

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Table 1. Isoforms of NO synthase

Type	Cosubstrates, cofactors	Regulated by:	M_r of denatured protein (SDS–PAGE)	Present in:
Ia (soluble)	NADPH, BH_4, FAD/FMN*,	Ca^{2+}/calmodulin	155 kDa [16]	Brain, cerebellum [15–17], N1E-115 neuroblastoma cells [10, 12]
Ib (soluble)	NADPH	Ca^{2+}/calmodulin	Unknown	Endothelial cells [13]
Ic (soluble)	NADPH, BH_4, FAD	Ca^{2+} (*not* calmodulin)	150 kDa [21]	Neutrophils [21, 22]
II (soluble)	NADPH, BH_4, FAD/FMN, GSH (thiols)	Unknown (induced by endotoxin/cytokines)	125–135 kDa [23] 150 kDa [48]	Macrophages [23–28,†], Enzyme activities with probably similar characteristics can be induced in hepatocytes, Kupffer cells, vascular smooth muscle cells, fibroblasts, endothelial cells, lung and liver [29–37]
III (particulate)	NADPH, BH_4	Ca^{2+}/calmodulin	135 kDa [38]	Endothelial cells [13, 38, 39, 65]
IV (particulate)	NADPH	Unknown (induced by endotoxin/cytokines)	Unknown	Macrophages†

All isoenzymes use L-arginine as a substrate and all are inhibited by N^G-methyl-L-arginine and N^G-nitro-L-arginine. Abbreviations: BH_4, 5,6,7,8-tetrahydrobiopterin; FAD, flavin-adenine-dinucleotide; FMN, flavin-mononucleotide; GSH, reduced glutathione; NADPH, nicotinamide-adenine-dinucleotide phosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

* Unpublished observations.

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was a short-lived humoral substance [53, 54] which could directly stimulate purified soluble guanylyl cyclase [55, 56]. In 1987 it was reported that NO can account for the biological activity of EDRF [57, 58] even though other labile NO-containing substances cannot be ruled out. Analogous to the macrophage system, L-arginine was established as a substrate for EDRF/NO synthesis in endothelial cells [59, 60]. In 1989, we reported that endothelial cell homogenates synthesized NO-like material that stimulated cyclic GMP levels in RFL-6 detector cells [19] and others found that the homogenates produced L-citrulline from L-arginine [61]. Subsequent studies described the NO-forming activity in endothelial cells as being cytosolic and regulated by Ca^{2+} and calmodulin [62, 63]. Others, however, found NO generation by a presumably membrane-associated endothelial enzyme with somewhat unusual characteristics. It was not dependent on exogenous Ca^{2+}, its activity was only observed at 0° and not at 37°, and it did not seem to require O_2 [64]. When we analyzed the subcellular fractions of cultured endothelial cells, we found about 95% of the EDRF/NO-synthesizing activity in the particulate fraction, whereas the cytosolic fraction contained only about 5% of the total enzymatic activity [13]. Re-investigation of this distribution in *native* endothelial cells harvested mechanically from bovine aortae demonstrated that almost all of the EDRF/NO synthase activity was localized in the particulate fraction [39]. The particulate and the small cytosolic activities were both sensitive to Ca^{2+}, the major increase in activity occurring between 100 and 500 nM free Ca^{2+} (see Fig. 1c). Activity of the particulate enzyme was not decreased significantly after washing it with 1 M KCl (which removes soluble proteins), but the activity could be solubilized with the detergent CHAPS (3-[{3-cholamidopropyl}dimethylammonio]-1-propanesulfonate) [13, 39]. We then purified the particulate EDRF/NO synthase from both native and cultured bovine aortic endothelial cells [38, 65]. Purified particulate EDRF/NO synthase migrated at a molecular mass of 135 kDa in denaturing SDS–PAGE and appeared to be identical in native and cultured cells. With L-arginine as the substrate, the pure enzyme synthesized EDRF/NO (detected by the stimulation of soluble guanylyl cyclase in RFL-6 cells [20]) and citrulline (detected by conversion of [^{3}H]L-arginine [16]). For activity the enzyme required Ca^{2+}/calmodulin, NADPH and 5,6,7,8-tetrahydrobiopterin [38, 65]. The purified EDRF/NO synthase relaxed endothelium-denuded rabbit aortic strips when added to the tissues together with substrate and cofactors. The vascular relaxation was dependent on the presence of superoxide dismutase which is consistent with EDRF/NO as the mediator [65]. This particulate enzyme, which is responsible for most of the EDRF/NO synthesis in endothelial cells, was termed isoform III of NO synthase (Table 1). The *cytosolic* enzyme found in small amounts in cultured endothelial cells is also Ca^{2+}/calmodulin dependent [13], but it does not seem to be identical with isoform Ia from the brain. Unlike the brain enzyme which is very susceptible to inhibition by calmodulin antagonists [12, 16], the activity of the cytosolic endothelial enzyme is only partially inhibited by these compounds [13, 63]. Furthermore, the elution profile of the predominant cytosolic EDRF/NO synthase from DEAE anion exchange columns differs from the brain enzyme (isoform Ia) in that it elutes later and in the presence of calmodulin (Fig. 2). On Superose 6 gel permeation chromatography the cytosolic endothelial enzyme eluted similarly to the solubilized particulate enzyme [65] but differently from the brain enzyme (isoform Ia). Consequently, we classify this enzyme as isoform Ib of NO synthase (Table 1).

NO synthase in neutrophil polymorphonuclear granulocytes

Neutrophils release NO-like material [66–69] which has been detected by its ability to relax vascular smooth muscle [66], to inhibit platelet aggregation [68, 70], or by the measurement of nitrite, the oxidation product of NO [67, 69]. Some investigators found that NO production in neutrophils could be stimulated with receptor agonists such as *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine or leukotriene B_4 [68, 69], but others failed to confirm this finding [67] (probably because their neutrophils were already maximally activated). Recently, the NO synthase from neutrophils has been characterized and purified [21, 22]. The enzyme which was isolated from the cytosolic fraction of these cells migrated with a molecular mass of 150 kDa in denaturing SDS–PAGE. The same molecular weight was found for the native protein on Superose 12 gel permeation chromatography [21]. Therefore, the authors concluded that NO synthase from neutrophils was a monomeric protein with a molecular mass of 150 kDa [21]. Its cofactor requirement was similar to the other known NO synthases; NADPH, 5,6,7,8-tetrahydrobiopterin and flavin-adenine-dinucleotide were required for activity [21]. The enzyme was reported to be regulated by Ca^{2+}, but unlike the brain enzyme (isoform Ia), the activity of the purified neutrophil enzyme was independent of calmodulin and was not inhibited by calmodulin antagonists [21]. The enzyme also seems to be dependent on Mg^{2+} [22], another difference from isoform Ia. Although these observations have not yet been confirmed by others, we tentatively propose that the constitutive soluble neutrophil enzyme be classified as isoform Ic (Table 1).

Enzymes synthesizing NO-like non-adrenergic non-cholinergic neurotransmitter

A variety of smooth muscle tissues such as the rat anococcygeus muscle [4], the bovine retractor penis muscle [71], and structures throughout the gastrointestinal tract [72] and the trachea [73] show autonomic non-adrenergic non-cholinergic (NANC) innervation. In some tissues, the smooth muscle relaxation that followed stimulation of the NANC nerves was shown to be mediated by cyclic GMP [74], but the identity of the inhibitory neurotransmitter(s) released by these neurons was unknown. During recent years, however, evidence accumulated showing that many of the NANC neurons may release NO or an NO-containing substance. This evidence was based on the inhibition of the NANC response by hemoglobin and superoxide anion, the instability of the transmitter, the potentiation of the NANC

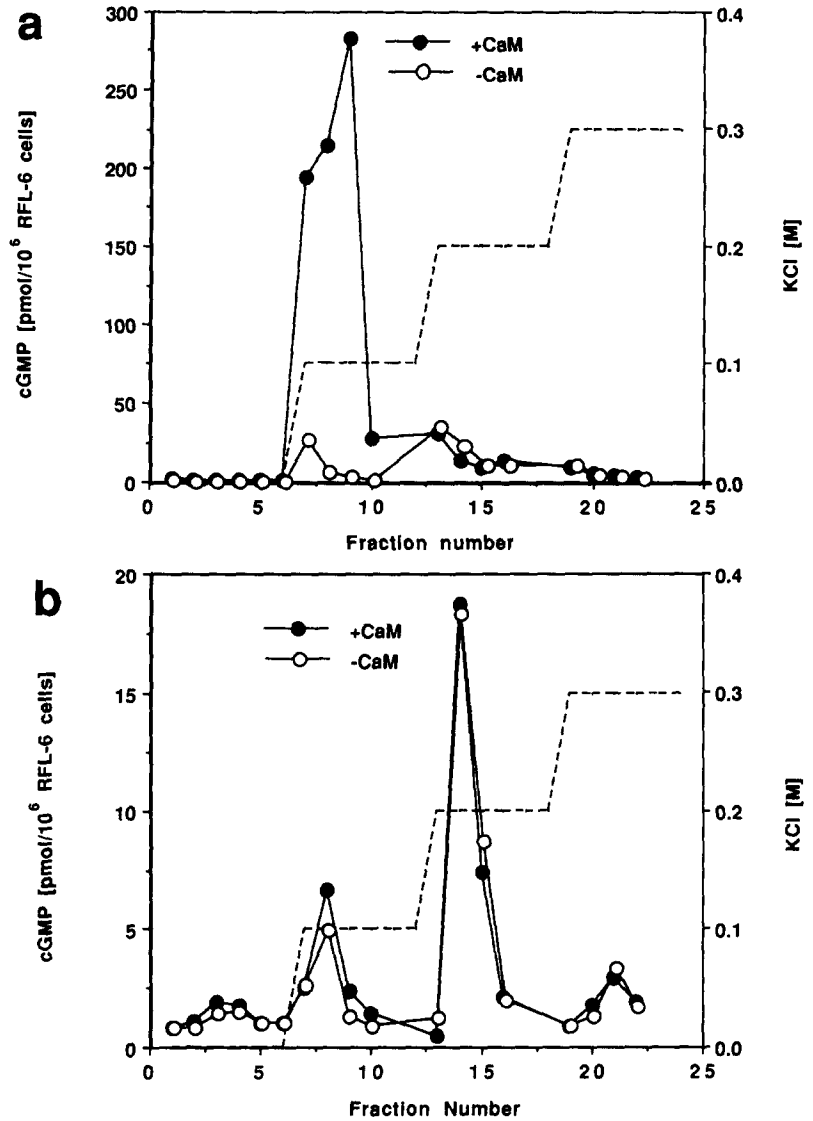

Fig. 2. Elution profile of cytosolic NO synthases from rat brain (panel a) and endothelial cells (panel b) on DEAE anion exchange chromatography. DEAE Sephacel columns were equilibrated with ice-cold Tris/HCl buffer (50 mM, pH 7.4) containing EDTA (0.1 mM), EGTA (0.1 mM), 2-mercaptoethanol (12 mM) and glycerol (10%, v/v). The cytosolic fractions from rat brain (a) or cultured bovine aortic endothelial cells (b) (10 mg of protein each) were loaded onto the columns, and 0.8-mL fractions were collected throughout the experiment. The columns were washed with equilibration buffer and eluted with the same buffer containing increasing concentrations of KCl (0.1 to 0.3 M, dashed line, right ordinate). Fractions (100-μL aliquots) were analyzed for NO synthase activity on RFL-6 cells [19, 20] in the presence or absence of calmodulin (CaM, 100 nM). L-Arginine (100 μM), NADPH (100 μM) and superoxide dismutase (20 units/mL) were present in the incubation mixture and the RFL-6 detector cells were treated with 3-isobutyl-1-methyl xanthine (300 μM). The brain enzyme (panel a) eluted from the column at 0.1 KCl, and the activity was completely CaM dependent (because endogenous CaM only elutes from this column at KCl concentrations ≥0.2 M [12]). The predominant cytosolic endothelial enzyme activity, however, co-eluted with CaM at 0.2 M KCl and, consequently, was independent of *exogenous* CaM (panel b).

response by L-arginine and its inhibition by N^G-methyl- and N^G-nitro-derivatives of L-arginine [75–79]. Recently, we partially purified and characterized the NO-forming enzymes from two NANC-innervated tissues—the rat anococcygeus muscle [80] and the bovine retractor penis muscle [80]. Both tissues contained particulate and soluble activity that produced NO-like material from L-arginine (as determined by the stimulation of soluble guanylyl cyclase in RFL-6 detector cells [20]. The enzymes also converted L-arginine to L-citrulline (as assayed by the conversion of [^{3}H]L-arginine [16]. The particulate activity was solubilized with the detergent CHAPS [cf. 13], and both the solubilized particulate and the cytosolic activities were partially purified by DEAE anion exchange chromatography. Both types of enzymes required the cofactors NADPH and 5,6,7,8-tetrahydrobiopterin and their activity was clearly

dependent on Ca^{2+} and inhibited by calmodulin antagonists [80]. Further purification and characterization of these enzymes are required to determine whether they are the same as, or different from, established isoforms of NO synthase.

Summary and concluding remarks

NO synthase exists in several isoforms, four of which have been purified and characterized extensively. All isoforms use L-arginine as a substrate and all seem to require NADPH and 5,6,7,8-tetrahydrobiopterin as cofactors. Isoform Ia is a constitutive cytosolic Ca^{2+}/calmodulin-regulated enzyme that has been purified from brain [15–17]. Isoform Ib is a cytosolic enzyme from endothelial cells which is also regulated by Ca^{2+}/calmodulin [13, 62, 63], but whose chromatographic behavior differs from the brain enzyme. In addition, other investigators have isolated a soluble, Ca^{2+}-dependent enzyme from neutrophils which, however, was reported to be calmodulin independent [21]. We tentatively propose that this enzyme be classified as isoform Ic. Isoform II is a cytosolic, Ca^{2+}-independent enzyme that can be induced in macrophages by endotoxin and/or cytokines. In addition to the cofactors mentioned above, isoform II requires flavin-adenine-dinucleotide and thiols [23–28]. Inducible enzymes in cells other than macrophages are also Ca^{2+} independent. However, further work is required to determine whether they share all the characteristics of the macrophage enzyme. Isoform III is a particulate Ca^{2+}/calmodulin-dependent enzyme that is responsible for most of the EDRF/NO synthesis in endothelial cells [13, 38, 39]. The exact nature of the NO synthases in peripheral neurons producing non-adrenergic non-cholinergic transmitter remains to be determined, but they seem to share many of the properties of isoforms Ia and III. To further characterize the different enzymes, antibodies have now been generated to some isoforms. Their lack of cross-reactivity with other isoforms substantiates the different protein structures of the isoforms. Isoform Ia from brain has recently been cloned [81]. Efforts are underway in several laboratories including our own to clone the other isoforms. This should provide the definitive answer regarding the number of isoforms and their primary structure. Some recent reports have suggested that constitutive NO synthases (in brain and endothelial cells) and inducible NO synthase (in macrophages) may differ in their sensitivity to inhibition by L-arginine analogs. N^G-Nitro-L-arginine was found to be more potent than N^G-amino-L-arginine and N^G-methyl-L-arginine in inhibiting NO synthesis in brain and endothelial cells. In induced macrophages, however, N^G-amino-L-arginine and N^G-methyl-L-arginine were more potent inhibitors than N^G-nitro-L-arginine [82, 83]. If these differences in potency hold true for the isolated constitutive and induced enzymes in these and other cells, comparative studies with different inhibitors could provide important clues relative to the enzyme type responsible for NO synthesis in complex systems [82, 83]. The availability of the different isoforms of NO synthase in pure form now allows the development and testing of new selective inhibitors of the different enzymes. Such compounds are likely to be important tools for future research because the different isoenzymes have distinct functions in physiological and pathophysiological events.

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