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Subcellular Localization of Neuronal Nitric Oxide Synthase in Rat Small Intestine

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ABSTRACT. The subcellular localization of neuronal nitric oxide synthase (NOS I, EC 1.14.13.39) was investigated in the longitudinal muscle/myenteric plexus (LM/MP) preparation of rat small intestine. The presence of NOS I, inducible nitric oxide synthase (NOS II), and endothelial nitric oxide synthase (NOS III) was assessed after homogenization and low-speed centrifugation in a postnuclear supernatant by immunological detection after PAGE and Western blotting. Only NOS I was clearly present, whereas NOS II and NOS III were below detection limits. After high-speed centrifugation of the postnuclear supernatant, soluble and particulate fractions were obtained, and the presence of NOS I in these fractions was investigated by measurement of NOS I immunoreactivity and enzyme activity. We found that 90 ± 1% of NOS I immunoreactivity and 97 ± 1% of NOS enzyme activity were confined to the soluble fraction of the tissue. Further immunological analysis demonstrated that washing the particulate fraction revealed detectable amounts of NOS I only after concentration of the washing supernatant. Most particulate NOS I remained in the pellet and therefore represents cell organelle-associated enzyme. No NOS I immunoreactivity could be detected as a soluble protein within organelles of the cell. Particulate NOS I could in part be solubilized by Triton X-100 treatment, and the detection of Triton X-100-soluble NOS I was dependent on the antibody used. In conclusion, our results indicate that NOS I in the LM/MP preparation of rat small intestine is mainly soluble and that the particulate NOS I is partly an intrinsic membrane protein and can partly be solubilized by detergent treatment. BIOCHEM PHARMACOL **60**;1:145–153, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. NOS I; subcellular localization; LM/MP preparation; L-citrulline assay; PAGE

NO† is synthesized from L-arginine by NOS (EC 1.14.13.39) with the simultaneous production of equimolecular amounts of L-citrulline [1]. Three different isoforms of NOS are known: neuronal (NOS I) and endothelial (NOS III) nitric oxide synthase, which are constitutive, and inducible (NOS II) NOS [2]. In the central and peripheral nervous system, the presence of NOS I in neurones has been demonstrated by immunological studies [2, 3]. In the gastrointestinal tract, NOS I, which is present in nitrergic neurones, synthesizes the nitrergic neurotransmitter responsible to a large extent for non-adrenergic, non-cholinergic smooth muscle relaxation [4, 5]. Although there is little doubt that NO is a neurotransmitter, nitrergic neurotransmission deviates at least in one important aspect from classic neurotransmission. Whereas neurotransmitters such as noradrenaline and acetylcholine are stored in vesicles and are released by exocytosis when action potentials reach the nerve endings, it is generally accepted that the nitrergic neurotransmitter is synthesized on demand within the cell and diffuses through the plasma membrane towards its effector enzyme, soluble guanylate cyclase [6]. Because NO is a short-living free radical, the actions of NO are thought to be limited to the vicinity of the production site. Therefore, the precise cellular localization of NOS I is of major importance.

Contradictory results concerning the subcellular localization of NOS I have been reported. Brain NOS I was originally reported to be mainly soluble [2]. Hecker *et al.* [7], however, showed that up to 60% of total NOS activity in the rat cerebellum was present in a particulate fraction. Using ultracentrifugation methods, these authors concluded that this particulate NOS I was mainly associated with the endoplasmatic reticulum. Several studies reveal the presence of alternatively spliced NOS I mRNA in different species [8–11], sometimes leading to a truncated protein [9]. Transcription and translation of these splice variants is believed to play a role in tissue- and development-specific regulation of NOS I expression. Different NOS I splice variants may also have different subcellular localizations due to the presence or absence of certain association domains.

Very recently, the association of NOS I with membranes

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† *Abbreviations:* NO, nitric oxide; NOS I, neuronal nitric oxide synthase; NOS II, inducible NOS; NOS III, endothelial NOS; PDZ, PSD-95, discs-large, zona occludens-1; PSD-95, postsynaptic density-95 protein; LM/MP, longitudinal muscle/myenteric plexus; PMSF, phenylmethylsulfonyl fluoride; P_{1-4}, particulate fraction 1–4; S_{1-4}, soluble fraction 1–4; LDH, lactate dehydrogenase; and L-NOARG, N^G-nitro-L-arginine.

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by protein–protein interactions has been suggested. This interaction is mediated by its PDZ domain (PSD-95, *Drosophila* septate junction protein discs-large, epithelial tight junction protein zona occludens-1) [12–14]. In cerebral neurones, the PDZ domain of NOS I is linked with the second PDZ domain of PSD-95 [15], which in turn is associated with the *N*-methyl-D-aspartic acid (NMDA) receptor through interaction of its first PDZ domain with the COOH-terminal motif of the receptor. In the brain, NO indeed mainly acts as a retrograde transmitter released from a postsynaptic neuron upon stimulation of NMDA receptors and diffusing to the presynaptic neuron [16].

In peripheral neurones, nitrergic neurotransmission acts in an anterograde way. Little information is available on the subcellular distribution of NOS I in peripheral neurones. In the pig urethra [17] and the cat esophagus [18], α-latrotoxin-evoked relaxation was partially reduced by NOS inhibition. Since it is assumed that α-latrotoxin causes massive release of vesicle-stored neurotransmitters, these results could be explained by the vesicular presence of NOS I. However, biochemical data with regard to this hypothesis are lacking.

In view of the importance of the site of synthesis with respect to the action of NO and the lack of knowledge on the distribution of NOS I in the gastrointestinal tract, the aim of our study was to investigate the subcellular localization and distribution of NOS I in the peripheral nitrergic neurones of the gastrointestinal tract. The tissue studied was the rat small intestine, in which functional evidence for nitrergic neurotransmission was demonstrated previously [19, 20]. Particulate and soluble fractions were obtained via ultracentrifugation, and the distribution of NOS was determined via an immunological and enzyme activity assay. Subsequently, the particulate NOS I was studied in more detail. A preliminary account of these results has been given before [21].

MATERIALS AND METHODS

LM/MP Preparation and Cerebellum

Male Wistar rats of 200–540 g were obtained from Janssen Pharmaceutica or from Iffa Credo. The animals were killed by decapitation. After laparotomy, the small intestine 20 cm distally from the antrum to 10 cm proximally to the ileocecal junction was removed rapidly. After the lumen was rinsed in the direction of peristalsis, the ileum was mounted on a glass rod. The longitudinal muscle with the myenteric plexus was gently wiped off with cotton wool soaked in homogenization buffer 1 (250 mM sucrose, 10 mM tris(hydroxymethyl)aminomethane, 10 mM $MgCl_2$, 2 mM EDTA, 1 μM leupeptin, 1 μg/mL of aprotinin, 0.2 mM PMSF, pH 7.4). The LM/MP preparations were immediately immersed in ice-cold homogenization buffer 1 until homogenization, which was performed less than 30 min after preparation. Material from 4 to 8 rats was used for one experiment. Cerebellum of rat was rapidly removed after decapitation and immersed in ice-cold homogenization buffer 1 until homogenization.

Homogenization and Differential Centrifugation

The tissue was minced and homogenized in homogenization buffer 2 (250 mM sucrose, 10 mM tris(hydroxymethyl)aminomethane, 10 mM $MgCl_2$, 2 mM EDTA, Complete™ EDTA-free [protease inhibitor cocktail], pH 7.4) in 5 to 10 volumes (v/w) at 4° with a Potter-Elvehjem homogenizer fitted with a low-clearance Teflon pestle. The homogenate was centrifuged in a SS-34 rotor of a Sorvall RC-5B centrifuge at 300 *g* for 10 min at 4°. The pellet (nuclei and remaining intact tissue) was discarded. The supernatant (postnuclear supernatant, PNS) was centrifuged in a T65 rotor of a Beckman L2-65B centrifuge at 82,500 *g* for 45 min at 4° yielding a microsomal pellet P_1, containing the organelles of the cell, and a supernatant S_1, containing the soluble material of the cell. The P_1 fraction was resuspended in homogenization buffer 2 and used for analysis.

In one series of experiments, the ultracentrifugation scheme mentioned above was extended; an overview of this scheme is presented in the Results section. Briefly, the P_1 fraction was resuspended in homogenization buffer 2 (7–9 mL) by gentle homogenization and recentrifuged at 82,500 *g* for 45 min at 4°. The resulting P_2 fraction, which is a washed P_1 fraction, was resuspended in hypotonic homogenization buffer 3 (10 mM tris(hydroxymethyl)aminomethane, 10 mM $MgCl_2$, 2 mM EDTA, Complete™ EDTA-free [Protease Inhibitor Cocktail], pH 7.4) and subjected to at least 3 freeze–thaw cycles. The lysed P_2 fraction was recentrifuged under the same conditions as P_1, which gave rise to a P_3 fraction containing the membranes and a S_3 fraction containing the soluble material originally present within subcellular particles. The P_3 pellet was resuspended in homogenization buffer 3 supplemented with 0.1% (v/v) Triton X-100, incubated for 30 min at 4°, and centrifuged again under the same conditions. The final P_4 pellet consisted of membranes with intrinsic detergent-resisting membranous proteins. The S_4 supernatant contained the detergent-soluble proteins. Concentration of samples was done by either freeze-drying under vacuum or with a filter macrosolute concentrator Minicon-B15 (Amicon) based on a multiple ultrafilter with a cut-off rate of 15 kDa.

Immunodetection of NOS I, NOS II, and NOS III Quantification of NOS I

The proteins of a given fraction were separated by PAGE under denaturating conditions [22], blotted onto a nitrocellulose membrane [23], and NOS was immunologically detected as follows. All incubations were performed at room temperature on a three-dimensional rotating plate. After overnight postcoating with 5% (w/v) dry skimmed milk powder or 10% (v/v) Western blocking reagent in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl,

1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4), the blot was incubated for 2 hr with polyclonal rabbit anti-NOS antiserum. After extensive rinsing with PBS/0.05% (v/v) Tween 20, the blot was incubated for 2 hr with goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated antibodies (Sigma, 1:5000 diluted in PBS/0.05% (v/v) Tween 20). The NOS isoforms were detected by enhanced chemiluminescence, performed according to the manufacturer's instructions. No difference could be seen between the two postcoating solutions used. For the immunological detection of NOS I, NOS II, and NOS III, polyclonal rabbit antibodies were used: for NOS I, an antibody from Sigma, diluted at 1:5000, or from Alexis, diluted at 1:1000; for NOS II, an antibody from Alexis, diluted at 1:1500; and for NOS III, an antibody from Alexis, diluted at 1:1500. All antibodies were diluted in PBS/0.05% (v/v) Tween 20.

The NOS I antibody from Sigma was generated in rabbit with a peptide corresponding to amino acids 250–271 of the N-terminal region of the NOS I protein. The coding sequence for these amino acids lies entirely within exon 2 of the *NOS I* gene. The immunization peptide used in the case of the Alexis NOS I antibody corresponds to an N-terminal 2-kDa peptide. It is also likely that this peptide is encoded by exon 2. Huber *et al.* [9] reported the presence of 3 mRNA splice variants in the LM/MP preparation of rat small intestine: nNOSα-a, nNOSα-b, and nNOSβ. The first two mRNAs differ only in the 5′ untranslated region and will therefore, upon translation, result in the same protein. In the latter mRNA, exon 2 is deleted. From these data, we can infer that the antibodies used can detect only one alternatively spliced mRNA transcript, derived from nNOSα-a or nNOSα-b mRNA, leading to a single NOS I immunoreactive band, hereafter called NOS I.

The NOS I immunoreactive bands were quantified by densitometry (LKB Bromma 2202 Ultroscan laser densitometer). Peak heights were taken as arbitrary units. As a rule, each sample was electrophorized in at least 5 dilutions and the densitometric value was plotted against the dilution factor, indicating the amount of protein loaded on the gel. Only the dilutions in which the densitometric value was within the linear area of the curve were used for calculation of the average value for that particular sample. In order to determine the subcellular distribution of NOS I over the S_1 and P_1 fractions, the densitometric value of the NOS I bands was corrected for the volume, and the proportional distribution of NOS I immunoreactivity between the S_1 and P_1 fractions was calculated. Only S and P samples electrophorized on the same gel, hence treated identically in the immunodetection procedure, were used for calculation.

Enzyme Activity Assays

LDH. The designation of the subcellular fraction as soluble (S_1) was confirmed using the activity of a typical marker enzyme for the soluble fraction, i.e. LDH (EC 1.1.1.27). The activity of LDH was measured according to Bergmeyer [24] with small volume adaptations. Briefly, 50 μL of a subcellular fraction was mixed with 1.425 mL phosphate–pyruvate solution (0.05 M phosphate buffer, pH 7.5; 310 μM pyruvate) and 25 μL 8 mM NADPH. The extinction (E) was continuously monitored at 340 nm and ΔE/min was used to calculate LDH units/mg protein. One unit is defined as the amount of LDH which changes the E of NADPH at 340 nm by 0.001 in 1 min [24]. Each sample was measured in duplicate in at least two dilutions. Taking into account the total volume of respective subcellular fractions, total LDH units of each fraction were calculated. The proportional distribution over the S_1 and P_1 fractions was then determined.

5′-NUCLEOTIDASE. The presence of membrane-bound enzymes in the S_1 fraction was estimated by determination of the 5′-nucleotidase (EC 3.1.3.5) activity distribution between the S_1 and P_1 fractions. The activity of 5′-nucleotidase was determined as follows: a 100-μL sample was incubated with 80 μL 5′-AMP solution (50 mM 5′-AMP, 0.3 M tris(hydroxymethyl)aminomethane, 12.5 mM $MgCl_2$, pH 7.8) for 30 min at 37°. The reaction was stopped by addition of 45 μL ice-cold trichloroacetic acid (50%). After an incubation of 30 min on ice, proteins were pelleted by centrifugation for 6 min at 15,800 *g*. The concentration of liberated P_i was determined by incubation of 100 μl supernatant with 1 mL ammonium molybdate (0.42% [w/v] in 1 M H_2SO_4)/ascorbic acid (10% [w/v]) mixture (1:6) at 37°. Absorbance of samples and the standard curve were measured at 820 nm. Each sample was measured in at least two dilutions. Enzyme activity was expressed as μg liberated P_i/mg protein/hr. Taking into account total volumes of respective fractions, the total 5′-nucleotidase activity of each fraction was calculated, and the proportional distribution between the S_1 and P_1 fractions was determined.

NOS. NOS activity was assessed by a slight modification of the L-citrulline assay [25]. In brief, 25 μL of the subcellular fraction was incubated with 100 μL incubation mixture (25 mM tris(hydroxymethyl)aminomethane, 10 μM 1 μCi/mL of [G-$^3H_4$]L-arginine, 3 μM tetrahydrobiopterin, 100 nM calmodulin, 1 μM flavin mononucleotide, 1 μM flavin adenine dinucleotide, 1 mM NADPH, pH 7.4) for 60 min at 37° in the presence or absence of 100 μM L-NOARG. After incubation, 1 mL of ice-cold stop buffer (200 mM HEPES, 20 mM EDTA, pH 5.5) was added, and L-citrulline was separated from L-arginine on Dowex 50X8-400 (Na^+ form). The flow-through was collected and the radioactivity measured by liquid scintillation counting. Within the linear part of the enzyme activity–protein concentration curve, NOS I activity was assessed in three dilutions. Enzyme activity was expressed as pmol L-citrulline/min/mg protein. For the calculation of the cellular distribution of NOS activity, the total amount of L-citrulline/min produced in each fraction was calculated,

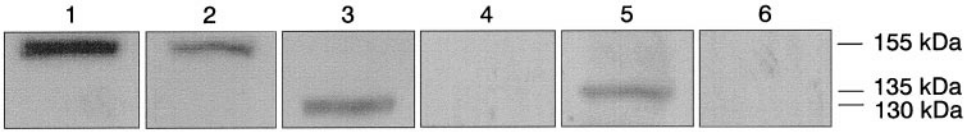

FIG. 1. Immunodetection of NOS I, NOS II, and NOS III in the postnuclear supernatant (PNS) of the LM/MP preparation of rat small intestine. Lanes 1, 3, and 5 are controls for NOS I (PNS of rat cerebellum), NOS II (cell lysate of induced RAW 264.7 cells), and NOS III (cell lysate of the human aortic endothelial cell line HUV-EC-C), respectively. The postnuclear supernatant of rat small intestine is repeated in lanes 2, 4, and 6. Lanes 1 and 2 were incubated with primary antibodies against NOS I, lanes 3 and 4 against NOS II, and lanes 5 and 6 against NOS III. Molecular weights of NOS I (155), NOS II (130), and NOS III (135) reactivity are indicated.

and the proportional distribution of NOS activity between the S_1 and P_1 fractions was determined.

In preliminary experiments, the Ca^{2+} dependence of the NOS enzyme activity was determined, using the concentration-dependent inhibition of NOS activity with EGTA. Ten microliters of various EGTA concentrations was included in the incubation mixture, and a complete inhibition of the enzyme activity was obtained at a concentration of 2 mM EGTA. Incubation in the presence of $CaCl_2$ (more than 70 μM final concentration) led to a decrease in activity. This inhibition by higher concentrations of Ca^{2+} was described previously by Knowles *et al.* [26]. Therefore, NOS activity was assessed in the absence of additional Ca^{2+}.

Determination of Protein Concentration

Protein concentrations were measured according to the method of Bradford [27] with the modification of Macart and Gerbaut [28]. Each sample was assessed in at least two dilutions within the range of the calibration curve.

Drugs

Sucrose, leupeptin, aprotinin, PMSF, Triton X-100, NADPH, tetrahydrobiopterin, flavin mononucleotide, flavin adenine dinucleotide, polyclonal rabbit anti-NOS I antiserum (N 7155), goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated antiserum (A 0545), Na_2HPO_4, Tween 20, L-NOARG, Dowex 50X8-400, and EGTA were obtained from Sigma. Alexis provided polyclonal rabbit anti-NOS I (210-502-R100), anti-NOS II (210-504-R100), and anti-NOS III (210-505-R100) antiserum. Tris(hydroxymethyl)aminomethane, $MgCl_2$, NaCl, KCl, KH_2PO_4, and $CaCl_2$ were purchased from Merck. EDTA, Complete™ EDTA-free (protease inhibitor cocktail), Western blocking reagent, and HEPES were obtained from Boehringer Mannheim. Sodium pyruvate was obtained from Life Technologies and [G-$^3H_4$]L-arginine from Amersham. Calmodulin was from Calbiochem. Cell lysates of induced RAW264.7 (ATCC TIB71) macrophages and of the aortic endothelial cell line HUV-EC-C (ATCC CRL-1730) were obtained from Transduction Laboratories. All solid drugs were dissolved in deionized water except for EGTA (10 mM), which was dissolved in 0.1 M NaOH, and PMSF (0.2 M), which was dissolved in pure ethanol.

RESULTS

Presence of NOS I, NOS II, and NOS III in LM/MP Preparation of Rat Small Intestine

A representative result of the immunological detection of NOS I, NOS II, and NOS III in the postnuclear supernatant of the LM/MP preparation is depicted in Fig. 1. Only NOS I could be detected: a clear band at the 155-kDa level of the pattern was present when antibodies against NOS I were used, and the location on the gel of this band was identical to NOS I present in cerebellum. When an antibody against NOS II was used, a 130-kDa band was seen in the cell lysate of induced RAW 264.7 murine macrophages, but not in the postnuclear supernatant of the LM/MP preparation. Similarly, in cell lysate of the human aortic endothelial cell line HUV-EC-C, a clear 135-kDa band was detected when the antibody against NOS III was used; in the postnuclear supernatant fraction of the LM/MP preparation, this band was not present.

Subcellular Localization and Distribution of NOS I

A representative example of the presence of NOS I immunoreactivity in the particulate and soluble fractions is shown in Fig. 2. Upon incubation with NOS I antibodies, a 155-kDa band was found in both fractions. The staining method for NOS I immunoreactivity present in both the soluble and particulate fractions showed linearity (Fig. 3). At a dilution factor of 1/15 (protein load below 2 μg) for the S_1 fraction and 1/20 (protein load 3 μg) for the P_1 fraction, a linear relationship was obtained between the amount of protein loaded on the gel and the staining intensity. At lower dilution factor, hence higher protein load, deviations from linearity were obtained.

Average values for LDH enzyme activity, 5′-nucleotidase activity, NOS immunoreactivity, and NOS enzyme activity in the postnuclear supernatant are given in Table 1, as well as the distribution among the S_1 and P_1 fractions and the

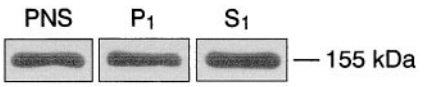

FIG. 2. NOS I immunoreactivity in subcellular fractions of the LM/MP preparation of the rat small intestine. A representative result of NOS I immunoreactivity in the PNS and the P_1 and S_1 fractions is given. Molecular weight of the NOS I reactivity is indicated.

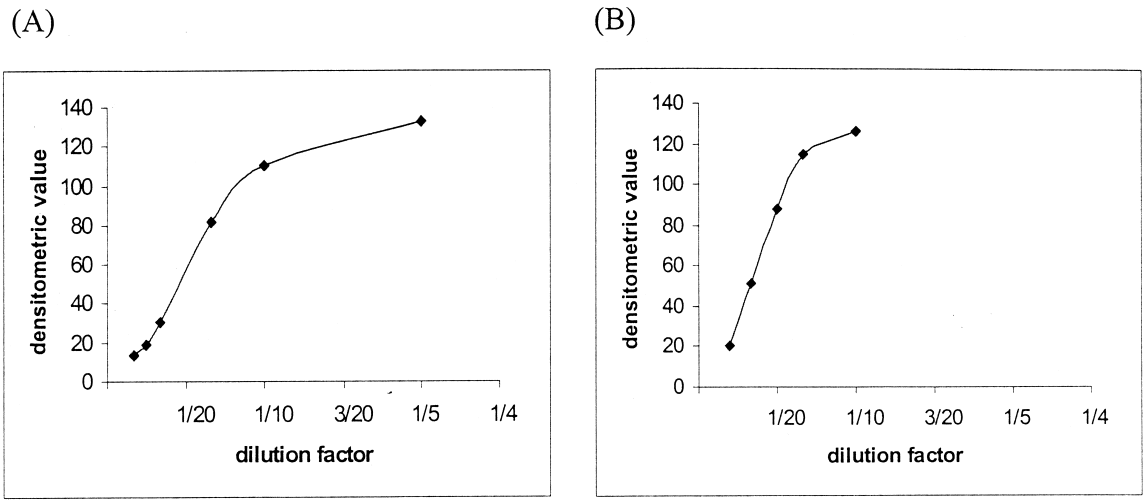

FIG. 3. Representative example of the relation between the densitometric value of NOS I immunoreactivity and the dilution factor of the sample. The densitometric value of the NOS I-specific band in the S_1 fraction with a protein concentration of 1.183 mg/mL (A) and the P_1 fraction with a protein concentration of 2.477 mg/mL (B) for different dilution factors of the sample is given. In this specific case, taking the volumes of S_1 and P_1 into account, the distribution of NOS I between S_1 and P_1 was 90% and 10%, respectively.

recovery. LDH activity was nearly completely confined to the soluble fraction of the tissue. Sixty-four percent of 5′-nucleotidase activity was confined to the particulate fraction. Only 4 ± 0.3% (mean ± SEM, N = 3) of 5′-nucleotidase activity in the S_1 fraction could be sedimented by recentrifugation for 60 min at 102,000 *g*. Ninety percent of NOS I immunoreactivity and 97% of NOS enzyme activity were present in the supernatant S_1. These percentages were significantly different (two-sided χ^2 test, $P = 0.045$). The recoveries for LDH enzyme activity, 5′-nucleotidase activity, proteins, and NOS immunoreactivity varied between 71 and 98% in the individual experiments; for NOS enzyme activity, recovery varied between 34 and 93%. Recentrifugation of the S_1 fraction for 60 min at 102,000 *g* resulted in an additional sedimentation of only 6 ± 0.2% (mean ± SEM, N = 3) of the soluble NOS I immunoreactivity.

Incubation with 100 μM L-NOARG significantly reduced enzyme activity in all subcellular fractions to 6% or less of its control value (Fig. 4A). Furthermore, NOS activity decreased with increasing concentrations of EGTA; when 2 mM EGTA was included in the incubation mixture, slight to no residual NOS activity could be measured (Fig. 4B).

Characterization of Particulate NOS I

In Fig. 5A, the scheme of the extended centrifugation, applied to further characterize particulate NOS I, is represented. In Fig. 5B, the results of immunological detection with the Alexis antibody of NOS I in the different fractions are depicted. In all particulate fractions, a band at 155-kDa corresponding to NOS I was present. The decrease in immunoreactivity from P_1 to P_4 was due to dilution of the sample, since a part of the P fraction was taken for analysis after each centrifugation, while the remainder was used to proceed as in Fig. 5A. Immunoreactivity in the soluble fractions obtained after washing (S_2), lysis (S_3), and detergent treatment (S_4) remained, even after concentration of the samples below detection limits. The extended centrifugation scheme was also repeated twice for detection with the Sigma NOS I antibody. The same results were obtained as with the Alexis antibody except for 2 points: we were now able to detect NOS I in the S_2 fraction, (although only

TABLE 1. Distribution of LDH enzyme activity, 5′-nucleotidase activity, NOS I immunoreactivity, NOS enzyme activity, and protein content in the LM/MP preparation of rat small intestine

	PNS	Recovery	P_1	S_1
LDH enzyme activity (N = 4)	8357 ± 478	95 ± 2	1 ± 0	99 ± 0
5′-Nucleotidase enzyme activity (N = 3)	194 ± 61	82 ± 7	64 ± 2	36 ± 2
NOS I immunoreactivity (N = 3)	347 ± 34	83 ± 1	10 ± 1	90 ± 1
NOS enzyme activity (N = 4)	65 ± 5	61 ± 12	3 ± 1	97 ± 1
Protein (N = 8)	8 ± 0	87 ± 1	15 ± 1	85 ± 1

The absolute value is given for postnuclear supernatant (PNS; LDH enzyme activity, U/mg protein; 5′-nucleotidase enzyme activity, μg liberated P_i/mg protein/hr; NOS I immunoreactivity, arbitrary units/mg protein; NOS enzyme activity, pmol L-citrulline/min/mg protein; protein, mg protein/g tissue). Recovery and distribution between P_1 and S_1 are given as percentages. For LDH enzyme activity, 5′-nucleotidase enzyme activity, NOS I immunoreactivity, and NOS I enzyme activity, the distribution between the P_1 and S_1 fractions was calculated as described in Materials and Methods. The distribution of protein was calculated by expressing the amount of protein in the P_1 and S_1 fractions as percentage of the sum of the total amount of protein in both fractions. The mean ± SEM is given.

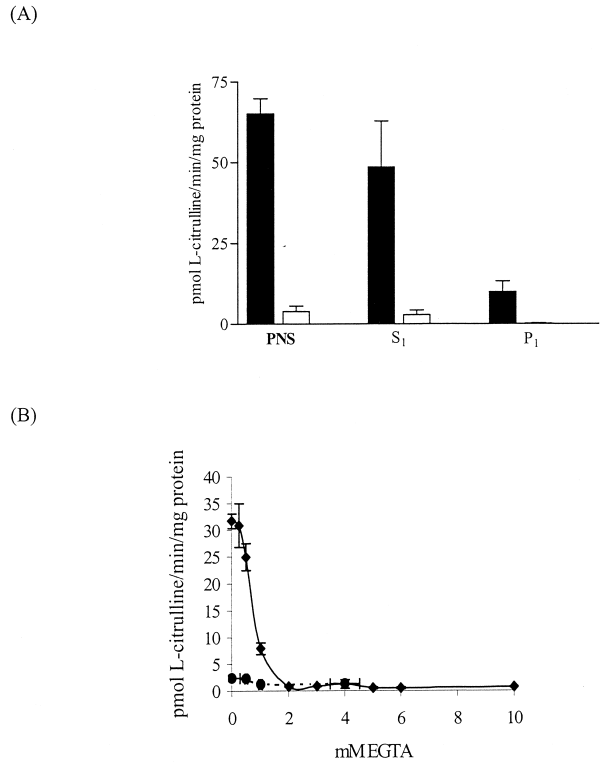

FIG. 4. Inhibition of NOS enzyme activity by L-NOARG and EGTA. (A) Inhibition of NOS activity by 100 μM L-NOARG. NOS activity (pmol L-citrulline/min/mg protein) in the PNS and the S_1 and P_1 fractions was assayed in the absence (full bars) or presence (open bars) of L-NOARG. The mean ± SEM (N = 4) is given. Enzyme activity was reduced by 94.0%, 94.2%, and 98.5%, respectively. (B) Dependence of NOS enzyme activity on the EGTA concentration. NOS activity (pmol L-citrulline/min/mg protein) in the S_1 fraction was assayed in the absence (full line) or presence (dotted line) of 100 μM L-NOARG with varying concentrations of EGTA. The mean ± SEM (N = 3) is given.

after concentration) and in the S_4 fraction containing the detergent-soluble proteins. Densitometric analysis revealed that 7 to 10% of NOS I immunoreactivity in the P_1 fraction originated from trapped cytosolic material and could, after washing, be found in the concentrated S_2 fraction. A distribution of about 50–50% between the detergent-resistant (P_4) and detergent-soluble (S_4) proteins (54.2% for experiment 1 and 55.1% for experiment 2) was calculated.

DISCUSSION

In this study, we have ascertained the presence of NOS I in the LM/MP preparation of rat small intestine using biochemical methods, thereby extending the earlier functional evidence presented by Kanada *et al.* [19] and Smits and Lefebvre [20]. Since we could not detect NOS II and NOS III in the LM/MP preparation, we conclude that NOS I is responsible for the generation of the nitrergic neurotransmitter in this tissue. However, it is still possible that the nitrergic transmitter is not, or not solely, synthesized in neurones but also in other cell types. The LM/MP preparation also contains smooth muscle cells, and the presence of a constitutive NOS isoform in gastrointestinal smooth muscle cells has been proposed [29, 30]. Recent results obtained in freshly dispersed gastric smooth muscle cells suggest that the muscular NOS is NOS III [31], but this isoform was not detected in the LM/MP preparation of the rat small intestine in this study.

In a study by Huber *et al.* [9], the presence of three alternatively spliced NOS I mRNAs in LM/MP preparation of rat small intestine was demonstrated. These different mRNAs encoded for two different proteins with weights of 155 and 135. For reasons explained in Materials and Methods, our NOS I antibodies could only detect the 155-kDa NOS I band. Since it has been proposed by different authors that the expression of NOS I splice variant mRNA and protein is spatially and developmentally regulated [8–11] and the age of Wistar rats used in the study of Huber *et al.* [9] was not stated, we cannot exclude the possibility that the absence of a NOS I splice variant in our LM/MP preparation was developmentally regulated.

NOS I was originally reported to be mainly soluble [2], while the other constitutive NOS isoform, NOS III, is almost entirely linked to the cell membrane of endothelial cells via an N-terminal palmitoylation sequence [32, 33]. Since NO is known to be a very short-living neurotransmitter, an association of a significant portion of NOS I with cell membranes could contribute to the regulation of the action of NOS. Confirming the results of Huber and co-workers [9], we detected the 155-kDa full-length NOS I both in soluble and particulate fractions. Our results indicate that in peripheral nitrergic neurones, NOS I is mainly present as a soluble enzyme, with only a small part being found in the membranes. Upon PAGE and Western blot analysis, no difference in molecular weight could be detected between the soluble and particulate forms of NOS I, which thus likely represents the same protein. This immunological equivalency was also found in rat brain [34].

The results of the L-citrulline assay indicate that particulate NOS represents only a small part of total NOS activity. This might suggest that particulate NOS has no major physiological influence; however, it has been demonstrated for other enzymes such as protein phosphokinase C that part of the soluble enzyme migrates to the membranes and becomes particulate upon activation of the cell [35]. Whether or not a comparable mechanism occurs for NOS I upon activation of the neuronal cell remains to be investigated.

The significant difference in proportional distribution obtained by the immunological and enzyme activity assays (about 10% vs 3%, respectively, found to be particulate) could be caused by different reasons. First, the small percentage of particulate NOS I found in the LM/MP preparation may be associated with the endoplasmatic reticulum due to *de novo* synthesis. These NOS I molecules might not have the fully developed configuration for enzyme activity, but might display full immunological activity. Second, the accessibility of co-factors to NOS I

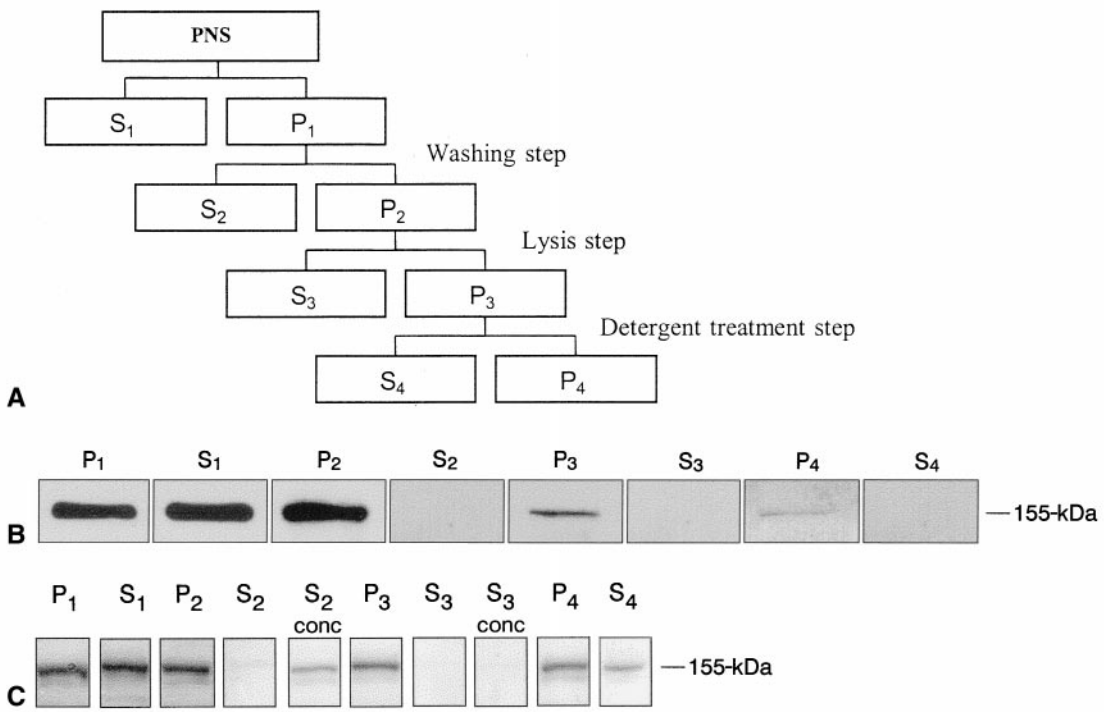

FIG. 5. Results from the extended ultracentrifugation. (A) Scheme of the extended ultracentrifugation. The whole experiment was repeated twice with both antibodies; all samples were assayed as described in Materials and Methods. (B) Representative example of NOS I immunoreactivity when detection was performed with the Alexis NOS I antibody in P_1 to P_4 and S_1 to S_4. Molecular weight of the NOS I reactivity is indicated. (C) Representative example of NOS I immunoreactivity when detection was performed with the Sigma NOS I antibody in P_1 to P_4 and S_1 to S_4. To the right of S_2 and S_3, the concentrated samples $S_{2,conc}$ and $S_{3,conc}$ respectively are shown. Molecular weight of the NOS I reactivity is indicated.

might be limited due to steric hindrance of the membrane-associated enzyme. As a result, particulate NOS I would be immunologically detectable but not have full enzymatic activity. Third, the dimerization process for particulate NOS I could be limited by membrane rigidity, once more resulting in a lower enzymatic activity for particulate NOS I. Finally, soluble alternatively spliced NOS I transcripts such as nNOSβ in the study of Huber *et al.* [9] could not be detected with our antibodies, but could tip the balance further in the direction of the soluble fraction in the enzyme activity assay.

The results of the distribution of LDH activity between S_1 and P_1 indicate that nearly no soluble LDH is present in the P_1 fractions. This suggests that the P_1 fraction was not contaminated with soluble material. In agreement with this result, NOS I immunoreactivity could be detected in the washing supernatant S_2 only after extensive concentration of the supernatant fluid. Moreover, the detection of NOS I in the concentrated S_2 fraction was only possible when the most sensitive antibody was used. Therefore, by far the major part of NOS I immunoreactivity in P_1 represents true particulate-bound enzyme and is not derived from contaminating soluble enzyme. Since only 4% of soluble 5′-nucleotidase and 6% of soluble NOS I immunoreactivity could be sedimented by recentrifugation of the S_1 fraction at higher speed, the immunologically determined NOS I distribution is not largely influenced by remaining sedimentable NOS I in the S_1 fraction.

A lower sensitivity, hence higher detection limit, could be a possible explanation for the absence of detection of NOS I in the concentrated S_2 fraction with the Alexis NOS I antibody. In the case of the 155-kDa band detected in the S_4 fraction, this can, however, not be the only explanation. The absence of detection of detergent-solubilized NOS I when the Alexis antibody was used could be due to the fact that Triton X-100 solubilization destroys the epitopes recognized by this antibody. The immunization peptide of the Alexis antibody was, according to the data sheet, an N-terminal 2-kDa peptide. The N-terminal domain of NOS I contains the PDZ domain, the most plausible candidate to cause an interaction between NOS I and the membrane or membrane-bound proteins. Triton X-100 weakening of the NOS I enzyme from the membranes could cause alterations in the PDZ domain, resulting in a loss of immunoreactivity for the Alexis antibody. The Sigma antibody was generated after immunization with amino acids 251–270 from rat brain NOS I. This sequence lies outside the PDZ domain region as reported by Tochio *et al.* [13] (PDZ domain-containing region between amino acids 11–133), Hillier *et al.* [14] (PDZ domain-containing region between amino acids 1–130), and Brenman *et al.* [15] (PDZ domain-containing region between amino acids 1–195).

As mentioned earlier, the results of Werkström *et al.* [17] and Ny *et al.* [18] suggest a possible localization of NOS I within vesicles. If NOS I were present as a soluble enzyme

in organelles of the cell, relaxation evoked by electrical stimulation or α-latrotoxin treatment could be caused by docking of the NOS I-containing vesicles, releasing the enzyme into the extracellular environment. Our results of the extended centrifugation scheme, however, do not support this hypothesis. Indeed, NOS I could not be detected in the S_3 fraction, the fraction containing the soluble material originally present within the organelles. Our results with the detergent treatment indicate that particulate NOS I is bound to membranes and is approximately 50% detergent-resistant. It is, however, still possible that particulate NOS I is present in or associated with the membrane of a vesicle, which fuses with the neuronal plasma membrane upon stimulation by α-latrotoxin. This hypothesis is in agreement with both our results and those of Werkström *et al.* [17] and Ny *et al.* [18].

The question as to which type of membrane the particulate NOS I is associated with thus remains to be answered. In rat cerebellum, particulate NOS I was assumed to be associated with the endoplasmatic reticulum [7]. In addition, an association with the plasma membrane in which the enzyme is present as a dimer should be considered, because in skeletal muscle and brain, the PDZ domain of NOS I causes an association with the plasma membrane via protein–protein interactions [15]. Further experiments will be needed to establish to which type of subcellular particle the particulate NOS I is bound in the LM/MP preparation of rat small intestine.

In conclusion, we found that in the LM/MP preparation of the rat small intestine NOS I is mainly present as a soluble enzyme. Particulate NOS I is not present within the organelles of the cell and is a partly intrinsic membrane enzyme. Further experiments will be needed to elaborate the nature of the membrane to which the particulate enzyme is bound.

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