Identification of a specific amino acid cluster in the calmodulin-binding domain of the neuronal nitric oxide synthase

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Abstract The calmodulin (CaM) binding domain of rat neuronal nitric oxide synthase (nNOS) was analyzed using 3 synthetic peptides corresponding to different regions of the middle portion of the enzyme. One corresponding to nNOS 732–754 gave complete inhibition of NOS enzyme activity with an IC_{50} of about 1 μM. Kinetic analysis indicated that the inhibition was not competitive with respect to L-arginine and the peptide produced a Ca^{2+} dependent, electrophoretic mobility shift of CaM on 1 M urea gels. A specific hydrophobic/basic amino acid cluster in the rat nNOS sequence, Lys^{732}LysLeu, that was critical for its CaM binding was also identified in this study.

Key words: Calmodulin; Neuronal nitric oxide synthase; Peptide

1. Introduction

Nitric oxide synthases (NOSs) catalyze the oxidation of L-arginine to form L-citrulline and nitric oxide (NO), a ubiquitous paracrine substance with diverse physiological roles [1]. The activity of the constitutive neuronal NOS (nNOS) [2,3] and endothelial NOS (eNOS) [4,5] is Ca^{2+}/calmodulin (CaM) dependent. There is also an inducible NOS (iNOS) [6,7] which is a Ca^{2+} insensitive enzyme containing CaM as a tightly bound subunit. For enzyme regulation of NOSs, there are at least two important points to consider in terms of structure and function. First, there is the CaM binding domain, identified as the amino acid sequences 725–754 (nNOS) [8], 504–532 (iNOS) [9] and 493–512 (eNOS) [10] (numbering based on positions within each NOS isoform). Secondly, there is the unique complexity of NOSs as homodimers [11,12]. Oligomerization may play a significant role not only for enzyme regulation but also for signal transduction. In the present study, we have defined the crucial amino acid cluster for CaM binding in nNOS using peptide chemistry studies and site-directed mutagenesis.

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Abbreviations: CaM, calmodulin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; NOS, nitric oxide synthase; nNOS, iNOS, eNOS, neuronal, inducible, and endothelial isoforms of nitric oxide synthase; CaM-kinase, Ca^{2+}/calmodulin dependent protein kinase; PVDF, polyvinylidene; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2. Materials and methods

2.1. cDNA construction and mutagenesis

A cDNA for rat brain nNOS was introduced into the *Eco*RI- and *Bam*HI-digested pVL1393 transfer vector resulting in pVLnNOS. The *Eco*RI/*Bam*HI fragment of the cDNA for the nNOS was inserted into M13mp18 to produce single strand DNA for mutagenesis. Two different oligonucleotides, 5′-CGCCTTGACCCTATCGGCCTCGGCCTGCCCCAT-3′ and 5′-CTTGACGGCCTCTGCCTCATCATCAAAGCCGATAGCTCG-3′, designed to provide mutations of (Met^{749}/Lys^{751}) to (Glu^{749}/Asp^{751}) and (Lys^{732}LysLeu) to (Asp^{732}AspGlu) (underlined codons), respectively, were synthesized. The Amersham oligonucleotide-directed mutagenesis system (version 2.1) was used to generate mutant cDNAs. Mutant clones were isolated, and cDNA sequences were reconfirmed by dideoxynucleotide sequencing (Sequenase version 2.0, U.S. Biochem.). The mutant cDNAs were ligated into pVLnNOS and transfected into insect Sf9 cells.

2.2. Purification of expressed nNOS protein

Expression proteins in Sf9 cells and purification of expressed wild-type or mutant nNOS on 2′,5′-ADP-Sepharose were performed as described previously [13]. Protein concentrations were determined by the method of Bradford using BSA as the standard [14].

2.3. NOS enzymatic assays

NOS activity of purified enzyme or cell lysates was assayed by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, as described previously [2].

2.4. CaM-kinase Iα assays

Assay conditions were 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 100 μM [γ-^{32}P]ATP, 50 μM syntide-2, 1 μM CaM, 1 mM $CaCl_2$, and 300 nM recombinant CaM-kinase Iα in a final volume of 20 μl at 30°C. The reaction was initiated by the addition of CaM-kinase Iα and terminated after 10 min by spotting aliquots (16 μl) onto phosphocellulose paper (Whatman P-81), followed by washing in 75 mM phosphoric acid.

2.5. Gel electrophoresis and Western blot analysis

One-dimensional SDS-PAGE was carried out according to the method of Laemmli [15]. The electrophoretic transfer of proteins from SDS-PAGE gels to PVDF membranes was performed as described by Towbin et al. [16]. For immunodetection of the transferred proteins, the procedure of Burnette [17] was used except that the second antibody was linked to horseradish peroxidase. Antigen-antibody complexes were visualized by reacting the bound peroxidase with a chemiluminescence reagent (Du Pont).

2.6. Gel mobility shift assays

High affinity binding of peptides was analyzed by gel mobility shift assays of CaM on 1 M urea-15% polyacrylamide gels. Gels were run in the presence of either 0.1 mM $CaCl_2$ or 2 mM EGTA in order to determine the Ca^{2+} dependence.

2.7. CaM overlays

Wild-type and mutant nNOS proteins were resolved on 7.5% SDS-PAGE and electrophoretically transferred onto PVDF membranes. The membranes were blocked with 100 mM NaCl, 50 mM Tris (pH 7.5), 1 mg/ml BSA, 0.05% Tween 20 in the presence of 1 mM $CaCl_2$ for 30 min at room temperature. Biotinylated CaM was then added at a final concentration of 0.5 μg/ml in Tris buffer followed by incubation for 30 min. After washing with the buffer, membranes were in-

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cubated with avidin-biotin-peroxidase (Vector Laboratories, Inc.), washed extensively, and then developed with the chemiluminescence reagent (Du Pont).

2.8. Materials

nNOS CaM binding peptide 23 (KKLAEAVKFSAKLMGQAMAKRVK), 16 (KFSAKLMGQAMAKRVK), and 12 (KLMGQAMAKRVK) were obtained from Nippon Shinyaku Co. (Kyoto, Japan). The cDNA for rat brain nNOS was a generous gift of Dr. Solomon H. Snyder (The Johns Hopkins University School of Medicine, Baltimore, MA). [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Dupont-New England Nuclear. L-[^{3}H]Arginine and ECL detection kits were purchased from Amersham Corp. Restriction enzyme and DNA-modifying enzymes were from Takara Shuzo. 2′,5′-ADP-Sepharose was obtained from Pharmacia Biotec Inc. Electrophoresis reagents and the Bradford protein dye reagent were products of Bio-Rad. Anti-nNOS antibody was from Transduction Laboratories (Lexington, KY). All other materials and reagents were of the highest quality available from commercial suppliers.

3. Results and discussion

3.1. Identification of nNOS CaM binding sequence by gel mobility shift assays

The CaM binding domain of rat brain nNOS was earlier identified to be formed by the sequence of amino acids 725–754 (nNOS) [8] based on a computer search for hydrophobicity, charge, and amphiphilic helical moment. For further studies of the CaM binding domain in nNOS, we initially synthesized a 23 residue peptide ($nNOS_{732-754}$; peptide 23) (Fig. 1) and tested its ability to produce an electrophoretic mobility shift of CaM on polyacrylamide gel electrophoresis in 1 M urea. In the presence of Ca^{2+}, the electric mobility of CaM was retarded by peptide 23 (Fig. 1A), suggesting that it contains residues necessary for binding of Ca^{2+}/CaM by nNOS. Peptide 23, however, did not form a complex with CaM in the presence of EGTA, indicating the complex formation to be Ca^{2+} dependent (Fig. 1B). The sequence was next shortened in the COOH-terminal direction to make a 16 residue ($nNOS_{739-754}$; peptide 16) and a 12 residue peptide ($nNOS_{743-754}$; peptide 12) (Fig. 1) for determination of specific amino acid residues critical for CaM binding. No complex formation was detected with either in the presence of Ca^{2+} even at a 3:1 molar ratio of peptide to CaM (Fig. 1A).

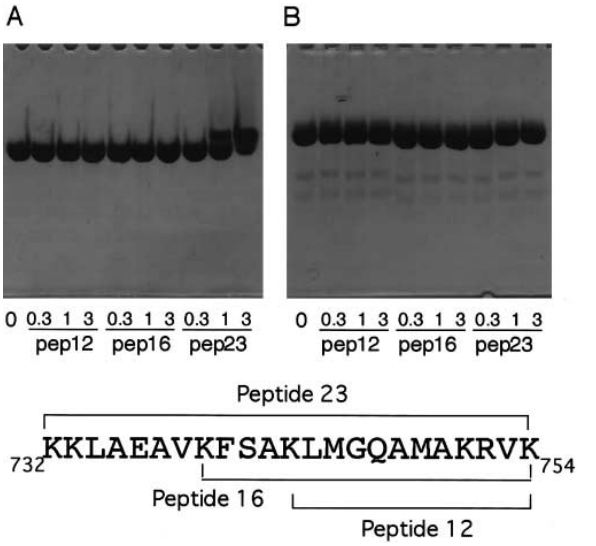

Fig. 1. Electrophoretic mobility shift of CaM by synthetic peptides. CaM (10 μM) was incubated with the indicated synthetic peptide in the presence of 0.1 mM $CaCl_2$ (A) or 2 mM EGTA (B). Samples were then electrophoresed on 1 M urea-15% polyacrylamide gels containing either 0.1 mM $CaCl_2$ or 2 mM EGTA. The relative mobilities of CaM and CaM-peptide complexes were visualized by Coomassie staining. Molar ratios of peptide:CaM of 0:1, 0.3:1, 1:1, and 3:1 are indicated. Note the mobility of CaM in the presence of $CaCl_2$ is faster than that in the presence of EGTA without peptides. The sequences of the nNOS synthetic peptides are shown below the gels. Peptides were synthesized corresponding to the indicated nNOS sequence.

3.2. Inhibition of CaM-dependent enzyme activity by nNOS peptides

We tested nNOS peptides as inhibitors of NOS enzyme activity using L-[^{3}H]arginine as the substrate. As shown in Fig. 2A, peptide 23 strongly inhibited recombinant nNOS activity with an IC_{50} of about 1 μM. The concentration (0.1 μM) of CaM was chosen to be more than sufficient for activation of NOS (data not shown) to ensure that inhibition would be seen if the mechanism was competition with CaM. Indeed, increasing the concentration of CaM resulted in a decrease in the inhibitory action of peptide 23 (data not shown), while increasing the concentration of substrate did not affect NOS activity (Fig. 2B). Neither peptide 12 nor peptide 16 showed any significant effects on NOS enzyme activity at the 0.1 μM concentration of CaM (Fig. 2A). To assess the inhibition by peptide 23 of another CaM dependent enzyme, its effects on recombinant CaM-kinase Iα activity were determined. In the presence of Ca^{2+}/CaM, peptide 23 did inhibit CaM-kinase Iα with an IC_{50} (7 μM) larger than its IC_{50} for nNOS (1 μM) (Fig. 2C). Peptides 12 and 16, in contrast, did not show any significant influence at the 0.1 μM concentration of CaM (Fig. 2C).

3.3. Identification of amino acids crucial for CaM binding

In line with the concept that the hydrophobic/basic composition determining the propensity to form an amphiphilic helix is important for interaction with CaM, the above results indicated that Lys^{732}LysLeu in the nNOS are critical residues for CaM binding. To further investigate this hypothesis, we mutated two clusters of hydrophobic/basic residues in nNOS to produce a double mutant (Met^{749}/Lys^{751} to Glu^{749}/Asp^{751}) and a triple mutant (Lys^{732}LysLeu to Asp^{732}AspGlu) (Table 1). The specific activity of wild-type nNOS was 16.1 ± 0.20 nmol L-citrulline produced/min/mg protein, as determined by L-arginine to L-citrulline conversion. The double mutant showed significant loss of CaM activation (4.36 ± 0.19 nmol L-citrulline produced/min/mg protein) and the triple mutant demonstrated a severe loss (Fig. 3), even with up to a 30:1 molar excess of CaM (data not shown). The CaM binding of each mutant was significantly decreased as determined by the CaM gel overlay technique, with relative CaM binding affinities were wild-type > double mutant > triple mutant (Fig. 3).

This study used multiple approaches to identify the region of the nNOS that is involved in CaM binding. It was shown that the latch domain of CaM is an important interaction site which plays a critical role in nNOS activation [18]. Since peptide 23 did form a complex with CaM in the presence of Ca^{2+}, the difference in IC_{50} between nNOS (1 μM) and CaM-kinase Iα (7 μM) (Fig. 2) might be due to interaction with the latch domain of CaM. On the basis that the hydrophobic/basic composition determining the propensity to form an amphiphilic helix is thought to be important for the interaction

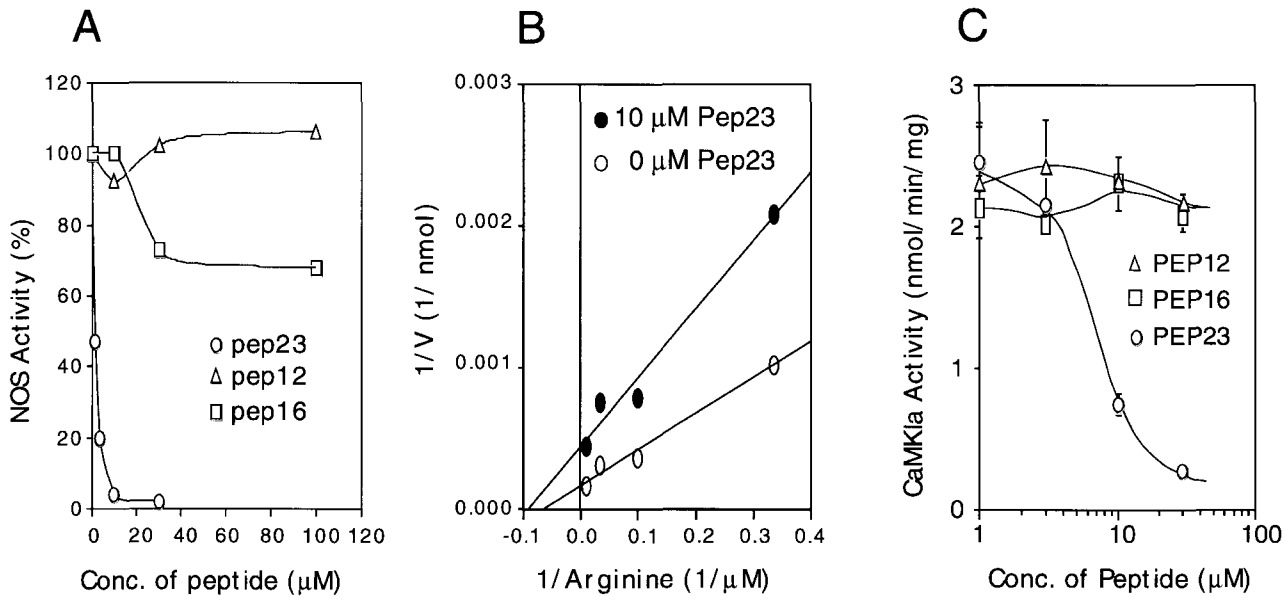

Fig. 2. Effects of synthetic peptide derived from nNOS on CaM dependent enzyme activity. A: Effect of nNOS peptides on nNOS activity. Recombinant nNOS (100 nM) was assayed with 10 μM L-[^{3}H]arginine, 0.1 μM CaM, 1 mM $CaCl_2$, 10 μM BH_4, 100 μM NADPH, and the indicated concentrations of peptide 23 (○), peptide 16 (□), or peptide 12 (△) for 10 min at 30°C. The data were normalized to give percentages of the control value, defined as the total activity of NOS. B: Kinetic analysis of nNOS inhibition. Recombinant nNOS (100 nM) was assayed in either the presence (●) or the absence (○) of 10 μM peptide 23 under the same conditions as described in A with 3–100 μM L-[^{3}H]arginine. C: Effect of nNOS peptides on CaM-kinase Iα activity. Recombinant CaM-kinase Iα (300 nM) was assayed with 50 μM syntide-2, 1 mM $CaCl_2$, 1 μM CaM, and the indicated concentrations of peptide 23 (○), peptide 16 (□), or peptide 12 (△) for 10 min at 30°C. The data are the means of two experiments.

with CaM, we focused on the hydrophobic/basic cluster in $nNOS_{732-754}$. The $iNOS_{504-532}$ region has been identified as critical for CaM binding of murine iNOS [9]. Our results are similar to those described in the literature in that the $eNOS_{493-512}$ peptides F498A, K499A, and L511A lost their capacity for high affinity binding of CaM, as evidenced by loss of CaM mobility shift on 4 M urea gels and significantly lower potencies for inhibition of eNOS than the wild-type peptide [10] (Table 1). Even though the specific hydrophobic/basic amino acid cluster critical for CaM binding by nNOS was identified in this study to be Lys^{732}LysLeu, other hydrophobic/basic clusters within the nNOS sequence might also be important determinants of CaM binding, including Ala^{737}ValLysPhe and Ala^{742}LysLeuMet. The triple mutant of nNOS could be a useful molecular biological tool for studying the activation mechanism of nNOS, since this does not interfere with subunit interaction but should influence electron transfer through at least one subunit of the enzyme (Y. Watanabe and H. Hidaka, submitted).

Table 1
Alignment of putative CaM binding domains

	Triple Mutant	Double Mutant
	732	749
	DDE	E D
nNOS(Rat)	KRRAIGFKKLAEAVKFSAKLMGQAMAKRVKATILYA	
nNOS(Human)	••••••••••••••••••••••••••••••••••••	
	504	532
iNOS(Human)	KRREIPLKVLVKAVLFACMLMRKTMASRVRVTILFA	
iNOS(Mouse)	R••••RFR••••V•F••S•••••V••••••A•VL••	
	498	511
eNOS(Human)	RKK---TFKEVANAVKISASLMGTVMAKRVKATILYG	
eNOS(Bovine)	•••---••••••••••••••••••L•••••••••••A	

The proposed CaM binding domains are for rat nNOS, bovine eNOS, and murine iNOS (solid dots denote identical residues). Amino acids determined to be critical for CaM binding are boxed [10]. Mutated

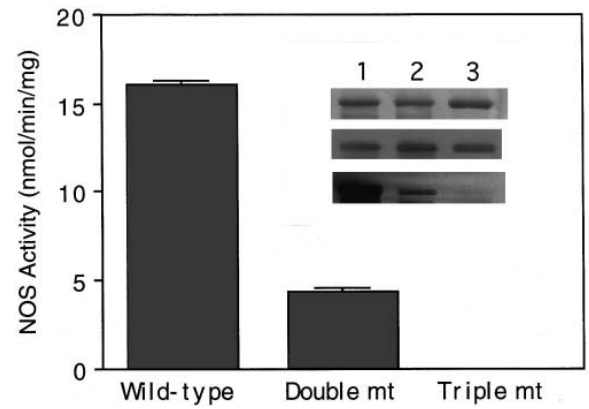

Fig. 3. Enzyme activity of purified wild-type and mutant nNOS proteins. Equivalent amounts (100 nM) of wild-type enzyme, double, and triple mutants were used and NOS activity was assayed by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline with 10 μM L-[^{3}H]arginine, 1 μM CaM, 1 mM $CaCl_2$, 10 μM BH_4, and 100 μM NADPH for 10 min at 30°C. The data are the means of duplicate determinations from two experiments. The inset shows Coomassie (top), Western blot (middle), and CaM overlay (bottom) analyses of purified nNOS mutants. Lane 1, wild-type nNOS; lane 2, double mutant nNOS; lane 3, triple mutant nNOS. Purified recombinant nNOS proteins including mutant enzymes (2 μg of each protein) were separated on 7.5% SDS-PAGE and analyzed by Coomassie blue staining, Western blotting with anti-nNOS, and reaction with biotinylated CaM in the presence of 1 mM $CaCl_2$ as described in Section 2.

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