Articles

Interaction of Calmodulin with the Inducible Murine Macrophage Nitric Oxide Synthase

Regina Stevens-Truss[‡] and Michael A. Marletta*[,‡,§]

Interdepartmental Program in Medicinal Chemistry, College of Pharmacy, and Department of Biological Chemistry, School of Medicine, University of Michigan, Ann Arbor, Michigan 48109-1065

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ABSTRACT: The inducible isoform of nitric oxide synthase (iNOS) isolated from murine macrophages copurifies with calmodulin (CaM) as a tightly bound subunit. The exact function of this tightly bound CaM, however, has not been elucidated. In order to probe the function of this unusually strong interaction between iNOS and CaM, a 30 amino acid peptide derived from the putative CaM binding site of iNOS was synthesized. Cross-linking and autoradiographic analyses demonstrated that the peptide and CaM form a 1:1 complex as well as several higher molecular weight complexes. When assayed in the presence of a 12-fold excess of peptide to iNOS, over 90% of the enzymatic activity was inhibited. This inhibition could be prevented with the addition of exogenous bovine CaM to the assay mixture, in a concentration-dependent manner. Native PAGE and Western blot analysis of iNOS treated with peptide revealed the formation of a peptide−CaM complex with CaM derived from iNOS. Moreover, EGTA (5 mM) caused a 30% maximal inhibition of activity that was reversed by the addition of exogenous Ca^{2+} in a concentration-dependent fashion, suggesting a role for Ca^{2+} in this interaction. EGTA also changed the native PAGE mobility of iNOS and increased the intensity of a band which comigrates with CaM. These results demonstrate that the binding interaction between CaM and iNOS is tight but reversible, requires Ca^{2+}, and is atypical from other known CaM−enzyme interactions.

The free radical nitric oxide (•NO) has emerged in the last decade as a new signaling agent, which is produced by a variety of cells (Marletta, 1994; Nathan & Xie, 1994). The functions of •NO are diverse as are the cellular sources from which it is produced, ranging from regulation of vascular tone and neuronal signaling to immune response to infection. •NO is a product of the oxidation of the amino acid L-arginine by the enzyme nitric oxide synthase (NOS, EC 1.14.13.39)[1] (Marletta, 1993, 1994). The reaction requires molecular oxygen and NADPH and proceeds via the intermediate N^G-hydroxy-L-arginine (Stuehr et al., 1991b; Pufahl et al., 1992; Klatt et al., 1993). All NOSs isolated to data are homodimers containing a protoporphyrin IX heme (McMillan et al., 1992; Stuehr & Ikeda-Saito, 1992; White & Marletta, 1992) and 1 equiv each of FMN and FAD (Hevel et al., 1991; Mayer et al., 1991; Stuehr et al., 1991a) per subunit. In addition, all isoforms have been shown to have a requirement for (6*R*)-tetrahydro-L-biopterin (H_4B) (Hevel & Marletta, 1992; Schmidt et al., 1992). NOSs can be classified based on their mode of regulation as either constitutive or inducible. The constitutive isoforms, exemplified by those isolated from neuronal and endothelial cells, are regulated by the reversible binding of Ca^{2+}/calmodulin (CaM), while the inducible isoforms, of which the best characterized is isolated from murine macrophages, are regulated transcriptionally by the action of cytokines (Nathan, 1992; Marletta, 1994).

The inducible isoforms were originally characterized as Ca^{2+}/CaM independent. Nathan and co-workers (Cho et al., 1992), however, have shown that the macrophage-inducible NOS (iNOS) has CaM as a tightly bound subunit. CaM has been shown to be responsible for regulating the activity of a large number of enzymes including several kinases, calcium pumps, adenylate cyclase (O'Neil & DeGrado, 1990), and more recently NOS (Bredt & Snyder, 1990). The exact mode by which CaM exerts its action, however, is not completely understood. Typically, as the intracellular levels of calcium are elevated, Ca^{2+} binds to CaM with an affinity of 10^{-6} M, bringing about a conformational change. Interaction of CaM with most target enzymes occurs after Ca^{2+} binding. Peptides corresponding to the CaM binding region of many enzymes have been found to have affinities for CaM in the nanomolar range. Smooth muscle myosin light chain kinase (SMMLCK), for example, binds CaM reversibly with an affinity of 1 nM (Lukas et al., 1986). Skeletal muscle phosphorylase-*b* kinase which is comprised of four subunits (termed α, β, γ, and δ) has CaM as its δ subunit. The γ subunit of this enzyme binds CaM with an affinity of 6.5

* To whom correspondence should be addressed at the College of Pharmacy, 428 Church St., University of Michigan, Ann Arbor, MI 48109-1065.

‡ Interdepartmental Program in Medicinal Chemistry.

§ Department of Biological Chemistry, School of Medicine.

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[1] Abbreviations: NOS, nitric oxide synthase; iNOS, inducible macrophage nitric oxide synthase; H_4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; HEPES, 4-(2-hydroxyethyl)piperazineethanesulfonic acid; BSA, bovine serum albumin; LPS, bacterial lipopolysaccharide; IFN-γ, interferon-γ; DTT, dithiothreitol; TCA, trichloroacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CaM, calmodulin; PVDF, poly(vinylidene difluoride); HRP, horseradish peroxidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium.

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nM (Dasgupta et al., 1989). Neuronal NOS, which binds CaM similarly to SMMLCK in a freely reversible fashion, has been shown to have a CaM affinity of ~1 nM (Vorherr et al., 1993; Sheta et al., 1994). The binding affinity of the iNOS for CaM, however, has not been determined, but this interaction has been speculated to be Ca^{2+} independent.

The CaM binding domains of target enzymes, although highly variable in primary sequence, typically possess a consensus motif which is required for binding. These domains consist primarily of apolar residues and basic residues, and fold to form amphiphilic α-helices (O'Neil & DeGrado, 1990; Vorherr et al., 1993). All NOSs identified to date have been shown to possess such a sequence (Bredt et al., 1991; Lamas et al., 1992; Xie et al., 1992). Despite the homology in their CaM binding sequences, neuronal and macrophage NOSs (53% homology; 43% identity) appear to have different binding affinities for CaM. As stated above, CaM binds to neuronal NOS in the classical, reversible Ca^{2+}-dependent manner, whereas binding of CaM to the macrophage-inducible NOS isoform is seemingly irreversible and appears to be Ca^{2+} independent. The role of Ca^{2+} in the tight binding of CaM to iNOS, however, has not been extensively investigated. The binding of Ca^{2+}/CaM to the neuronal NOS has been shown to modulate the transfer of electrons from NADPH through the flavins to the heme (Abu-Soud & Stuehr, 1993). Because of the difficulty in separating CaM from iNOS, it has been impossible to determine if CaM plays a similar role for this isoform.

Herein, we report that the tight binding of CaM to iNOS requires Ca^{2+}. Moreover, we show that the bound CaM is involved in the regulation of iNOS. Whether CaM is playing a mechanistic or a structural role is under investigation. The results presented also demonstrate that the interaction between iNOS and CaM is unusual and may be more complicated than the interaction between other enzymes and CaM.

EXPERIMENTAL PROCEDURES

Materials and General Methods. Macrophage RAW 264.7 cells were purchased from American Type Culture Collection (ATCC TIB 71). Calf serum was obtained from HyClone Laboratories. H_4B was purchased from Dr. B. Schircks Laboratory (Jona, Switzerland) and prepared in 15 mM HEPES (pH 7.5) containing 100 mM DTT. 2′,5′-ADP−Sepharose 4B was purchased from Pharmacia−LKB Biotechnology Inc. DEAE-Biogel-A, AG 50W-X8 cation exchange resin, Bradford protein dye reagent, and 4−20% Tris−glycine ready gels were obtained from Bio-Rad. All Tricine buffers and Tricine ready gels (10−20%) were purchased from Novex. L-[U-^{14}C]Arginine (specific activity = 319 mCi/mmol) and ECL-chemiluminescence reagents were purchased from Amersham Corp. Ecolume scintillation cocktail was obtained from ICN-Flow. CaM, CaM monoclonal antibodies (6D4 clone), BSA, HEPES, DTT, NADPH, $NADP^+$, glycerol, L-arginine, DMEM (with L-glucose), LPS, penicillin, streptomycin, and all other reagents were purchased from Sigma Chemical Co. [^{125}I]Calmodulin was obtained from DuPont. PVDF membrane was purchased from Schleicher & Schuell. Polyclonal antibodies to purified iNOS were developed in rabbits by Cocalico Biologicals, Inc. IFN-γ was a generous gift from Genentech Inc.

Peptide Synthesis. The 30 amino acid residue peptide (RRREIRFRVLVKVVFFASMLMRKVMASRVR), which corresponds to the putative CaM binding domain (residues 503−532) of the iNOS (Xie et al., 1992), was obtained as a lyophilized powder from the University of Michigan Protein Core Facility, Ann Arbor, MI. The peptide was synthesized using FMOC chemistry on an Applied Biosystems Peptide Synthesizer. The peptide was purified by reverse-phase high-performance liquid chromatography on a C18 Vydac column (4.6 mm i.d. × 25 cm) run with a gradient of 25−85% acetonitrile in water with detection at 220 nm and was determined to be 92% pure. The molecular mass of the peptide was determined to be 3741 Da by electrospray mass spectroscopy (theoretical mass = 3738 Da). Prior to use, the peptide was dissolved in 100 mM HEPES, pH 7.5, and the concentration determined using the Bradford microassay with BSA as the standard.

iNOS Purification. Cell culture procedures were carried out as previously described (Tayeh & Marletta, 1989), with the following modifications: RAW 264.7 cells were grown to confluency on 150 × 25 mm culture plates. The cells were then activated (10−12 plates/L of media) for 18−20 h with *Escherichia coli* LPS (100 ng/mL) and recombinant murine IFN-γ (5 units/mL) in DMEM containing 10% heat-inactivated calf serum, 4 mM L-glutamine, penicillin (50 units/mL), and streptomycin (50 μg/mL). iNOS was purified from the 100000*g* supernatant of 20−30 plates (550−820 units; 1 unit = 1 nmol of •NO/min, measured using the hemoglobin assay) as previously described (Hevel & Marletta, 1992) with the following modifications: H_4B (7 μM) was included throughout the purification and a 50K cutoff ultrafiltration membrane (Filtron) was used in the concentration step. Enzyme purified in this fashion has been shown to be stoichiometric with H_4B (1 H_4B per subunit) (Hevel & Marletta, 1992). In almost all cases, iNOS was stored frozen at −80 °C in HEPES (100 mM, pH 7.5) with 20% glycerol and 5−10 μM H_4B. This method yielded protein which was deemed greater than 98% pure as judged by native PAGE or SDS−PAGE stained with a combination of Coomassie Blue R-250 and Crocein Scarlet (to be described under native PAGE electrophoresis section). Protein concentrations were determined by Bradford microassay using BSA as the standard and an iNOS molecular mass of 130 kDa.

NOS Activity Assays. Enzymatic activity was determined using either the hemoglobin assay or the citrulline assay. (A) The hemoglobin assay measures •NO formation by following the oxidation of oxyhemoglobin to methemoglobin as an increase in absorbance at 401 nm as previously described (Hevel & Marletta, 1994). Units of iNOS activity were calculated using an extinction coefficient of 60 000 M^{-1} cm^{-1}. (B) Citrulline formation was measured as previously described (Hevel & Marletta, 1994) with the following modifications: iNOS (typically 20−60 nM) was incubated with L-[U-^{14}C]arginine (200 μM, specific activity = 3 μCi/μmol), H_4B (40−50 μM), DTT (200−400 μM), and glycerol (0.8−1.3%) in HEPES (100 mM, pH 7.5) for 2 min at 37 °C. Reactions were initiated with NADPH (200 μM) and proceeded for an additional 2 min at 37 °C before being terminated with TCA (10 μL of 6 N) and placed immediately on ice. Final assay volumes were 300 μL. The assay mixture was neutralized using HEPES (1.5 M, pH 7.5, 250 μL) and applied to an AG 50W-X8 cation exchange column (1 mL, sodium form) to separate citrulline from unreacted arginine. The eluate and the water washes (3 × 1 mL) were

collected directly into Ecolume scintillant (15 mL) and counted. Citrulline recovery was typically greater than 96% under these conditions.

Native PAGE Analysis. Electrophoresis was performed following the procedures outlined in the instruction manual for Bio-Rad ready gels. Briefly, 4−20% Tris−glycine gels were electrophoresed under nondenaturing conditions at 160 V for 70 min at 4 °C, using the Bio-Rad Mini-PROTEAN II system. Following electrophoresis, gels were fixed with 40% methanol/10% acetic acid for at least 2 h, stained with a combination of Coomassie−Crocein scarlet dye mixture (27% 2-propanol, 10% acetic acid, 0.04% Coomassie brilliant blue R-250, 0.05% Crocein scarlet, and 0.5% $CuSO_4$; see Bio-Rad instruction manual for IEF gels, fixing/staining section) for several hours, and destained in 10% methanol/5% acetic acid. The gels were then dried and scanned using a computing densitometer from Molecular Dynamics (Model 300A). Images were then analyzed using the program Image Quant (v. 3.0).

Western Blot Analysis. Samples were electrophoresed as described above. Following electrophoresis, protein was transferred to a PVDF membrane. Transfer was performed at 250 mA for 2 h at 4 °C in electroblot buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). Membranes were fixed for 1 h with 0.2% glutaraldehyde and blocked with 2% BSA for 18−24 h at 4 °C. The membranes were either (a) probed with monoclonal antibodies to CaM (6D4 clone; 1:1000 dilution) for 18−24 h at 4 °C and incubated with goat anti-mouse HRP-conjugated IgG (secondary antibody) for 2 h at room temperature or (b) probed with polyclonal antibodies to iNOS (1:1500 dilution) for 1 h at room temperature and incubated with goat anti-rabbit HRP-conjugated IgG for 1 h at room temperature. Target proteins were detected either by chemiluminescence or by color development with 4-chloro-1-naphthol. The blots were scanned and data analyzed as described above.

Treatment of iNOS with EGTA. iNOS activity was determined using the hemoglobin assay as described above. iNOS (1.0−1.5 μM) was incubated with varying concentrations of EGTA (0−20 mM) in 100 mM HEPES, pH 7.5, for 30 min at 4 °C. Following incubation, the samples were separated into two aliquots. One aliquot was assayed for activity; the other was subjected to native PAGE electrophoresis and/or Western blot analysis (with CaM monoclonal antibodies) as described above. To determine whether Ca^{2+} could reverse the inhibition by EGTA, samples were incubated with 0−4 mM Ca^{2+} for an additional 30 min at 4 °C following EGTA treatment and assayed for activity in the same way as the EGTA-only samples. The activity remaining in each sample after treatment was expressed as a percentage of the total activity in untreated samples.

Synthetic Peptide Effect on iNOS Activity. Activity was determined using the citrulline assay described above. iNOS (20−60 nM) was incubated as described in the assay section with varying concentrations of peptide (0−30-fold excess over iNOS) for 2 min at 37 °C, followed by initiation of the reaction with NADPH. For the competition experiments, iNOS was incubated with peptide and bovine CaM for 2 min at 37 °C before enzymatic activity was initiated with NADPH. iNOS activity was expressed as a percentage of the total activity present in untreated samples. Values were corrected by that of a noninitiated control. Samples of iNOS incubated with peptide (0−20-fold excess over iNOS) for 10−30 min at 4 °C were also subjected to native PAGE and Western blot analysis as described above.

Cross-linking Experiments. Bovine CaM (100 μM) with or without peptide (300 μM) was incubated for 20 min at 4 °C in 100 mM HEPES, pH 7.5, in the presence or absence of 2 mM Ca^{2+} (final volume = 100 μL). Following this incubation, dimethyl suberimidate (4 mM) dissolved in 200 mM HEPES (pH 7.5) was added to the reaction mixture in five separate aliquots (0.8 mM each) at 37 °C, all within 10 min of the initial addition. The reaction mixture was then incubated for 30 min at 37 °C. Samples were diluted 1:2 with 2× Tricine−SDS sample buffer (Novex) and heated at 95 °C for 5 min. Aliquots (10 μL) of each sample was run on a 10−20% Tricine precast gel (Novex) for 60 min at 160 V at room temperature using the Novex Xcell II mini-cell and Novex Tricine−SDS running buffer (pH 8.8). Gels were then fixed for 1 h in 40% methanol/10% acetic acid and stained with Coomassie−Crocein scarlet stain as described in the native PAGE section.

Autoradiographic Determination of CaM−Peptide Complex Formation. Bovine [^{125}I]CaM (12 mM, 25 cpm/fmol) was incubated with varying concentrations of peptide (0−192 μM) in 100 mM HEPES, pH 7.5 (final volume = 30 μL), for 10 min at 4 °C. Native sample buffer (5 μL) was then added to the samples, and 30 μL aliquots of this mixture were subjected to native PAGE analysis as described previously. The gels were dried overnight and exposed on Reflection NEF-496 autoradiography film (DuPont) for 7 days at −80 °C using an image intensifying screen.

RESULTS

Effect of EGTA on iNOS Activity. To ascertain whether Ca^{2+} is involved in the tight interaction of iNOS and CaM, the effect of the Ca^{2+} chelator EGTA on enzymatic activity was examined. EGTA caused a moderate decrease of iNOS activity measured using the hemoglobin assay. Maximal inhibition was approximately 30% at 5 mM EGTA (Figure 1, black shaded bars) and did not increase further even at concentrations of EGTA up to 20 mM (data not shown). The inhibition was reversed by Ca^{2+} in a concentration-dependent fashion (Figure 1, grey and white shaded bars). The observed EGTA inhibition cannot be attributed to the presence of neuronal NOS in the enzyme preparation, since there is no detectable constitutive NOS activity in RAW 264.7 cells (Hevel et al., 1991). Ca^{2+} alone did not increase the activity of the purified iNOS and, if anything, appeared to slightly inhibit the reaction (Figure 1, 0 mM EGTA). Similar results were obtained with EGTA treatment when activity was determined using the citrulline assay (data not shown).

Effects of EGTA on iNOS Electrophoretic Mobility. On native PAGE analysis of purified iNOS, we regularly observe a band which comigrates with CaM (Figure 2A, lane 4). The electrical field, therefore, appears to contribute to the dissociation of CaM from the iNOS. This dissociation, however, is enhanced by treatment of the enzyme with EGTA. As iNOS is treated with increasing concentrations of EGTA, the intensity of the CaM band increases, as can be clearly seen on Western blot analysis (Figure 2B). The intensity of each band was calculated by densitometry, and the data are presented in the figure legend. EGTA treatment of the enzyme caused a concomitant change in the electro-

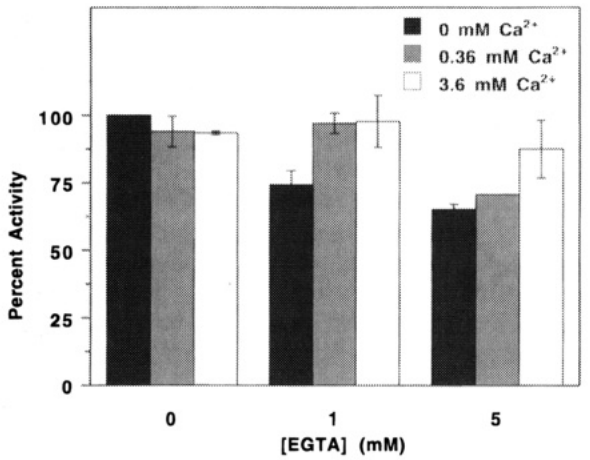


FIGURE 1: Effect of EGTA and Ca^{2+} on iNOS activity. iNOS (1.0−1.2 μM) was incubated with EGTA (0, 1, or 5 mM) in the presence or absence of Ca^{2+} (0, 0.36, or 3.6 mM). Activity was determined using the hemoglobin assay as described under Experimental Procedures. iNOS activity in the absence of EGTA was used as the total activity present in the sample (100%), and the activity as a function of EGTA treatment was determined as a fraction of the total. Values of percent shown are the means ± standard error of the means: those for the 0 mM Ca^{2+} (bars shaded black) are from four separate experiments, while the Ca^{2+}-containing samples (grey and white bars) are from two separate experiments.

phoretic mobility of the iNOS monomer and dimer bands. With increasing EGTA concentration, iNOS did not migrate into the gel and was visible as a strongly-stained band at the top of the gel (Figure 2A, lanes 5 and 6). Increasing gel running times did not cause this band to migrate further (data not shown). Similar to the activity results, Ca^{2+} was able to reverse the EGTA electrophoretic effects: Ca^{2+} caused a disappearance of the CaM band and a reappearance of the iNOS monomer and dimer bands (Figure 2A,B). Because EGTA treatment appeared to remove CaM from the iNOS as observed on native PAGE (maximal dissociation observed at 5 mM EGTA) but enzymatic activity was not completely inhibited (Figure 1), a separate native PAGE experiment was carried out under assay conditions. EGTA-treated iNOS samples were incubated with arginine, H_4B, and NADPH (at concentrations used in activity assays) prior to being electrophoresed. The results were the same as those of EGTA-treated samples alone, including the change in iNOS migration (data not shown). Bands above dimeric iNOS that can be seen in Figure 2A correspond to oligomeric iNOS; these have been frequently observed on native PAGE at high enzyme concentrations. Since it is clear that EGTA treatment removes some bound CaM, the nonmigrating complex could be due to a polymeric complex formed by iNOS dimers that are now bridged by a CaM bound to another dimer.

Synthetic Peptide−CaM Complex Formation. To determine if the 30 amino acid synthetic peptide constructed to mimic the CaM binding site of iNOS would form a complex with CaM, cross-linking experiments were performed in the presence and absence of Ca^{2+}. As can be seen in Figure 3, the peptide formed a complex with CaM with a calculated molecular mass of 24.4 kDa. This mass closely approximates the expected molecular mass (24.9 kDa) for a complex formed by one CaM molecule (molecular mass = 19.4 kDa) and one peptide molecule (molecular mass = 5.5 kDa). A second, fainter band was also visible with a calculated molecular mass of 28.0 kDa, suggesting a complex involving

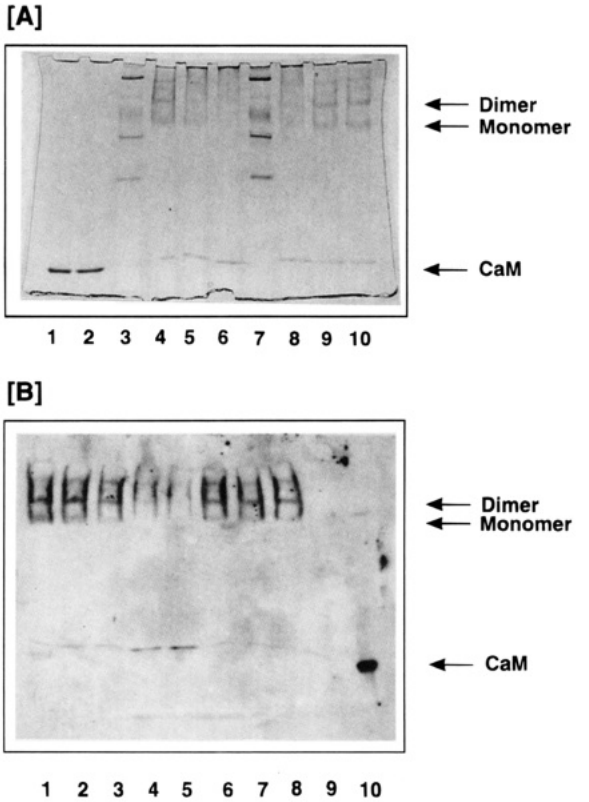


FIGURE 2: EGTA dissociation of the CaM−iNOS complex. Native PAGE electrophoresis of iNOS on BioRad 4−20% Tris−glycine gradient gels run in the absence of SDS. iNOS was incubated with EGTA ± Ca^{2+} first and then subjected to electrophoresis for 70 min (see Experimental Procedures). (A) Representative gel stained with Coomassie/Crocein scarlet (see Experimental Procedures). Lane 1, 2.5 μg of bovine CaM; lane 2, 2.5 μg of bovine CaM + 2 mM Ca^{2+}; lanes 3 and 7, native PAGE molecular mass standards (Pharmacia) (values in kilodaltons are 660, 440, 232, 140, and 67); lane 4, 3 μg of iNOS, untreated; lane 5, 3 μg of iNOS treated with 1 mM EGTA; lane 6, 3 μg of iNOS treated with 5 mM EGTA; lane 8, 2.7 μg of iNOS treated with 1 mM EGTA plus 0 mM Ca^{2+}; lane 9, 2.7 μg of iNOS treated with 1 mM EGTA plus 0.36 mM Ca^{2+}; lane 10, 2.7 μg of NOS treated with 1 mM EGTA plus 3.6 mM Ca^{2+}. The percent intensities for the scanned CaM bands were as follows: lane 4, 100%; lane 5, 246%; lane 6, 313%; lane 8, 160%; lane 9, 64%; lane 10, 68%. (B) Protein transferred to PVDF membrane, probed with monoclonal antibodies to CaM, and detected by chemiluminescence. Lanes 1−8 contain 3 μg of iNOS: lane 1, 0 mM EGTA; lane 2, 0.1 mM EGTA; lane 3, 1 mM EGTA; lane 4, 5 mM EGTA; lane 5, 10 mM EGTA; lane 6, 0 mM EGTA + 3.6 mM Ca^{2+}; lane 7, 1 mM EGTA + 3.6 mM Ca^{2+}; lane 8, 5 mM EGTA + 3.6 mM Ca^{2+}. Lane 9, native PAGE molecular mass standards (Pharmacia), not visible; Lane 10, 5 μg of bovine CaM. The percent intensity of the scanned CaM bands for each lane was 100%, 275%, 207%, 602%, and 915% for lanes 1, 2, 3, 4, and 5, respectively. Scanning of lanes 6 through 8 was not possible since the bands were not detectable above background.

two molecules of peptide with one molecule of CaM, as well as a third band at a calculated molecular mass of 34 kDa, suggestive of a 3:1 peptide to CaM complex. A CaM dimer band (molecular mass = 38.4 kDa) was present in all lanes containing CaM: the intensity of this band was greatest in the presence of peptide and excess Ca^{2+} (Figure 3, lane 3). A nonmigrating band (absent in the CaM-only sample, lane 2) is also visible in the samples which contained peptide (Figure 3, lanes 3 and 4). Ca^{2+} appeared to increase the formation of this nonmigrating band and to cause the greatest complex formation between the peptide and CaM (Figure 3, lane 3).

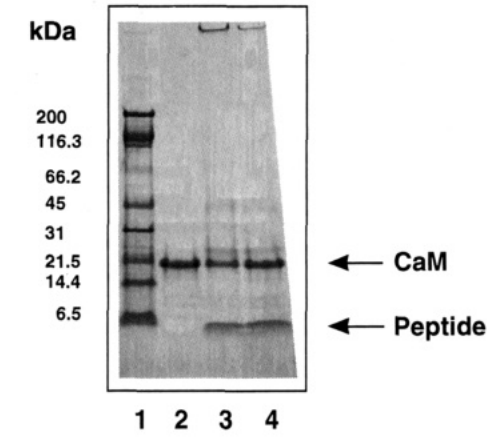


FIGURE 3: Cross linking of CaM to synthetic peptide. Following cross-linking of the complex with DMS, samples were treated with SDS sample buffer, run on a 10−20% Tris−Tricine gradient gel (Novex), and stained with Coomassie−Crocein scarlet dye described under Experimental Procedures. All lanes contained 10 μg of bovine CaM. Lane 1, molecular mass standards (Biorad). The band corresponding to the 97.5 kDa band is not labeled. Lane 2, CaM and Ca^{2+} only; lane 3, CaM, peptide, and Ca^{2+}; lane 4, CaM and peptide.

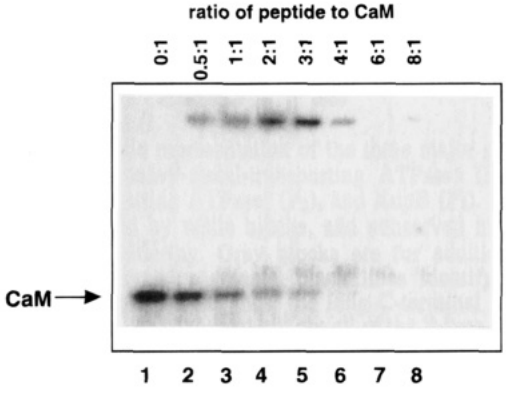


FIGURE 4: Native PAGE analysis of the interaction of CaM with the synthetic peptide. Shown is the autoradiogram depicting the complexes formed between [^{125}I]CaM and the synthetic peptide. All lanes contained 12 μM CaM. Lane 1, 0 μM peptide; lane 2, 6 μM peptide; lane 3, 12 μM peptide; lane 4, 24 μM peptide; lane 5, 36 μM peptide; Lane 6, 48 μM peptide; lane 7, 72 μM peptide; lane 8, 96 μM peptide.

Use of Radiolabeled CaM to Follow the CaM−Peptide Complex. Autoradiographic studies of the interaction of the synthetic peptide with [^{125}I]CaM revealed that a nonmigrating band is formed between CaM and the peptide. A strongly labeled band appears at the top of every lane which contained peptide and CaM (Figure 4). The intensity of this band diminishes as the concentration of peptide is increased and completely disappears at a 6:1 peptide to CaM ratio. Maximal formation of this nonmigrating band occurs at a 2−3:1 peptide to CaM ratio. Another complex with lower label intensity migrates close to uncomplexed CaM. Maximal formation of this complex is observed at a 4:1 peptide to CaM ratio, which is also the concentration at which most of the free [^{125}I]CaM is complexed (Figure 4, lane 6). At ratios above 4:1 peptide to CaM, the intensity of the peptide−CaM complexes appears to lessen and disappear. Although the total amount of labeled CaM loaded in each lane was the same, at the higher peptide concentrations there appeared to be less labeled CaM. We hypothesized that the loss was the result of precipitation; therefore, each sample was centrifuged in a table-top microcentrifuge prior to being

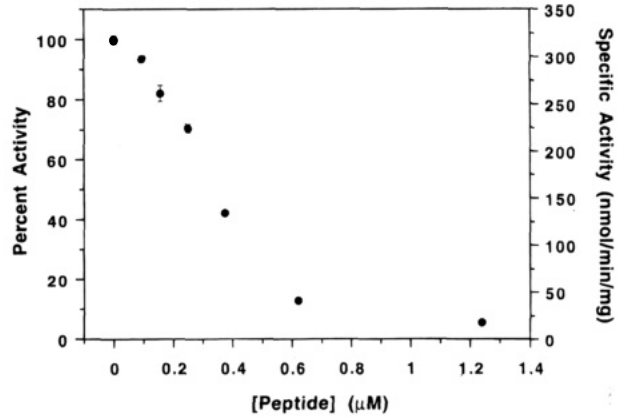


FIGURE 5: Effects of synthetic peptide on iNOS activity. iNOS activity was determined using the citrulline assay as described under Experimental Procedures. iNOS (40 nM) was incubated with increasing concentrations of the synthetic peptide (0−1.24 μM) and assayed as described under Experimental Procedures, for 2 min at 37 °C. In this representative experiment, each concentration was performed in duplicate; the values of percent activity shown are the means ± standard error of the means. The activity in untreated samples was used as 100%, and the activity in treated samples was expressed as a fraction of the total.

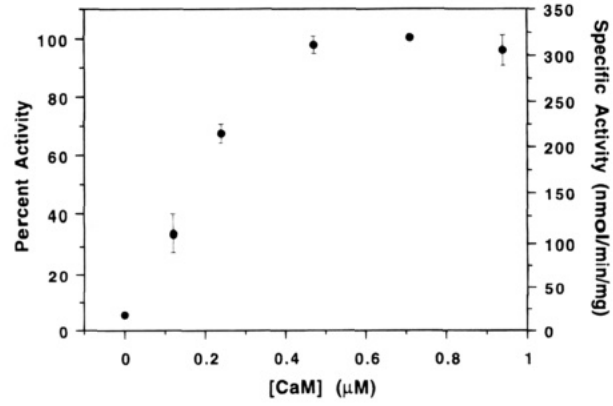


FIGURE 6: Effect of exogenous CaM on peptide inhibition of iNOS activity. iNOS (40 nM) was incubated with peptide (1.24 μM) and increasing concentrations of bovine CaM and assayed for 2 min at 37 °C as described under Experimental Procedures. In this representative experiment, each concentration was performed in duplicate; the values of percent activity shown are the means ± standard error of the means. The activity in untreated samples was used as 100%, and the activity in treated samples was expressed as a fraction of the total.

electrophoresed. Equal aliquots of the supernatant were loaded onto the gel, and the remaining sample in the tube was counted. The lost ^{125}I counts were found in the tubes coincident with increasing concentration of peptide (data not shown).

Effects of Synthetic Peptide on iNOS Activity. The activity of purified iNOS was determined in the presence of the synthetic peptide. Figure 5 shows that the peptide was able to almost completely inhibit the activity of iNOS in a concentration-dependent manner. Maximal inhibition, however, was not observed until the peptide concentration exceeded the iNOS concentration by 12-fold. Exogenous bovine CaM was able to prevent the inhibition of iNOS activity observed in the presence of the peptide, also in a concentration-dependent manner (Figure 6). Concentrations of CaM required to completely prevent peptide inhibition of the iNOS, however, were typically between 30% and 50% of the concentration of peptide used. As a control, BSA

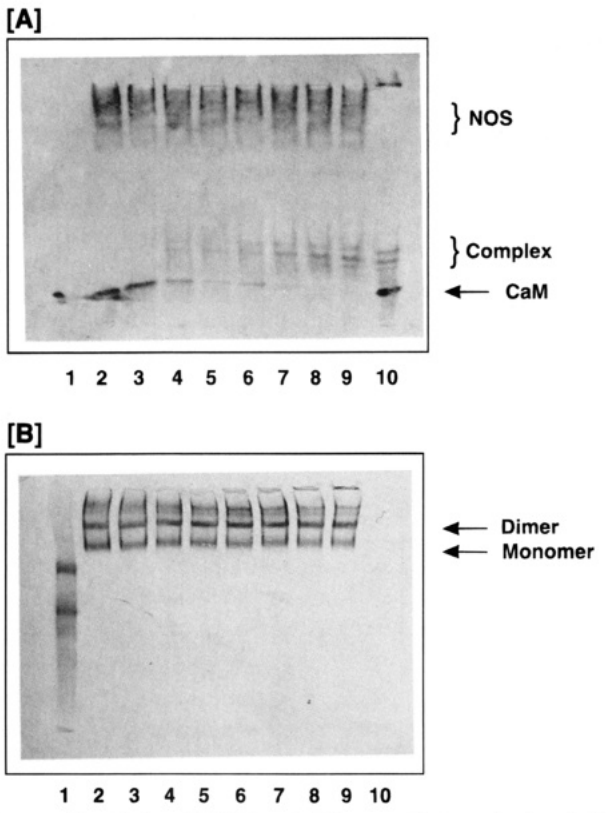
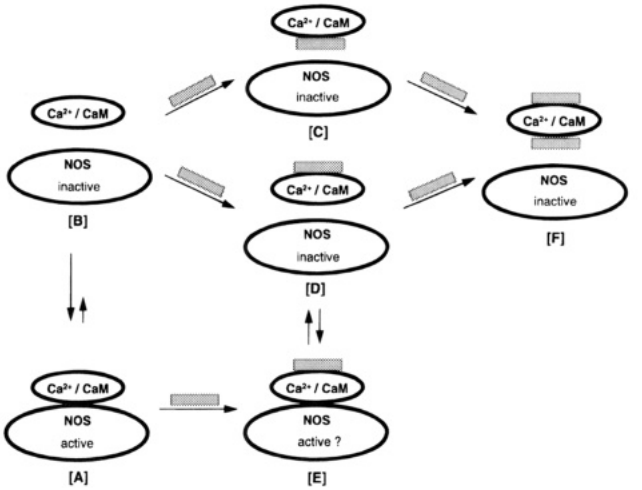


FIGURE 7: Native PAGE and Western blot analysis of the interaction between the synthetic peptide and iNOS. (A) Western blot probed with monoclonal antibodies to CaM and visualized by chemiluminescence. (B) Western blot similar to that in (A) except each lane contained only 5 μg of iNOS (This blot had been previously probed with monoclonal antibodies to CaM. The membrane was then stripped and reprobed with polyclonal antibodies to iNOS and visualized using 4-chloro-1-naphthol). iNOS samples were incubated with peptide for 10−30 min at 4 °C, prior to being electrophoresed and subjected to Western blot analysis (see Experimental Procedures). Lane 1, molecular mass markers (BioRad, prestained) [not visible in (A)]: lanes 2 through 8 contained 2.3 μM iNOS (15 μg): lanes 2, 0 μM peptide; lane 3, 4.6 μM peptide; lane 4, 9.2 μM peptide; lane 5, 13.8 μM peptide; lane 6, 18.4 μM peptide; lane 7, 23 μM peptide; lane 8, 36.8 μM peptide; lane 9, 46 μM peptide; lane 10, 5.9 μM bovine CaM + 6.4 μM peptide.

was not able to reverse the observed peptide inhibition (data not shown).

Native PAGE and Western Blot Analysis of Peptide-Treated iNOS. To determine what migrational effects the peptide was causing, native PAGE and Western blot analysis of iNOS treated with the peptide were performed. As can be seen in Figure 7A, the peptide forms a complex with CaM endogenous to iNOS. The same bands can be observed for bovine CaM complexed with the peptide (Figure 7A, lane 10). The CaM band which had previously been observed in untreated iNOS samples (Figure 7A, lanes 2) disappears with increasing concentrations of peptide, and several new bands appear at higher mass (Figure 7A, lanes 4 through 9). The complexes appear at lower peptide concentrations as a doublet (Figure 7A, lanes 4 through 6) and at higher peptide concentratioins as a multiplet of closely migrating bands (lanes 7 through 9). It is interesting to note that these complexes form without the addition of excess Ca^{2+}. In order to visualize these complexes, however, the lanes had to be overloaded; thus, it is difficult to visualize the individual iNOS bands at the top of the gel. Figure 7B demonstrates that iNOS monomer and dimer bands, however, are essentially unaffected by treatment with peptide. Figure 7A also reveals a nonmigrating band which forms in the bovine CaM plus peptide lane (lane 10). A nonmigrating band is also visible in lanes 6 through 9 of Figure 7B.

Scheme 1: Model for the Interaction of CaM with iNOS and the Peptide[a]



[a] Since CaM binds tightly to iNOS, the equilibrium between [A] and [B] is shown lying toward the formation of [A]. Chemical cross-linking experiments support the formation of 1:1 and 2:1 peptide−CaM complexes and, therefore, are consistent with structures [C], [D], [E], and [F]. Stippled bars represent the peptide. Letters in brackets denote the different species possible. For simplicity, only one subunit of iNOS is represented in this model.

DISCUSSION

Typical control of cellular processes by Ca^{2+} and CaM involves a signal-stimulated rise in intracellular Ca^{2+} levels from a basal concentration of ~100 nM to concentrations of ~1 μM. Generally, binding of Ca^{2+} to CaM takes place at this elevated level of intracellular Ca^{2+} (James et al., 1995). CaM then undergoes a conformational change upon Ca^{2+} binding which is required for interaction with target enzymes. This binding is typically reversibly controlled as the intracellular levels of Ca^{2+} are returned to resting levels. In the case of the iNOS from murine macrophages, CaM copurifies with the enzyme as a tightly bound subunit, presumably occurring at the basal level of intracellular Ca^{2+}. Indeed, since there was no observed dependence on Ca^{2+} or CaM, and given the role of this particular NOS isoform in the host response to infection, control of the classical CaM type was thought not to be necessary. While the cDNA sequence from this isoform predicted a potential CaM binding site (Lowenstein et al., 1992; Lyons et al., 1992; Xie et al., 1992), the finding that this interaction would be essentially irreversible was unexpected (Cho et al., 1992). The goal of the studies reported here was to explore the function of this tightly bound CaM and begin to understand the nature of this unusual interaction. This tight interaction of CaM with a protein is not without precedent. Phosphorylase-*b* kinase is a tetrameric enzyme where the δ subunit has been shown to be CaM (Cohen et al., 1978).

Scheme 1 illustrates the experimental design that formed the basis for our studies and also serves as a summary of our results. Evidence supports the interaction of CaM with iNOS to be essentially an irreversible one, shown as [A] in Scheme 1. The design of our experiments was to increase

the concentration of CaM-free iNOS shown as [B] in order to assess both structure and function. Therefore, our initial experiments set out to determine the role that Ca^{2+} might play in the equilibrium between [A] and [B]. Experiments involving chelation of Ca^{2+} by EGTA indeed suggest a role for Ca^{2+} in this tight binding complex. The maximal inhibition of enzymatic activity by EGTA was only 30% even at very high concentrations (5 mM) (Figure 1); however, Ca^{2+} was able to reverse the observed inhibition (Figure 1). It would appear from these studies that the affinity for Ca^{2+} by the CaM−iNOS complex is much greater than that of EGTA for Ca^{2+} and probably is the basis for the conclusion that the interaction of CaM with iNOS is independent of Ca^{2+} (Cho et al., 1992). The dissociated complex [B] could be observed by analysis with native PAGE (Figure 2A, lane 4). This dissociation increases with EGTA treatment and is reversed by Ca^{2+} addition (Figure 2A,B). Our conclusions are that binding of CaM to the iNOS is stronger in the presence of Ca^{2+}, but that these can still associate in the absence of Ca^{2+}. Since the electrical field appears to facilitate the dissociation of CaM from the iNOS, we could attribute the difference between the observed dissociation and the loss of activity to a change in binding affinity. The affinity of the two proteins appears to be lowered by treatment with EGTA, judged by an increase in the CaM band that was observed on native PAGE electrophoresis. However, in solution, as is the case in the enzymatic assay, association is possible. These experiments also illustrate the apparent structural effect of bound CaM judged by the change in the native PAGE mobility of iNOS (Figure 2A).

Based on the observation under native PAGE conditions that some dissociation of the CaM−iNOS complex occurs, we attempted to drive the equilibrium toward free iNOS by complexing the endogenous CaM with the peptide derived from iNOS. Precedent for a complex of CaM with target protein sequences is well-known and has been reported with peptides derived from other CaM-regulated enzymes (Ikura et al., 1992; Vorherr et al., 1993). The 30 amino acid peptide derived from iNOS does indeed form a complex with CaM as observed under native PAGE conditions (Figure 4); however, the binding behavior is clearly not simple. A CaM−peptide complex was observed at a molar ratio of 1:1 peptide to CaM. The amount of this complex increased in a concentration-dependent manner as the ratio of peptide to CaM increased. In addition, a high molecular weight band is observed in the autoradiogram that increases in intensity up to a ratio of 2:1 peptide to CaM and then decreases. The apparent loss in total radioactivity observed in Figure 4 is due to an insoluble complex. Based on our observation that the peptide−CaM complex seemed to precipitate at high concentrations of peptide (Figures 4 and 7A, lane 10), [^{125}I]-CaM was incubated with increasing amounts of peptide. Each sample was centrifuged, and radioactivity was measured in the supernatant and the pellet. At concentrations above a ratio of 2:1 peptide to CaM, the ^{125}I counts were found in the pellet (data not shown). The cross-linking experiments provide further evidence that a peptide−CaM complex forms (Figure 3). As described under results, molecular mass values corresponding to species other than the 1:1 complex can be observed. These results support the formation of a 2:1 peptide−CaM complex shown as [F] in Scheme 1. Analogously, species [E] could potentially form where the 2:1 complex consists of 1 peptide and 1 iNOS bound to CaM. Multiple complexes of other peptides, such as melittin and mastoparan, with CaM have been reported (Linse et al., 1986; Mukherjea & Beckingham, 1993).

Most importantly, the peptide inhibits iNOS activity in a concentration-dependent manner (Figure 5). Maximal inhibition requires about a 12-fold excess of peptide to iNOS. As shown in Figure 6, this inhibition can be reversed by the addition of exogenous CaM (0.3−0.5 molar ratio to peptide). This reversal of the inhibition by CaM is consistent with a CaM-free form of iNOS as the inhibited form of the enzyme ([C] and [D] in Scheme 1). However, at this time we cannot rule out structure [E] in Scheme 1 nor can we speculate on the affinity of iNOS for the CaM−peptide complex in [E] or the enzymatic activity of [E]. If [A] binds the peptide to form [E] and the iNOS in [E] has reduced affinity for CaM, then [D] would be formed by that route, namely, [A] → [E] → [D]. In the most critical test of the model, the peptide was able to form a complex with iNOS-derived CaM. In the native Western blot probed with CaM antibody shown in Figure 7A, one can clearly see that with increasing concentrations of peptide the iNOS-derived CaM band forms several higher migrating species with the peptide, which are also formed with bovine CaM. The freely dissociated CaM band has essentially disappeared in lane 7 (10-fold excess of peptide); however, the CaM−peptide complex bands continue to increase with further increases in peptide concentration (lanes 8 and 9). The diffuse iNOS bands at the top of the blot, however, are essentially unchanged. This may mean that at concentrations of peptide less than 10-fold greater than iNOS, CaM is still bound to the enzyme, as shown in [E] (Scheme 1). This interpretation is complicated by the fact that we have found that the monoclonal antibodies against CaM cross-react with CaM-free iNOS. Interestingly, however, greater than 50% loss of enzymatic activity is observed at a 10-fold excess of peptide to iNOS (Figure 5). These results together suggest that the peptide ultimately leads to the formation of [C], [D], and [F] and that species [E] in Scheme 1 may indeed form and is expected to be catalytically inactive. In Figure 7B, the same experiment was probed with iNOS antibody, showing that the peptide does little, if anything, to the monomer/dimer bands of iNOS. The results in Figures 3, 4, and 7A indicate that more than one peptide−CaM complex is formed whether bovine CaM or CaM derived from iNOS is treated with peptide. Therefore, based on these results, the structures shown as [C], [D], and [F] of Scheme 1 are speculated to exist. Although the experiments were designed based on a simple model of mass action, our results suggest that this does not completely explain our observations. Since CaM copurifies bound to iNOS, the K_d must be small and the $t_{1/2}$ for dissociation relatively long. Yet maximal inhibition by the peptide is observed after 2 min, suggesting that inhibition is not simply due to direct competition of the peptide with CaM dissociated from iNOS (Scheme 1, [B]). Formation of an initial complex such as [E] illustrated in Scheme 1 could explain this apparent kinetic problem if CaM has reduced affinity for iNOS in this ternary complex or if this species is catalytically inactive.

The results presented here establish the importance of this tightly bound CaM moiety in the catalytic activity of this inducible NOS isoform. Given the unique nature of this interaction, it is possible that selective inhibition of this activity is possible. Current studies are directed toward a

further characterization of the interaction of the peptide with CaM, as well as to compare this interaction with that of other CaM binding peptides derived from proteins that have a classical reversible interaction with CaM.

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