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Activation and inactivation of neuronal nitric oxide synthase: characterization of Ca^{2+}-dependent [^{125}I]Calmodulin binding

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Abstract

Constitutive isoforms of nitric oxide synthase (NOS) are activated by transient binding of Ca^{2+}/Calmodulin. Here, we characterize the binding of Calmodulin to purified neuronal NOS (nNOS). [^{125}I]Calmodulin bound to a single class of non-interacting and high affinity sites on nNOS. [^{125}I]Calmodulin binding achieved rapid saturation, was linear with nNOS concentration, and exhibited a strict dependence on [Ca^{2+}]. Neither affinity nor extent of [^{125}I]Calmodulin binding was affected by L-arginine, NADPH or Tetrahydrobiopterin. Native Calmodulin and engineered Calmodulin homologs [i.e., duplicated N-terminal (CaMNN)] potently displaced [^{125}I]Calmodulin. CaMNN supported nNOS catalysis, but required ~ five-fold more Ca^{2+} for comparable activity with native Calmodulin. Taken with results from kinetic analyses of [^{125}I]Calmodulin association and dissociation, our findings suggest four sequential steps in activation of nNOS by Calmodulin: (1) Ca^{2+} binds to Calmodulin's C-lobe, (2) the C-lobe of Calmodulin binds NOS, (3) Ca^{2+} binds to the N-lobe of Calmodulin, and (4) the N-lobe binds to nNOS. Activation of nNOS only occurs after completion of step (4), with the displacement of nNOS's autoinhibitory insert. Upon intracellular Ca^{2+} sequestration, deactivation of nNOS would proceed in reverse order.

Keywords: Nitric oxide (NO) synthase, neuronal; Calmodulin; Ca^{2+}; Radioligand binding

1. Introduction

Nitric oxide (NO) is a key cell signaling molecule, which is synthesized from L-arginine by a family of three mammalian NO synthase (NOS) gene products (Nathan and Xie, 1994; Griffith and Stuehr, 1995). While NOS isoforms differ in their localization and modes of regulation, all share a requirement that Calmodulin be bound for enzyme activity. Indeed, appreciation of a requirement for Calmodulin was essential to the first purification of a NOS isoform (Bredt and Snyder, 1990). A 20–30 amino acid Calmodulin-binding sequence that is both basic and hydrophobic is prototypic of Ca^{2+}/Calmodulin activated enzymes (Cohen and Klee, 1988). In NOSs, this Calmodulin-binding sequence resides at the juncture between N-terminal oxygenase and C-terminal reductase domains (Bredt and Snyder, 1990; Liu and Gross, 1996). All NOSs are obligate homodimers (Griffith and Stuehr, 1995) and thus have a possibility for binding two molecules of Calmodulin per dimer. Indirect evidence suggests that Calmodulin binding triggers NOS catalysis by eliciting a reorientation of heme and flavin cofactors within oxygenase and reductase domains, respectively, thereby promoting interdomain and intradomain electron flux (Abu-Soud and Stuehr, 1993; Abu-Soud et al., 1994). Calmodulin was shown to gate electron flux in trans, i.e., between the reductase and oxygenase domains of companion subunits, providing a molecular explanation for the experimental finding that dimerization is essential to nNOS function (Siddhanta et al., 1998). The ability of Calmodulin to gate electron flux is associated with the displacement of a putative regulatory polypeptide within the FMN-binding domain of nNOS (Salerno et al., 1997).

Neuronal NOS and eNOS, named for the neuronal and endothelial cells in which they were first described, are constitutively expressed isoforms of NOS, which are catalytically dormant until Calmodulin binding is triggered by transient elevations in intracellular Ca^{2+}. Basal levels of

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intracellular Ca^{2+} are typically 50–100 nM, whereas during cell activation Ca^{2+} levels approach 1 μM. Accordingly, in vitro studies have shown that nNOS and eNOS are both inactive at basal intracellular levels of Ca^{2+}, and activity increases as Ca^{2+} levels rise with EC_{50} values for Ca^{2+} of 250 to 350 nM (Bredt and Snyder, 1990; Schmidt et al., 1991). Thus, nNOS and eNOS are perfectly poised for regulation by changing levels of intracellular Ca^{2+} concentration, in the physiological range. In contrast, the inducible isoform of NOS (iNOS) contains Calmodulin that is bound tightly, even at low resting levels of intracellular Ca^{2+} (Cho et al., 1992), explaining the apparent Ca^{2+}-independence of this isoform.

Calmodulin is a 17-kDa protein composed of an 8-turn central α-helix, flanked by globular N- and C-terminal Ca^{2+}-binding lobes (Babu et al., 1988). Two helix-loop-helix structural motifs (EF-hands) are present per lobe, each with a capacity for binding a single Ca^{2+} atom (Manalan and Klee, 1984). Binding of Ca^{2+} causes compaction of the lobes, exposing hydrophobic and acidic residues, which engage in binding to cognate regulatory sites on target protein (Weinstein and Mehler, 1994). Since strong positive cooperativity for Ca^{2+} binding occurs within lobes, but little or none between lobes, Calmodulin species will predominate that contain neither or both Ca^{2+} atoms bound to a given lobe (Weinstein and Mehler, 1994). Notably, the C-lobe of Calmodulin binds Ca^{2+} with 5- to 10-fold higher affinity than the N-lobe (Linse et al., 1991). Once Calmodulin is bound to a protein target, the affinity of Ca^{2+} for each lobe can increase substantially and differentially (Johnson et al., 1996).

Both the Ca^{2+}-bound N- and C-lobes of Calmodulin are independently able to bind nNOS (Persechini et al., 1994). While the C-lobe compacts into its nNOS-binding mode at lower concentrations of Ca^{2+} than needed for N-lobe compaction, binding of the N-lobe was reported to be essential for nNOS activation (Persechini et al., 1996a). Factors that regulate Calmodulin binding to nNOS and the precise sequence of Calmodulin-binding events involved in NOS activation and deactivation await definition.

The present studies were performed to elucidate details of Calmodulin binding and control of nNOS activity. We demonstrate that Calmodulin binds in a Ca^{2+}-dependent manner to a single class of non-interacting sites on nNOS. Calmodulin initially binds to nNOS via its C-lobe, and subsequently activates catalysis by engaging its N-lobe. A sequential model for activation and deactivation of nNOS is inferred that has important implications for our appreciation of physiological nNOS activation/deactivation kinetics.

2. Materials and methods

2.1. Materials

High purity bovine brain Calmodulin and protein S-100 were purchased from Calbiochem (San Diego, CA). Bolton-Hunter labeled [^{125}I]Calmodulin (sp. act. 1395 Ci/mmol) was obtained from NEN Life Sciences (Boston, MA). Rat recombinant nNOS was expressed in *Escherichia coli* and purified as previously described (Roman et al., 1995). The concentration of nNOS was quantified based on spectrophotometric determination of heme chromophore content, after heme reduction with dithiotheritol and CO binding (McMillan and Masters, 1993). Engineered Calmodulin analogs, CaMNN (in which Calmodulin residues 82–148 have been replaced by residues 9–75) and CaMCC (in which Calmodulin residues 9–75 have been replaced by residues 82–148) were kindly provided by Dr. A. Persechini and prepared as previously described (Persechini et al., 1996a). Tetrahydrobiopterin was purchased from Schirks Laboratory (Jona, Switzerland). Fura-2 and Ca^{2+} standard solutions were from Molecular Probes (Eugene, OR). Microtiter plates with GF/B filtration membranes were from Millipore (Bedford, MA). Troponin C, nitrate reductase, lactic dehydrogenase and all other chemicals were from Sigma (St. Louis, MO).

2.2. Binding of [^{125}I]Calmodulin

Binding assays were performed in 96-well microfiltration plates containing GF/B membranes that had been prewashed with buffer A [Tris–HCl (50 mM), $CaCl_2$ (100 μM; omitted in experiments where effects of calcium concentration were under investigation) and β-lactoglobulin (0.5%), pH 7.6]. Incubations contained buffer A, Dithiotheritol (1 mM), [^{125}I]Calmodulin (1 nM) (except for saturation analyses) and desired additions in a final volume of 100 μl. Addition of nNOS (final concentration 10 nM) typically initiated the 15-min binding reaction, conducted at 23 °C. Binding experiments were terminated by rapid vacuum filtration followed by two washes with 100 μl of ice-cold buffer A. Plates were air-dried and 25 μl scintillation cocktail was added to each well (OptiPhase SuperMix, Wallac). Bound radioactivity was determined using a Microbeta Plus liquid scintillation counter (Wallac). Nonspecific binding was defined by inclusion of either EGTA (5 mM) or unlabeled Calmodulin (1 μM) and typically accounted for $<10\%$ of total [^{125}I]Calmodulin bound. Ca^{2+}-EGTA buffers were prepared to approximate the desired test concentrations of free Ca^{2+} using a standard protocol (Fabiato and Fabiato, 1979). Actual free Ca^{2+} concentration of buffers was specified by ratiometric fluorometry using fura-2 (1 μM) and a Fluorolog-2 spectrofluorimeter (SPEX; Edison, NJ).

2.3. Preparation of MOPS/Ca^{2+} buffers

Calcium buffers were prepared by mixing 3-(*N*-Morphlino)propanesulfonic acid (MOPS) (10 mM) pH 7.2, KCl (100 mM) EGTA (10 mM) with MOPS/KCl/EGTA and Ca^{2+} (10 mM).

2.4. Assay of NOS activity based on NADPH consumption

NADPH consumption by nNOS was assessed at 25 °C in 96-well microtiter plates, as previously described (Gross, 1996). Incubation mixtures contained Tris–HCl (50 mM), $CaCl_2$ (100 μM), Tetrahydrobiopterin (10 μM), NADPH (500 μM), L-arginine (500 μM), Dithiotheritol (1 mM) and Calmodulin (100 nM) (in the absence or presence of CaMCC (100 nM), or CaMNN (100 nM) (pH 7.6), in a final volume of 100 μl. Reactions were initiated by the addition of nNOS (100 nM) and the rate of A_{340} decrease was measured at 15-s intervals for a period of 30 min in a kinetic microplate spectrophotometer (Molecular Devices; Menlo Park, CA).

2.5. Assay of NOS activity based on NOx production

NO synthesis was determined from nitrite + nitrate (NOx) accumulation in reaction mixtures. NOx was quantified by the method of Griess, following enzymatic reduction of nitrate to nitrite and oxidation of residual NADPH.

2.6. Analysis of radioligand binding data

Binding parameters were assessed by computer-assisted non-linear least square regression analysis, using the Prism2 program (GraphPad Software) to fit the equation:

$$B = (B_{max} + T + K_d)/2 - \{[(B_{max} + T + K_d)/2]^2 - B_{max}T\}^{1/2}$$

B is the amount of bound [^{125}I]Calmodulin (in dpm), B_{max} is the maximal binding density for Calmodulin on nNOS, T is the total concentration of [^{125}I]Calmodulin (in dpm) and K_d is the dissociation constant of Calmodulin. This equation derives from the basic equilibrium formula $[L][B_{max}]/[B] = K_d$, under the specialized condition where free ligand concentration (L) is significantly less than T, due to formation of complexes, B. Analysis of association and dissociation kinetics of Calmodulin binding and comparison of one-site vs. two-site binding models was performed using the *Ligand* program (Biosoft, Cambridge, UK) and Prism3.

3. Results

Preliminary experiments with a solution-phase radioligand assay using [^{125}I]Calmodulin demonstrated that upon vacuum filtration through GF/B membranes, nNOS/[^{125}I]Calmodulin complexes are retained while blank binding is less than 0.01% of free [^{125}I]Calmodulin. A high single-pass extraction efficiency of nNOS/[^{125}I]Calmodulin complexes by GF/B membranes was indicated by our observation that two additional re-passages of the flow-through resulted in < 5% additional retention of radiolabelled complexes on the GF/B filters (beyond that measured after the initial passage). Thus, filtration through 96-well GF/B filter-bottom microtiter plates was validated as an effective and reliable means to quantify solution-phase binding of [^{125}I]Calmodulin to purified nNOS.

3.1. Affinity, specificity and influence of substrates/cofactors on Calmodulin binding to nNOS

As shown in Fig. 1A, the binding of [^{125}I]Calmodulin (1 nM) to nNOS (10 nM) progressed in a monophasic manner with time, reaching apparent equilibrium with a $t_{1/2}$ of < 2 min at 23 °C. In the presence of 0.1-pmol [^{125}I]Calmodulin, complex formation increased linearly with added [nNOS], until saturation was approached with >95% of added [^{125}I]Calmodulin engaged in complex with nNOS (Fig. 1B). The hyperbolic fit observed in Fig. 1B reveals that

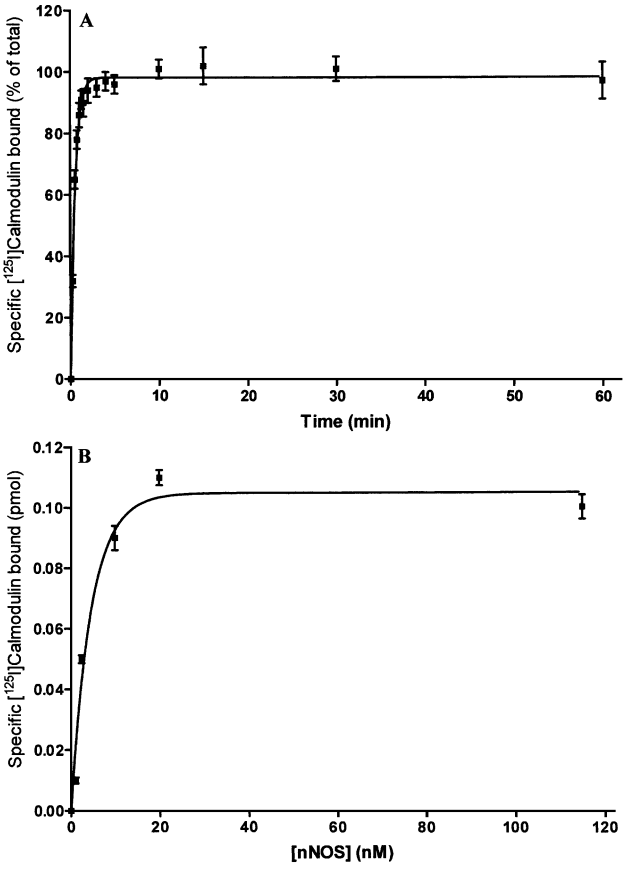

Fig. 1. Binding of [^{125}I]Calmodulin to purified rat nNOS. Panel A: Time course of [^{125}I]Calmodulin (1 nM) binding to nNOS (10 nM) at 23 °C (for details, see Materials and methods). The reaction was initiated by the addition of nNOS and terminated by rapid vacuum filtration at the indicated times. Data points represent means ± S.E.M. of specific [^{125}I]Calmodulin bound ($n = 5$). Panel B: Effect of increasing [nNOS] on the formation of nNOS/[^{125}I]Calmodulin complexes, after 30-min incubation with 0.1 pmol [^{125}I]Calmodulin (1 nM final concentration). Data points represent means ± S.E.M. for triplicate determinations of specifically bound [^{125}I]Calmodulin. Similar results were obtained in three independent experiments.

[^{125}I]Calmodulin bound to a finite number of specific binding sites on nNOS.

Analysis of saturation binding experiments revealed that [^{125}I]Calmodulin binds to a single class of high affinity binding sites on nNOS with a $K_d = 4.70 \pm 0.47$ nM ($n = 8$; Fig. 2). Maximal binding was observed at a stoichiometry of [^{125}I]Calmodulin to nNOS heme chromophore that approached 1.0 (Fig. 2).

Substrates and cofactors of nNOS were tested for their influence on [^{125}I]Calmodulin binding. Inclusion of 10 μM concentrations of NADPH, Tetrahydrobiopterin and L-arginine, individually and in combinations, resulted in no significant effect on [^{125}I]Calmodulin binding (results not shown); notably these concentrations all exceed the respective K_m values for ligand binding (Griffith and Stuehr, 1995). Higher concentrations of NADPH and L-arginine (0.1 and 1 mM each) were similarly without effect on [^{125}I]Calmodulin binding (results not shown).

Specificity of the Calmodulin binding site on nNOS was investigated by comparing the ability of native Calmodulin, other calcium-binding proteins, and engineered Calmodulin analogs, to compete for binding of [^{125}I]Calmodulin. To this end, the relative ability of Troponin C and protein S-100 to compete with [^{125}I]Calmodulin was examined (Fig. 3). Troponin C was an effective competitor but exhibited relatively low potency, with an IC_{50} value of 5.9 μM. On the other hand, S-100 was completely inactive as a Calmodulin competitor at concentrations up to 30 μM. Native Calmodulin and engineered Calmodulin analogs that contain either duplicated C-lobes or N-lobes, namely CaMCC and CaMNN, inhibited [^{125}I]Calmodulin binding to nNOS with K_i values of 4.37 ± 1.12 ($n = 7$), 31.01 ± 5.03 ($n = 3$) and 4.97 ± 0.86 ($n = 6$) nM, and Hill coefficients of 1.34 ± 0.22, 0.77 ± 0.17 and 1.30 ± 0.20, respectively (Fig. 4).

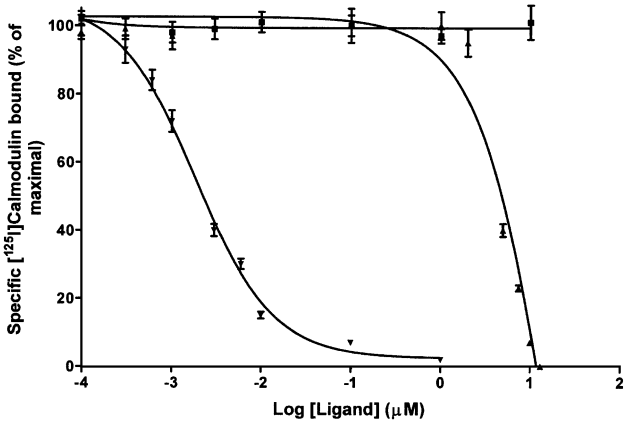

Fig. 3. Specificity of [^{125}I]Calmodulin binding to nNOS: competition for binding by other Ca^{2+} binding proteins. Calmodulin (inverted filled triangle), Troponin C (filled triangle) or protein S-100 (filled square) were added at the indicated concentrations to incubation mixtures containing [^{125}I]Calmodulin (1 nM) and nNOS (10 nM). Incubations were performed as described in Materials and methods and data points are mean ± S.E.M. values of triplicate determinations. Similar results were obtained in four independent experiments.

Activity studies showed that CaMNN faithfully mimics the ability of native Calmodulin to promote electron transfer in nNOS, monitored as an increase in NADPH consumption rate (data not presented). On the other hand, an increase in NADPH consumption was not observed at a concentration of CaMCC that occupies >80% of Calmodulin binding sites on nNOS. Indeed, the addition of CaMCC in 10-fold molar excess over native Calmodulin resulted in a >70% inhibition of NADPH consumption (results not shown).

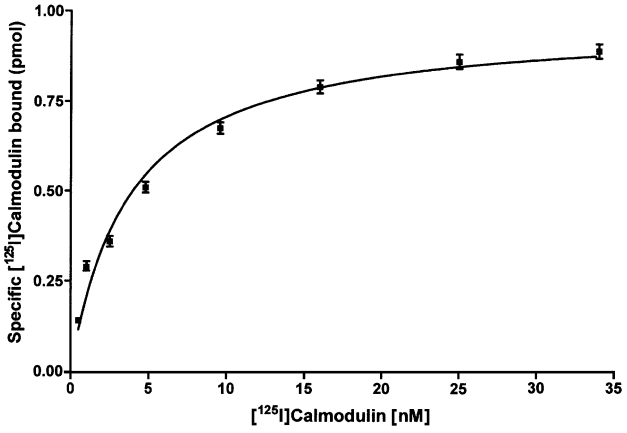

Fig. 2. Saturation binding of [^{125}I]Calmodulin to nNOS. Increasing concentrations of [^{125}I]Calmodulin were incubated with nNOS (10 nM) and binding reactions were terminated after 30 min incubation. The K_d for specific [^{125}I]Calmodulin bound to nNOS was determined by nonlinear regression analysis using an algorithm that accounts for depletion of free ligand as a result of binding (for details, see Materials and methods). Eight independent analyses yielded similar results, with $r^2 > 0.95$ and a mean K_d value of 4.70 ± 0.47 nM.

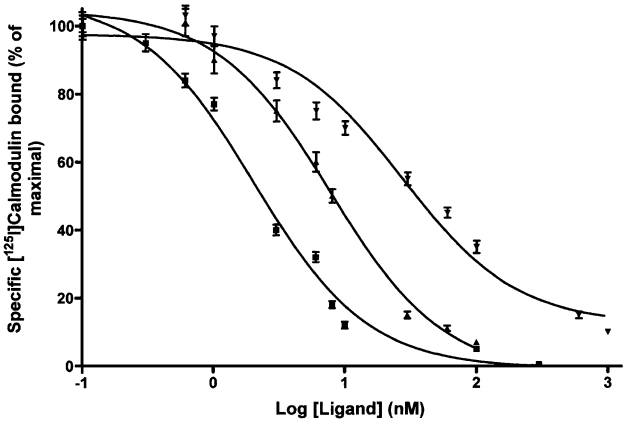

Fig. 4. Competition by engineered Calmodulin proteins with [^{125}I]Calmodulin for binding to nNOS. CaMNN (residues 82–148 replaced by residues 9–75; filled triangle), CaMCC (residues 9–75 replaced by residues 82–148; filled inverted triangle) or native Calmodulin (filled square) was incubated at the indicated concentrations with nNOS (10 nM) and [^{125}I]Calmodulin (1 nM) as described in Materials and methods. Data represent mean ± S.E.M. values of triplicate determinations. Similar results were obtained in three to seven separate experiments.

3.2. Calcium-dependence of Calmodulin binding and nNOS activation

The calcium-dependence of [^{125}I]Calmodulin binding to nNOS was directly assessed using a series of calcium-EGTA buffers for precise control of free Ca^{2+} concentration. The amount of [^{125}I]Calmodulin bound to nNOS under equilibrium conditions increased as a function of free [Ca^{2+}], with half-maximal binding achieved at 202 ± 61 nM ($n = 5$; dashed line, closed circles in Fig. 5). Minimal Calmodulin binding was detectable at < 16 nM free [Ca^{2+}] and binding approached a maximal level at 1 μM [Ca^{2+}]. Further increase in free [Ca^{2+}] from 10 μM to 1 mM resulted in a 24% reduction from maximal Calmodulin bound ($n = 2$, data not shown). The Hill coefficient for Ca^{2+}-dependence of [^{125}I]Calmodulin binding to nNOS was 1.22 ± 0.24, not significantly different from unity. As shown in Fig. 5, the curve which reflects the Ca^{2+}-dependence of nNOS activity (solid line, filled triangles) lies to the right of that for Ca^{2+}-induced Calmodulin binding exhibiting an EC_{50} value of 447 ± 13 nM, ($n = 5$). Furthermore, the EC_{50} of Ca^{2+} for activation of nNOS by CaMNN was found to be 2.66 ± 0.42 μM ($n = 3$, Fig. 5), more than five-fold higher than that for native Calmodulin.

3.3. Calcium-dependence of kinetics for Calmodulin binding to nNOS

On-rates for [^{125}I]Calmodulin binding to nNOS were found to increase progressively with increasing Ca^{2+} concentration; $t_{1/2}$ values of 1.549 ± 0.163, 0.603 ± 0.201 and 0.414 ± 0.098 min were observed with 0.08, 0.23 and 6.2 μM Ca^{2+}, respectively (in the presence of 1 nM Calmodulin and 10 nM nNOS; $n = 3$ for each concentration). Accuracy of kinetic measurements is limited by an assay dead time estimated to approach 0.1 min. Increasing [Ca^{2+}] also enhanced the levels of equilibrium [^{125}I]Calmodulin binding to nNOS, achieving approximately 22%, 56% and 100% of maximal levels, respectively (data not shown).

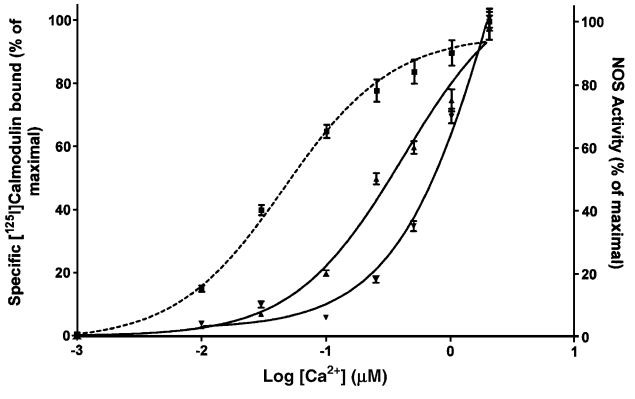

Fig. 5. Effect of free Ca^{2+} concentration on binding of [^{125}I]Calmodulin (100 nM) to nNOS (100 nM) and activation of nNOS by native Calmodulin and CaMNN. Ca^{2+} concentrations were controlled using Ca^{2+}-EGTA buffers and were quantified by FURA-2 fluorescence, as described in Materials and methods. Calmodulin binding data are indicated by the dashed line and closed circles and is represented on the ordinate as % total binding. Activity of nNOS (100 nM) was measured as nitrite production during a 30-min period of incubation with Calmodulin (100 nM; filled triangle) or CaMNN (100 nM; filled inverted triangle) and buffer yielding the indicated concentration of free Ca^{2+}. All points are means ± S.E.M. of triplicate determinations.

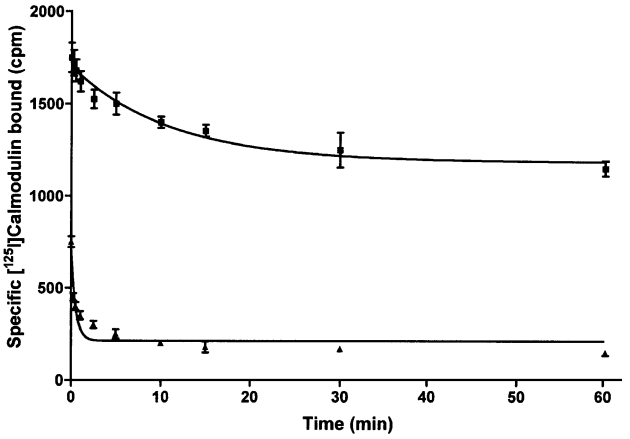

Fig. 6. Dissociation of [^{125}I]Calmodulin from complexes with nNOS formed at differing concentrations of free Ca^{2+}. [^{125}I]Calmodulin (1 nM) was incubated with nNOS (10 nM) for 15 min at 0.23 μM (filled triangles) and 6.2 μM (filled squares) Ca^{2+} ion concentrations. Dissociation was induced by addition of 1 μM native unlabeled Calmodulin. Points are means ± S.E.M. values of triplicate determinations.

Rates of [^{125}I]Calmodulin dissociation from complexes with nNOS were determined upon addition of a 1000-fold molar excess of unlabeled Calmodulin. When [^{125}I]Calmodulin/nNOS complexes were formed in the presence of a concentration of Ca^{2+} that elicits maximal Calmodulin binding and nNOS activation (6.2 μM), dissociation of [^{125}I]-Calmodulin occurred at a monophasic exponential rate with $t_{1/2} = 54.2 \pm 7.6$ min (Fig. 6 and summary data in Table 1). An indistinguishable monophasic dissociation rate was observed when [^{125}I]Calmodulin/nNOS complexes were formed using a still greater excess of Ca^{2+} (100 μM; dissociation $t_{1/2} = 48.8 \pm 13.2$ min). However, when [^{125}I] Calmodulin/nNOS

Table 1
Influence of [Ca^{2+}] on the kinetics of [^{125}I]Calmodulin dissociation from nNOS

[Ca^{2+}] (μM)	N	Site 1		Site 2		r^2
		$t_{1/2}$ (min)	% Total sites	$t_{1/2}$ (min)	% Total sites	
100	3	48.8 ± 13.22	100	–	–	0.931
6.2	3	54.24 ± 7.58	100	–	–	0.921
0.23	4	24.59 ± 7.59	52.6 ± 12.23	0.74 ± 0.36	47.4 ± 15.70	0.994

[^{125}I]Calmodulin (1 nM) was incubated for 30 min at 23 °C with nNOS (10 nM) to elicit complex formation. Dissociation of complexes was initiated by the addition of excess unlabeled Calmodulin (1 μM). All values are means ± S.E.M. N denotes the number of experiments performed and the mean correlation coefficient for non-linear least squares fit to one-site or two-site models of dissociation are indicated by r^2. Data were analyzed using *Ligand* software (Biosoft; Cambridge, UK).

complexes were formed in the presence of 0.23 μM Ca^{2+}, a concentration that supports approximately 50% of maximal Calmodulin binding, two distinct temporal phases of dissociation were observed (Fig. 6 and Table 1). Whereas 52.6% of the [^{125}I]Calmodulin/nNOS complexes formed at 0.23 μM Ca^{2+} dissociated within 2 min ($t_{1/2} = 0.74 \pm 0.36$ min; $n = 4$), the remaining 47.4% dissociated at a substantially slower rate ($t_{1/2} = 24.6 \pm 7.6$ min; $n = 4$) that was not significantly different from the singular dissociation rate observed with high [Ca^{2+}].

In additional experiments, the rate of dissociation of [^{125}I]Calmodulin/nNOS complexes was assessed after free [Ca^{2+}] was diminished to a specified level by chelation with EGTA. Rapid reduction of calcium concentration from 6.2 to 0.23 μM, concomitant with a 10-fold dilution of incubates, elicited biphasic dissociation kinetics; a rapid component involved >50% of complexes ($t_{1/2} = 0.205$ min, $n = 3$), and a much slower phase followed ($t_{1/2}$ of 13.993 min, $n = 3$; data not shown).

4. Discussion

While the pivotal role of Calmodulin as a regulator of NOSs is well established, knowledge of the quantitative relationship between binding and NOS activation is limited. Prior analyses of Calmodulin binding to target proteins, including nNOS, have utilized indirect methods such as activation of enzyme activity or altered intrinsic protein fluorescence resulting from Calmodulin occupancy (Bredt and Snyder, 1990; Sheta et al., 1994). As a consequence of limited assay sensitivity, these approaches usually require that the target protein is present in great excess over the K_d for Calmodulin binding; this results in diminished precision in determining Calmodulin binding affinity due to uncertainties in the calculation of free [Calmodulin]. Moreover, the use of activity as a surrogate marker for binding assumes a 1:1 ratio between binding and activity, which is not necessarily the case. Alternatively, direct binding of [^{125}I]Calmodulin has been investigated using protein targets bound to nitrocellulose membranes (Edlund et al., 1996; Hubbard and Klee, 1987) or by Western blot analysis of protein/Calmodulin immunoprecipitates (Presta et al., 1997; Ruan et al., 1996). This solid-phase approach is limited in practice to the analysis of few samples, is neither highly reproducible nor amenable to the analysis of binding kinetics, and can give results that poorly reflect actual binding events in solution. For greater precise, high-throughput and rapid analysis of Calmodulin binding to purified nNOS, we developed a solution-phase radioligand assay using [^{125}I]Calmodulin in a 96-well microfiltration plate format.

[^{125}I]Calmodulin binds to high affinity binding sites on nNOS with a maximal stoichiometry of 1.0. A goodness-of-fit to a single hyperbolic function, indicates that Calmodulin binds to each subunit of the nNOS homodimer with an equivalent affinity. Thus, although Calmodulin binding has been demonstrated to act both within and between nNOS subunits to promote electron flux (Abu-Soud and Stuehr, 1993; Abu-Soud et al., 1994; Siddhanta et al., 1996), no evidence for site-to-site cooperativity was detected. The observed K_d of 4.70 nM for Calmodulin binding to holo-nNOS confirms and refines an earlier estimate of 1 nM, based on binding-induced changes in intrinsic tryptophan fluorescence of nNOS (Sheta et al., 1994), and is in accord with reports of a 1–3 nM affinity for Calmodulin binding to nNOS-derived peptides (Vorherr et al., 1993; Zhang and Vogel, 1994).

The observation that none of the substrates or co-factors examined affected [^{125}I]Calmodulin binding suggests that substrate/cofactor occupancy of the active site of nNOS does not elicit allosteric effects on key Calmodulin-binding residues sufficient to manifest as a macroscopic effect on Calmodulin binding affinity. In contrast, Tetrahydrobiopterin is reported to be an allosteric modulator of recombinant rat nNOS and required for high affinity binding of arginine analogs (Alderton et al., 1998; Liu and Gross, 1996; Roman et al., 1995). Moreover, maximal [^{3}H]Tetrahydrobiopterin binding to nNOS has been reported to require arginine occupancy (Alderton et al., 1998; Gorren et al., 1996; Liu and Gross, 1996). Thus, although Calmodulin binding clearly alters the active site environment of nNOS, allowing for electron gating from FMN to heme, our findings suggest that active site substrates do not elicit a detectable effect on Calmodulin binding characteristics.

Calmodulin binding site on nNOS was found to be specific, as evident from the ability of native Calmodulin to potently compete for binding of [^{125}I]Calmodulin whereas the ubiquitous calcium binding protein of brain, S-100, was completely inactive. Troponin C, previously shown to bind to Calmodulin-activated enzymes such as nNOS (Su et al., 1995), was an effective competitor but exhibited relatively low potency. The Calmodulin analogs CaMCC and CaMNN potently inhibited [^{125}I]Calmodulin binding to nNOS with K_I values in the low nanomolar range. The six- to seven-fold lower affinity of CaMCC for nNOS, relative to native Calmodulin and CaMNN, suggests that the C-lobe of Calmodulin may not dock effectively with binding sites on nNOS that interact normally with the N-lobe of native Calmodulin. Moreover, a diminished Hill coefficient for binding CaMCC relative to these for the binding of native Calmodulin and CaMNN, suggests that the C-lobe of Calmodulin may actually sustain anti-cooperative interactions with sites on nNOS that interact with the N-lobe of native Calmodulin. In contrast, the similar affinity and Hill coefficients for binding CaMNN and native Calmodulin suggest that the N-lobe of Calmodulin can effectively interact with sites on nNOS that normally interact with the C-lobe of native Calmodulin. While the order of CaMNN and CaMCC binding affinity described above is opposite to that previously reported (Persechini et al., 1996a), it is notable that the latter determination was indirect, i.e., deduced from enzyme activity measurements rather than direct analysis of binding.

The observations that CaMNN activated while CaMCC inhibited nNOS activity, are in accord with the earlier finding (Persechini et al., 1996a) that a Calmodulin with duplicated C-lobes can bind but not activate nNOS, whereas a Calmodulin with duplicated N-lobes supports both binding and catalytic activity. Since 2-D NMR studies have demonstrated that the N-terminal lobe of Calmodulin binds to the C-terminal aspect of the Calmodulin binding site of nNOS (Zhang et al., 1995), we infer that it is this specific interaction that is crucial for activation of nNOS by Calmodulin. Notably, it is also the C-terminal aspect of the Calmodulin binding site that is in closest proximity to the FMN-domain regulatory control-element whose displacement by Calmodulin was identified and proposed to mediate calcium-dependent NOS activation (Salerno et al., 1997). Function of the proposed regulatory control element was recently confirmed for both eNOS (Nishida and Ortiz de Montellano, 1999) and nNOS (Daff et al., 1999). Thus, the N-lobe of Calmodulin is essential for nNOS activity and, when bound to nNOS, is poised to engage in putative control-element interactions that may serve to release the proposed autoinhibition of nNOS activity.

[^{125}I]Calmodulin binding to nNOS under equilibrium conditions increased as a function of free [Ca^{2+}] and exhibited a Hill coefficient not significantly different from unity. Residual Calmodulin binding was observed at < 16 nM free [Ca^{2+}] and while binding reached maximal level at 1 μM [Ca^{2+}], elevation of free [Ca^{2+}] to 1 mM resulted in a marked reduction in Calmodulin binding, consistent with the decline in nNOS activity reported by Bredt and Snyder (1990) at similarly high concentrations of Ca^{2+}. This loss of Calmodulin binding with high [Ca^{2+}] may involve binding of Ca^{2+} to additional weak affinity sites on Calmodulin that have recently been identified (Gilli et al., 1998). Surprisingly, the EC_{50} of calcium for support of nNOS catalytic activity (i.e., 447 nM) was significantly greater than that required for support of [^{125}I]Calmodulin binding (i.e., 202 nM).

The conspicuous non-identity of binding and activation curves can be reconciled by the existence of at least two modes of Calmodulin binding to nNOS: at low levels of Ca^{2+} (< 100 nM) a Calmodulin/nNOS complex can form that is devoid of catalytic activity, whereas at higher levels of Ca^{2+} the activated nNOS complex results. We questioned the molecular identity of these two postulated complexes. Given that the C-lobe of Calmodulin compacts into its protein-binding conformation at 5- to 10-fold lower Ca^{2+} than that needed for N-lobe compaction (Linse et al., 1991), and the C-lobe alone is insufficient for nNOS activation, we hypothesized that the catalytically non-productive complex may result from exclusive binding of Calmodulin's C-lobe. However, when Ca^{2+} concentrations are sufficiently high to compact both C- and N-lobes, permitting each lobe to simultaneously bind nNOS, critical N-lobe interactions may elicit the activated nNOS complex. Consistent with the higher concentration of Ca^{2+} required for compaction and binding of N-lobes (relative to C-lobes), the EC_{50} of Ca^{2+} for activation of nNOS by CaMNN was found to be approximately five-fold higher than that for native Calmodulin. Taken together, these findings suggested that the existence of two distinct nNOS/Calmodulin complexes: one that is catalytically inactive, formed at low levels of Ca^{2+} and involves C-lobe interactions only, and a second that is catalytically active, formed at higher concentrations of Ca^{2+} and involves both C- and N-lobe interactions. This "stepwise" binding hypothesis was tested by studies of the effects of Ca^{2+} concentration on Calmodulin binding kinetics.

Levels of free Ca^{2+} dictate the extent to which the C- and N-lobes of Calmodulin are compacted in a conformation suitable for nNOS binding. Association rates for [^{125}I]-Calmodulin binding to nNOS were quantified at each of several free [Ca^{2+}]. As expected, on-rates progressively increased with increasing Ca^{2+} concentration, concomitant with enhanced levels of [^{125}I]Calmodulin binding to nNOS. When the rates of [^{125}I]Calmodulin dissociation from complexes with nNOS were determined upon addition of large excess of unlabeled Calmodulin, and in the presence of [Ca^{2+}] of either 6.2 or 100 μM, dissociation occurred at a monophasic exponential rate. Nevertheless, when [Ca^{2+}] was lowered to 0.23 μM, a concentration that supports approximately 50% of maximal Calmodulin binding, two distinct temporal phases of dissociation were observed. Close to half of the complexes formed at 0.23 μM Ca^{2+} dissociated within 2 min, the remaining at a rate not significantly different from that observed with high [Ca^{2+}]. Further experiments were carried out by reducing free [Ca^{2+}] to a specified level by chelation with EGTA, presenting a mode akin to actual cellular regulation and therefore affording a more physiologically relevant approach to the analysis of Calmodulin dissociation. Rapid reduction of Ca^{2+} concentration resulted in a biphasic dissociation kinetics with similar distribution of sites (ca. 1:1) and a fast (< 0.5 min) phase, and a second stage demonstrating a much higher $t_{1/2}$ value.

Biphasic dissociation kinetics of insect Calmodulin from a variety of Calmodulin-binding peptides have similarly been observed using stopped-flow measurements after calcium-chelation (Brown et al., 1997); dissociation by a combination of partially calcium-occupied Calmodulin and calcium-devoid Calmodulin was indicated to be responsible for the biphasic phenomenon. Studies of chelator-induced calcium dissociation from Calmodulin/nNOS complexes, in which calcium levels were driven to essentially zero (Persechini et al., 1996b), demonstrated that of four bound calcium ions, two dissociate rapidly from the N-lobe of Calmodulin, at a rate exceeding 1000 s^{-1}, whereas the additional two dissociate slowly from the C-lobe, at 1 s^{-1}. Thus, ample evidence favors the view that Calmodulin can dissociate from nNOS, and perhaps other Calmodulin-binding proteins, in either of two modes depending on free [Ca^{2+}]: one in which only the C-lobe of Calmodulin has bound calcium and a second in which both N- and C-lobes of Calmodulin are devoid of calcium. Reciprocally, Calmodulin association

with nNOS should similarly involve binding of two distinct species whose relative concentrations would be determined by free Ca^{2+}, one with Ca^{2+} bound only to the C-lobe of Calmodulin (most abundant at low intracellular Ca^{2+}) and a second with Ca^{2+} bound to both N- and C-lobes of Calmodulin (most abundant at high intracellular $[Ca^{2+}]$). Interestingly, a small-angle scattering study of Calmodulin binding to myosin light chain kinase (MLCK) demonstrated that Calmodulin binding to this enzyme occurs at substoichiometric $[Ca^{2+}]$ (Krueger et al., 1998). While Calmodulin was shown to bind to MLCK at Ca^{2+} concentrations lower than 2 mol/mol of Calmodulin, compaction and activation required saturating $[Ca^{2+}]$.

Taken together, our findings are reconciled by a sequential model that describes the relationship between binding and activation of nNOS by Calmodulin. The model reflects an appreciation of the independent contributions of the two lobes of Calmodulin to formation, stabilization and catalytic activation of Calmodulin/nNOS complexes. Analyses of $[^{125}I]$Calmodulin/nNOS association and dissociation kinetics suggest four successive and distinct steps in binding/activation of nNOS by Calmodulin. In step 1, an intracellular Ca^{2+} transient results in an increase in $[Ca^{2+}]_i$, and preferential binding of 2 mol of Ca^{2+} to EF-hand pairs within the C-terminal lobe of Calmodulin. This results in a compaction of the C-lobe and exposure of residues that are acidic and hydrophobic for high-affinity binding interactions, in step 2, with the N-terminal aspect of the Calmodulin binding site on nNOS. The resulting species is proposed to explain catalytically inactive $[^{125}I]$Calmodulin/nNOS complexes detected at low levels of Ca^{2+} (i.e., ≤ 100 nM). As $[Ca^{2+}]_i$ continue to rise, additional 2 mol of Ca^{2+} can saturate EF-hand pairs within the N-terminal lobe of nNOS-bound Calmodulin (step 3). This triggers in situ compaction of the N-lobe of Calmodulin, revealing amino acid residues that can engage the C-terminal aspect of the Calmodulin binding site on nNOS (step 4). Activation of catalytic activity would occur only after completion of this final step. Based on reported structures of Calmodulin bound to cognate peptides from myosin light chain kinase (Meador et al., 1992, 1993) and Calmodulin-dependent protein kinase II (Ikura et al., 1992), it is presumed that the lobes of Ca^{2+}-saturated Calmodulin envelope the Calmodulin-binding peptide of nNOS, resulting in nNOS activation via reorientation of inter- and intra-subunit heme and flavin cofactors. This structural reorganization is proposed to occur in association with a displacement of the autoinhibitory control peptide. It is notable that three-dimensional homology-based modeling indicates that the control peptide occupies space that is adjacent to or overlapping the site where Calmodulin's N-lobe interacts with nNOS (Salerno et al., 1997). The identities of residues within the N-lobe of Calmodulin that may contribute to displacement of the autoinhibitory peptide in nNOS await specification.

Upon intracellular Ca^{2+} sequestration, deactivation of nNOS would proceed in reverse order. Since Ca^{2+} dissociates orders of magnitude faster from the N-lobe than the C-lobe of nNOS-bound Calmodulin (Persechini et al., 1996b), and N-lobe binding is essential for nNOS activity, Ca^{2+} dissociation from the N-lobe would control nNOS activity. Thus, the initial phase of Ca^{2+} dissociation from the N-lobe of Calmodulin would result in inactive nNOS with Calmodulin bound only by C-lobe interactions. As the affinity of Ca^{2+} for Calmodulin may be significantly increased for NOS-bound Calmodulin, relative to NOS-free Calmodulin (Persechini et al., 1996b), the concentration of free Ca^{2+} that elicits half-maximal complex dissociation may be significantly less than that required for half-maximal complex formation. Thus, hysteresis in the Ca^{2+}-dependence for nNOS binding and unbinding of Calmodulin is anticipated. Our finding that significant levels of catalytically inactive $[^{125}I]$Calmodulin/nNOS complexes can be formed in vitro in the presence of 50–100 nM Ca^{2+}, makes it likely that these species would also predominate in cells at resting levels of intracellular $[Ca^{2+}]$. Our findings lend direct support for the view that rapid Ca^{2+} dissociation from the N-terminal lobe of Calmodulin would elicit enzyme inactivation without triggering dissociation of the Calmodulin-nNOS complex, proposed in earlier stopped-flow fluorescence studies of calcium dissociation from Calmodulin, bound to a nNOS-derived peptide (Persechini et al., 1996b).

An important implication of the above scenario is that some nNOS would be replete with ineffectually bound Calmodulin in cells at rest, and thus reside in a state that is primed and ready for rapid activation upon exposure to an intracellular calcium transient. Evolution may have favored pre-bound Calmodulin as a means to endow nNOS with an enhanced temporal responsiveness to physiological stimulus-evoked changes in free $[Ca^{2+}]$ in neurons, skeletal muscle and other nNOS-containing tissues. This situation is not likely to be unique to nNOS; it has been speculated earlier that in some other Calmodulin-regulated enzymes high C-lobe binding affinity may result in Calmodulin binding in vivo, at resting levels of free $[Ca^{2+}]_i$ (Meador et al., 1992).

In conclusion, we have used a novel radioligand-binding assay to perform the first direct quantitative analysis of Calmodulin binding to a NOS isoform. Conceivably, this technique will also have utility for the analysis of Calmodulin interactions with other high-affinity Calmodulin-binding proteins. In addition to defining K_d, Ca^{2+}-dependence and the potential influence of cofactors and other calcium-binding proteins on Calmodulin/nNOS interactions, this approach has enabled us to perform a direct assessment of the relationship between Calmodulin binding and nNOS activation. Our findings reveal that Calmodulin can bind to nNOS at low basal levels of intracellular calcium without triggering NO production. Molecular details of subsequent interactions, specifically those involving the N-lobe of Calmodulin that are critical for NOS activation, will be the key to a future understanding of how Calmodulin gates electron flux in NOSs.

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