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ACTIVATION AND INACTIVATION OF NEURONAL NITRIC OXIDE SYNTHASE: CHARACTERIZATION OF CALCIUM-DEPENDENT [^{125}I]CALMODULIN BINDING

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SUMMARY

Constitutive isoforms of nitric oxide synthase (NOS) are activated by transient binding of Ca^{2+}/calmodulin (CaM). Here, we have developed a solution phase radioligand binding assay to characterize the binding of CaM to purified neuronal NOS (nNOS). [^{125}I]CaM bound to a single class of non-interacting and high affinity sites on nNOS homodimers with K_d = 4.7\ nM. [^{125}I]CaM binding at 23°C achieved rapid saturation, was linear with nNOS concentration, and exhibited a strict dependence on [Ca^{2+}] (EC_{50} = 202 nM). Neither affinity nor extent of [^{125}I]CaM binding was affected by nNOS substrates and cofactors L-arginine, NADPH or tetrahydrobiopterin. Native CaM and engineered CaM homologs with duplicated C-terminal (CaMCC) and N-terminal (CaMNN) lobes displaced [^{125}I]CaM with K_i values in the low nM range. CaMNN supported nNOS catalysis, but required ~5-fold more calcium for comparable activity with native CaM, while nNOS activity was inhibited by CaMCC. Taken with results from kinetic analyses of [^{125}I]CaM association and dissociation at various concentrations of free Ca^{2+}, our finings suggest four sequential steps in activation of nNOS by CaM: (1) Ca^{2+} binds to CaM's C-lobe, (2) the C-lobe of CaM binds NOS, (3) Ca^{2+} binds to the N-lobe of nNOS-bound CaM, (4) the N-lobe of CaM binds 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, resulting in partial occupancy of inactive nNOS by CaM even at basal levels of Ca^{2+}.

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 (CaM) be bound for enzyme activity. Indeed, appreciation of a requirement for CaM was essential to the first purification of a NOS isoform (Bredt and Snyder, 1990). A 20 - 30 amino acid CaM-binding sequence that is both basic and hydrophobic is prototypic of Ca^{2+}/CaM activated enzymes (Cohen and Klee, 1988). In NOSs, this CaM-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 CaM per dimer. Indirect evidence suggests that CaM 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 et al., 1993, 1994). CaM 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 CaM 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 CaM binding is triggered by transient elevations in intracellular Ca^{2+}. Basal levels of 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 CaM 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.

CaM is a 17 kD 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, CaM 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 CaM binds Ca^{2+} with 5- to 10-fold higher affinity than the N-lobe (Linse et al., 1991).. Once CaM 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 CaM 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 CaM binding to nNOS and the precise sequence of CaM-binding events involved in NOS activation and deactivation await definition.

The present studies were performed to elucidate details of CaM binding and control of nNOS activity. We demonstrate that CaM binds in a calcium-dependent manner to a single class of non-interacting sites on nNOS. CaM 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.

MATERIALS AND METHODS

Materials High purity bovine brain calmodulin and protein S-100 were purchased from Calbiochem (San Diego, CA). Bolton-Hunter labeled [^{125}I]CaM (sp. act. 1395 Ci/mmol) was obtained from NEN Life Sciences (Boston, MA). Rat recombinant nNOS was expressed in *E. 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 dithiothreitol and CO binding (McMilla and Masters, 1993). Engineered CaM analogs, CaMNN (in which CaM residues 82-148 have been replaced by residues 9-75) and CaMCC (in which CaM 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 (BH_4) was purchased from Schirks Laboratory (Jona, Switzerland). Fura-2 and calcium 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, MS).

Binding of [^{125}I]Calmodulin Binding assays were performed in 96-well microfiltration plates containing GF/B membranes that had been pre-washed 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, DTT (1 mM), [^{125}I]CaM (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-minute 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). Non-specific binding was defined by inclusion of either EGTA (5 mM) or unlabeled CaM (1 µM) and typically accounted for <10% of total [^{125}I]CaM 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).

Preparation of MOPS/Ca^{2+} buffers

Calcium buffers were prepared by mixing MOPS (10 mM) pH 7.2, KCl (100 mM) EGTA (10 mM) with MOPS/KCl/EGTA and Ca^{2+} (10 mM).

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), BH_4 (10 µM), NADPH (500 µM), L-arginine (500 µM), DTT (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).

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.

Analysis of Radioligand Binding Data Binding parameters were assessed by computer-assisted non-linear least squares regression analysis, using the Prism2 program (GraphPad Software Inc.) 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]CaM (in dpm), B_{max} is the maximal binding density for CaM on nNOS, T is the total concentration of [^{125}I]CaM (in dpm) and K_d is the dissociation constant of CaM. 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 CaM binding and comparison of one- site vs. two-site binding models was performed using the *Ligand* program (Biosoft, Cambridge, UK) and Prism2.

RESULTS AND DISCUSSION

While the pivotal role of CaM as a regulator of NOSs is well established, knowledge of the quantitative relationship between binding and NOS activation is limited. Prior analyses of CaM binding to target proteins, including nNOS, have utilized indirect methods such as activation of enzyme activity or altered intrinsic protein fluorescence resulting from CaM 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_m for CaM binding; this results in diminished precision in determining CaM binding affinity due to uncertainties in the calculation of free [CaM]. 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]CaM 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/CaM 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 CaM binding to purified nNOS, we sought to develop a solution-phase radioligand assay using [^{125}I]CaM in a 96-well microfiltration plate format.

Preliminary experiments demonstrated that upon vacuum filtration through GF/B membranes, nNOS/[^{125}I]CaM complexes are retained while blank binding is less than 0.01% of free [^{125}I]CaM. A high single-pass extraction efficiency of nNOS/[^{125}I]CaM 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 radiolabeled 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]CaM to purified nNOS.

Affinity, specificity and influence of substrates/cofactors on CaM binding to nNOS. As shown in Fig. 1A, the binding of [^{125}I]CaM (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]CaM, complex formation increased linearly with added [nNOS], until saturation was approached with >95% of added [^{125}I]CaM engaged in complex with nNOS (Fig. 1B). The hyperbolic fit observed in Fig. 1B reveals that [^{125}I]CaM bound to a finite number of specific binding sites on nNOS.

Analysis of saturation binding experiments revealed that [^{125}I]CaM binds to a single class of high affinity binding sites on nNOS with a K_d = 4.70 ± 0.47 nM (n=8; Fig. 2). Maximal binding was observed at a stoichiometry of [^{125}I]CaM to nNOS heme chromophore that approached 1.0 (Fig. 2). The observed maximal stoichiometry of 1.0, and goodness-of-fit to a single hyperbolic function, indicates that CaM binds to each subunit of the nNOS homodimer with an equivalent affinity. Thus, although CaM binding has been demonstrated to act both within and between nNOS subunits to promote electron flux (Abu-Soud et al., 1993, 1994; Siddhanta et al., 1996b), no evidence for site-to-site cooperativity was detected. The observed K_d of 4.70 nM for CaM 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 CaM binding to nNOS-derived peptides (Vorherr et al., 1993; Zhang and Vogel, 1994).

Substrates and cofactors of nNOS were tested for their influence on [^{125}I]CaM binding. Inclusion of 10 μM concentrations of NADPH, BH_4 and L-arginine, individually and in combinations, resulted in no significant effect on [^{125}I]CaM 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]CaM binding (results not shown). These findings suggest that substrate/cofactor occupancy of the

active site of nNOS does not elicit allosteric effects on key CaM-binding residues sufficient to manifest as a macroscopic effect on CaM binding affinity. In contrast, BH_4 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 CaM 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 CaM binding characteristics.

Specificity of the CaM binding site on nNOS was investigated by comparing the ability of native CaM, other calcium-binding proteins, and engineered CaM analogs, to compete for binding of [^{125}I]CaM. Since previous studies suggested that calcium-binding proteins displace [^{125}I]CaM from CaM-activated enzymes such as nNOS (Su et al., 1995), the relative ability of troponin C and protein S-100 to compete with [^{125}I]CaM 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, the ubiquitous calcium binding protein of brain, S-100, was completely inactive as a CaM competitor at concentrations up to 30 µM. Thus, the CaM binding site on nNOS exhibits a high degree of selectivity for CaM.

Native CaM and engineered CaM analogs that contain either duplicated C-lobes or N-lobes, namely CaMCC and CaMNN, inhibited [^{125}I]CaM binding to nNOS with Ki values of 4.37 ± 1.12 (n=7), 31.01 ± 5.03 (n=3) and 4.97 ± 0.86 (n=6) nM, respectively (Fig. 4). The 6-7 fold lower affinity of CaMCC for nNOS, relative to native CaM and CaMNN, suggests that the C-lobe of CaM may not dock effectively with binding sites on nNOS that interact normally with the N-lobe of native CaM. Moreover, a diminished Hill coefficient for binding CaMCC (0.77 ± 0.17), relative to the Hill coeficients for the binding of native CaM (1.34 ± 0.22) and CaMNN (1.30 ± 0.20), suggests that the C-

lobe of CaM may actually sustain anti-cooperative interactions with sites on nNOS that interact with the N-lobe of native CaM. In contrast, the similar affinity and Hill coefficients for binding CaMNN and native CaM suggest that the N-lobe of CaM can effectively interact with sites on nNOS that normally interact with the C-lobe of native CaM. While the order of CaMNN and CaMCC binding affinity described above is opposite to that previously reported for nNOS (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.

Activity studies showed that CaMNN faithfully mimics the ability of native CaM 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 CaM binding sites on nNOS. Indeed, the addition of CaMCC in 10-fold molar excess over native CaM resulted in a >70% inhibition of NADPH consumption (results not shown). These observations are in accord with the earlier finding (Persechini et al., 1996a) that a CaM with duplicated C-lobes can bind but not activate nNOS, whereas a CaM with duplicated N-lobes supports both binding and catalytic activity. Since 2-D NMR studies have demonstrated that the N-terminal lobe of CaM binds to the C-terminal aspect of the CaM binding site of nNOS (Zhang et al., 1995), we infer that it is this specific interaction that is crucial for activation of nNOS by CaM. Notably,0 it is also the C-terminal aspect of the CaM binding site that is in closest proximity to the FMN-domain regulatory control-element whose displacement by CaM was identified and proposed to mediate calcium-dependent NOS activation (Saleno et al., 1997). Function of the proposed regulatory control element was recently confirmed for both eNOS (Nishida and de Montellano, 1999) and nNOS (Daff et al.,1999). Thus, the N-lobe of CaM 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.

Calcium-dependence of CaM binding and nNOS activation. The calcium-dependence of [^{125}I]CaM 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]CaM 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, open circles in Fig. 5). Minimal CaM 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 CaM bound (n = 2, data not shown), consistent with the decline in nNOS activity reported by Bredt and Snyder (1990) at similarly high concentrations of Ca^{2+}. This loss of CaM binding with high [Ca^{2+}] may involve binding of Ca^{2+} to additional weak affinity sites on CaM that have recently been identified (Gilli et al., 1998). The Hill coefficient for Ca^{2+}-dependence of [^{125}I]CaM binding to nNOS was 1.22 ± 0.24, not significantly different from unity. Surprisingly, the EC_{50} of calcium for support of nNOS catalytic activity was significantly greater than that required for support of [^{125}I]CaM binding. As shown in Fig 5, the curve which reflects the Ca^{2+}-dependence of nNOS activity (solid line, filled circles) lies to the right of that for Ca^{2+}-induced CaM binding exhibiting an EC_{50} value of 447 ± 13 nM, (n = 5).

The conspicuous non-identity of binding and activation curves can be reconciled by the existence of at least two modes of CaM binding to nNOS: at low levels of Ca^{2+} (< 100 nM) a CaM/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 CaM 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 CaM'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 2.66 ± 0.42 μM (n = 3), approximately five-fold higher than that for native CaM. Taken together, these findings suggested that the existence of two distinct nNOS/CaM 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 CaM binding kinetics.

Calcium-dependence of kinetics for CaM binding to nNOS. Levels of free Ca^{2+} dictate the extent to which the C- and N-lobes of CaM are compacted in a conformation suitable for nNOS binding. Association rates for [^{125}I]CaM binding to nNOS were quantified at each of several free [Ca^{2+}]. As expected, on-rates 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 CaM 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]CaM binding to nNOS, achieving approximately 22, 56 and 100% of maximal levels, respectively (data not shown).

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

[^{125}I]CaM/nNOS complexes were formed using a still greater excess of Ca^{2+} (100 µM; dissociation $t_{1/2}$ = 48.8 ± 13.2 min). However, when [^{125}I]CaM/nNOS complexes were formed in the presence of 0.23 µM Ca^{2+}, a concentration that supports approximately 50% of maximal CaM binding, two distinct temporal phases of dissociation were observed (Fig. 6 and Table 1). Whereas 52.6% of the [^{125}I]CaM/nNOS complexes formed at 0.23 µM Ca^{2+} dissociated within 2 min ($t_{1/2}$ = 0.74 ± 0.36 min; n = 3), the remaining 47.4% dissociated at a substantially slower rate ($t_{1/2}$ = 24.6 ± 7.6 min; n=3) 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]CaM/nNOS complexes was assessed after free [Ca^{2+}] was diminished to a specified level by chelation with EGTA; this is akin to actual cellular regulation and therefore affords a more physiologically relevant approach to the analysis of CaM dissociation. 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).

Biphasic dissociation kinetics of insect CaM from a variety of CaM-binding peptides have similarly been observed using stopped-flow measurements after calcium-chelation (Brown et al., 1998); dissociation by a combination of partially calcium-occupied CaM and calcium-devoid CaM was indicated to be responsible for the biphasic phenomenon. Studies of chelator-induced calcium dissociation from CaM/nNOS complexes, in which calcium levels are driven to essentially zero (Persechini et al., 1996b), demonstrated that of 4 bound calcium ions, two dissociate rapidly from the N-lobe of CaM, 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 CaM can dissociate from nNOS, and perhaps other CaM-binding proteins, in either of two modes depending on free [Ca^{2+}]: one in which only the C-lobe of CaM has bound calcium and a second in which both N- and C-lobes of CaM are devoid of

calcium. Reciprocally, CaM 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 CaM (most abundant at low intracellular Ca^{2+}) and a second with Ca^{2+} bound to both N- and C-lobes of CaM (most abundant at high intracellular Ca^{2+}). Interestingly, a small-angle scattering study of CaM binding to myosin light chain kinase (MLCK) demonstrated that CaM binding to this enzyme occurs at substoichiometric [Ca^{2+}] (Krueger et al., 1998). While CaM was shown to bind to MLCK at Ca^{2+} concentrations lower than 2 mol/mol of CaM, 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 CaM (Fig. 7). The model reflects an appreciation of the independent contributions of the two lobes of CaM to formation, stabilization and catalytic activation of CaM/nNOS complexes. Analyses of [^{125}I]CaM/nNOS association and dissociation kinetics suggest four successive and distinct steps in binding/activation of nNOS by CaM. In step 1, an intracellular Ca^{2+} transient results in an increase in $[Ca^{2+}]_i$, and preferential binding of 2 mols of Ca^{2+} to EF-hand pairs within the C-terminal lobe of CaM. 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 CaM binding site on nNOS. The resulting species is proposed to explain catalytically inactive [^{125}I]CaM/nNOS complexes detected at low levels of Ca^{2+} (i.e., $\leq$ 100 nM). As $[Ca^{2+}]_i$ continues to rise, additional 2 mols of Ca^{2+} can saturate EF-hand pairs within the N-terminal lobe of nNOS-bound CaM (step 3). This triggers *in situ* compaction of the N-lobe of CaM, revealing amino acid residues that can engage the C-terminal aspect of the CaM binding site on nNOS (step 4). Activation of catalytic activity would occur only after completion of this final step. Based on reported structures of CaM bound to cognate peptides from myosin light chain kinase (Meador et al., 1992, 1993) and CaM-dependent protein kinase II (Ikura et al., 1992), it is presumed

that the lobes of Ca^{2+}-saturated CaM envelope the CaM-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 CaM's N-lobe interacts with nNOS (Salerno et al., 1997). The identity of residues within the N-lobe of CaM 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 CaM (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 CaM would result in inactive nNOS with CaM bound only by C-lobe interactions. As the affinity of Ca^{2+} for CaM may be significantly increased for NOS-bound CaM, relative to NOS-free CaM (Persechini et al., 196b), 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 CaM is anticipated. Our finding that significant levels of catalytically inactive [^{125}I]CaM/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 CaM would elicit enzyme inactivation without triggering dissociation of the CaM-nNOS complex, proposed in earlier stopped-flow fluorescence studies of calcium dissociation from CaM, 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 CaM 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 CaM 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 CaM-regulated enzymes high C-lobe binding affinity may result in CaM 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 CaM binding to a NOS isoform. Conceivably, this technique will also have utility for the analysis of CaM interactions with other high-affinity CaM-binding proteins. In addition to defining K_d, Ca^{2+}-dependence and the potential influence of cofactors and other calcium-binding proteins on CaM/nNOS interactions, this approach has enabled us to perform a direct assessment of the relationship between CaM binding and nNOS activation. Our findings reveal that CaM 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 CaM that are critical for NOS activation, will be the key to a future understanding of how CaM gates electron flux in NOSs.

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

[Ca^{2+}] (μM)	*N*	Site 1 $t_{1/2}$ (min)	Site 1 % total sites	Site 2 $t_{1/2}$ (min)	Site 2 % total sites	r^2
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]CaM (1 nM) was incubated for 30 minutes at 23°C with nNOS (10 nM) to elicit complex formation. Dissociation of complexes was initiated by the addition of excess unlabeled CaM (1 μM). All values are means ± SEM. The number of experiments performed is denoted by *N* 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).

FIGURE LEGENDS

Figure 1. Binding of [^{125}I]CaM to purified rat nNOS. *Panel A:* Time course of [^{125}I]CaM (1 nM) 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 ± SEM of specific [^{125}I]CaM bound (n = 5). *Panel B:* Effect of increasing [nNOS] on the formation of nNOS/[^{125}I]CaM complexes, after a 30 min incubation with 0.1 pmol [^{125}I]CaM (1 nM final concentration). Data points represent means ± SEM for triplicate determinations of specifically-bound [^{125}I]CaM. Similar results were obtained in 3 independent experiments.

Figure 2. Saturation binding of [^{125}I]CaM to nNOS. Increasing concentrations of [^{125}I]CaM were incubated with nNOS (10 nM) and binding reactions were terminated after a 30 min incubation. The Kd for specific [^{125}I]CaM 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*). The computer-fit binding parameters are given on the plot. Eight independent analyses yielded similar results, with $r^2 > 0.95$ and a mean K_d value of 4.70 ± 0.47 nM. The inset depicts a Scatchard plot of the given data, consistent with a single class of binding sites and a stoichiometry of 1 mol [^{125}I]CaM bound per mol of nNOS.

Figure 3. Specificity of [^{125}I]CaM binding to nNOS: competition for binding by other calcium binding proteins. Calmodulin (circles), Troponin C (stars) or protein S-100 (diamonds) were added at the indicated concentrations to incubation mixtures containing [^{125}I]CaM (1 nM) and nNOS (10 nM). Incubations were performed as described in *Materials and Methods* and data points are mean ± SEM values of triplicate determinations. Similar results were obtained in 4 independent experiments.

Figure 4. Competition by engineered CaM proteins with [^{125}I]CaM for binding to nNOS. CaMNN (residues 82-148 replaced by residues 9-75; squares), CaMCC (residues 9-75 replaced by residues 82-148; triangles) or native CaM (circles) were incubated at the indicated concentrations with nNOS (10 nM) and [^{125}I]CaM (1 nM) as described in *Materials and Methods*. Data represent mean ± SEM values of triplicate determinations. Similar results were obtained in 3-7 separate experiments.

Figure 5. Effect of free calcium concentration on binding of [^{125}I]CaM (100 nM) to nNOS (100 nM) and activation of nNOS by native CaM and CaMNN. Ca^{2+} concentrations were controlled using calcium-EGTA buffers and were quantified by FURA-2 fluorescence, as described in *Materials and Methods*. CaM binding data are indicated by the dashed line and open circles and are 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 CaM (100 nM; filled circles) or CaMNN (100 nM; filled squares) and buffer yielding the indicated concentration of free Ca^{2+}. All points are means ± SEM of triplicate determinations.

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

References

Abu-Soud, H.M., Yoho, L.L., Stuehr D.J., 1994. Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. J. Biol. Chem. 269, 32047-32050.

Abu-Soud, H.M., Stuehr, D.J., 1993. Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. Proc. Natl. Acad. Sci. USA. 90, 10769-10772.

Alderton, W.K., Boyhan, A. Lowe, P.N., 1998. Nitroarginine and tetrahydrobiopterin binding to the haem domain of neuronal nitric oxide synthase using a scintillation proximity assay. Biochem. J. 332, 195-201

Babu, Y.S., Bugg, C.E. Cook, W.J., 1988. Structure of calmodulin refined at 2.2 A resolution. J. Mol. Biol. 204, 191-204.

Bredt, D.S., Snyder, S.H., 1990. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. USA. 87, 682-685.

Brown, S.E., Martin, S.R. Bayley, P.M., 1997. Kinetic control of the dissociation pathway of calmodulin-peptide complexes. J. Biol. Chem. 272, 3389-33897

Cho, H.J., Xie, Q.W., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Nathan, C., 1992. Calmodulin is a subunit of nitric oxide synthase from macrophages. J. Exp. Med. 176, 599-604.

Cohen,, P.K. Klee, C.B., 1988. *Calmodulin: Molecular aspects of cellular regulation,* Elsevier, Amsterdam.

Daff, S., Sagami, I., Shimizu, T., 1999. The 42-amino acid insert in the FMN domain of neuronal nitric-oxide synthase exerts control over Ca(2+)/calmodulin-dependent electron transfer. J. Biol. Chem. 274, 30589-30595.

Edlund, M., Blikstad, I., Obrink, B., 1996. Calmodulin binds to specific sequences in the cytoplasmic domain of C-CAM and down-regulates C-CAM self-association. J. Biol. Chem. 271, 1393-1399.

Fabiato, A., Fabiato, F., 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. J Physiol (Paris) 75, 463-505.

Gorren, A.C., List, B.M., Schrammel, A., Pitters, E., Hemmens, B., Werner, E.R., Schmidt, K., Mayer, B., 1996. Tetrahydrobiopterin-free neuronal nitric oxide synthase: evidence for two identical highly anticooperative pteridine binding sites. Biochemistry 35, 16735-16745.

Gilli, R., Lafitte, D., Lopez, C., Kilhoffer, M., Makarov, A., Briand, C. Haiech, J., 1998. Thermodynamic analysis of calcium and magnesium binding to calmodulin. Biochemistry 37, 5450-5456.

Griffith, O.W., Stuehr D.J., 1995. Nitric oxide synthases: properties and catalytic mechanism. Annu. Rev. Physiol. 57, 707-736.

Gross, S.S., 1996. Microtiter plate assay for determining kinetics of nitric oxide synthesis. Methods Enzymol. 268, 159-168.

Hubbard, M.J. Klee, C.B., 1987. Calmodulin binding by calcineurin. Ligand-induced renaturation of protein immobilized on nitrocellulose. J. Biol. Chem. 262, 15062-15070.

Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B., Bax, A., 1992. Solution structure of a calmodulin-target peptide complex by multidimensional NMR. Science 256, 632-63

Johnson, J.D., Snyder, C., Walsh, M., Flynn, M., 1996. Effects of myosin light chain kinase and peptides on Ca^{2+} exchange with the N-and C-terminal Ca2+ binding sites of calmodulin. J. Biol. Chem. 271**,** 761-767.

Krueger, J.K., Bishop, N.A., Blumenthal, D.K., Zhi, G., Beckingham, K., Stull, J.T., Trewhella, J., 1998. Calmodulin binding to myosin light chain kinase begins at substoichiometric Ca^{2+} concentrations: a small-angle scattering study of binding and conformational transitions. Biochemistry 37, 17810-17817.

Linse, S., Helmersson, A., Forsen, S., 1991. Calcium binding to calmodulin and its globular domains. J. Biol. Chem. 266, 8050-8054.

Liu, Q., Gross S.S., 1996. Binding sites of nitric oxide synthases. Methods Enzymol. 268, 311-324.

Manalan, A.S., Klee, C.B., 1984. Calmodulin. Adv. Cyclic Nucleotide Protein Phosphorylation Res..18, 227-278.

McMillan, K., Masters, B.S., 1993. Optical difference spectrophotometry as a probe of rat brain nitric oxide synthase heme-substrate interaction. Biochemistry 32: 9875-9880.

Meador, W.E., Means, A.R., Quiocho, F.A., 1992. Target enzyme recognition by calmodulin: 2.4 A structure of a calmodulin-peptide complex. Science 257, 1251-1255.

Meador, W.E., Means, A.R., Quiocho, FA., 1993. Modulation of calmodulin plasticity in molecular recognition on the basis of x-ray structures. Science 262, 1718-1721.

Nathan, C., Xie Q.W., 1994. Regulation of biosynthesis of nitric oxide. J. Biol. Chem. 269, 13725-13728.

Persechini, A., McMillan, K., Leakey, P., 1994. Activation of myosin light chain kinase and nitric oxide synthase activities by calmodulin fragments. J. Biol. Chem. 269**,** 16148-16154.

Persechini, A., Gansz, K.J., Paresi, R.J., 1996a. Activation of myosin light chain kinase and nitric oxide synthase activities by engineered calmodulins with duplicated or exchanged EF hand pairs. Biochemistry 35, 224-228.

Persechini, A., White, H.D., Gansz, K.J., 1996b. Different mechanisms for Ca2+ dissociation from complexes of calmodulin with nitric oxide synthase or myosin light chain kinase. J. Biol. Chem. 271**,** 62-67.

Presta, A., Liu, J., Sessa, W.C. Stuehr, D.J., 1997. Substrate binding and calmodulin binding to endothelial nitric oxide synthase coregulate its enzymatic activity. Nitric Oxide 1, 74-87.

Rodriguez-Crespo, I., Nishida, C.R., Knudsen, G.M., de Montellano, P.R., 1999. Mutation of the five conserved histidines in the endothelial nitric-oxide synthase hemoprotein domain. No evidence for a non-heme metal requirement for catalysis. J. Biol. Chem. 274, 14692-14698.

Roman, L.J., Sheta, E.A., Martasek, P., Gross, S.S., Liu, Q. Masters, B.S., 1995. High-level expression of functional rat neuronal nitric oxide synthase in Escherichia coli. Proc Natl Acad Sci USA. 92, 8428-8432.

Ruan, J., Xie, Q.W., Hutchinson, N., Cho, H., Wolfe, G.C., Nathan, C., 1996. Inducible nitric oxide synthase requires both the canonical calmodulin-binding domain and additional sequences in order to bind calmodulin and produce nitric oxide in the absence of free Ca^{2+}. J. Biol. Chem. 271, 22679-22686.

Salerno, J.C., Harris, D.E., Irizarry, K., Patel, B., Morales, A.J., Smith, S M., Martasek, P., Roman, L. J., Masters, B.S., Jones, C.L., Weissman, B.A., Lane, P., Liu, Q. Gross, S.S., 1997. An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. J. Biol. Chem. 272, 769-777.

Schmidt, H.H., Pollock, J.S., Nakane, M., Gorsky, L.D., Forstermann, U., Murad, F., 1991. Purification of a soluble isoform of guanylyl cyclase-activating-factor synthase. Proc.Natl.Acad.Sci.USA. 88, 365-369.

Sheta, E.A., McMillan, K., Masters, B.S., 1994. Evidence for a bidomain structure of constitutive cerebellar nitric oxide synthase. J. Biol. Chem. 269, 15147-15153.

Siddhanta, U., Wu, C., Abu-Soud, H.M., Zhang, J., Ghosh, D.K., Stuehr, D.J., 1996. Heme iron reduction and catalysis by a nitric oxide synthase heterodimer containing one reductase and two oxygenase domains. J. Biol. Chem. 271, 7309-7312.

Siddhanta, U., Presta, A., Fan, B., Wolan, D., Rousseau, D.L., Stuehr, D.J., 1998. Domain swapping in inducible nitric-oxide synthase. Electron transfer occurs between flavin and heme groups located on adjacent subunits in the dimer. J. Biol. Chem. 273, 18950-1898.0

Su, Z., Blazing, M.A., Fan, D., George, S.E., 1995. The calmodulin-nitric oxide synthase interaction. Critical role of the calmodulin latch domain in enzyme activation. *J*. Biol. Chem. 270**,** 29117-29122

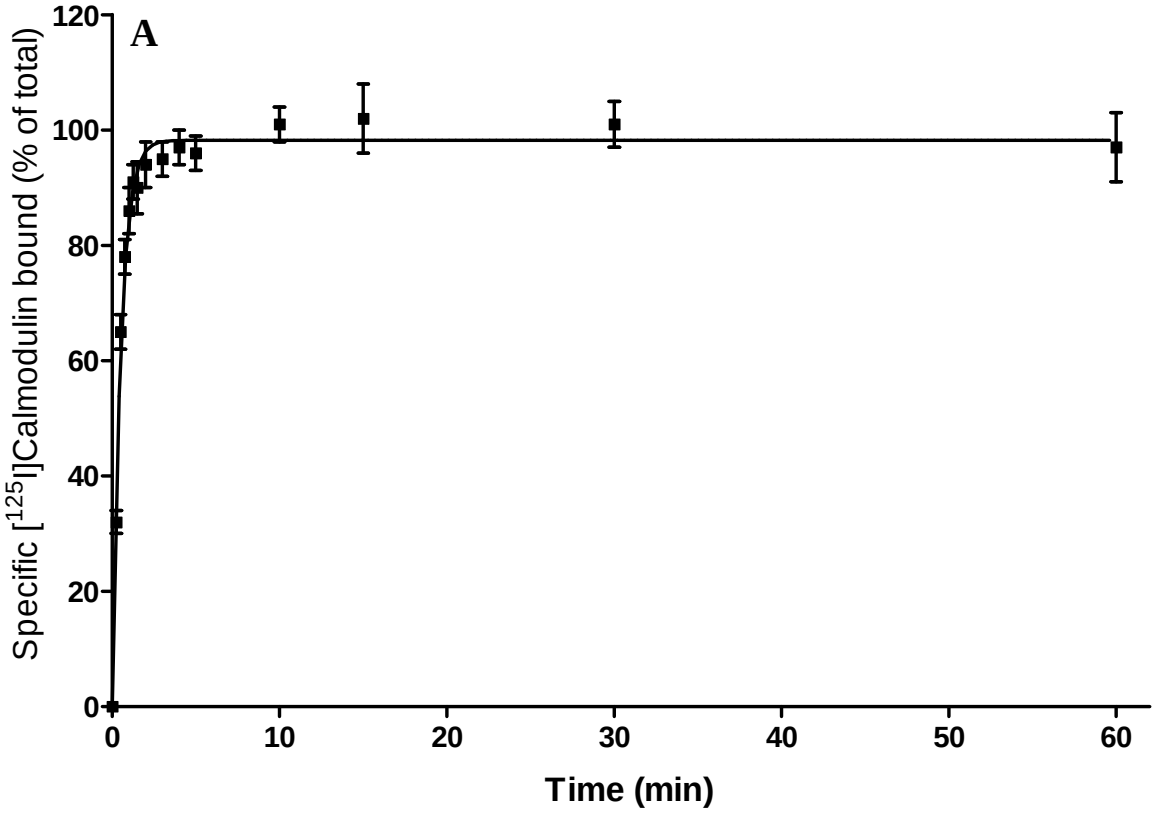
Vorherr, T., Knopfel, L., Hofmann, F., Mollner, S., Pfeuffer, T., Carafoli, E., 1993. The calmodulin binding domain of nitric oxide synthase and adenylyl cyclase. Biochemistry 32, 6081-6088.

Weinstein, H., Mehler, E.L., 1994. Ca(2+)-binding and structural dynamics in the functions of calmodulin. Annu. Rev. Physiol. 56, 213-236.

Zhang, M., Vogel, H.J., 1994. Characterization of the calmodulin-binding domain of rat cerebellar nitric oxide synthase. J. Biol. Chem. 269**,** 981-985

Zhang, M., Yuan, T., Aramini, J.M., Vogel H.J., 1995. Interaction of calmodulin with its binding domain of rat cerebellar nitric oxide synthase. A multinuclear NMR study. J. Biol. Chem. ***270,*** 20901-20907.

Fig. 1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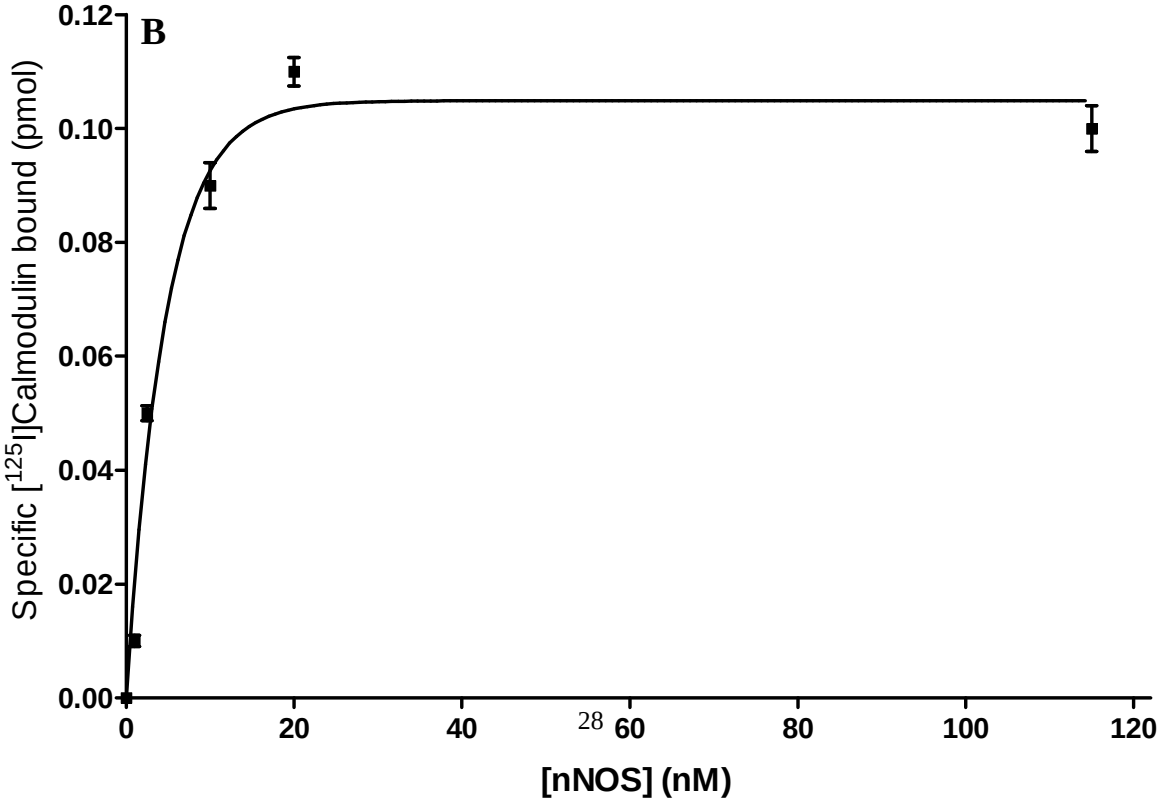
60
40
20
0
0
10
20
30
40
50
60
Time (min)


B
Specific [125I]Calmodulin bound (pmol)
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40
60
80
100
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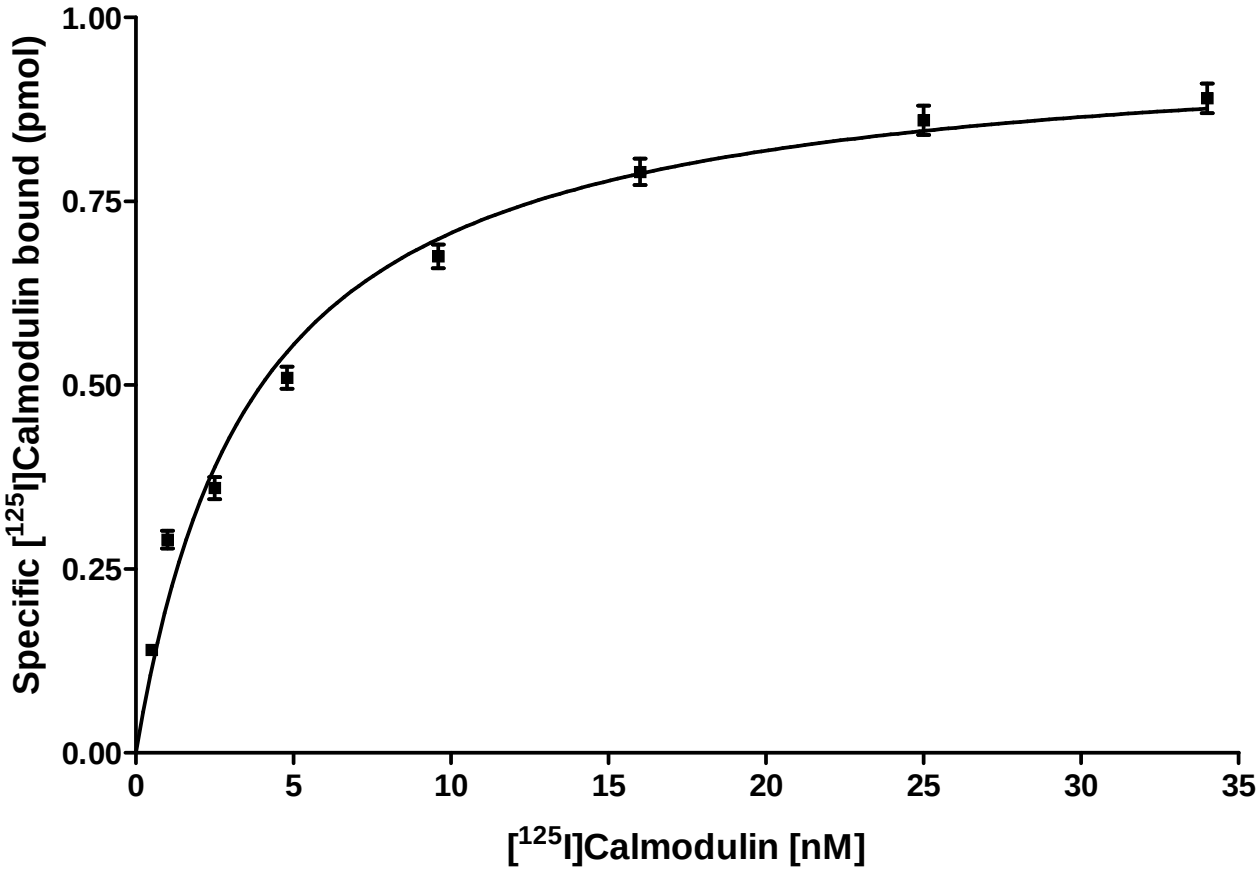
[nNOS] (nM)

Fig. 2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0.25
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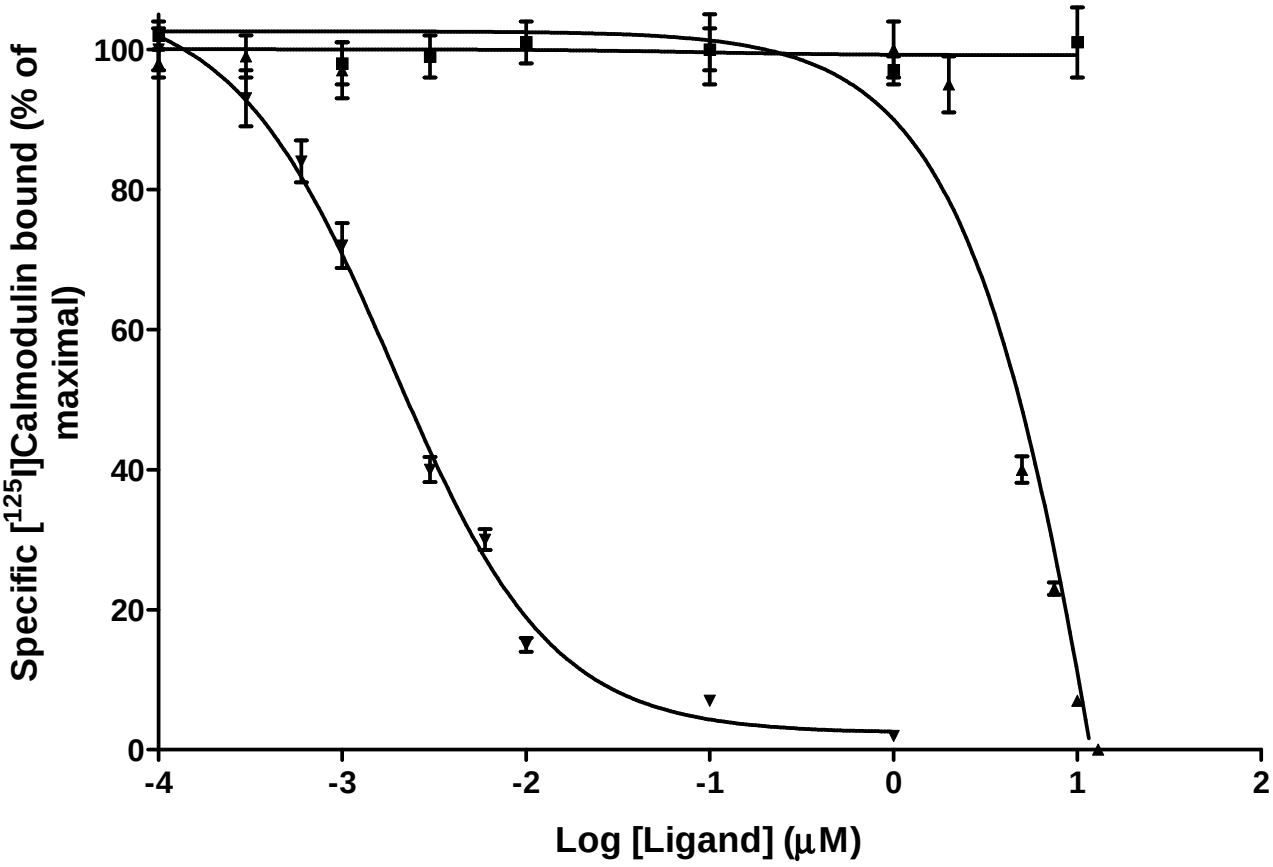
25
30
35
[125I]Calmodulin [nM]

Fig. 3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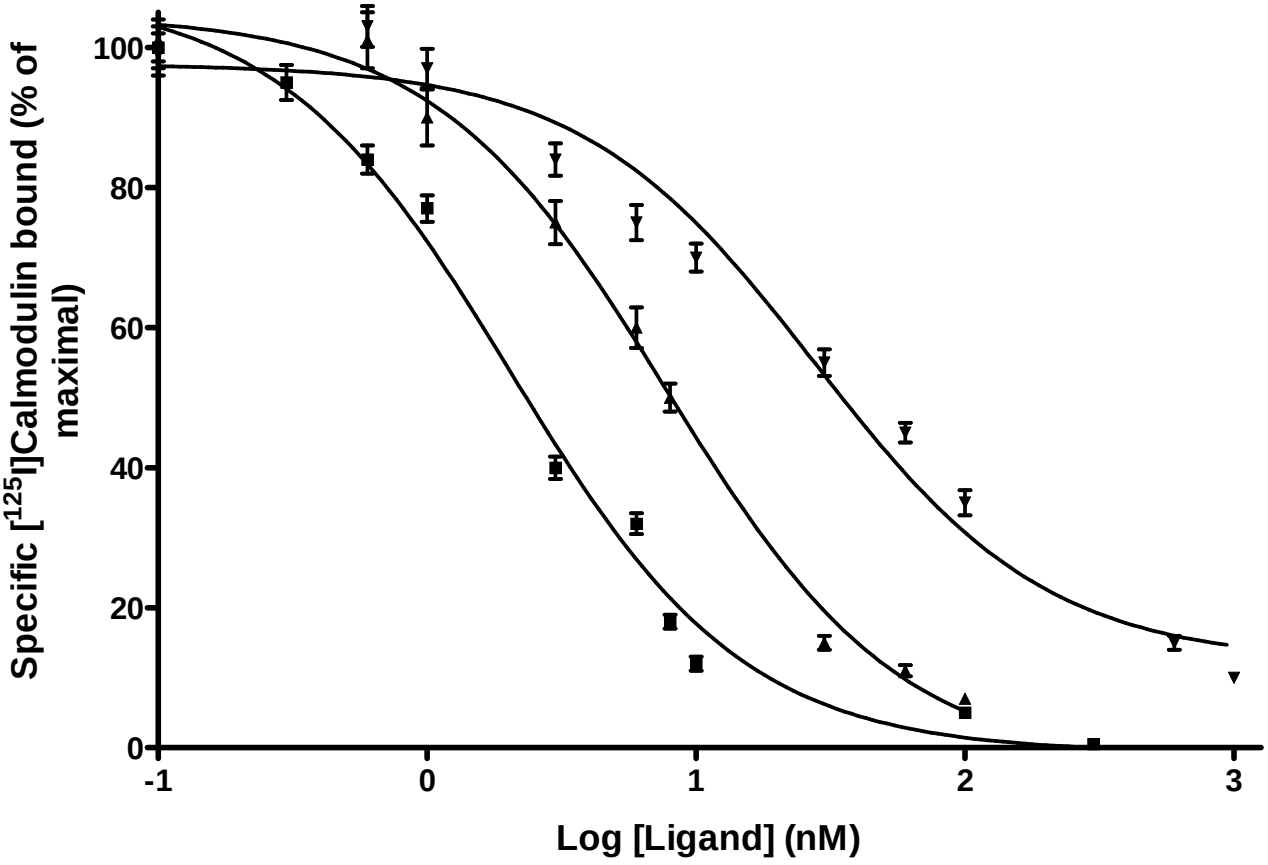
0
1
2
Log [Ligand] (μM)

Fig. 4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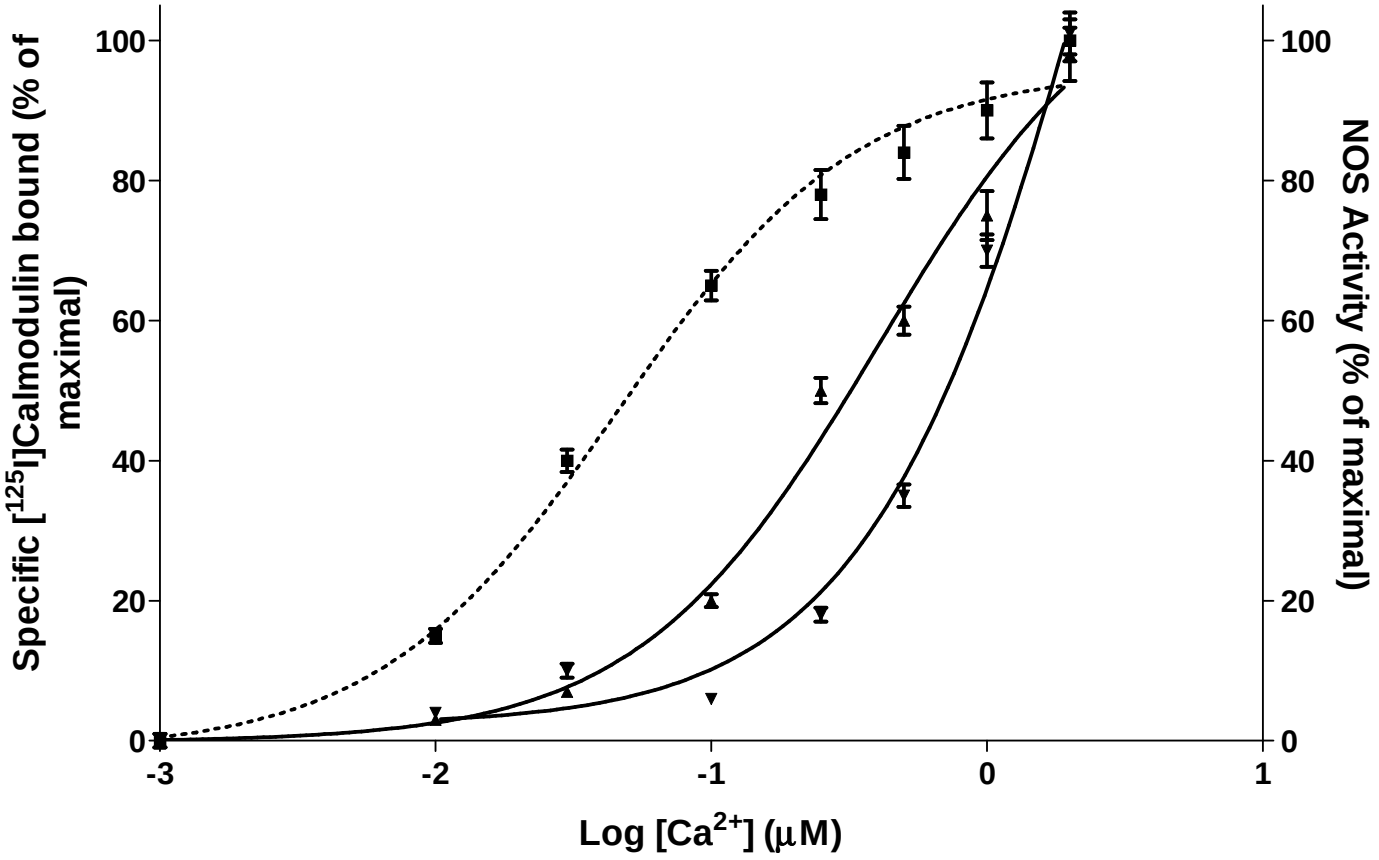
Log [Ligand] (nM)

Fig. 5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NOS Activity (% of maximal)
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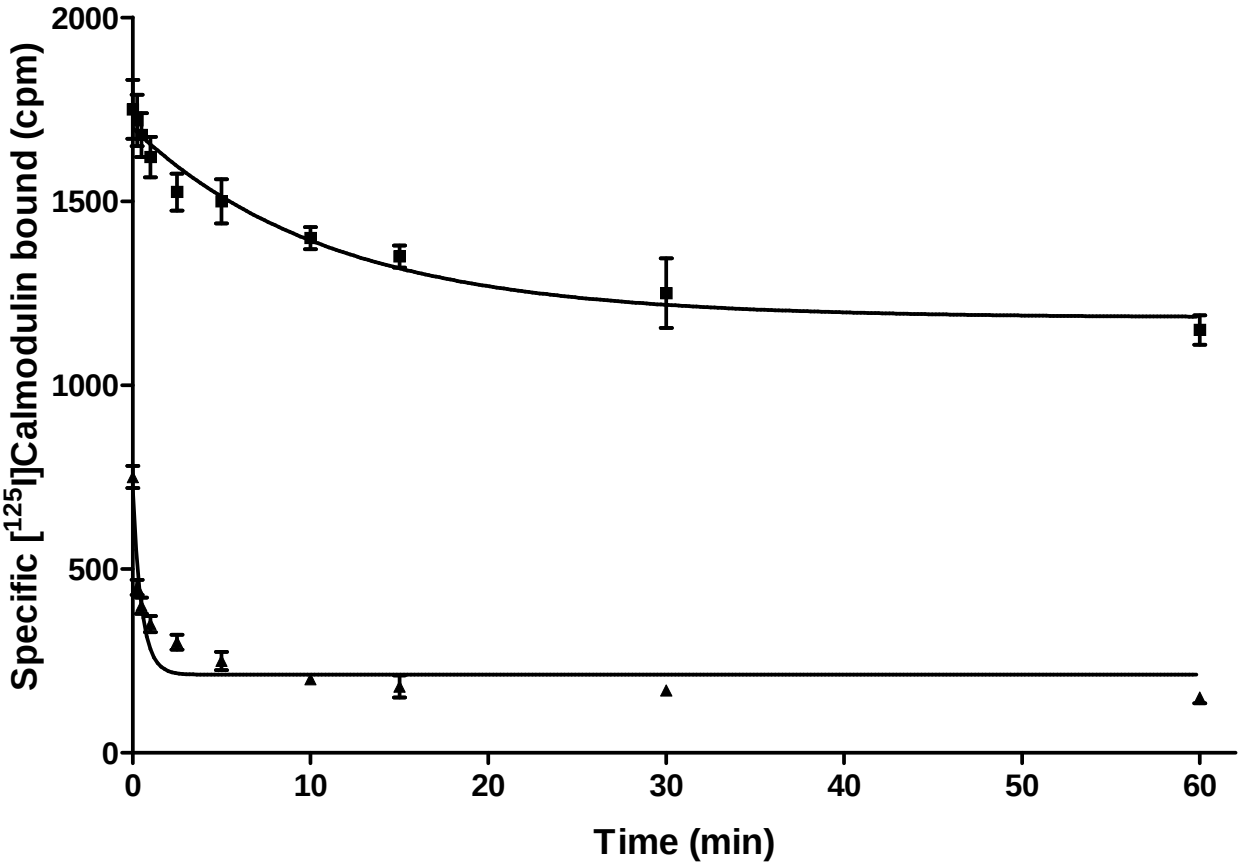
60
40
20
0
-3
-2
-1
0
1

Fig. 6


Specific [125I]Calmodulin bound (cpm)
2000
1500
1000
500
0
0
10
20
30
40
50
60
Time (min)