Calmodulin-dependent Regulation of Inducible and Neuronal Nitric-oxide Synthases

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Neuronal and endothelial nitric-oxide synthases depend upon Ca\(^{2+}\)/calmodulin for activation, whereas the activity of the inducible nitric-oxide synthase is Ca\(^{2+}\)-independent, presumably due to tightly bound calmodulin. To study these different mechanisms, a series of chimeras derived from neuronal and inducible nitric-oxide synthases were analyzed. Chimeras containing only the oxygenase domain, calmodulin-binding region, or reductase domain of inducible nitric-oxide synthase did not confer significant Ca\(^{2+}\)-independent activity. However, each chimera was more sensitive to Ca\(^{2+}\) than the neuronal isofrom. The calmodulin-binding region of inducible nitric-oxide synthase with either its oxygenase or reductase domains resulted in significant, but not total, Ca\(^{2+}\)-independent activity. Co-immunoprecipitation experiments showed no calmodulin associated with the former chimera in the absence of Ca\(^{2+}\). Trifluoperazine also inhibited this chimera in the absence of Ca\(^{2+}\). The combined interactions of calmodulin bound to inducible nitric-oxide synthase calmodulin-binding region with the oxygenase domain may be weaker than with the reductase domain. Thus, Ca\(^{2+}\)-independent activity of inducible nitric-oxide synthase appears to result from the concerted interactions of calmodulin with both the oxygenase and reductase domains in addition to the canonical calmodulin-binding region. The neuronal isofrom is not regulated by a unique autoinhibitory element in its reductase domain.

NO with an unpaired electron reacts with protein targets primarily through their thio or heme groups and acts as a messenger or modulator molecule in many biological systems (1–4). It is produced from L-arginine with L-citrulline as a co-product in a reaction catalyzed by NOS\(^1\) that requires NADPH, FAD, FMN, BH4, calmodulin, and heme (1–4).

Three NOS isoforms were originally identified based on the tissue source: nNOS, eNOS, and iNOS (5). All NOSs contain three domains: an oxygenase domain at the N-terminal half and a reductase domain at the C-terminal half connected by a calmodulin-binding region in the middle of the molecule. All isoforms are highly related with at least 50–60% identity and are classified into two categories based on their dependence on Ca\(^{2+}\) for activity. When Ca\(^{2+}\) binds to calmodulin, the complex binds to nNOS or eNOS and stimulates nitric oxide production. In contrast, when iNOS expression is induced upon stimulation of cells with cytokines or endotoxins, it is fully active, even when Ca\(^{2+}\) levels in cells are low. The Ca\(^{2+}\)-independent activity of iNOS is associated with calmodulin tightly bound to the enzyme (6).

Calmodulin binds proteins through IQ (IQXXRGGXXR) motifs in a Ca\(^{2+}\)-independent manner or through a canonical calmodulin-binding region in a Ca\(^{2+}\)-dependent way (7). However, iNOS does not have a typical IQ motif. Moreover, all of the putative calmodulin-binding regions from nNOS, eNOS, and iNOS satisfy the criteria for properties of a canonical calmodulin-binding region, a basic amphipathic α-helical sequence containing 12 basic and hydrophobic residues. Calmodulin activates nNOS at two points in the electron transfer sequence: electron transfer into the flavins and interdomain electron transfer between the flavins and the hemes (9). The association of calmodulin with nNOS may tether the oxygenase and the reductase domain or some subdomains close together for electron transfer by which calmodulin acts as a molecular switch (10).

The calmodulin-binding sequence in iNOS is necessary but not sufficient for Ca\(^{2+}\)-independent activity (11, 12). Replacement of the calmodulin-binding sequence of eNOS or nNOS with the corresponding sequence from iNOS resulted in a chimera that was still Ca\(^{2+}\)-dependent. It was proposed that, in addition to the canonical calmodulin-binding region, sites in the reductase domain confer Ca\(^{2+}\)-independent binding of calmodulin, which is presumably responsible for the iNOS Ca\(^{2+}\)-independent activity. Alternatively, Salerno et al. (13) proposed an autoinhibitory segment in the PMN-binding domains of nNOS and eNOS, but not iNOS. Hence, calmodulin binding may displace this unique autoinhibitory segment, resulting in catalysis. These two models for Ca\(^{2+}\)/calmodulin regulation of NOS activities were investigated by the characterization of chimeric enzymes made from nNOS and iNOS.

MATERIALS AND METHODS

Construction of Mutant and Chimeric NOSs—nNOS cDNA (a gift from Solomon H. Snyder) was cut with EcoRI and subcloned into the pcMV5 EcoRI site. Mouse iNOS cDNA (a gift from Richard C. Lyons) was cut by HindIII and BgII and subcloned into pCMV5. A XhoI site was introduced into NOS cDNA at amino acids 796–797 by oligonucleotide-directed mutagenesis without changing any amino acid. A XhoI site was also introduced into iNOS cDNA at amino acids 574–575 without changing any amino acid by the two-step polymerase chain reaction method (14) through the ApoI–ApoI 900-base pair fragment of iNOS cDNA. nNOS and iNOS cDNAs were generated by exchanging the XhoI–XhoI fragments of XhoI-nNOS and XhoI-iNOS cDNAs. A HpaI site was introduced into XhoI-nNOS cDNA at amino acids 696–697 or XhoI-iNOS cDNA at amino acids 475–476 to generate nNOS\(^{\text{Leu747}}\) (Leu changed to Val at residue 696) and iNOS\(^{\text{Leu747}}\) (Leu changed to Val at residue 475) for making other chimeric NOSs by the
two-step polymerase chain reaction method. The specific activity and dependence on different [Ca\(^{2+}\)] for activity of nNOS\(_{L696V}\) and iNOS\(_{L475V}\) were the same as their respective wild-type NOSs (see below). The chimeric NOS constructs were made by two-step polymerase chain reaction using nNOS\(_{L696V}\), iNOS\(_{L475V}\), and appropriate NOS chimeras as templates. Buffer containing magnesium and manganese was used to lyse cells at 4 °C. Lysates (10 μg of cDNA by DEAE-dextran and chloroquine as described previously (15)). The cells were shocked with 10% dimethyl sulfoxide in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum before removing chloroquine for a 3-min incubation at room temperature followed by a final wash step. The subsequent transfectants were cultured with Dulbecco's modified Eagle's medium (Cellgro Co.) plus 10% fetal bovine serum, streptomycin, penicillin, and fungizone (Life Technologies, Inc.) in the presence or absence of 1 μM t-NAME (Sigma), 5 μM hemin (Sigma), and 100 μM BH4 (Research Biochemical, Co.) to obtain active enzymes.

**NOS Enzyme Activity Assays**—To measure NOS activity at different Ca\(^{2+}\) concentrations, EGTA/Ca\(^{2+}\)-buffered buffers were prepared from buffers containing 2 mM EGTA, 100 mM Hapes, pH 7.4, with or without 300 μM free Ca\(^{2+}\) (method 1). The final free Ca\(^{2+}\) concentrations were determined by atomic absorptionometry with the fluorescent dyes fluo-3 or Ca\(^{2+}\) Green SN (Molecular Probes, Inc., Eugene, OR). Calibrated Ca\(^{2+}\)-concentrations (Molecular Probes) were used to confirm the free Ca\(^{2+}\) concentrations measured.

Cells in one 100-mm tissue culture dish were harvested at 40–48 h post-transfection in 100 μl of lysis buffer (100 mM Hapes, pH 7.4, 80 μM BH4, 5 mM dithiothreitol, and protease inhibitors: 0.54 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A) after trypsinization and washing cells twice with phosphate-buffered saline. For those transfected cells cultured in the presence of t-NAME, BH4, and hemin, the cells were incubated with regular media for 1 h before trypsinization. Sonication (30% output, 10 s) was used to lyse cells at 4 °C. Lysates (10 μl) were added to 190 μl of EGTA/Ca\(^{2+}\)-buffered solution containing 0.55 μM calmodulin, 600,000 cpm [\(^{3}H\)arginine (60 Ci mmol\(^{-1}\)), 2 μM β-NADPH, 11 μM FAD, 11 μM FMN, 22 μM BH4, and 1.1 μM hemin. Assays were performed at 25 °C in duplicate within a predetermined time for linear reactions for each NOS. The reaction was stopped by adding 0.8 ml of prechilled stop solution (2 mM EDTA, 2 mM EGTA, and 100 mM Hapes, pH 5.5) and then applied to a 1-ml Dowex-50 (Sigma) column and washed with 2 ml of stop solution. Enzyme activities were measured through conversion of [\(^{3}H\)arginine to [\(^{3}H\]citrulline by liquid scintillation spectrometry. Ca\(^{2+}\)-dependent NOS activity was also measured by another method (method 2). Transfected COS-7 cells were harvested as above in 0.3 ml of lysis buffer/100-mm culture dish. Cells were lysed by sonication at 4 °C, and 50 μl of transfected COS-7 lystate was added in duplicate to 50 μl of an assay solution containing 100 mM Hapes pH 7.4, 4 mM \([\beta\text{-NADPH}, 20 \mu M \text{FAD}, 20 \mu M \text{FMN}, 30 \mu M \text{calmodulin, and 140,000 cpm[\(^{3}H\]arginine (60 Ci mmol\(^{-1}\)] with 200 μM CaCl\(_2\), or 5 mM EGTA. After incubation at 37 °C at different times to obtain linear reactions, 0.8 ml of prechilled stop solution (2 mM EDTA, 2 mM EGTA, and 100 mM Hapes, pH 5.5) was added, and the solution was passed through a 0.5-ml Dowex-50 (Sigma) column. The column was washed with 1 ml of stop solution, and the enzyme activities were measured through conversion of [\(^{3}H\]arginine into [\(^{3}H\]citrulline by liquid scintillation spectrometry. Mock transfectants with vector alone (pCMV5) showed the same radioactivity as the blank control (no cell lysate), indicating no significant NOS activities in COS-7 cells.

Activities were also measured in cell lysates in the presence of 100 μM Ca\(^{2+}\) and 800,000 cpm [\(^{3}H\]arginine (60 Ci mmol\(^{-1}\)) in 100 μl of the reaction mixture described above (method 2). The lysates were pre-treated with Dowex-50 resin equilibrated with lysis buffer to remove endogenous arginine. However, this pretreatment had negligible effects on NOS activities. The maximal activities of chimeric enzymes were calculated by Lineaweaver-Burk analysis, assuming an average K\(_{m}\) value of 6 μM for t-arginine from published data (16–25). The amount of protein was estimated with appropriate standards from purified nNOS and iNOS by Western blot analysis using antibodies against peptides derived from amino acids 1422–1435 of human nNOS (differ by only one amino acid from rat nNOS) and from amino acids 1130–1141 of iNOS. Results from these measurements show estimated activities in nmol of citrulline/min/mg of NOS as follows: nNOS, 100; nNOS\(_{L696V}\), 102; iNOS, 40; iNOS\(_{L475V}\), 59; nNOS\(_{L696V}\)-iNOS-L475V; 16; iNOS-N\(_{725,730}\), 15; nNOS-I\(_{776,-1144}\), 41; nNOS-I\(_{776,-1144}\). For catalytic activity, NOSs must be dimeric (21, 26, 27). Heme, BH4, and t-NAME were supplemented to cell growth media for expressing some of the NOS chimeras when needed. Transfectants were tested as described above for maintaining proper structure. More than 95% of iNOS expressed under these conditions was dimeric (data not shown) as analyzed by fast protein liquid chromatography (28, 29).

**Immunoprecipitation Assays**—Lysates were prepared as above from COS-7 cells transfected with wild type or chimeric NOSs with the above transfection and lysis buffer containing Mg\(^{2+}\) and Mn\(^{2+}\) for activity. Surprisingly, it was more sensitive to [Ca\(^{2+}\)] than those required to activate nNOS. Based on results with nNOS-I\(_{776,-1144}\) and iNOS-L\(_{475V}\) containing a conserved point mutation designed to make some of the chimeric NOSs were also catalytically active, and their dependence on different [Ca\(^{2+}\)] were the same as their respective wild-type enzymes (Fig. 2). Thus, the introduced mutations did not affect the respective enzymes’ sensitivity to different [Ca\(^{2+}\)] or maximal activity.

**NOS Activities**—To investigate mechanisms responsible for differences in calmodulin binding and Ca\(^{2+}\)-dependent activity, a series of chimeras and point mutants derived from nNOS and iNOS were constructed and transiently expressed in COS-7 cells (Fig. 1). As described under “Materials and Methods,” all expressed chimeric enzymes were catalytically active with activities ranging from 100 to 30% relative to iNOS. nNOS-L\(_{696V}\) and iNOS-L\(_{475V}\) containing a conserved point mutation designed to make some of the chimeric NOSs were also catalytically active, and their dependence on different [Ca\(^{2+}\)] were the same as their respective wild-type enzymes (Fig. 2). Thus, the introduced mutations did not affect the respective enzymes’ sensitivity to different [Ca\(^{2+}\)] or maximal activity.

**NOS Chimeras with Individual iNOS Oxygenase Domain, Calmodulin-binding Region, or Reductase Domain**—Although the canonical calmodulin-binding sequences of nNOS (amino acids 725–755) and iNOS (amino acids 503–533) are not identical, they display features that are considered characteristic of Ca\(^{2+}\)-dependent, calmodulin-binding regions (8, 30, 31). nNOS-I\(_{776,-1144}\) with the calmodulin-binding region of iNOS and the respective reductase and oxygenase domains of NOSs did show Ca\(^{2+}\)-independent activity like iNOS (Fig. 3). However, this chimera was more sensitive to activation at lower [Ca\(^{2+}\)] than those required to activate nNOS. Based on results with nNOS-I\(_{776,-1144}\) and iNOS-L\(_{475V}\) containing a conserved point mutation designed to make some of the chimeric NOSs were also catalytically active, and their dependence on different [Ca\(^{2+}\)] were the same as their respective wild-type enzymes (Fig. 2). Thus, the introduced mutations did not affect the respective enzymes’ sensitivity to different [Ca\(^{2+}\)] or maximal activity.
of the iNOS reductase domain, nNOS-I749–1144, also showed greater sensitivity to [Ca\(^{2+}\)] than nNOS (Fig. 3). These results indicate that neither the iNOS oxygenase domain, the iNOS calmodulin-binding region, nor the iNOS reductase domain alone confer significant Ca\(^{2+}\)-independent activity similar to that of iNOS. However, all of these substitutions affect enzyme activity by increasing sensitivity to [Ca\(^{2+}\)] relative to nNOS.

NOS Chimeras with iNOS Calmodulin-Binding Region and either iNOS Oxygenase or iNOS Reductase Domains—Chimeric NOSs with combinations of iNOS oxygenase or iNOS reductase domains with the iNOS calmodulin-binding sequence were constructed to investigate the influences of these domains on Ca\(^{2+}\) regulation. Results in Fig. 4 show that the iNOS calmodulin-binding region with either the iNOS oxygenase domain or the iNOS reductase domain (nNOS-I1–533 and nNOS-I504–1144) significantly increased Ca\(^{2+}\)-independent NOS activity compared with the chimeras containing only one of the domains (Fig. 3). Furthermore, there was also a similar increase in sensitivity to activation at different [Ca\(^{2+}\)] with both nNOS-I504–1144 and nNOS-I1–533 (Fig. 4). Thus, the concerted actions of iNOS calmodulin-binding region with either the iNOS oxygenase or the reductase domains were similar in regard to Ca\(^{2+}\)-dependent activities.

The Flanking Regions of iNOS Canonical Calmodulin-Binding Region—In the case of the phosphorylase kinase \(\gamma\)-subunit, calmodulin binds to two noncontiguous regions spanning 70 residues (one forms an \(\alpha\)-helix with a canonical calmodulin-binding sequence, and the other forms a \(\beta\)-hairpin structure) located at the C-terminal end of the \(\gamma\)-subunit, resulting in tight association of calmodulin as an integral \(\delta\)-subunit (32,
33). Secondary structure predictions on the iNOS and nNOS calmodulin-binding region and flanking regions (amino acids 475–575 and 696–796, respectively) suggest a structure similar to those in phosphorylase kinase γ-subunit for iNOS but not nNOS (data not shown).

To investigate if the flanking regions of the iNOS canonical calmodulin-binding sequence contribute to iNOS Ca$^{2+}$-independent activity, nNOS-I$^{1-575}$-533 was expressed. Unfortunately, it was catalytically inactive (data not shown). Alternatively, a series of chimeric NOSs with swapping points between the canonical calmodulin-binding region and its flanking regions were expressed in COS-7 cells (Fig. 5). Replacement of the reductase domain of iNOS alone with that of nNOS (iNOS to nNOS-I$^{1-475}$) did not significantly change the enzymatic response to different Ca$^{2+}$ concentrations of components in the reaction buffers (method 1). Data are means ± S.D. for at least three experiments. Error bars smaller than symbols are not shown.

The iNOS canonical calmodulin-binding region from nNOS-I$^{1-533}$ was replaced with that of nNOS (nNOS-I$^{1-533}$ to nNOS-I$^{1-503}$), the enzyme activity became completely Ca$^{2+}$-dependent and was similar to that of nNOS. Replacement of the N-terminal flanking region of the canonical calmodulin-binding region of iNOS from nNOS-I$^{1-503}$ with that of nNOS (nNOS-I$^{1-503}$ to nNOS-I$^{1-477}$) did not significantly change the Ca$^{2+}$ sensitivity of the NOS activity. Thus, the immediate N- and C-terminal flanking regions of the iNOS canonical calmodulin-binding sequence appear not to be involved in Ca$^{2+}$-regulation of enzyme activity.

Concerted Domain Actions for Ca$^{2+}$-independent Activity in iNOS—To confirm that the concerted actions of iNOS calmodulin-binding region with the iNOS oxygenase or reductase domains enhance Ca$^{2+}$-independent activity, the Ca$^{2+}$-dependent activities of the chimeric NOSs were assayed with different concentrations of components in the reaction buffers (method 2; see “Materials and Methods”). Ca$^{2+}$-independent activity...
Each factor was examined individually or in combination with iNOS-N725–755, nNOS-I1–533, nNOS-I503–533, nNOS-I504–1144, nNOS-I576–1144, and nNOS-I749–1144 were investigated. Lysates of transfected COS-7 cells were immunoprecipitated with anti-nNOS or anti-iNOS antibodies in the presence of 100 μM CaCl₂ or 2.5 mM EGTA, in the presence or absence of 1 mM TFP. Co-immunoprecipitation results showed that calmodulin dissociates in the absence of Ca²⁺ from nNOS and nNOS-I533, containing the iNOS calmodulin-binding region and oxygenase domain (Fig. 8). The dissociation of calmodulin from nNOS-I533 in the presence of EGTA was surprising, since this chimera had significant enzymatic activity under these conditions (Figs. 4 and 6). In the presence of Ca²⁺, TFP did not prevent calmodulin binding to nNOS or nNOS-I533 (Fig. 8), although it inhibited nNOS activity but not nNOS-I533 activity. In contrast, TFP had no effect in the presence or absence of Ca²⁺ on the association of calmodulin with iNOS and nNOS-I504–1144. Thus, the Ca²⁺-independent activity derived from iNOS calmodulin-binding region and its reductase domain (nNOS-I504–1144) was associated with calmodulin binding. In contrast, the Ca²⁺-independent activity derived from iNOS calmodulin-binding region and iNOS oxygenase domain (nNOS-I533) was not associated with high affinity calmodulin binding.

**DISCUSSION**

Synthetic peptides that bind calmodulin provide important structural information about molecular determinants for specific interactions (31, 34–37). However, multiple regions of a protein may interact with calmodulin for effective regulation, and calmodulin binding per se is necessary but not sufficient for activation of many enzymes (38, 39). Moreover, unique calmodulin-binding sequences with atypical structural properties may be overlooked (40, 41). Previous results obtained on chimeras with the respective calmodulin-binding sequences from iNOS substituted in nNOS or nNOS showed the sequence was necessary but not sufficient for Ca²⁺-independent activity or calmodulin binding (11, 12). Similar results are presented herein with chimera nNOS-I503–533 activity, which was Ca²⁺-dependent. However, the sensitivity of nNOS-I503–533 activity to [Ca²⁺] was greater than that obtained with nNOS. When the calmodulin-binding sequence of nNOS was substituted into iNOS, the chimeric iNOS-N725–755 activity was still Ca²⁺-dependent, but its sensitivity to activation by [Ca²⁺] was also greater than nNOS. These results support the concept that there are sites in addition to the canonical calmodulin-binding sequence that contribute to Ca²⁺ regulation of NOS activity.

As expected, substitution of the iNOS oxygenase or reductase domains alone into nNOS did not result in Ca²⁺-independent NOS activity, consistent with the view that the iNOS calmodulin-binding region is necessary, but not sufficient, for Ca²⁺-independent NOS activity (11, 12). However, substitution of either iNOS oxygenase or reductase domains increases the Ca²⁺ sensitivity of the respective chimeric NOS activities with a greater effect observed with the reductase domain (nNOS-I504–1144). Even the nNOS-I504–1144 chimera, containing only the C-terminal half of the iNOS reductase domain, was more sensitive to [Ca²⁺] for activity than nNOS. The functional interaction between iNOS calmodulin-binding sequence and its reductase domain in terms of Ca²⁺ regulation was also recently reported (42). However, the functional interaction with the
CO-immunoprecipitation of calmodulin with different NOS enzymes was performed. The Ca$^{2+}$-dependent and Ca$^{2+}$-independent activities of iNOS, nNOS-I$_{1-533}$, and nNOS-I$_{504-1144}$ were measured in the presence of 2 mM EGTA and 1 mM TFP, respectively. L-NAME (200 mM) completely abolished nNOS and iNOS activities in the presence of 1 mM TFP either in the presence or absence of Ca$^{2+}$. The Ca$^{2+}$-dependent and Ca$^{2+}$-independent activities of iNOS, nNOS-I$_{1-533}$, and nNOS-I$_{504-1144}$ were both normalized to 100% in the absence of TFP, respectively. L-NAME (200 mM) completely abolished nNOS, iNOS, nNOS-I$_{1-533}$, and nNOS-I$_{504-1144}$ activities in the presence of 1 mM TFP either in the presence or absence of Ca$^{2+}$. Data shown are means ± S.D. of at least three experiments. Error bars smaller than symbols are not shown.

It was recently proposed that an autoinhibitory sequence exists in the reductase domain of nNOS and eNOS (13). These Ca$^{2+}$-dependent NOSs contain unique sequences in their respective FMN-binding domains not shared by iNOS (residues 820–880 in nNOS). Synthetic peptides derived from this insert in eNOS inhibited calmodulin binding and Ca$^{2+}$ activation by binding to either eNOS or nNOS. However, the insert from nNOS lacks a RRKRK motif thought to be important for autoinhibition in eNOS (13). In contrast to results obtained with the synthetic peptides from eNOS, synthetic peptides derived from the putative autoinhibitory region of nNOS provided only modest inhibition of calmodulin binding to nNOS and no significant inhibition of nNOS activity. Our results are also not consistent with a proposed autoinhibitory function for this insert in nNOS. For example, chimera nNOS-I$_{1-1144}$ contains the reductase domain of iNOS, but its activity is inhibited in the absence of Ca$^{2+}$. Also, chimeras containing the nNOS reductase domain with iNOS canonical calmodulin-binding region and oxygenase domain (nNOS-I$_{1-533}$ and nNOS-I$_{504-1144}$) showed significant Ca$^{2+}$-independent activities (50–73%). Niibida and Ortiz de Montellano (42) recently reported that substitution of the reductase domain of nNOS into iNOS resulted in the retention of significant Ca$^{2+}$-independent activity. The evidence that the insert in the FMN domain may function as an autoinhibitory element for eNOS is more compelling (13) and may point to a structural and functional distinction between nNOS and eNOS. Differences in the Ca$^{2+}$ regulation of a class of structurally related, calmodulin-dependent enzymes (skeletal and smooth muscle myosin light chain kinases) have been previously noted (43, 44).

Ca$^{2+}$-dependent activation of nNOS was associated with calmodulin binding, a finding similar to many previously published results. TFP inhibits the activities of other Ca$^{2+}$/calmodulin-regulated enzymes, e.g., myosin light chain kinase, phosphodiesterase, calcineurin, etc. (45–48). Previous reports showed that TFP abolishes nNOS but not iNOS activity, which is confirmed herein (49–51). However, inhibition of nNOS activity by TFP in the presence of Ca$^{2+}$ is not associated with dissociation of calmodulin. The inactivation of nNOS by TFP may be similar to the inactivation of another calmodulin-dependent enzyme, myosin light chain kinase. TFP does not cause dissociation of calmodulin from the kinase, and inhibi-

**Fig. 7.** Inhibition of NOS activities with trifluoperazine and L-NAME. NOS activities in lysates of COS-7 cells transfected with nNOS, iNOS, nNOS-I$_{1-533}$, and nNOS-I$_{504-1144}$ were measured in the presence of 300 μM free Ca$^{2+}$ or 2 mM EGTA by method 1 at the indicated concentrations of TFP and L-NAME. A, nNOS activity was sensitive to inhibition by TFP. B, iNOS activity was resistant to inhibition by TFP either in the presence or absence of Ca$^{2+}$ relative to nNOS. C, activity of nNOS-I$_{1-533}$ was resistant to inhibition by TFP in the presence but not in the absence of Ca$^{2+}$. D, activity of nNOS-I$_{504-1144}$ was resistant to inhibition by TFP in the presence or absence of Ca$^{2+}$. It was recently proposed that an autoinhibitory sequence exists in the reductase domain of nNOS and eNOS (13). These Ca$^{2+}$-dependent NOSs contain unique sequences in their respective FMN-binding domains not shared by iNOS (residues 820–880 in nNOS). Synthetic peptides derived from this insert in eNOS inhibited calmodulin binding and Ca$^{2+}$ activation by binding to either eNOS or nNOS. However, the insert from nNOS lacks a RRKRK motif thought to be important for autoinhibition in eNOS (13). In contrast to results obtained with the synthetic peptides from eNOS, synthetic peptides derived from the putative autoinhibitory region of nNOS provided only modest inhibition of calmodulin binding to nNOS and no significant inhibition of nNOS activity. Our results are also not consistent with a proposed autoinhibitory function for this insert in nNOS. For example, chimera nNOS-I$_{1-1144}$ contains the reductase domain of iNOS, but its activity is inhibited in the absence of Ca$^{2+}$. Also, chimeras containing the nNOS reductase domain with iNOS canonical calmodulin-binding region and oxygenase domain (nNOS-I$_{1-533}$ and nNOS-I$_{504-1144}$) showed significant Ca$^{2+}$-independent activities (50–73%). Niibida and Ortiz de Montellano (42) recently reported that substitution of the reductase domain of nNOS into iNOS resulted in the retention of significant Ca$^{2+}$-independent activity. The evidence that the insert in the FMN domain may function as an autoinhibitory element for eNOS is more compelling (13) and may point to a structural and functional distinction between nNOS and eNOS. Differences in the Ca$^{2+}$ regulation of a class of structurally related, calmodulin-dependent enzymes (skeletal and smooth muscle myosin light chain kinases) have been previously noted (43, 44).

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The presence of Ca$^{2+}$ is required for activation of iNOS. However, full Ca$^{2+}$-independent activity of iNOS requires additional binding to the canonical calmodulin-binding region as well as both the reductase and oxygenase domains. Additional investigations involving biophysical and structural studies are necessary to verify this model.

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