The results suggest that CaMKII significantly inhibited iNOS-specific activity following cytokine induction. In chronic and acute CaMKII modulators, there was increased nuclear iNOS localization in resting cells but inhibited ionophore-stimulated iNOS trafficking in cultured rat aortic vascular smooth muscle cells (VSMCs). Immunofluorescence and confocal microscopy demonstrated colocalization of iNOS and CaMKIIβ2 in perinuclear distribution and concentration in aggresome-like structures identified by colocalization with γ-tubulin. Furthermore, CaMKIIβ2 communoprecipitated with iNOS in a CaMKII activity-dependent manner. Addition of Ca2+-mobilizing stimuli expected to activate CaMKII: a purinergic agonist (UTP) or calcium ionophore (ionomycin) caused a general redistribution of iNOS from cytosolic to membrane and nuclear fractions. Similarly, adenal expression of a constitutively active CaMKIIβ2 mutant altered iNOS localization, shifting iNOS from the cytosolic fraction. Suppression of CaMKIIβ2 using an adenovirus expressing a short hairpin, small interfering RNA in VSMCs inhibited proliferation (8), and the production of iNOS in VSM has the same effect.

Recent discoveries of posttranslational modifications on iNOS raise the possibility of complex iNOS regulation separate from transcriptional regulation (13, 19, 23, 24, 28). iNOS is primarily found in the cytosolic fraction of cells, has been associated with Golgi membranes (23), and has also been identified in the nucleus of brown adipocytes under β-adrenergic stimulation (13) and in the nuclei of rat neutrophils under quiescent conditions (28). Kolodziejska et al. (19) provided evidence for iNOS trafficking dependent on microtubules and colocalization with the microtubule organizing complex in perinuclear aggresome-like structures. Aggresomal localization of protein is usually described as a mechanism for storing excess or misfolded protein ultimately destined for degradation (7), although in the case of iNOS, evidence suggests that this compartment functions as a reservoir for latent iNOS (19). Palmitoylation of Cys3 in iNOS (28) and tyrosine phosphorylation (24) of iNOS are among the posttranslational mechanisms that affect iNOS localization. Mutations to the Cys3 palmitoylation site have been shown to cause a mislocalization of iNOS protein in C57BL/6 mice and obliteration of iNOS activity (28). Although it is not fully clear how iNOS trafficking is regulated, it appears that iNOS activity is dependent on proper trafficking and subcellular localization (5, 13, 19, 24, 28, 38). iNOS localization and trafficking have not been examined in VSM, where it has important physiological and pathophysiological functions.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is an ubiquitous, structurally complex multifunctional protein kinase (CaMKII) on inducible nitric oxide synthase (iNOS). CaMKII isoform expression and function have been studied to some extent in differentiated VSM, where both γ- and δ-isofoms have been detected (21, 25, 40) and γ-isoforms linked to contractile regulation (27, 34). In undifferentiated cultured rat aortic VSMCs, the primary isoform has been shown to be the δ-isoform, which has been linked to signaling pathways controlling cell migration (25, 26, 40) and proliferation (11). Limited studies have localized CaMKIIβ2 isoform to intracellular membranes colocalizing with endoplasmic reticulum (ER) markers in VSMCs (4) and with Golgi membranes in astrocytes (32). Furthermore, activation studies in cultured VSM indicate a close functional cou-

Address for reprint requests and other correspondence: H. A. Singer, Center for Cardiovascular Sciences, Albany Medical College, 43 New Scotland Ave., Albany, NY 12208 (e-mail: singerh@mail.amc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
pling of activated CaMKII δ2 with Ca\(^{2+}\) released from intracellular Ca\(^{2+}\) stores (12, 26). Ionomycin-induced activation of CaMKII in cultured VSMCs results in redistribution of both CaMKII δ2 and ER markers, consistent with an ER stress response. CaMKII function on intracellular membranes is not known.

Interactions between endothelial NOS (eNOS) or neuronal NOS (nNOS) proteins with CaMKII have been demonstrated in other cell systems. eNOS is palmitoylated, proving vital for the proper targeting of the enzyme in caveolar domains at the plasma membrane (30), and eNOS activity may be regulated by CaMKII in these domains. nNOS is also palmitoylated and interacts with scaffolding proteins such as PSD-95 necessary for nNOS trafficking to postsynaptic densities (3). nNOS interaction with PSD-95 and enzymatic activity may be regulated by CaMKII (35). To our knowledge, no one has investigated potential regulation of iNOS trafficking and/or activity by CaMKII. In the present study we reexamined the distribution of CaMKII δ2 in VSMCs and compared it with iNOS distribution, induced in response to cytokine stimulation. The results indicate a role for CaMKII in the regulation of iNOS trafficking and activity in VSM.

EXPERIMENTAL PROCEDURES

Antibodies and materials. Production and characterization of the antibody specific for CaMKII δ2 have been described previously (29). Monoclonal and polyclonal antibodies to iNOS and nucleolin were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-mouse and anti-rabbit secondary horseradish peroxidase-conjugated antibodies and ECL reagent were purchased from Amersham Biosciences (Little, UK); KN-93, KN-92, and ionomycin were purchased from Calbiochem (La Jolla, CA). Protein A beads were purchased from Pierce (Richmond, CA). All cell culture media and supplies were purchased from Fisher Scientific (Pittsburgh, PA). All other materials were purchased from Sigma.

Cell culture. VSMCs were enzymatically dispersed from thoracic aortas of male Sprague-Dawley rats (200–300 g) as previously described (30). Cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. Cell confluence of between 50% and 80% was used in cell culture in passages 3–9. Before experimentation, cells were grown arrested by replacing growth media (9) with DMEM/F-12 medium supplemented with 0.4% FBS for 16 h at 37°C and 5% CO₂. Use of experimental animals as a source of cells was reviewed and approved by the Albany Medical College Institutional Animal Care and Use Committee.

Induction of iNOS in VSMCs. Induction of iNOS was performed as previously described (31). Following growth arrest, fresh 0.4% FBS or 10% FBS media were added as indicated, with the following cytokine cocktail for 24 h: IL-1β (final concentration of 20 ng/ml), IFN-γ (final concentration 200 U/ml), and TNF-α (final concentration of 20 ng/ml). After this induction period, the cells were washed with 0.4% FBS growth media and used for analysis or treated with various inhibitors/activators as described in each experiment.

Cell lysates, immunoprecipitation, and immunoblotting. The CaMKII activator ionomycin was added at a concentration of 0.5 μM and incubated for 3 min. The CaMKII inhibitor KN-93 was added at a concentration of 30 μM and left on for the duration of the experiment. Cells were lysed on ice with NP-40 lysis buffer (4°C) 50 mM Tris, pH 7.4, 50 mM NaF, 0.1 mM NaVO₄, 0.5% NP-40, 0.1 mM phenylmethylsulfonyl fluoride, and 0.2 U/ml aprotinin) at 0.5 ml per well in a six-well plate. Immunoprecipitations consisted of a 1-h incubation with antibody and then a 1-h incubation with 40 μl of protein A beads.

Immunoprecipitates were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose. The membranes were blocked in 5% nonfat dry milk with Tris-buffered saline containing 0.2% Tween 20 (TBST). After the blocking was completed, the designated antibody was added to the mixture and incubated at room temperature for 1 h or 4°C overnight while being shaken at 150 rpm. The membranes were then washed three times with TBST for 10 min each and blocked again. After this, the secondary horseradish peroxidase-conjugated antibody was added and incubated for 1 h at room temperature, membrane washed three times with TBST, and developed using ECL reagent. ML-B film (Medlink Imaging; Elmsford, NY) was exposed to the membrane and developed in a SRX-101A film processor (Konica Minolta; Wayne, NJ).

Adenoviral infection and constructs. The CaMKII δ2 small interfering RNA (siRNA), designated here as deltakd (delta knockdown) was previously described (20). Adenovirus was added to confluent cells before serum starvation at a multiplicity of infection (MOI) of 150. This virus was incubated on the cells for 90–96 h. The adenovirus carrying the constitutively active CaMKII δ2 mutant construct (T287D), also previously described (19), was added at an MOI of 50 and incubated for 24–48 h. The cells were then washed, serum starved overnight, and induced for iNOS expression. As a negative control, an adenovirus encoding a green fluorescent protein-specific siRNA was added to confluent cells before serum starvation at an MOI of 50. This virus was incubated for the same times as the experimental virus it was compared against and induced for iNOS expression in the same fashion.

Activity assays. Nitrite readings were taken by gas phase chemiluminescence on a nitric oxide analyzer (NOA 280; Sievers Instruments, Boulder, CO) using peak integration, and nitrite concentrations were determined using nitrate as a standard.

Immunofluorescence. Confluent VSMCs on coverslips induced to express iNOS were washed after 20–24 h, and any activators or inhibitors were added with fresh media at that time. The cells were then washed with 1× PBS and fixed with 4% paraformaldehyde for 30–40 min at room temperature, followed by three 5-min washes with 1× PBS. The cells were then permeabilized with 0.2% Triton X-100 for 5 min at room temperature and washed three times for 5 min each with 1× PBS containing 1% BSA. Cells were then blocked with 0.5% fish gelatin for 30 min at room temperature, washed three times with 1× PBS-Triton, and then labeled with 5 μg of primary antibody for 2 h at room temperature. Cells were then washed three times with 1× PBS-Triton and labeled with secondary antibody for 2 h at room temperature. The cells were then washed three times at room temperature, and coverslips were mounted on slides, viewed, and photographed.

Cell fractionation. Cells were lysed by douncing with a pestle in a hypotonic buffer consisting of 250 mM sucrose, 10 mM MOPS, 2.5 mM EGTA, 2 mM EDTA, 0.2 mM PMSF, and 0.85 μg/ml aprotinin. The lysates were then centrifuged at 2,000 rpm for 10 min. The cell debris pellet at the bottom of the tube was discarded, and the spin was repeated. The resulting soft pellet (side of tube) was separated from the rest of the postnuclear supernatant that was carefully pipetted into 1.5-ml polycarbonate tubes. The soft pellet was washed and centrifuged twice at 10,000 rpm for 10,000 rpm. At this point, the soft pellet containing nuclei was resuspended in 200 μl of the hypotonic buffer, and nuclei were lysed by pipetting with a 25-gauge needle. The resulting nuclear-enriched lysate was then centrifuged at 10,000 rpm, and the pellet was discarded. The nuclear lysate was frozen at −20°C until use. The polycarbonate tubes containing the postnuclear lysate were centrifuged at 70,000 rpm for 40 min at 4°C. The supernatant (cytosolic fraction) was removed and stored at −20°C until use, whereas the pellet was washed in 200 μl of NP-40 cell lysis buffer containing 0.5% SDS and centrifuged again at 70,000 rpm. The wash buffer was discarded, and the pellet (washed membrane fraction) was resuspended in 200 μl of hypotonic buffer (with 1 mM DTT added), homogenized through a 25-gauge needle, and frozen at −20°C until use.
use. Fraction purity was determined by Western blotting for fraction-specific protein nucleolin (nuclear fraction), Golgi marker gm130 (membrane fraction), and β-actin (cytosolic fraction).

RESULTS

CaMKII β2 and iNOS colocalize and coimmunoprecipitate in VSMCs. iNOS was induced for 24 h in growth-arrested cultured rat aortic VSMCs with a cytokine mixture containing IL-1β (20 ng/ml), IFN-γ (200 U/ml), and TNF-α (20 ng/ml). Figure 1 demonstrates the typical distribution of iNOS and CaMKII β2 in VSMCs using immunofluorescence and confocal microscopy. iNOS, which was undetectable in control cells (Fig. 1C), was found to be diffusely distributed throughout the cytokine-induced cells (Fig. 1, A and B), excluding the nucleus, with a clear concentration in perinuclear regions and in structures reminiscent of Golgi-adjacent, aggresome-like structures as defined in previous studies using C2C12 (23), RT-4, human embryonic kidney 293, A-172, A-549, and RAW-264.7 cells (19). Cells stained for CaMKII β2 indicated a similar distribution, although the aggresome-like distribution was not as apparent (Fig. 1, A and B). Overlays of iNOS and CaMKII β2 immunofluorescence reproducibly indicated a strong degree of colocalization, particularly in the perinuclear areas.

Colocalization of iNOS and CaMKII β2, suggested by the immunofluorescence approach, was confirmed by immunoprecipitation. CaMKII β2 was immunoprecipitated from VSMCs that had been induced with cytokines, and the resolved precipitates were probed for iNOS (Fig. 2A). After induction, iNOS was communoprecipitated with CaMKII β2. A reverse immunoprecipitation using the iNOS antibody consistently pulled down a fraction of CaMKII β2 (Fig. 2C).

iNOS interaction with CaMKII β2 is regulated by CaMKII activity. The effect of CaMKII activity on the degree of association was tested. Acute activation of CaMKII by addition of the Ca^{2+} ionophore ionomycin to the cells for 3 min significantly decreased the amount of coimmunoprecipitating iNOS (Fig. 2, A and B). Pretreatment of the cells with the CaMKII inhibitor KN-93 for 30 min, but not its inactive analog KN-92, prevented the acute ionomycin-induced decrease in

Fig. 1. Inducible nitric oxide synthase (iNOS) and CaMKII β2 colocalize in perinuclear regions. A: z sections of confocal images. B and C: immunofluorescence (IF) images. Vascular smooth muscle (VSM) cells (VSMCs) in A and B were induced with a cytokine cocktail for 24 h in media with no growth serum, fixed, and then stained with anti-iNOS (green) and anti-CaMKII β2 (red). VSMCs in C were fixed and stained without cytokine stimulation. IF staining controls with only primary or secondary antibodies were negative (not shown).
coimmunoprecipitating iNOS. The conditions of ionomycin treatment used here have been previously shown to activate CaMKII in these cells using either activity assays (1) or Western blotting with an antibody specific to the threonine 287 autophosphorylated form of the kinase (20). Similarly, the concentrations of KN-93 used in this study have been previously shown to maximally inhibit CaMKII activation in these cells (22).

Chronic treatment of the VSMCs with KN-93 alone after the iNOS induction phase also significantly enhanced the CaMKII-iNOS interaction (Fig. 3), suggesting that the interaction was also sensitive to basal activity of the kinase (i.e., unstimulated by acute addition of ionomycin). As expected, suppression of CaMKIIβ expression before iNOS induction using a siRNA specific for the δ-isoform reduced both the content of CaMKIIβ in the cells and proportionately decreased

Fig. 2. CaMKII and iNOS coimmunoprecipitate. A, top: cell lysates were immunoprecipitated with an antibody specifically recognizing CaMKIIδ2, and the precipitates were immunoblotted with an antibody specific for iNOS (IB:iNOS). In the bottom panels, aliquots of preimmune lysates were blotted with iNOS and β-actin antibodies to demonstrate equal levels of iNOS induction. B: Immunoblots from A quantified by densitometry. Values plotted are means ± SE; n = 5 experiments; io, ionomycin. C: reverse immunoprecipitation using anti-iNOS to immunoprecipitate followed by immunoblotting with anti-CaMKIIβ. All samples have been stimulated with cytokines for iNOS production, except the one labeled uninduced.

Fig. 3. iNOS interaction with CaMKIIβ is enhanced following treatment with the CaMKII inhibitor KN-93. VSMCs were infected with an adenovirus encoding small interfering RNA (siRNA) targeting CaMKIIβ or a control siRNA for green fluorescent protein (SIGFP). After 96 h, the cells were serum starved overnight and then induced for iNOS expression for 24 h. After this, KN-93 was added for 16 h to the appropriate well with fresh media. A: iNOS from a CaMKIIβ immunoprecipitation. The membrane was then stripped and re-probed for CaMKIIβ. The bottom panel shows preimmune lysates. B: densitometric analysis of the blots in A; inclusive of at least three experiments. The overall level of cellular iNOS remained constant in these experiments; the average suppression of CaMKII was 69% (as quantified by densitometry; not shown).
coimmunoprecipitating iNOS (Fig. 3). The results of the immunoprecipitation experiments confirm the immunofluorescence colocalization result and suggest that the association of iNOS and CaMKII\(\alpha_2\) may be regulated by the state of CaMKII activation.

**iNOS and CaMKII localization are affected by CaMKII activity.** The distribution of iNOS in the VSMCs shown in Fig. 1 includes concentration in a perinuclear area consistent with previously reported aggresome-like structures (19). Since aggresomes have been previously shown to colocalize with \(\gamma\)-tubulin in microtubule organizing centers, we compared iNOS and \(\gamma\)-tubulin distribution in the VSMCs (Fig. 4A). A strong degree of overlap in staining is consistent with the interpretation of iNOS concentration in an aggresomal-like compartment. Inhibition of CaMKII activity with KN-93 after the iNOS induction phase enhanced localization of iNOS in the aggresome-like structures (Fig. 4C). CaMKII was also confirmed to colocalize in this compartment, and levels of the protein also increased with lowered kinase activity. iNOS localization was quantified in Fig. 5, with 57% of control cytokine-stimulated cells demonstrating concentrated iNOS immunoreactivity in aggresome-like structures, whereas 75% of the cells pretreated with KN-93 demonstrated this pattern.

As an alternative to pharmacological inhibition of CaMKII activity, we evaluated the consequences of siRNA suppression of CaMKII on iNOS localization. VSMCs were infected with an adenovirus encoding a siRNA for CaMKII\(\alpha_2\) (deltakd) for 96 h, and the cells were then induced for iNOS, fixed, and stained. On a cell-to-cell basis, CaMKII\(\alpha_2\) knockdown using this protocol was variable between cells, as shown between the

![Fig. 4. Changes in iNOS localization dependent on CaMKII\(\alpha_2\).](image)

**Fig. 4.** Changes in iNOS localization dependent on CaMKII\(\alpha_2\). **A:** iNOS (green) and \(\gamma\)-tubulin (red) colocalization by immunofluorescence; \(\gamma\)-tubulin is known to colocalize to aggresomes, adjacent to the Golgi. **B–D:** iNOS (green) and CaMKII\(\alpha_2\) (red) immunofluorescence. Images from **B** and **C** were from the experiment demonstrating iNOS localization in VSMCs (**B**) or cells to which KN-93 was added for 4 h (**C**). VSMCs in **D** were infected with an adenovirus encoding a siRNA for CaMKII\(\alpha_2\) for 96 h. CaMKII\(\alpha_2\) knockdown visualized cell to cell was variable, as indicated in the two adjacent cells. Interestingly, cells with CaMKII\(\alpha_2\) knockdown had a different pattern of iNOS localization (diffuse/nuclear) when compared with the cells expressing higher amounts of CaMKII\(\alpha_2\) (aggresome-like). All cells shown in **A–D** have been stimulated with cytokines for iNOS production.
with more CaMKII demonstrated diffuse iNOS localization (upper cell), whereas cells
mobilizing stimulus, significantly fewer cells demonstrated iNOS localization in aggresomes when compared with control cells (57% vs. 31%, respectively). On the basis of the opposing
of CaMKII activity inhibition compared with lack of expression, we propose that inactive CaMKII may facilitate recruitment to or stabilization of iNOS to aggresome-like structures, perhaps through formation of a protein complex.

Because inhibition of CaMKII activity enhanced aggresomal-like localization of iNOS, we tested the effect of CaMKII activation on this localization. In cells stimulated with either a pharmacological (ionomycin) or physiological (UTP) Ca2+ mobilizing stimulus, significantly fewer cells demonstrated iNOS localization in aggresomes when compared with control cells (57% vs. 31%, respectively). On the basis of the opposing effects of CaMKII activity inhibition compared with lack of expression, we propose that inactive CaMKII may facilitate recruitment to or stabilization of iNOS to aggresome-like structures, perhaps through formation of a protein complex.

Intriguingly, those cells in which CaMKII knockdown, significantly fewer cells demonstrated iNOS localization in aggresomes when compared with control cells (57% vs. 31%, respectively). On the basis of the opposing effects of CaMKII activity inhibition compared with lack of expression, we propose that inactive CaMKII may facilitate recruitment to or stabilization of iNOS to aggresome-like structures, perhaps through formation of a protein complex.

Because inhibition of CaMKII activity enhanced aggresomal-like localization of iNOS, we tested the effect of CaMKII activation on this localization. In cells stimulated with either a pharmacological (ionomycin) or physiological (UTP) Ca2+ mobilizing stimulus, significantly fewer cells demonstrated this pattern of localization (Fig. 5). Furthermore, cells infected with an adenovirus encoding a constitutively active form of CaMKII (T287D) exhibited a similar decrease in aggresome-like localization of iNOS. These results coupled with the results from Fig. 2 suggest that the complex between iNOS and CaMKII dissociates when CaMKII is activated.

**Table 1. iNOS distribution in VSM cell fractions**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nuclear Fraction,%</th>
<th>Membrane Fraction,%</th>
<th>Cytosolic Fraction,%</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP control</td>
<td>0.5±0.03</td>
<td>18.5±1.3</td>
<td>83.0±2.0</td>
</tr>
<tr>
<td>KN-92 control</td>
<td>0.6±0.03</td>
<td>17.4±5.9</td>
<td>82.0±5.7</td>
</tr>
<tr>
<td>KN-93</td>
<td>0.1±0.01*</td>
<td>9.9±0.8*</td>
<td>90.0±1.1*</td>
</tr>
<tr>
<td>CAdelta</td>
<td>11.0±1.7*</td>
<td>21.0±2.3</td>
<td>68.0±8.6*</td>
</tr>
<tr>
<td>Deltakd</td>
<td>2.0±0.1†</td>
<td>20.0±1.1</td>
<td>78.0±0.6†</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>2.0±0.2*</td>
<td>25.0±1.0*</td>
<td>73.0±2.0*</td>
</tr>
<tr>
<td>UTP</td>
<td>1.5±0.5†</td>
<td>22.0±1.3*</td>
<td>76.5±2.6†</td>
</tr>
<tr>
<td><strong>Acute treatments</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deltakd + ionomycin</td>
<td>3.0±0.3‡</td>
<td>19.0±2.1‡</td>
<td>78.0±0.3‡</td>
</tr>
</tbody>
</table>

Values are means ± SE (n ≥ 3 experiments). Inducible nitric oxide synthase (iNOS) was induced in vascular smooth muscle (VSM) cells. Twenty-four hours after induction, cells were washed, and fresh media were added with or without ionomycin for 3 min. The cells were then fractionated, and iNOS was immunoprecipitated from the fractions and quantified by densitometry of immunoblot signals. CAdelta, constitutively active CaMKII δ; deltailk, small interfering RNA knockdown of CaMKII δ; GFP, green fluorescent protein.

\*P < 0.01; \*P < 0.05; †not significant compared with deltailk samples.

To determine whether CaMKII could regulate iNOS activity, nitrite accumulation was measured in the cultures following iNOS induction as a function of manipulations (KN-93, deltailk, and constitutively active CaMKII δ), resulting in altered CaMKII expression or activity (Fig. 6A). Nitrite accumulation in noninduced cells was low, and the induced nitrite accumulation was blocked by treatment with aminoguanidine (data not shown), suggesting that the measured products were primarily derived from iNOS-dependent production of NO. Total iNOS protein content was not significantly affected by the experimental treatments (Fig. 6B).

**DISCUSSION**

Posttranslational modifications that control function and localization have been found on each of the NOS enzymes (23, 24, 13, 19, 28). In particular, lipid modifications and trafficking of both eNOS and nNOS to specific subcellular microdomains have been found to be important components of activity and changes in nuclear- and membrane-associated iNOS following perturbations in CaMKII activity mirror (reciprocally) changes in cytosolic/aggresomal iNOS localization. Suppression of CaMKII δ expression with siRNA (deltailk) also resulted in a marked redistribution of iNOS to the nuclear and membrane fractions. Addition of ionomycin to these CaMKII-deficient cells had no further effects on iNOS localization (Table 1). This result also mirrors the aggresomal data shown in Fig. 5, and supports the idea that CaMKII protein and activity regulates iNOS trafficking in VSMCs.

CaMKII affects iNOS activity in VSM. To determine whether CaMKII could regulate iNOS activity, nitrite accumulation was measured in the cultures following iNOS induction as a function of manipulations (KN-93, deltailk, and constitutively active CaMKII δ), resulting in altered CaMKII expression or activity (Fig. 6A). Nitrite accumulation in noninduced cells was low, and the induced nitrite accumulation was blocked by treatment with aminoguanidine (data not shown), suggesting that the measured products were primarily derived from iNOS-dependent production of NO. Total iNOS protein content was not significantly affected by the experimental treatments (Fig. 6B).

**DISCUSSION**

Posttranslational modifications that control function and localization have been found on each of the NOS enzymes (23, 24, 13, 19, 28). In particular, lipid modifications and trafficking of both eNOS and nNOS to specific subcellular microdomains have been found to be important components of activity and changes in nuclear- and membrane-associated iNOS following perturbations in CaMKII activity mirror (reciprocally) changes in cytosolic/aggresomal iNOS localization. Suppression of CaMKII δ expression with siRNA (deltailk) also resulted in a marked redistribution of iNOS to the nuclear and membrane fractions. Addition of ionomycin to these CaMKII-deficient cells had no further effects on iNOS localization (Table 1). This result also mirrors the aggresomal data shown in Fig. 5, and supports the idea that CaMKII protein and activity regulates iNOS trafficking in VSMCs.

CaMKII affects iNOS activity in VSM. To determine whether CaMKII could regulate iNOS activity, nitrite accumulation was measured in the cultures following iNOS induction as a function of manipulations (KN-93, deltailk, and constitutively active CaMKII δ), resulting in altered CaMKII expression or activity (Fig. 6A). Nitrite accumulation in noninduced cells was low, and the induced nitrite accumulation was blocked by treatment with aminoguanidine (data not shown), suggesting that the measured products were primarily derived from iNOS-dependent production of NO. Total iNOS protein content was not significantly affected by the experimental treatments (Fig. 6B).

**DISCUSSION**

Posttranslational modifications that control function and localization have been found on each of the NOS enzymes (23, 24, 13, 19, 28). In particular, lipid modifications and trafficking of both eNOS and nNOS to specific subcellular microdomains have been found to be important components of activity and changes in nuclear- and membrane-associated iNOS following perturbations in CaMKII activity mirror (reciprocally) changes in cytosolic/aggresomal iNOS localization. Suppression of CaMKII δ expression with siRNA (deltailk) also resulted in a marked redistribution of iNOS to the nuclear and membrane fractions. Addition of ionomycin to these CaMKII-deficient cells had no further effects on iNOS localization (Table 1). This result also mirrors the aggresomal data shown in Fig. 5, and supports the idea that CaMKII protein and activity regulates iNOS trafficking in VSMCs.

CaMKII affects iNOS activity in VSM. To determine whether CaMKII could regulate iNOS activity, nitrite accumulation was measured in the cultures following iNOS induction as a function of manipulations (KN-93, deltailk, and constitutively active CaMKII δ), resulting in altered CaMKII expression or activity (Fig. 6A). Nitrite accumulation in noninduced cells was low, and the induced nitrite accumulation was blocked by treatment with aminoguanidine (data not shown), suggesting that the measured products were primarily derived from iNOS-dependent production of NO. Total iNOS protein content was not significantly affected by the experimental treatments (Fig. 6B).

**DISCUSSION**

Posttranslational modifications that control function and localization have been found on each of the NOS enzymes (23, 24, 13, 19, 28). In particular, lipid modifications and trafficking of both eNOS and nNOS to specific subcellular microdomains have been found to be important components of activity and changes in nuclear- and membrane-associated iNOS following perturbations in CaMKII activity mirror (reciprocally) changes in cytosolic/aggresomal iNOS localization. Suppression of CaMKII δ expression with siRNA (deltailk) also resulted in a marked redistribution of iNOS to the nuclear and membrane fractions. Addition of ionomycin to these CaMKII-deficient cells had no further effects on iNOS localization (Table 1). This result also mirrors the aggresomal data shown in Fig. 5, and supports the idea that CaMKII protein and activity regulates iNOS trafficking in VSMCs.
function. CaMKII has been reported to regulate both eNOS and nNOS activity (6, 32). In the case of nNOS, CaMKII may affect its trafficking to postsynaptic densities by regulating nNOS interaction with scaffolding proteins such as PSD-95 (32). When compared with eNOS and nNOS, iNOS trafficking is less completely characterized, and little is known about stimuli and posttranslational events that influence its localization and activity. Several recent studies have indicated the importance of palmitoylation in iNOS processing and activation (23). We show here that CaMKII\textsubscript{2} influences iNOS localization and activity in cultured VSMCs.

iNOS and CaMKII\textsubscript{2} were found to colocalize (Fig. 1) and coimmunoprecipitate (Fig. 2) in primary VSMCs. Immunofluorescence and confocal microscopy indicated the presence of iNOS concentrated in a perinuclear distribution and colocalizing with \(\gamma\)-tubulin, consistent with previous reports describing iNOS localization in aggresome-like structures (19). The distribution is also consistent with previous reports localizing iNOS to Golgi membranes, where it is palmitoylated, a modification required for activity (23). CaMKII\textsubscript{2} subcellular localization has not been studied carefully, although previous reports from this lab using VSMC (4) and studies in astrocytes (32) suggest a concentration of the kinase in perinuclear areas, associated with ER and Golgi markers. Coimmunoprecipitation of iNOS and CaMKII\textsubscript{2} suggest their localization in a complex, although at this time it is not known whether or not this is a direct interaction or an interaction involving other unidentified proteins. On the basis of studies of nNOS regulation, it is tempting to speculate that a protein scaffold analogous to PSD-95 may be involved in formation of a multiprotein complex including iNOS and CaMKII.

To probe further whether the interaction between CaMKII and iNOS is functional, we evaluated the effects of manipulating CaMKII activity or content on iNOS localization and activity. Acute activation of the kinase with a Ca\textsuperscript{2+} ionophore, ionomycin, decreased interaction between CaMKII\textsubscript{2} and iNOS, an effect blocked by the CaMKII inhibitor KN-93, but not its inactive analog. The latter results strongly suggest that the ionomycin effects on localization were, in fact, attributable to CaMKII activity and not, for example, a nonspecific stress response that might disrupt ER or Golgi structure. The fact that prolonged incubation with KN-93 alone after the iNOS induction phase enhanced iNOS/CaMKII\textsubscript{2} interaction suggests that basal CaMKII activity in growing cells plays a function in iNOS targeting.

Interestingly, CaMKII activation with ionomycin or expression of a constitutively active mutant of CaMKII\textsubscript{2} decreased the number of cells exhibiting a distinct aggresome-like structure localized with iNOS and CaMKII. Conversely, addition of an inhibitor of CaMKII activity appears to increase iNOS localization in such structures (Figs. 4 and 5). A similar phenomenon of reduced aggresome-like localization of iNOS was observed in cells lacking CaMKII\textsubscript{2} following suppression with siRNA. Thus both CaMKII activity and content may affect trafficking of iNOS into the aggresome-like structure. Although aggresomes are usually thought of as sites of misfolded protein accumulation and degradation, it has been proposed that, in the case of iNOS, the structure may serve as a reservoir for functional iNOS (19).

Cell fractionation was used as an alternative approach to assess CaMKII-dependent changes in intracellular iNOS distribution. Acute activation of CaMKII with ionomycin or a G protein receptor-coupled agonist, UTP, increased the relative content of iNOS in the membrane and nuclear fractions (Table 1). A similar effect was observed following overexpression of constitutively active CaMKII\textsubscript{2}. Conversely, exposure to KN-93 caused an increase in cytosolic iNOS, with reductions in the nuclear and membrane fractions. Results using this approach paralleled those from the immunofluorescence approach, suggesting iNOS localized in aggresome-like structures might be recovered in the cytosolic fraction. Together, these approaches indicate that the activity of CaMKII modulates iNOS localization.

There are several reports suggesting nuclear NOS localization in other cell systems (13, 28), similar to what we have observed here in primary VSMCs following expression of constitutively active CaMKII of activation of the endogenous kinase with Ca\textsuperscript{2+}-mobilizing stimuli. Although the functional significance of nuclear localized NOS is not clear, it is possible that locally generated NO could differentially affect the redox environment or posttranslational modification of proteins through nitrosylation in specific subcellular domains and
thereby affect specific cellular functions. High concentrations of nuclear NO in VSMC could affect gene transcription or cell proliferation, resulting in VSMC cytostasis or cytotoxicity.

Conventional thought dictates that iNOS activity is largely unregulated following induction and similar or the same regardless of the cellular compartment (15). If this were the case, then CaMKII-dependent changes in iNOS localization would not modulate iNOS activity. However, iNOS activity measured by accumulation of nitrate in the cell culture medium was decreased under conditions of CaMKII activation or depletion shown to affect iNOS localization. At the present time, we cannot propose a specific molecular model linking CaMKII activity to regulation of iNOS trafficking and activity. Our general interpretation of the data is that regulated CaMKII activity is important for iNOS trafficking and consequently activity: inhibiting CaMKII activity changes the pattern of trafficking, including accumulation of a CaMKII/iNOS complex in aggresome-like structures with consequent decreases in iNOS activity; deleting CaMKII disrupts normal iNOS trafficking and activation, perhaps interfering with posttranslational modification and activation by palmitoylation; and overexpressing constitutively active CaMKII disrupts normal trafficking through dysregulated CaMKII activity. A common mechanistic link, consistent with proposed mechanisms of nNOS regulation, may be through CaMKII activity-regulated interactions of iNOS with protein complexes involved in vesicular transport. If aggresome-like structures contain a pool of activatable iNOS, as proposed (19), CaMKII activation could provide a mechanism for mobilizing this pool.

However, certain factors could complicate this interpretation. One includes the possibility that the overexpression of a constitutively active multifunctional kinase or knockdown of CaMKII by siRNA could have “off target” effects. In addition, the possibility of acute modulation of iNOS activity by CaMKII was not addressed in these studies. Immediate changes in iNOS activity are difficult to assess with the assay used, and, therefore, any changes in activity due to acute and transient activation of CaMKII would not be readily detected. Both eNOS and nNOS have been suggested to be CaMKII substrates (30, 35), but this has not yet been evaluated, or at least reported, in the case of iNOS. Although our results suggest that CaMKII-dependent regulation of iNOS trafficking may affect the active pool of iNOS, we cannot yet rule out direct effects of CaMKII on iNOS activity independent of localization per se.

iNOS can be upregulated in VSM in response to acute injury (strokes, stent placement, balloon catheter, and vascular transplants), infection and sepsis, and chronic maladies such as hypertension. VSM-localized iNOS is generally considered vasculoprotective due to inhibitory effects of NO on VSMC migration and proliferation. The results of these studies suggest that CaMKII activity is not addressed in these studies. Immediate changes in iNOS activity are difficult to assess with the assay used, and, therefore, any changes in activity due to acute and transient activation of CaMKII would not be readily detected. Both eNOS and nNOS have been suggested to be CaMKII substrates (30, 35), but this has not yet been evaluated, or at least reported, in the case of iNOS. Although our results suggest that CaMKII-dependent regulation of iNOS trafficking may affect the active pool of iNOS, we cannot yet rule out direct effects of CaMKII on iNOS activity independent of localization per se.

iNOS and CaMKII and the biological significance of this with respect to VSM cell responses to injury.

GRANTS
This work was supported by a fellowship from the National Institutes of Health (T32-HL-07194) for R. J. Jones and in part by the National Heart, Lung, and Blood Institute Grant R01-HL-49426 to H. A. Singer.

REFERENCES


