Differences in eNOS Activity Because of Subcellular Localization Are Dictated by Phosphorylation State Rather than the Local Calcium Environment*

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Nitric oxide (NO) produced in the endothelium via the enzyme endothelial nitric-oxide synthase (eNOS) is an important vasoactive compound. Wild-type (WT) eNOS is localized to the plasma membrane and perinuclear/Golgi region by virtue of N-terminal myristoylation and palmitoylation. Acylation-deficient mutants (G2AeNOS) remain cytosolic and release less NO in response to Ca\(^{2+}\)-elevating agonists; a disparity that we hypothesized was attributed to the greater distance between G2AeNOS and plasma membrane Ca\(^{2+}\) influx channels. The reduced activity of G2AeNOS versus WT was reversed upon disruption of cellular integrity with detergents or sonication. NO production from both constructs relied almost exclusively on the influx of extracellular Ca\(^{2+}\), and elevating intracellular Ca\(^{2+}\) to saturating levels with 10 \(\mu\)M ionomycin in the presence of 10 \(\mu\)M extracellular Ca\(^{2+}\) equalized NO production. To identify the contribution of calcium to the differences in activity between these enzymes, we created Ca\(^{2+}\)/CaM-independent eNOS mutants by deleting the two putative autoinhibitory domains of eNOS. There was no difference in NO production between WT and G2A-targeted Ca\(^{2+}\)-independent eNOS, suggesting that Ca\(^{2+}\) was the factor responsible. When eNOS constructs were fused in-frame to the bioluminescent probe aequorin, membrane-bound probes were exposed to higher [Ca\(^{2+}\)] in unstimulated cells but upon ionomycin stimulation, the probes experienced equal amounts of Ca\(^{2+}\). The WT and G2A enzymes displayed significant differences in the phosphorylation state of Ser\(^{617}\), Ser\(^{635}\), and Ser\(^{1179}\), and mutating all three sites to alanine or restoring phosphorylation and palmitoylation. Acylation-deficient mutants (G2AeNOS) fail to undergo fatty acylation, specifically myristylation of the glycine at position 2, which then leads to palmitoylation of the cysteines at positions 15 and 26 (11). This fatty acylation has been reported to target eNOS to both the plasma membrane and the perinuclear/Golgi region (12, 13). Mutant eNOS constructs, in which the glycine at position 2 is mutated to alanine (G2AeNOS), fail to undergo fatty acylation, and as a consequence remain cytosolic (14). This mistargeting has profound consequences for NO production, as both basal and stimulated NO production by G2AeNOS is markedly reduced compared with WT eNOS (14, 15). However, when G2AeNOS constructs are purified and examined in vitro with maximal substrate and cofactors, no differences in catalytic activity are observed (14). The mechanisms underlying the difference in catalytic activity in distinct intracellular environments remain unclear.

When inducible NOS (iNOS) is expressed with the targeting sequences for both WT and G2AeNOS, the production of NO is completely unaffected by intracellular location (16). As iNOS and eNOS require similar amounts of L-arginine, BH\(_4\), and NADPH for activity, it seems unlikely that local variations in these cofactors are responsible for the altered activity of targeted eNOS constructs. In contrast, iNOS and eNOS are diagnostically opposed in their dependence on Ca\(^{2+}\)/CaM, with NO production from iNOS driven by Ca\(^{2+}\)/CaM even at basal levels of intracellular Ca\(^{2+}\), and is thus to all practical purposes Ca\(^{2+}\)-independent (17). Therefore, intracellular [Ca\(^{2+}\)] represents a plausible candidate that mediates the differences in NO production observed between WT eNOS and G2AeNOS.

One of the major sources of Ca\(^{2+}\) entry into non-excitable cells is the opening of non-selective cation channels in the plasma membrane in response to the depletion of intracellular Ca\(^{2+}\) stores, a phenomenon known as capacitative calcium entry (CCE) (18). CCE is the predominate source of agonist-regulated Ca\(^{2+}\) entry into endothelial cells and is also believed to play a major role in shear stress-stimulated Ca\(^{2+}\) entry (19–21). It is now widely accepted that the influx of external Ca\(^{2+}\), specifically that provided by CCE, is also the predominate source of the Ca\(^{2+}\) required by eNOS for sustained activation and NO production (22). Elevations in intracellular Ca\(^{2+}\), particularly in response to Ca\(^{2+}\) influx, are rarely homogeneous throughout the cell. For instance, invok-

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2 The abbreviations used are: NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; WT, wild type; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; AEQ, aequorin; CCE, capacitative calcium entry; CaM, calmodulin; iNOS, inducible nitric-oxide synthase; BAEC, bovine aortic endothelial cells; BH\(_4\), tetrahydrobiopterin.
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ing CCE in A7r5 cells produces a rather modest increase in cytosolic Ca\(^{2+}\), while producing localized [Ca\(^{2+}\)] up to 300 times greater at the plasma membrane (23). Indeed, it has previously been demonstrated that the peak Ca\(^{2+}\) transient response reported upon invoking of CCE is higher for WTeNOS than a myristoylation-deficient eNOS expressed in COS cells (22). However, whether this disparity in local [Ca\(^{2+}\)] is sufficient to explain the large differences in NO production between WTeNOS and G2AeNOS over sustained periods of time remains to be elucidated. Indeed, NO production and calcium elevation are elevated above baseline several minutes beyond the initial influx of CCE-driven calcium (24, 25). Therefore, the aim of the present study was to determine whether proximity to plasma membrane Ca\(^{2+}\) entry channels and the subsequent differences in localized [Ca\(^{2+}\)] experienced by the plasma membrane and the cytosol, were the sole determinant of the disparity between the NO production by membrane-bound or cytosolic eNOS constructs.

MATERIALS AND METHODS

Cell Culture and Transfection—Both COS-7 cells and bovine aortic endothelial cells (BAECs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing L-glutamine, penicillin, streptomycin, and 10% (v/v) fetal bovine serum. COS-7 cells were transfected by using Lipofectamine™ 2000 according to the manufacturer’s instructions (Invitrogen). BAECs were transfected with constructs (WTeNOS, G2AeNOS, or aequorin constructs) that had been inserted into the pDEST adenoviral vector (Invitrogen).

Generation of eNOS Constructs—A cytosolic bovine eNOS construct (G2AeNOS) was constructed by mutating the alanine at position 2 of wild-type bovine eNOS (WTeNOS) to a glycine, preventing fatty acylation of the enzyme, as previously described (14). The eNOS constructs with reduced Ca\(^{2+}\)/CaM dependence were based on a Ca\(^{2+}\)/CaM-independent construct (Δ45/Δ14eNOS) of human eNOS first characterized by Chen and Wu (26). Two significant portions of the sequence of human eNOS that were not shared by the Ca\(^{2+}\)-insensitive NOS isoform iNOS, specifically the residues of the two putative autoinhibitory control elements: 594–606/614–645 (Δ45) and 1165–1178 (Δ14) were deleted. The constructs for the present study were created by deleting the corresponding residues in bovine eNOS, specifically 596–608/616–648 (Δ45) and 1167–1180 (Δ14). cDNA fragments containing the mutated regions were generated by PCR using WTeNOS cDNA as a template, and a pair of primers were used for each region: 5′-GAGCTGGAATCTTTGTA ACTCTTCATCTCCATCAG-GGCAG-3′ and 5′-GAGTTAAGAAGATTCCGATTCACTGGTTCTGTTGTTCACTGGGAC -GC-3′ for the Δ45 mutation, 5′-GATGGCGCTCTCGAGGAAACGTTAGGGCCAAATT GC-3′ and 5′-CCTACGGTTTTCCCTGCAGGGCCGATCG-3′ for the Δ14 mutation. Each fragment was then sequentially subcloned into WTeNOS or G2AeNOS cDNA.

Phospho-null eNOS mutants were generated by mutating serines 617, 635, and 1179 to alanine on bovine eNOS using sequential PCR mutagenesis with the primers previously described (5, 27), and then subcloning the various fragments into the WT and G2A targeting sequence construct. The sequences of the phospho-null eNOS and the Ca\(^{2+}\)-independent eNOS constructs were confirmed by DNA sequencing at the Genomics Core Facility of the Medical College of Georgia.

NO Release from Intact Cells—Cells were transfected with the constructs of interest, and 48 h later were stimulated with the Ca\(^{2+}\) ionophore ionomycin (1 μM) for 30 min. Medium containing NO (primarily NO\(^{-}\)) was ethanol-precipitated to remove proteins and was refluxed in sodium iodide/glacial acetic acid to convert NO\(^{-}\) to NO. NO was then quantified via specific chemiluminescence following reaction with ozone (Sievers). All NO measurements were corrected by subtracting the signal detected from cells transfected with empty vector (pCDNA3).

NO Production in Disrupted Cell Preparations—COS-7 cells transfected with either WTeNOS or G2AeNOS were scraped into 1.5-ml Eppendorf tubes, pelleted, and resuspended at a density of ~1 × 10\(^7\) cells/ml in fresh serum-free DMEM supplemented with NADPH (100 μM). Cells undergoing sonication were then subjected to 3 × 1-s bursts with a sonic dismembrator (Fisher Model 100, setting: 3), after which the lysates were aliquoted and incubated for 30 min at 37 °C. Detergent-treated samples were treated with 1% Triton X-100 and then incubated for 30 min at 37 °C. The reaction was terminated by mixing samples with 2× volume of cold absolute ethanol before analysis for NO content (see above).

Generation of Aequorin (AEQ) Constructs—Four AEQ constructs were created; an HA-tagged aequorin for measurement of cytosolic Ca\(^{2+}\) (CYT-AEQ), an AEQ fused in-frame to the transmembrane domain of the T-cell surface glycoprotein CD8 (accession NM_001768) for measurement of Ca\(^{2+}\) at the plasma membrane (PM-AEQ), and AEQ fused in-frame to either WTeNOS (WTeNOS-AEQ) or G2AeNOS (G2AeNOS-AEQ) for measurement of the Ca\(^{2+}\) experienced by each construct (28).

Calibration of Aequorin Constructs to [Ca\(^{2+}\)]—COS-7 cells were transfected with the AEQ fusion proteins, and 48 h later, the cells were homogenized by sonication in medium of the following composition: 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 10 mM NaF, 1 mM orthovanadate, and 1× protease inhibitor mixture (Sigma). The lysates were then exposed to EGTA-buffered solutions of known Ca\(^{2+}\) concentrations, followed by total discharge of the AEQ with 10 mM Ca\(^{2+}\). The ratio of luminescence in the one second immediately after exposure to the Ca\(^{2+}\) solution was calculated as a proportion of the total luminescence emitted over the duration of the experiment (L/L\(_{max}\)) and plotted against each known concentration of Ca\(^{2+}\) to obtain a calibration curve.

Ca\(^{2+}\) Measurements Using Aequorin in COS Cells—COS-7 cells were transfected with one of the four aequorin constructs, and 48 h later, the aequorin was activated by incubating the cells in Ca\(^{2+}\)-free DMEM (BIOSOURCE) containing 5 μM coelenterazine (Sigma) for 1 h. Following this, loading medium was replaced with Ca\(^{2+}\)-free DMEM without coelenterazine. The cells were then exposed to Ca\(^{2+}\)-free DMEM (Ca\(^{2+}\)-DMEM (DMEM containing 1.5 mM free Ca\(^{2+}\)), or Ca\(^{2+}\)-DMEM with 1 μM ionomycin. Cells were placed in a luminescence plate reader (Lumistar Galaxy) and after the appropriate treatment time the remaining luminescence (L\(_{max}\)) was discharged by exposing the cells to a lysis buffer consisting of 10 mM Ca\(^{2+}\) and 0.3% Triton X-100 in dH\(_2\)O, and then expressed as a ratio of the total luminescence obtained from untreated cells (t = 0).

AEQ and its cofactor coelenterazine react in the presence of Ca\(^{2+}\) to emit a photon. This reaction results in the permanent oxidation of coelenterazine to coelenteramide, and therefore the emission of light in response to Ca\(^{2+}\) is a once-only reaction. The size of the available pool of luminescence therefore decreases constantly over time in direct proportion to the amount of Ca\(^{2+}\) present. We, therefore, interpreted the decrease in L\(_{max}\) as being indicative of exposure to Ca\(^{2+}\) over time, with a greater degree of burnout indicating that the AEQ probe had been exposed to more Ca\(^{2+}\).

Immunoprecipitation and Western Blotting—Cells were washed twice with phosphate-buffered saline and lysed in homogenization buffer of the following composition: 50 mM Tris-HCl, 0.1 mM EGTA, 0.1 mM EDTA, 0.1% SDS, 1% Nonidet P-40, 0.1% deoxycholic acid. Lysates were homogenized and cleared by centrifugation. After determining protein concentration (DC protein assay kit, Pierce), equal amounts of
protein were incubated with an excess of eNOS monoclonal antibody for 2 h at 4 °C. Protein A-Sepharose was then added, and the mixture incubated for another 1 h at 4 °C. The immune complexes were washed three times with lysis buffer and boiled in SDS-PAGE sample buffer (5 min), and then size-fractionated by SDS-PAGE. Blots were transferred to nitrocellulose membrane in standard transfer buffer for 18 h at 4 °C and 40 V, and then blocked in nonfat milk for 1 h. After blocking, membranes were incubated with phosphospecific antibodies raised to the individual phosphorylation sites (phospho-116 and phospho-Ser617, Upstate; phospho-Thr497, Cell Signaling Technologies; phospho-Ser635 and phospho-Ser1179, BD Transduction Laboratories) or with anti-eNOS monoclonal antibody (9D10, Zymed Laboratories) for 2 h, washed, incubated with secondary antibody conjugated to horseradish peroxidase (anti-mouse, Amersham Biosciences), washed again, and then developed using chemiluminescence (Amersham Biosciences).

2',5'-ADP-Sepharose Isolation of eNOS Constructs—Cells were washed twice with phosphate-buffered saline and homogenized as for the AEQ calibration experiments (see above). When required, membrane and cytosolic fractions were separated by centrifugation (100,000 × g, 90 min; Beckman ultracentrifuge), and the membrane fraction was resuspended in homogenization buffer supplemented with 1% Nonidet P-40. 2',5'-ADP-Sepharose beads (Amersham Biosciences) were then added to the fractions and rotated at 4 °C for 3 h. Beads were then washed twice in homogenization buffer before being prepared for Western blotting (see above).

Concentration-Response Curves to Ca2+/CaM with Isolated eNOS Constructs—COS-7 cells expressing WT and G2A eNOS constructs were homogenized and eNOS affinity-purified with 2',5'-ADP-Sepharose beads (as above). Following the 3-h rotation at 4 °C, beads were washed twice in HEPES-buffered saline (HBS; 135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl2, 11.6 mM HEPES, 11.5 mM glucose, pH 7.3) then twice in HBS supplemented with EGTA (2 mM) to remove all Ca2+/CaM from the sample, then washed twice again in HBS. Beads were then resuspended in treatment solutions consisting of HBS supplemented with NADPH (3 mM), BH4 (30 μM), L-arginine (100 μM), CaCl2 (1 mM), and varying concentrations of calmodulin (bovine heart calmodulin, Sigma). Reactions were terminated after 30 min by addition of 2× volume of ice-cold EtOH. The relative production of NO was determined by chemiluminescence as described above.

Confocal Imaging—To visualize the subcellular location of WT and G2A eNOS and eNOS-aequorin fusion protein within cells, we constructed additional fusion proteins encoding WT and G2A eNOS-GFP and WT and G2A-RFP-AEQ. COS-7 cells were then transfected with cDNAs encoding the eNOS fusion proteins of EGFP and monomeric RFP. 24–48 h later, cells were replated onto glass-bottomed culture dishes (MatTek). All imaging was performed using the LSM 510 Meta 3.2 Confocal Microscope (Zeiss). Magnification power was set at ×40 with oil.

Statistical Analysis—NO release data were expressed as mean ± S.E. AEQ burnout data were individually normalized to the I max, obtained for each AEQ fusion construct in Ca2+-free DMEM, then expressed as mean ± S.E. Overall differences between groups were analyzed using a two-way analysis of variance (ANOVA) with Student-Newman–Keuls posthoc analysis for determining differences between the means when more than two groups were compared. An independent Student’s t test was used when only two groups were compared. Comparisons between the Ca2+ calibration curves of each AEQ fusion construct were made using 2-way ANOVA. Differences were considered significant if p < 0.05.
and the myristylation-deficient, cytosolic G2AeNOS may be the proximity of each construct to store-operated Ca^{2+}-entry channels (22); a hypothesis supported by our finding that disruption of cellular integrity abolished the differences in NO production. To further investigate this hypothesis, it was first necessary to confirm that the entry of extracellular Ca^{2+} was the dominant stimulus for NO production by both constructs. Cells transfected with either WTeNOS or G2AeNOS were stimulated with 1 \mu M ionomycin (1 \mu M; 30 min) in DMEM containing 1.5 mM free [Ca^{2+}] by preincubation with BAPTA-AM (10 \mu M, 20 min), or in the absence of extracellular Ca^{2+} (EGTA; 1 mM). NO release was quantified using a chemiluminescence NO analyzer (b). NO release over 30 min from transfected COS-7 cells that were stimulated with either 1 \mu M ionomycin or (c) 10 \mu M ionomycin in DMEM containing increasing concentrations of extracellular Ca^{2+}. Data are presented as means ± S.E. (n = 4). *p < 0.05 versus WT targeting; #, p < 0.05 versus NO production in the presence of Ca^{2+}.

**FIGURE 3.** The activity of Ca^{2+}-independent eNOS mutants is unaffected by intracellular targeting. COS-7 cells were transfected with WTeNOS, G2AeNOS, WT(\Delta 45/\Delta 14)eNOS, and G2A(\Delta 45/\Delta 14)eNOS. a, basal NO release was determined over 30 min from transfected COS-7 cells in DMEM containing either 1.5 mM free Ca^{2+} or zero free Ca^{2+} (1 mM EGTA) using chemiluminescence. b, stimulated NO release from transfected COS-7 cells that were exposed to ionomycin (1 \mu M) for 30 min in DMEM containing either 1.5 mM free Ca^{2+} or zero free Ca^{2+} (1 mM EGTA). Data are presented as means ± S.E. (n = 4). *p < 0.05 versus construct with WT targeting; #, p < 0.05 versus NO production in the presence of Ca^{2+}.
in \([\text{Ca}^{2+}]\) handling, and not an inability of the G2A construct to produce NO per se, might be the key regulatory factor mediating the differences in activity. Additionally, treatment of cells with the superoxide scavenger Tiron (10 mM, 30 min) had absolutely no effect on the NO production by either construct, suggesting that the disparity in NO production was not caused by aberrant production of superoxide (data not shown).

**The Activity of \(\text{Ca}^{2+}/\text{CaM}\)-insensitive Constructs Is Unaffected by Subcellular Localization**—Once we had confirmed the dependence of both constructs on extracellular \(\text{Ca}^{2+}\) entry, and that NO production could be equalized simply by increasing intracellular \(\text{Ca}^{2+}\) to saturating levels, we attempted to demonstrate a direct link between intracellular \(\text{Ca}^{2+}\) and the altered NO production by the two constructs. To achieve this we created \(\text{Ca}^{2+}/\text{CaM}\)–independent deletion mutants of the WTeNOS and G2AeNOS constructs based on the constructs of Chen and Wu (26). Both deletion mutants were found to be almost entirely \(\text{Ca}^{2+}/\text{CaM}\)-independent as, in contrast to the native constructs, they displayed extremely high basal NO production. This was unaffected by chelation of extracellular \(\text{Ca}^{2+}\) (Fig. 3a) and only a slight (although significant) increase in NO production in response to ionomycin stimulation, which was sensitive to removal of extracellular \(\text{Ca}^{2+}\) (Fig. 3b).

The WTeNOS and G2AeNOS deletion mutants retained the same membrane/cytosol distribution as the parent enzymes (data not shown). However, in contrast to the original constructs, the WTeNOS and G2AeNOS \(\text{Ca}^{2+}/\text{CaM}\)-independent mutants produced equivalent amounts of NO both basally and in response to ionomycin stimulation (Fig. 3, a and b). The ability of the \(\text{Ca}^{2+}/\text{CaM}\)-independent constructs to produce equal amounts of NO regardless of their subcellular localization strongly suggested that the disparity between the NO production of the original WTeNOS and G2AeNOS constructs involved the interaction of the constructs with \(\text{Ca}^{2+}/\text{CaM}\).

**WTeNOS Experiences Higher \(\text{Ca}^{2+}\) in Unstimulated but Not Stimulated Cells**—As our observations so far had concurred with the hypothesis that the disparity between NO production by WTeNOS and G2AeNOS was caused by differences in localized \(\text{Ca}^{2+}\), we attempted to test the hypothesis by measuring the localized \(\text{Ca}^{2+}\) experienced by each construct in response to CCE. Aequorin fusion constructs were constructed as discussed under “Materials and Methods” (Fig. 4a) and were evaluated to ensure that none of the modifications altered the sensitivity of the Aequorin to \(\text{Ca}^{2+}\) (Fig. 4b). To ensure that the targeting of the eNOS constructs was unaffected by the fusion to Aequorin, we examined the subcellular distribution of the WTeNOS and WTeNOS-AEQ constructs expressed in COS-7 cells. When cell lysates were separated into cytosolic and membrane fractions by centrifugation, both WTeNOS and WTeNOS-AEQ were found in equal proportion, primarily in the membrane fraction. However, the bulk of the G2AeNOS and G2AeNOS-AEQ protein was found in equal proportion, primarily in the membrane fraction. In COS-7 cells transfected with WT and G2A variants of eNOS-GFP and eNOS-AEQ-RFP fusion proteins, we performed confocal microscopy to visualize the subcellular distribution of eNOS fusion proteins. As shown in Fig. 5b, G2AeNOS is found distributed throughout the cytosol, colocalizing completely with G2A-eNOS-AEQ. In contrast, the WTeNOS is found at discrete intracellular locations, primarily at the perinuclear Golgi (arrows) with less expression at the plasma membrane (arrowheads) and importantly it also colocalizes with the WTeNOS-AEQ. These results further suggest that the addition of AEQ does not disrupt
FIGURE 5. The fusion of AEQ to the C terminus of eNOS does not modify its subcellular targeting. 

a. Subcellular fractionation of cell lysates from COS-7 cells transfected with WTeNOS, WTeNOS-AEQ, G2AeNOS, or G2AeNOS-AEQ. Cytosolic (c) and membrane (m) fractions were separated by high speed centrifugation and blotted for eNOS (upper panels), the membrane-bound protein tyrosine phosphatase 1B (PTP1B, middle panels), or the cytosolic protein, β-tubulin (lower panels).

b. Live cell imaging of WT and G2A eNOS and eNOS-AEQ fusion proteins. COS-7 cells co-transfected with eNOS-GFP (left panels, green) or eNOS-AEQ-RFP (middle panel, red) containing either the WT or G2A targeting sequences, and examined using confocal microscopy to determine the extent of colocalization of the eNOS and eNOS-AEQ constructs (right panels, yellow).

c. NO release was quantified by chemiluminescence in the presence or absence of ionomycin (1 μM, 30 min). Data are presented as means ± S.E. (n = 3). *, p < 0.05 versus construct with WT targeting sequence.

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the subcellular targeting of WT or G2A eNOS. Finally, the functional effect of AEQ fusion on the eNOS constructs was determined. Although a slight reduction in NO production by both constructs was observed when fused to AEQ as, has been reported for eNOS-GFP fusion proteins (30), the relative differences between WTeNOS and G2AeNOS were unaffected by the addition of AEQ (Fig. 5c). Taken together, these results strongly suggest that the targeting of the two eNOS-AEQ constructs accurately reflects that of the native enzymes. When aequorin was expressed in unstimulated COS-7 cells either as a free cytosolic probe (CYT-AEQ) or fused to the transmembrane domain of CD8 as a plasma membrane probe (PM-AEQ), the localized \([Ca^{2+}]\) experienced, as interpreted by the burnout of the coelenterazine pool, was much greater for the plasma membrane probe than the cytosolic probe (Fig. 6a). These findings are consistent with our hypothesis that proximity to plasma membrane \(Ca^{2+}\) entry channels results in substantially higher localized \([Ca^{2+}]\). Likewise, when aequorin was fused to the C terminus of the eNOS constructs in unstimulated cells, the membrane-bound WTeNOS-AEQ construct experienced greater coelenterazine burnout than the cytosolic G2AeNOS-AEQ construct (Fig. 6b).

When COS-7 cells expressing the AEQ constructs were stimulated with ionomycin (1 \(\mu M\)) the coelenterazine burnout between the CYT-AEQ and PM-AEQ was not significantly different, suggesting that this stimulation results in both the plasma membrane and cytosolic probes being exposed to equivalent amounts of \(Ca^{2+}\) over time (Fig. 7a). Interestingly, the G2AeNOS-AEQ displayed significantly more coelenterazine burnout than the WTeNOS-AEQ probe (Fig. 7b), suggesting that over the 30 min of ionomycin stimulation the cytosolic G2AeNOS is actually exposed to more \(Ca^{2+}\) than the membrane-bound WTeNOS.

**Phosphorylation State Contributes to Differences in NO Production between the Two Constructs**—Both WT and G2A eNOS constructs experience similar exposure to intracellular \(Ca^{2+}\) upon ionomycin stimulation. However our earlier observations demonstrated that intracellular \(Ca^{2+}\) was undoubtedly implicated in the phenomenon. If \(Ca^{2+}\) was involved in the phenomenon, but the constructs did not experience different exposure to \(Ca^{2+}\), then the logical explanation was that the constructs were not equally responsive to \(Ca^{2+}\). A number of phosphorylation sites on eNOS are believed to increase enzyme sensitivity to intracellular \(Ca^{2+}\) (31–33). Indeed, when creating our own \(Ca^{2+}\)-independent eNOS mutants we deleted three such sites; specifically Ser\(^{617}\), Ser\(^{635}\), and Ser\(^{1179}\) (Fig. 8a). We therefore examined the differences in the phosphorylation state of the two constructs. When the eNOS constructs were immunoprecipitated and probed with phosphospecific antibodies (Fig. 8b), we observed a slight decrease in Ser\(^{635}\) phosphorylation, a marked decrease in Ser\(^{1179}\) phosphorylation, and virtually no Ser\(^{617}\) phosphorylation on the G2AeNOS compared with the WT enzyme, confirming earlier observations (10). As stimulation of eNOS has been reported to alter phosphorylation state (31, 34–36), we also examined the phosphorylation state of immunoprecipitates after stimulation with ionomycin (1 \(\mu M\), 30 min). We observed an increase in the phosphorylation of Ser\(^{617}\), Ser\(^{635}\), and Ser\(^{1179}\) in the stimulated cells.
Versus the unstimulated cells; however, there was little change in the relative differences between WTeNOS and G2AeNOS after ionomycin stimulation (Fig. 8b). Although it had not been removed in our Ca^{2+}-insensitive mutants, we also examined the phosphorylation state of Thr^{497}, a residue whose phosphorylation state has been intimately linked with CaM binding by the enzyme (8, 37). The Thr^{497} site was phosphorylated in WTeNOS but not G2AeNOS, an observation that was once more unchanged after stimulation with ionomycin. Moreover, the phosphorylation of Thr^{497} in the Ca^{2+}-insensitive deletion mutants was no different to that of the original constructs (data not shown), suggesting that the equalization of NO production in the deletion mutants was not a result of altered phosphorylation of the Thr^{497} site.

To examine whether differences in phosphorylation contributed to the altered NO production between the constructs, we mutated three phosphorylation sites (Ser^{617}, Ser^{635}, and Ser^{1179}) to alanine to create an eNOS construct that was phospho-null at these sites. Phospho-null constructs expressing either the WT or G2A targeting sequences produced equivalent amounts of NO when expressed in COS-7 cells (Fig. 8c).

To further confirm that phosphorylation state was responsible for the different catalytic activity between the two constructs, we examined the effect of calyculin A, an inhibitor of the protein phosphatases PP1 and PP2A. After incubation with calyculin A, phosphorylation of Ser^{617}, Ser^{635}, and Ser^{1179} was markedly increased (Fig. 9a), and NO production by both constructs was significantly increased (Fig. 9b). Importantly, treatment with calyculin A increased the stimulated NO production by G2AeNOS much more than that of WTeNOS (7-fold versus 2-fold), and in fact after calyculin A treatment stimulated NO production by G2AeNOS was equivalent with that of WTeNOS (Fig. 9b). Calyculin significantly increased basal NO production from both constructs; however, NO production from WT was elevated to a much greater degree than the G2A enzyme (4-fold versus 2-fold). This difference most likely results from the higher Ca^{2+} experienced by the WT enzyme versus the G2AeNOS under basal conditions (Fig. 6b).

When taken together, the above results strongly suggested that differences in ionomycin-stimulated NO production by WT and G2A eNOS was in fact because of differences in Ca^{2+}/CaM sensitivity as a result of differences in phosphorylation. We therefore sought to directly examine the Ca^{2+}/CaM-sensitivity of each WT and G2A eNOS. When both constructs were isolated on ADP-Sepharose beads and exposed to saturating levels of all other cofactors (NADPH, BH4, l-arginine, and Ca^{2+}), WTeNOS displayed a higher sensitivity to calmodulin than G2AeNOS (Fig. 9c), directly confirming that the two constructs have different sensitivity to Ca^{2+}/CaM.

Observations in COS Cells Are Mirrored in the More Physiologically Relevant Bovine Aortic Endothelial Cells (BAECs)—To confirm the physiological relevance of our findings in COS-7 cells, we extended some key experiments to primary cultures of BAECs. When either WTeNOS or G2AeNOS were overexpressed in BAECs, the NO production both basally and in response to stimulation was significantly less for cells overexpressing G2AeNOS compared with those overexpressing WTeNOS (Fig. 10b). Likewise when BAECs were transduced with WTeNOS-AEQ or G2AeNOS-AEQ, the WT construct reported higher Ca^{2+} exposure (based on coelenterazine burnout) than the G2A construct in unstimulated cells (Fig. 10c). When cells expressing the AEQ constructs were stimulated with ionomycin, the constructs did not report significantly different calcium concentrations (unlike the findings in COS cells). However the most significant finding was that WTeNOS is not exposed to more Ca^{2+} than the G2AeNOS, results that are consistent with the findings in COS cells (Fig. 10d).

Finally, we sought to examine the phosphorylation state of the eNOS constructs in BAECs. After transducing either WTeNOS or G2AeNOS in BAECs, we then separated the membrane and cytosolic fractions of the cells. Using ADP-Sepharose beads to isolate WTeNOS from membrane fractions and G2AeNOS from cytosolic fractions, we immunoblotted for the relevant phosphorylation sites (Fig. 10e). The phosphorylation state of each site was similar in BAECs as had been the case in COS-7 cells, once more suggesting that our findings in COS-7 cells are physiologically relevant.

**DISCUSSION**

The reduced activity of myristylation-deficient and thus incorrectly targeted eNOS constructs (G2AeNOS) has been known for some time (14); however, the mechanism behind this phenomenon remains poorly understood. In this report, we attempted to define the nature of the difference in catalytic activity between the two eNOS constructs by examining strain differences in phosphorylation and CaM binding. We found that G2AeNOS is more sensitive to CaM binding and has a lower Ca^{2+} equilibrium constant, resulting in a higher Ca^{2+} exposure for G2AeNOS constructs as compared with WTeNOS constructs. We found that G2AeNOS is also significantly less sensitive to phosphorylation of specific sites, which is consistent with a lower Ca^{2+}/CaM sensitivity. This decrease in Ca^{2+}/CaM sensitivity was linked to the reduced catalytic activity of G2AeNOS constructs as compared with WT eNOS constructs.

The low Ca^{2+} exposure and Ca^{2+}/CaM sensitivity of G2AeNOS constructs as compared with WT eNOS constructs suggests a physiological role for PKB/Akt and mTORC1 in regulating eNOS function. PKB/Akt and mTORC1 have both been shown to phosphorylate eNOS at Ser^{617} and Ser^{635} (11, 14, 27). These results are consistent with the lower Ca^{2+} exposure and Ca^{2+}/CaM sensitivity of G2AeNOS constructs as compared with WT eNOS constructs, which suggests a physiological role for PKB/Akt and mTORC1 in regulating eNOS function.

The increased phosphorylation of Thr^{497} and Ser^{1179} in WTeNOS constructs in response to stimulation is consistent with the reduced catalytic activity of WTeNOS constructs as compared with G2AeNOS constructs. This increase in phosphorylation is consistent with the lower Ca^{2+} exposure and Ca^{2+}/CaM sensitivity of WTeNOS constructs as compared with G2AeNOS constructs, which suggests a physiological role for PKB/Akt and mTORC1 in regulating eNOS function.
understood. It has long been known that intracellular Ca\textsuperscript{2+} is an absolute requirement for eNOS activation (38). Furthermore, it is now widely accepted that when sustained NO production is triggered by a number of Ca\textsuperscript{2+}-elevating agents such as the IP\textsubscript{3} generating agonists histamine, the SERCA inhibitor thapsigargin, or the Ca\textsuperscript{2+} ionophore A-23187, the major source of this Ca\textsuperscript{2+} is the influx of extracellular Ca\textsuperscript{2+} via CCE (22, 39, 40). The localization of WTeNOS in plasma membrane caveolae, sites where CCE channels have also been proposed to reside (41), raised the tempting hypothesis that the decreased NO production by G2AeNOS upon stimulation of CCE was simply a result of the construct experiencing lower localized [Ca\textsuperscript{2+}] than the WTeNOS because of greater spatial separation from Ca\textsuperscript{2+} influx channels. Indeed, the study of Lin et al. (22) conclusively demonstrated that upon activation of CCE, a WTeNOS construct did experience higher peak [Ca\textsuperscript{2+}] transient than a myristylation-deficient construct. Our previous observations that expression of WTeNOS and G2AeNOS targeting sequences fused to the Ca\textsuperscript{2+}-independent iNOS fail to demonstrate differences in NO production (16) also seemed to support this hypothesis. However, despite the peak differences in calcium influx, no direct link between has been made between local NO production and the more functionally relevant measurement of compartmentalized increases in intracellular calcium over time. Therefore, the aim of the present study was to directly demonstrate if the disparity in catalytic activity between the two constructs was caused by differences in localized [Ca\textsuperscript{2+}] upon stimulation with Ca\textsuperscript{2+}-elevating agonists, or if other regulatory factors were involved.

We first examined the importance of cellular integrity to the phenomenon. Three different Ca\textsuperscript{2+}-elevating agonists (ionomycin, thapsigargin, and ATP) all produced greater activation of WTeNOS than G2AeNOS in intact COS-7 cells. However when the cells were disrupted either by mechanical (sonication) or chemical (Triton X-100) means, G2AeNOS was significantly more active than WTeNOS. These findings confirmed that the relative activity of eNOS depends on factors present within the intracellular environment that each construct resides in, rather than a permanent change in enzyme activity.

Our hypothesis relied on the influx of extracellular Ca\textsuperscript{2+} as the major source of eNOS activation. As eNOS has been suggested to undergo Ca\textsuperscript{2+}-independent activation in response to certain stimuli (6, 33), we needed to ensure that all NO production by our constructs was dependent on Ca\textsuperscript{2+} influx. Removal of extracellular Ca\textsuperscript{2+} almost abolished NO production by both constructs, confirming that both relied on the influx of external Ca\textsuperscript{2+}. Interestingly, intracellular chelation of Ca\textsuperscript{2+} using BAPTA-AM failed to completely inhibit the activity of either construct, even though all such activity was dependent on Ca\textsuperscript{2+} influx. The inability of BAPTA-AM to affect near membrane Ca\textsuperscript{2+} responses has been previously reported (42), and possibly reflects the inability of BAPTA to immediately access and chelate all of the Ca\textsuperscript{2+} entering the cytosol through Ca\textsuperscript{2+} influx channels. Thus, the membrane-localized eNOS is likely to be relatively unaffected by BAPTA-AM even though cytosolic Ca\textsuperscript{2+} is significantly reduced. Further supporting this hypothesis is the observation that G2AeNOS, the construct furthest from the plasma membrane, was much more effectively inhibited by BAPTA-AM than WTeNOS. Another central tenet of our hypothesis was that if the two constructs were producing disparate amounts of NO because of their exposure to different levels of Ca\textsuperscript{2+}, then raising intracellular Ca\textsuperscript{2+} to saturating levels should equalize the NO production by both constructs. Cells with 1 mM ionomycin failed to display equalized NO production even in the presence of 10 mM extracellular Ca\textsuperscript{2+} (probably because of the fact that at this concentration ionomycin is thought to act almost exclusively by activating CCE (29), a Ca\textsuperscript{2+} entry pathway that can only produce Ca\textsuperscript{2+} influx as rapidly as plasma membrane Ca\textsuperscript{2+} channels will allow). However when the cells were stimulated with a 10-fold higher concentration of ionomycin (a concentration that facilitates Ca\textsuperscript{2+} entry independently of plasma membrane Ca\textsuperscript{2+} channels), NO production was equalized when extracellular Ca\textsuperscript{2+} was raised to sufficiently high concentration.
As our hypothesis was still supported by all observations so far, we then attempted to more directly determine the contribution of intracellular Ca$^{2+}$/CaM to the disparity in NO production between the constructs. To achieve this, we created Ca$^{2+}$/CaM-independent mutants based on those described by Chen and Wu (26). Interestingly, the WTeNOS and G2AeNOS Ca$^{2+}$/CaM-independent mutants showed absolutely no differences in NO production under any stimulation conditions observed, confirming our previous findings with targeted iNOS constructs (16).

FIGURE 10. WTeNOS and G2AeNOS expressed in BAECs behave similarly to those expressed in COS-7 cells. a, confluent BAECs were transduced with adenovirus encoding a control vector (lacZ), WTeNOS or G2AeNOS. Cells were lysed and immunoblotted for total eNOS. b, ionomycin-stimulated (1 μM, 30 min) NO release was measured from BAECs transduced with either WTeNOS or G2AeNOS by NO-specific chemiluminescence. c and d, consumption of coelenterazine was measured in BAECs transduced with WTeNOS-AEQ and G2AeNOS-AEQ fusion constructs either (c) basally or (d) in response to stimulation with 1 μM ionomycin. Measurements of the cumulative exposure of each construct to intracellular calcium were obtained by depletion of the coelenterazine pool as determined by $L_{\text{max}}$ at 0, 5, or 30 min normalized to $t = 0$. e, BAECs were transduced with WTeNOS and G2AeNOS. The cells lysed in hypotonic buffer and the membrane fraction and cytosolic fractions were separated by high speed centrifugation. WTeNOS was affinity-purified from the membrane fraction, and G2AeNOS from the cytosolic fraction, by 2’’,5’’-ADP-Sepharose beads. The relative phosphorylation of WTeNOS and G2AeNOS in BAECs was determined by immunoblotting with phosphospecific eNOS antibodies. Data are presented as means ± S.E. (n = 3–7), *, p < 0.05 versus constructs with wild-type targeting sequence.
This constitutes the first direct evidence that Ca\(^{2+}\) or CaM alone is responsible for the difference in activity.

After confirming that interaction of the constructs with Ca\(^{2+}\) was involved in the phenomenon, we next determined the localized [Ca\(^{2+}\)] experienced by each construct both basally and upon stimulation by fusing in-frame the bioluminescent Ca\(^{2+}\) probe aequorin (AEQ). Although Lin et al. (22) had demonstrated that the peak [Ca\(^{2+}\)] experienced after CCE stimulation was higher for WTeNOS than the G2A construct, this response was a transient (<30s) peak response to a protocol designed to maximally activate CCE. As we stimulated our cells with ionomycin for 30 min to measure NO production, we felt it would be more informative to measure the Ca\(^{2+}\) exposure during the 30-min stimulation with ionomycin rather than an immediate peak response to deliberate CCE activation. We therefore attempted to devise a method to observe Ca\(^{2+}\) exposure over more sustained time periods.

The reaction between AEQ and its cofactor coelenterazine in the presence of Ca\(^{2+}\) and molecular oxygen results in the emission of one photon and the irreversible oxidation of coelenterazine to coelenteramide. The emission of light in response to Ca\(^{2+}\) is thus a once-only reaction (43), and the size of the available pool of luminescence decreases constantly over time in direct proportion to the amount of Ca\(^{2+}\) present. The relative amounts of localized [Ca\(^{2+}\)] that each construct is exposed to over time can therefore be estimated by measuring the decrease in the size of the releasable pool of luminescence (L\(_{\text{max}}\)), as determined by discharging the remaining luminescence with lysis buffer. Using this technique, we observed that in unstimulated cells the PM-AEQ probe displayed a faster rate of burnout than CYT-AEQ. Likewise, WTeNOS-AEQ clearly displayed a faster rate of burnout than G2AeNOS-AEQ, indicating that the WTeNOS construct is exposed to higher levels of Ca\(^{2+}\) than the G2AeNOS construct in unstimulated cells. This probably reflects the constant transmembrane flux of Ca\(^{2+}\), which enters the cell because of the spontaneous opening of Ca\(^{2+}\) influx channels. It is rapidly pumped back out of the cytosol by plasma membrane Ca\(^{2+}\)-ATPases before having an opportunity to significantly elevate [Ca\(^{2+}\)] in the cytosol (23).

Upon 30 min of stimulation with ionomycin, the burnout between PM-AEQ and CYT-AEQ was not significantly different, suggesting that ionomycin stimulation elevates cytosolic [Ca\(^{2+}\)] to the point where both plasma membrane and cytosolic probes are equally exposed to Ca\(^{2+}\). When cells expressing the eNOS-AEQ fusion constructs were stimulated with ionomycin for 30 min, not only did the WTeNOS no longer display greater burnout than G2AeNOS, but in fact the membrane-bound WT probe displayed significantly less burnout than cytosolic G2A probe, suggesting that the G2A probe had been exposed to more Ca\(^{2+}\) over the stimulation period than the WT probe. This suggests that, even though the [Ca\(^{2+}\)] increase because of maximally activated CCE is transiently greater at the plasma membrane (as reported by Lin et al., Ref. 22), upon sustained (30 min) elevation of cytosolic Ca\(^{2+}\) (such as during ionomycin stimulation) the Ca\(^{2+}\) gradient between plasma membrane influx channels and the cytosol may be markedly reduced (or even non-existent) when observed as a Ca\(^{2+}\) exposure over time phenomenon (such as in these coelenterazine burnout experiments). This also supports the recent findings of Jobin et al. (44) using a FRET-based Ca\(^{2+}\) sensor fused to eNOS constructs.

Based on the AEQ burnout data, our hypothesis that the disparity in NO production was simply because of proximity to plasma membrane Ca\(^{2+}\) influx channels seemed insufficient to explain the phenomenon. The preceding data, however, strongly suggested that Ca\(^{2+}\) plays an important role in the phenomenon, and we therefore considered the possibility that the two constructs were differentially sensitive to Ca\(^{2+}\).

As our Ca\(^{2+}\)-independent deletion mutants (the only modification of the constructs, which had successfully equalized NO production) also deleted three key phosphorylation sites involved in sensitizing eNOS to calcium/calmodulin we next addressed whether this could explain the functional differences between the enzymes. Compared with the WT enzyme, G2A eNOS exhibited slightly reduced Ser\(^{617}\) phosphorylation, marked reduction of Ser\(^{1179}\) phosphorylation, and virtually no Ser\(^{635}\) phosphorylation. The phosphorylation of these residues is associated with reduced dependence on intracellular calcium (33), increased calcium sensitivity (31) and reduced dissociation of calcium-bound calmodulin (32). To directly test whether phosphorylation of these residues mediates the functional differences between these enzymes, we mutated all three residues to the nonphosphorylatable analogue, alanine. The marked difference in activity between the native WT and G2A enzymes was abolished in the equivalent constructs with triple (S617A, S635A, S1179A) mutations, suggesting that indeed a difference in phosphorylation mediates the functional differences in calcium sensitivity between these enzymes.

We then attempted the converse experiment, by equalizing the phosphorylation of WT and G2A eNOS using the phosphatase inhibitor, calyculin A. Treatment of cells with calyculin produced a marked increase in phosphorylation and an increase in both basal and stimulated NO production, for both constructs. Calyculin treatment equalized eNOS phosphorylation and stimulated production of NO between the WT and G2A eNOS. These results are consistent with the data obtained with phospho-null mutants and strongly support the contention that phosphorylation mediates the difference between these enzymes. Interestingly, phosphatase inhibition failed to equalize basal NO production between the constructs even though stimulated NO production was equalized. We believe that this reflects the differences in basal Ca\(^{2+}\) exposure between the two constructs that were observed using eNOS-aequorin fusion proteins.

As the two constructs differed in phosphorylation state, and either increasing or abolishing phosphorylation of the two constructs equalized NO production, it seemed likely that our previous results could be explained by these phosphorylation differences endowing the two constructs with different Ca\(^{2+}\)/CaM sensitivity. We therefore sought direct evidence that the two constructs were differentially sensitive to Ca\(^{2+}\)/CaM. Performing a concentration-response curve to CaM on isolated eNOS constructs in the presence of saturating Ca\(^{2+}\) conclusively demonstrated that the WT construct is more sensitive to Ca\(^{2+}\)/CaM than the G2A construct, supporting our conclusions.

Finally, we sought to confirm that our observations in COS-7 cells were mirrored in BAECs, a cell type which expresses endogenous eNOS and is thus a more physiologically relevant model. When BAECs over-expressing WTeNOS or G2AeNOS were stimulated, G2AeNOS-expressing cells produced significantly less NO than those expressing WTeNOS. When the WTeNOS-AEQ and G2AeNOS AEQ fusion constructs were expressed in BAECs, the basal burnout of WTeNOS-AEQ was once again greater than that of G2AeNOS-AEQ, confirming our results in COS-7 cells. Upon stimulation of BAECs expressing the AEQ fusion constructs, the WTeNOS and G2AeNOS constructs reported no significant difference in coelenterazine burnout. This differs slightly from the results obtained in COS-7 cells (where G2AeNOS displayed significantly higher burnout than WTeNOS) and may reflect either dimerization issues (G2AeNOS-AEQ being taken to the membrane after dimerizing with endogenous eNOS) or possibly some subtle differences in Ca\(^{2+}\) signaling between endothelial cells and COS-7 cells. Nonetheless, the pertinent finding: that stimulation with ionomycin does not produce greater burnout of the membrane-bound probe than
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the cytosolic probe, was confirmed in BAECs as well as COS-7 cells. In addition, immunoblotting demonstrated that the differences we observed in Ser617, Ser635, and Ser1179 phosphorylation of WT eNOS and G2AeNOS in COS-7 cells are preserved in BAECs, further supporting the physiological relevance of our findings in COS-7 cells.

In conclusion, we have presented direct evidence that the functional differences between the WT and myristylation-deficient, cytosolic G2AeNOS in COS-7 cells are preserved in BAECS, further supporting the physiological relevance of our findings in COS-7 cells.

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