Regulation by cAMP of Post-translational Processing and Subcellular Targeting of Endothelial Nitric-oxide Synthase (Type 3) in Cardiac Myocytes*

(Received for publication, October 1, 1996, and in revised form, February 4, 1997)

Laurent Belhassen, Olivier Feron‡, David M. Kaye, Thomas Michel§, and Ralph A. Kelly¶

From the Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115

Cardiac myocytes express the nitric-oxide synthase isoform originally identified in endothelial cells, termed eNOS or NOS3, where it plays a role in regulating myocyte responsiveness to both adrenergic and muscarinic cholinergic autonomic nervous system agonists. eNOS in endothelial cells has been shown to undergo extensive post-translational processing, and in cardiac myocytes as well as endothelial cells, eNOS has been shown to be targeted to plasmalemmal caveolae, a process that is dependent on myristoylation and palmitoylation. Other post-translational modifications essential for the correct subcellular targeting of eNOS have not been described previously. We demonstrate, using [35S]methionine pulse-chase experiments, that native eNOS in adult rat ventricular myocytes is initially translated as a nonpalmitoylated 150-kDa isoform, which is associated with cytosolic and intracellular membrane-enriched fractions. This is subsequently processed to a palmitoylated 135-kDa isoform, which is found only in a sarcolemma-enriched membrane fraction. Forskolin, an agent that elevates intracellular cAMP, rapidly inhibited processing of the 150-kDa isoform to the 135-kDa isoform and transport of eNOS to the sarcolemma, effects paralleled by protein kinase A-dependent phosphorylation of the larger eNOS isoform. Forskolin also decreased palmitoylation of the 135-kDa isoform, although it did not accelerate depalmitoylation of sarcolemmal eNOS, as determined by pulse-chase experiments with [3H]palmitate. Thus, post-translational processing of a 150-kDa isoform of myocyte eNOS appears to be necessary for intracellular trafficking of the enzyme to sarcolemmal caveolae. Both the post-translational processing and subcellular targeting of eNOS appear to be modified by changes in intracellular cAMP, an effect that may have important implications for cardiac myocyte responsiveness to autonomic agonists in vivo.

The constitutively expressed, calcium-sensitive isoform of nitric-oxide synthase originally described in large vessel endothelial cells, termed eNOS1 or NOS3, is now known to be expressed in a number of cell types. In the heart, eNOS is present within the endothelium of the epicardial and microvascular coronary vessels and the endocardium, but also in cardiac myocytes and in specialized cardiac pacemaker and conduction tissue, such as sinoatrial and atrioventricular nodal cells (Refs. 1–4; for a review, see Ref. 5). eNOS is activated at increased pacing frequencies in cardiac myocytes and also appears to regulate cardiac myocyte contractile responsiveness to both adrenergic and muscarinic cholinergic autonomic nervous system agonists (1–7). Although originally presumed to be constitutively expressed in most cell types, it is now known that eNOS expression is regulated. Inflammatory cytokines decrease endothelial cell eNOS mRNA abundance (8), and, as we have demonstrated previously, agonists that increase intracellular cAMP in cardiac myocytes in vitro or in myocytes in situ in intact hearts, gradually decrease eNOS mRNA, protein, and activity levels (9).

All NOS isoforms undergo some degree of subcellular compartmentation. Neuronal NOS (nNOS or NOS1) is associated with post-synaptic density protein-95 and -93 in neurons and with α1-syntrophin in skeletal muscle at an amino-terminal binding domain (PDZ domain) unique to this isoform (10, 11). The cytokeine-inducible NOS (iNOS or NOS2) appears also to undergo subcellular compartmentation, although neither the specific compartment nor the targeting sequence and/or relevant post-translational modification(s) are known (12). As in endothelial cells, eNOS in cardiac myocytes is targeted to specialized plasmalemmal and internal membrane-associated glycosphingolipid- and cholesterol-enriched microdomains that, in association with the integral transmembrane protein caveolin, form caveolae (13–15). In addition to plasmalemmal membranes, both these specialized lipid microdomains and caveolins are associated with intracellular compartments including a non-clathrin-coated “light vesicular fraction,” the Golgi apparatus, and the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (16–18). Efficient targeting of eNOS to these lipid microdomains, including plasmalemmal caveolae, requires co-translational myristoylation on an N-terminal glycine residue and post-translational palmitoylation of cysteines 15 and 26 (19–22). Palmitoylation appears to be dynamically regulated in endothelial cells, since agents such as bradykinin induce depalmitoylation and translocation of eNOS from plasmalemmal to cytosolic fractions.

* This work was supported by National Institutes of Health Grants HL52320 (to R. A. K.) and HL46457 (to T. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

‡ Supported by a fellowship from the Belgian American Educational Foundation and by a grant from the “Patrimoine Facultaire de l’UCL” (Belgium).

§ A Wyeth-Ayerst Established Investigator of the American Heart Association.

¶ To whom correspondence should be addressed: Cardiovascular Division, Brigham and Women’s Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115. Tel.: 617-732-7503; Fax: 617-732-5132; E-mail: rakelly@bwh.harvard.edu.

1 The abbreviations used are: eNOS or NOS3, endothelial isoform of nitric-oxide synthase; NOS, nitric-oxide synthase; NO, nitric oxide; ARVM, adult rat ventricular myocyte(s); ERGIC, endoplasmic reticulum Golgi intermediate compartment; nNOS or NOS1, neuronal isoform of nitric-oxide synthase; iNOS or NOS2, inducible isoform of nitric-oxide synthase; PAGE, polyacrylamide gel electrophoresis; IM, internal membrane-enriched; PM, plasmalemma-enriched membrane; C, cytosolic.
malemual caveolae to an intracellular compartment (13, 20). This may have important consequences for eNOS-dependent signaling in endothelial cells and cardiac myocytes, given the increasing evidence that these glycosphingolipid- and cholesterol-enriched microdomains and caveolae act to spatially constrain a number of transmembrane and intracellular signal transduction pathways (23–26). Finally, eNOS has been shown to undergo additional post-translational modifications, including phosphorylation by a calmodulin kinase (21). The importance of these post-translational modifications to subcellular targeting or to regulation of enzyme activity is not yet understood.

We report here that, in cardiac myocytes, eNOS is initially translated as a 150-kDa precursor that is subsequently processed to a 135-kDa protein. Subcellular fractionation studies revealed that the 150-kDa isoform of eNOS is found in intracellular compartments but not in a sarcomembrane fraction. Although both isoforms associate with caveolin-3, a muscle-specific caveolin, and therefore are likely present in glycosphingolipid- and cholesterol-enriched microdomains and in caveolae, only the 135-kDa isoform appears to be palmitoylated. Agents that elevate intracellular cAMP rapidly decrease the activity of eNOS, and therefore are likely present in the 135-kDa isoform and its translocation to the sarcolemma.

MATERIALS AND METHODS

Cell Culture and Labeling—Adult rat ventricular myocytes (ARVM) were isolated as described previously (27). Myocytes were cultured in a defined medium, termed ACCITT (described in Ref. 28). Cells were exposed to forskolin were diluted in dimethyl sulfoxide (Me2SO) (Sigma). For in vivo studies of eNOS palmitoylation, cells were incubated with 3H-palmitate (DuPont NEN). Drugs were applied at the end of this 2-h period. Control and treated plates were harvested at the same time. In vivo studies of eNOS palmitoylation were performed as described previously (13, 21). Briefly, ARVM were incubated for 2 h in ACCITT containing 1 mM nitro-L-arginine, an inhibitor of NOS activity. The specific signal was calculated by subtracting the background signal, determined in the presence of a NOS inhibitor. These results were normalized to protein content, as determined by a modified Lowry assay. The null hypothesis was rejected at \( p < 0.05 \).

RESULTS

Subcellular Localization of eNOS in ARVM—To evaluate the subcellular distribution of eNOS within cardiac myocytes, two separate cell fractionation techniques were used: a two-fraction technique that we have employed previously that yields "particulate" and "soluble" fractions. Briefly, ARVM were scrapped into buffer F and sonicated (Branson Sonifier 450; Branson Ultrasonic Corp., Danbury, CT; three 10-s bursts; output power = 1), and then ultracentrifuged (Beckman L5-65) at 100,000 \( \times g \) for 1 h, washed once in buffer F, and ultracentrifuged again at 100,000 \( \times g \) for 30 min. The pellet was resuspended in buffer F with 1% Triton X-100 ("particulate fraction"). 1% Triton X-100 (final concentration) was also added to the supernatant ("soluble fraction").

In other experiments, the protocol described by Mery et al. (29) was used, with modifications. After sonication, ARVM lysates were centrifuged at low speed (8,000 \( \times g \)) for 15 min. The pellet was washed once in buffer F and then resuspended in buffer F with 1% Triton X-100. This fraction is referred as the "plasmalemma-enriched fraction." The supernatant was then processed according to the protocol described above by Busconi and Michel (19). The pellet and the supernatant obtained are referred to as the "internal membrane-enriched" (IM) and the "cytosolic" (C) fractions. Antibodies to Na,K-ATPase (ABD Reagents) and to mannose-6-phosphate II (Babco) were used as markers for sarcolemmal and internal membranes (i.e. Golgi apparatus), respectively, by Western blot.

Immunoprecipitation—For eNOS immunoprecipitation as well as for Western blots, a monoclonal antibody against human eNOS was used (Transduction Labs), according to the protocol recommended by the manufacturer (i.e. 2 \( \mu g \) of eNOS antibody was combined with 500 \( \mu g \) of ARVM lysate) (27). Immunoprecipitations were performed as described previously (14) with minor modifications. Briefly, after subcellular fractionation, eNOS was immunoprecipitated with anti-eNOS antibody in buffer F plus detergents, as noted above, containing 4 \( \mu M \) tetrahydrobiopterin and 1 mM L-arginine. All samples were run on 12% SDS-PAGE gels, and membranes were subsequently incubated with an anti-caveolin-3 monoclonal antibody (Transduction Labs) and processed as described below.

SDS-PAGE and Immunoblotting—Unlabeled or \(^{32}P\)-labeled denatured proteins in Laemmli sample buffer (30) were loaded on 7.5% SDS-PAGE gels (Ready-Gels, Bio-Rad) and transferred onto polyvinylidene difluoride membranes (Bio-Rad). As described previously (9, 14), the membranes were blocked, incubated for 1 h with the primary antibodies, anti-eNOS, anti-iNOS, or anti-nNOS (Transduction Laboratories), or anti-NOS-\( \mu \) (a gift of Dr. David S. Brodtk), washed six times in TBST (Tris-buffered saline containing 0.1% Tween 20, Sigma), and incubated for an additional 30 min with the secondary antibody. Membranes were stripped in a buffer containing 50 mM Tris, 2% SDS, and 10 mM \( \beta \)-mercaptoethanol at 55 °C for 30 min, washed six times in TBST, and immunoblotted with another antibody. For \(^{32}P\)-labeled-proteins, this procedure was used to verify equivalent loading among lanes. The activity of \(^{32}P\)-labeled proteins was obtained by exposing the polyvinylidene difluoride membranes to x-ray film for 24 h after immunoblotting. \(^{35}S\)- or \(^3\)H-labeled denatured proteins in Laemmli sample buffer were loaded onto 7.5% SDS-PAGE gels (Ready-Gels, Bio-Rad). Gels were incubated in \( \text{En'Hance} \) (DuPont NEN), washed in water, and dried for 2 weeks at \(-70^\circ\). Measurements of eNOS Enzymatic Activity—NOS activity was assayed as described previously using anion exchange chromatography to measure the conversion of \( \text{L-}^{[14]N}\text{arginine into L-}^{[14]C}\text{tricarboxylate (11). Assays were always performed in parallel with and without 1 mM CaCl}_2 as a screen for "calcium-insensitive" (i.e. inducible) nitric-oxide synthase activity in ARVM lysates. Parallel reactions were performed also in buffer containing 1 mM nitro-L-arginine, an inhibitor of NOS activity. The specific signal was calculated by subtracting the background signal, determined in the presence of a NOS inhibitor. These results were normalized to protein content, as determined by a modified Lowry assay (Bio-Rad). The amount of eNOS enzymatic activity is also presented in some experiments normalized to the amount of eNOS protein in each fraction for each experiment, as determined by densitometric analyses of Western blot autoradiograms of equal amounts of ARVM lysates (i.e. 25 \( \mu g \) procured in parallel with lysates used to determine eNOS enzymatic activity.

Statistical Methods—Data are presented as means \( \pm \) S.E. Comparisons among groups were performed by Student's \( t \) tests where data were normally distributed or by a Mann-Whitney test for nonparametric data. The null hypothesis was rejected at \( p < 0.05 \).

11997
Regulation of eNOS Post-translational Processing by cAMP

FIG. 1. Two isoforms of native eNOS can be resolved in cellular lysates from ARVM. ARVM lysates were fractionated, and equal amounts of protein were loaded onto 7.5% SDS-PAGE and then immunoblotted to detect eNOS and caveolin-3 monoclonal antibodies. Membranes were then stripped and successively hybridized with monoclonal antibodies against mannose-6-phosphate receptor (a Golgi marker), and then against Na,K-ATPase (a sarcolemmal marker). A, a subcellular (two-fraction) separation technique that yields a particulate (P) and a soluble (S) fraction (see "Materials and Methods"), and both a 135- and 150-kDa isoform of eNOS could be resolved. As expected, Na,K-ATPase and mannose-6-phosphate receptor localized to the particulate fraction. Caveolin-3 also localized to the particulate fraction, although longer exposure revealed its presence also in the cytosolic fraction. B, the procedure of sequential immunoblotting with antibodies to eNOS and caveolin-3, followed by an antibody to mannosidase II, after which Na,K-ATPase was applied to ARVM lysates after processing using a three-fraction technique. eNOS could be detected in PM, C, and IM fractions. Na,K-ATPase localized almost exclusively to plasmalemmal-enriched membranes, whereas the higher molecular mass band of caveolin-3 localized to the internal membrane-enriched fraction. Caveolin-3 was found both in plasmalemmal and internal membrane-enriched fractions and after longer exposure times in the cytosolic fraction as well. Subcellular fractionations and immunoblots were performed three times with similar results.

clonal antibodies against eNOS and caveolin-3, and then stripped and reprobed successively with monoclonal antibodies against Na,K-ATPase and mannose-6-phosphate receptor. As expected, Na,K-ATPase localized to the particulate fraction, using the two-fraction protocol, or to the plasmalemma-enriched fraction, using the three-fraction protocol, and mannose-6-phosphate receptor localized to the particulate and the internal membrane-enriched fractions, respectively. As we have shown previously (1), about 80% of eNOS was in the particulate and 20% in the cytosolic fraction, using the two-fraction technique (Fig. 1A). When equal amounts of protein were loaded, however, there was no significant difference in the eNOS concentration between the plasmalemma-enriched and internal membrane-enriched fractions. Therefore, since the total amount of protein recovered from the sarcolemmal fraction is 6 times greater than in the IM fraction from the same cell lysate, the large majority of eNOS in cardiac myocytes must be localized to sarcolemmal membranes.

We have shown previously that eNOS is found in caveolae both in bovine aortic endothelial cells and in cardiac myocytes, in association with caveolin-1 and caveolin-3, respectively (14). Therefore, we also investigated the localization of caveolin-3, a muscle-specific caveolin, using both cell fractionation techniques. As shown in Fig. 1, caveolin-3 was present mainly in the particulate fractions using the two-fraction technique, and predominantly in the plasmalemma-enriched and internal membrane-enriched fractions using the three-fraction method, although a faint band could be observed in the cytosol as well. This observation was also consistent with data from other cell types on caveolin cycling between plasmalemmal and internal compartments (18).

Interestingly, we identified two bands in protein extracts from ARVM running at 135 and 150 kDa on 7.5% SDS-PAGE that were specific for eNOS by immunoblotting. After subcellular fractionation using the three-fraction technique, the lower molecular mass band (135 kDa) was exclusively localized to the plasmalemma-enriched membranes, whereas the higher molecular mass band (150 kDa) could be detected only in the cytosolic and IM fractions. As a control, we verified that doublet bands were recovered only after mixing of sarcolemma-enriched and IM fractions, or sarcolemma-enriched and cytosolic fractions, but not from mixing IM and cytosolic fractions (data not shown). These two bands were also recovered after immunoprecipitation with the eNOS monoclonal antibody (Transduction Lab) and were not detected after immunoblotting using either monoclonal iNOS (NOS2) or nNOS (NOS1)-specific antibodies, or by a polyclonal antibody directed against NOS-μ (data not shown). Although the reason(s) for these differences in the migration pattern of the two bands on 7.5% SDS-PAGE gel is still unclear, this provides a useful tool to determine eNOS subcellular localization (i.e. cytosol, internal membrane, or sarcolemma) as a function of its apparent molecular mass.

Using pulse-chase experiments with [35S]methionine labeling in cultured ARVM (see “Materials and Methods”) followed by immunoprecipitation with eNOS-specific antibodies, we determined that the 150-kDa eNOS band, localized as shown in Fig. 1B to the IM fraction, was processed and translocated to the plasma membrane (where the 135-kDa isoform localizes specifically). After a 1-h pulse labeling, ARVM were washed and incubated in buffer containing unlabeled methionine. As shown in Fig. 2 (A and B), radioactivity in eNOS immunoprecipitates prepared from whole myocyte lysates was first incorporated into a 150-kDa band, while minimal labeling of the 135-kDa band could be detected (the 1-h exposure to [35S]methionine is sufficient time for some newly synthesized eNOS to be processed to the 135-kDa isoform). After the chase, the 135-kDa band became labeled as the labeling of the 150-kDa isoform rapidly declined with a half-life of the larger M, isoform of approximately 1 h. These data suggest that the 135-kDa band is derived from the 150-kDa band and that, given the specific subcellular localization of each band as indicated in Fig. 1, eNOS appears to be synthesized with an apparent molecular mass of 150 kDa and then processed and transported to the sarcolemma in cardiac myocytes.

We also measured eNOS enzymatic activity in each subcellular fraction to determine whether these differences in electrophoretic mobility would affect its activity. Following subcellular fractionation, equal amounts of cell lysates from each fraction were used for determination of enzymatic activity and for a parallel eNOS immuno blot. All enzymatic assays were performed in the presence and absence of 1 mM CaCl₂ and in the presence and absence of 1 mM nitro-l-arginine to measure
Ca\textsuperscript{2+}-sensitive, NOS-specific activity. There was approximately 2-fold higher maximal NOS activity in the cytosolic and internal membrane-enriched fractions (85 ± 21 pmol/min, 178 ± 34 pmol/min, and 187 ± 97 pmol/min in PM, C, and IM fractions, respectively) compared with maximal NOS activity in the sarcolemma-enriched fraction, measured with saturating concentrations of substrate and co-factors. Calcium-sensitive NOS activity in each fraction has been corrected for the relative intensity of bands of the appropriate size identified on eNOS immunoblots performed in parallel on each subcellular fraction and no change in the distribution of caveolin-3 among those three fractions over this time period. Changes in native eNOS isoform distribution over 2 h with forskolin were accompanied by changes in maximal eNOS activity in each subcellular fraction using the three-fraction method, the rate of L-[3H]arginine conversion to L-[3H]citrulline was measured in each fraction. The figure illustrates combined data from four experiments expressed as a percentage of the native eNOS activity in each fraction in the absence of forskolin, normalized either to the amount of total protein in PM, C, and IM fractions (open bars) or to the relative amount of eNOS in each fraction (solid bars), determined by the hybridization intensity for eNOS in each fraction by parallel immunoblots performed with each experiment (mean ± S.E.; *, p < 0.01 compared with the PM fraction). The significant increase in eNOS activity mg of protein in subcellular fractions from myocytes pretreated with forskolin (open bars) is due to the increased eNOS content of these fractions (closed bars).

**FIG. 3.** Forskolin regulates post-translational processing of native eNOS in ARVM. A and B, myocyte cell lysates processed using the three-fraction subcellular fractionation technique were prepared from cells exposed to 10 \( \mu \)M forskolin for 2 h, resolved with 7.5% SDS-PAGE, and immunoblotted with both anti-eNOS and anti-caveolin-3-specific antibodies. As shown in A, the higher Mr isoform of eNOS was enriched in the C and IM fractions compared with the PM fraction in forskolin-pretreated cells, although there was no obvious shift in the distribution of caveolin-3 among these three fractions. Combined data from four experiments are shown in B for total eNOS abundance, expressed as a percentage of that present in plasmalemmal membranes from control, non-forskolin-treated cells (CON), following normalization for the protein content of each fraction (mean ± S.E.; *, p < 0.05 compared with the same fraction in control). C and D, to verify that forskolin inhibited post-translational processing of the 150-kDa band and its translocation to the sarcolemma, eNOS immunoprecipitates were prepared from whole myocyte lysates and resolved by 7.5% SDS-PAGE, from ARVM previously exposed to [35S]methionine for 1 h and then incubated in the presence of unlabeled methionine for up to 24 h in the continuous presence of 10 \( \mu \)M forskolin. A representative autoradiogram is shown in C, and data from three independent experiments are shown in D, with the intensity of the 150-kDa band (open bars) and 135-kDa band (solid bars) expressed as a fraction of the intensity of the 150-kDa band, in arbitrary units, at time 0 (i.e. immediately after a 1-h pulse with [35S]methionine). E, to determine whether the changes in native eNOS isoform distribution over 2 h with forskolin were accompanied by changes in maximal eNOS activity in each subcellular fraction using the three-fraction method, the rate of L-[\textsuperscript{3}H]arginine conversion to L-[\textsuperscript{3}H]citrulline was measured in each fraction. The figure illustrates combined data from five experiments expressed as a percentage of the native eNOS activity in each fraction in the absence of forskolin, normalized either to the amount of total protein in PM, C, and IM fractions (open bars) or to the relative amount of eNOS in each fraction (solid bars), determined by the hybridization intensity for eNOS in each fraction by parallel immunoblots performed with each experiment (mean ± S.E.; *, p < 0.01 compared with the PM fraction). The significant increase in eNOS activity mg of protein in subcellular fractions from myocytes pretreated with forskolin (open bars) is due to the increased eNOS content of these fractions (closed bars).
150-kDa band with forskolin (mean ± S.E. of data from four independent experiments).

Fraction technique. Fig. 3E shows eNOS activity, expressed both per mg of protein in each subcellular fraction (open bars) and as a function of the amount of immunoreactive eNOS present in each fraction (solid bars). Maximal enzyme activity was significantly increased after forskolin exposure in both the IM and cytosolic fractions, whereas no significant change in activity was observed in the sarcolemma-enriched fraction. These results paralleled the changes in subcellular localization of eNOS after exposure to forskolin, as determined by Western blotting (Fig. 3, A and B). To determine whether this increase in apparent V_max represented an increase in activity per mg of eNOS protein or an increase in the amount of eNOS protein, the measurement of enzymatic activity was normalized to the relative amount of eNOS in each fraction. As shown in Fig. 3E, the increase in eNOS activity per mg of protein in the cytosolic and internal membrane-enriched fractions following forskolin was due to the increased eNOS protein content in these fractions.

cAMP-dependent Phosphorylation of eNOS in Vivo—eNOS has a consensus sequence for PKA-dependent phosphorylation. In endothelial cells, phosphorylation of eNOS by bradykinin but not forskolin was associated with translocation of the enzyme from a particulate fraction to the cytosol (22). To determine whether forskolin would initiate protein kinase A-dependent phosphorylation of eNOS in intact ARVM, ARVM were incubated with [32P]orthophosphate and exposed to 10 μM forskolin (FK) for 20 min, with or without preincubation with 1 μM KT5720, an inhibitor of protein kinase A, and subsequently resolved by 7.5% SDS-PAGE. An eNOS immunoblot was performed on the same membrane used to prepare the autoradiogram shown in A. The autoradiograms shown are representative of four independent experiments.

Effect of forskolin on phosphorylation of the 150- and 135-kDa isoforms of eNOS. A, eNOS immunoprecipitates of whole ARVM lysates were prepared from myocytes labeled with [32P]orthophosphate for 2 h, in the presence or absence of 10 μM forskolin (FK) for the last 20 min of labeling, either without or with 1 μM KT5720 (KT), an inhibitor of protein kinase A, and subsequently resolved by 7.5% SDS-PAGE. B, An eNOS immunoblot was performed on the same membrane used to prepare the autoradiogram shown in A. The autoradiograms shown are representative of four independent experiments.

1.8, the majority of phosphorylated eNOS in these cells is the 150-kDa isoform.

cAMP and eNOS Palmitoylation in ARVM—Palmitoylation appears to be required for efficient targeting of eNOS to caveolae in COS cells transfected with wild-type or palmitoylation-deficient eNOS mutants (13, 20, 21). It has also been shown that palmitoylation is a dynamic process and that depalmitoylation is regulated by bradykinin in endothelial cells. Thus, we examined whether increased intracellular cAMP would affect eNOS palmitoylation in cardiac myocytes, by examining cell lysates from ARVM that had been exposed to [3H]palmitate for 2 h in the absence or presence of forskolin. As shown in Fig. 5A, the only [3H]palmitate-labeled eNOS band is the 135-kDa band associated with the sarcolemma-enriched fraction. No 150-kDa band could be detected, suggesting that this eNOS isoform is not palmitoylated during the period of biosynthetic labeling. It is possible that the assay described here for the detection of eNOS palmitoylation was insufficiently sensitive to detect low, but biologically relevant levels of acylation of the less abundant 150-kDa isoform. If this were true, some degree of [3H]palmitate labeling should have been apparent in the immunoprecipitated eNOS lane under control conditions (Fig. 5A, upper part, lane 3). Moreover, after 2 h of incubation in forskolin when the ratio of the 135-kDa isoform to the 150-kDa isoform had declined to only 1.2 (see Fig. 3B), there was no detectable palmitoylation of the 150-kDa band. Two bands corresponding to the 150- and 135-kDa isoforms can be identified in a parallel immunoblot performed on the eNOS immunoprecipitate from this experiment (Fig. 5A, lower part).

When myocytes were exposed to forskolin during [3H]palmitate labeling, palmitate labeling of the 135-kDa eNOS signal was markedly reduced. As shown in Fig. 3B, this cannot be explained solely by removal and degradation of the lower M₆ eNOS from the sarcolemma-enriched fraction caused by only a 2-h exposure to forskolin. This happened without a noticeable change in palmitoylation of nonimmunoprecipitated proteins remaining in the supernatant, suggesting that the forskolin-mediated effect may be relatively specific for eNOS palmitoylation. To determine whether elevated intracellular cAMP would also affect eNOS depalmitoylation, pulse-chase experiments were performed in myocytes labeled for 2 h with [3H]palmitate and subsequently incubated in buffer containing unlabeled palmitate in the presence or absence of 10 μM for-
The recognition that there were two distinct eNOS $M_r$ species was evident only when ARVM eNOS whole cell lysate immunoprecipitates were resolved by 7.5% SDS-PAGE, rather than on the 12% gels employed by this laboratory in previous studies of this cell type (1, 14). In addition, due to the approximately 8-fold greater absolute abundance of the 135-kDa isoform, a 150-kDa band was not observed until longer exposure times for Western blots were routinely used. Interestingly, in a report by Busconi and Michel (19), doublet bands corresponding to 150- and 135-kDa proteins could be resolved by 7% SDS-PAGE of cell lysates of COS-7 cells that had been transfected with either wild-type eNOS or myristoylation-deficient eNOS mutant cDNAs. Since this eNOS doublet was only observed in lysates from transfected COS-7 cells and not in lysates from bovine aortic endothelial cells that express a native eNOS, where only the 135-kDa isoform could be resolved with either 7 or 12% SDS-PAGE gels, it was presumed that the higher $M_r$ band represented either a post-translational modification of eNOS that was more prominent in COS-7 cells or an anomalous processing of the transfected eNOS cDNA in these cells. Neither band, however, was localized to the particulate fraction in COS-7 cells transfected with the myristoylation-deficient mutant.

Several explanations are possible for the slower migrating 150-kDa band on SDS-PAGE. Based on the eNOS mRNA sequence, the predicted size of the protein is 135 kDa. Nevertheless, as noted above, transfection of COS-7 cells with the full-length eNOS cDNA yielded both 135- and 150-kDa bands (19). While differences in phosphorylation constitute one possible explanation, it is worth noting that increased phosphorylation by PKA in response to forskolin (Fig. 4) did not affect migration of either the 135- or 150-kDa band. Palmitoylation is also an unlikely explanation, since this post-translational modification has not been shown to affect eNOS mobility on 7% SDS-PAGE (19–22). Incomplete reduction of disulfide bonds is also an unlikely explanation, since the loading conditions are identical for both eNOS bands, and both bands can be recovered when plasmalemmal and internal membrane samples are combined. Finally, a consensus sequence for glycosylation does exist on eNOS, although glycosylation of this enzyme has not yet been reported. The identification of the post-translational processing responsible for the presence of the two apparent eNOS $M_r$ species is being actively investigated.

The addition of an initial low speed centrifugation step following preparation of ARVM lysates by sonication, followed by the same subcellular fractionation technique used in the report by Busconi and Michel (19) on the remaining supernatant, permitted the preparation of sarcolemmal membrane-enriched fractions identified by the presence of Na,K-ATPase only in this fraction. This technique does not permit quantitative separation of "plasma membranes" from "internal membranes" and "cytosolic" fractions of these cells. Nevertheless, it was in this fraction exclusively that the 155-kDa fraction was identified, as shown in Fig. 1B. The identity of the subcellular compartments containing the 150-kDa isoform is not resolved here, but these compartments are likely to include, based on the co-localization of mannosidase II in this fraction, portions of the Golgi apparatus or ERGIC complex as had been reported previously for eNOS in bovine aortic and bovine pulmonary microvascular endothelial cells (15, 31).

Our finding that caveolin-3 can be detected in both the plasmalemmal membrane- and internal membrane-enriched fractions is consistent with previous reports by us and by others that eNOS (at least the 135-kDa isoform) is associated with plasmalemmal caveolae in endothelial cells and cardiac myocytes (13–15). This is because caveolae (and, therefore, caveolins and associated glycosphingolipid- and cholesterol-enriched microdomains) have been shown to participate in intracellular trafficking into and from the Golgi apparatus and the ERGIC as well as from subplasmalemmal light vesicular fractions (16–18, 32, 33). However, the observation that only the 135-kDa isoform appears to be palmitoylated, as shown in Fig. 5, suggests that palmitoylation is necessary for eNOS association with sarcolemmal caveolae, since only the 135-kDa isoform, but not the higher $M_r$ eNOS species, could be identified in Na,K-ATPase-enriched myocyte subcellular fractions. This is consistent with a recent report from this laboratory, using a detergent-free technique for the isolation of plasmalemmal caveolae only, that showed that palmitoylation-deficient eNOS mutants expressed in COS-7 cells were not enriched in a caveolar fraction prepared from plasma membranes compared with COS-7 cells transfected with wild-type eNOS (13). The data reported in the present manuscript are also consistent with the observation by Garcia-Cardena (15) that native, acylated eNOS in rat pulmonary microvascular endothelial cells is present within glycosphingolipid-, cholesterol- and caveolae-enriched plasmalemmal microdomains isolated using a method of in situ perfusion of rat lungs with a solution of positively charged colloidal silica particles (34). These authors also noted that a palmitoylation-deficient eNOS mutant expressed in NIH 3T3 cells co-localized with caveolins in internal membranes (with a perinuclear distribution consistent with Golgi or ERGIC localization) but not with caveolins in the plasma membrane (15). This was in contrast to wild-type eNOS, which co-localized with caveolin in both internal and plasmalemmal membranes. As shown in the present study, palmitoylation of the mature 135-kDa isoform is associated with trafficking of native eNOS to the sarcolemma in myocytes.

The observation that forskolin, an agent that increases intracellular cAMP by directly activating adenyl cyclase, could interrupt processing of the 150-kDa eNOS precursor to the lower $M_r$ isoform, has not previously been reported. Indeed, as shown in Fig. 3C, little $[35]$methionine-labeled 135-kDa band could be identified in 7.5% SDS-PAGE gels of lysates that had been prepared from forskolin-treated cardiac myocytes during chase periods as long as 24 h after pulse labeling. In contrast, in the absence of forskolin, most of the 150-kDa band is processed to the 135-kDa form within 4 h, following a $[35]$methionine pulse, as shown in Fig. 2. Therefore, increased intracellular cAMP appears to prevent the trafficking of mature, palmitoylated native eNOS to sarcolemmal caveolae.

Interestingly, Mineo et al. (35) have recently shown that the recruitment of Raf-1 to plasmalemmal caveolae in rat-1 cells, following their stimulation with EGF, could be blocked by treating these cells with either forskolin or with 8-bromo-cAMP, a nonhydrolyzable cell-permeant cAMP analogue. Thus, elevated cAMP may interrupt trafficking to plasmalemmal caveolae of a number of proteins involved in signal transduction, including both growth factor-dependent and NO-dependent signaling pathways. Given the emerging evidence in support of a role for endogenous production of NO in mediating muscarinic cholinergic regulation of cardiac myocyte responsiveness to β-adrenergic agonists (1–5), the data reported here suggest that even short term elevations in intracellular cAMP may rapidly uncouple NO-dependent signaling by inhibiting post-translational processing of eNOS and its translocation to plasmalemmal caveolae.
Acknowledgment—We thank Dr. Kazuhiro Sase for helpful discussions.

REFERENCES
1. Balligand, J.-L., Kobzik, L., Han, X., Kaye, D. M., Belhassen, K., O’Hara, D. S., Kelly, R. A., Smith, T. W., and Michel, T. (1995) J. Biol. Chem. 270, 14582–14586
2. Han, X., Shimoni, Y., and Giles, W. R. (1994) J. Physiol. 476, 309–314
3. Han, X., Shimoni, Y., and Giles, W. R. (1995) J. Gen. Physiol. 106, 45–65
4. Han, X., Kobzik, L., Balligand, J.-L., Kelly, R. A., and Smith, T. W. (1996) Circ. Res. 78, 998–1008
5. Kelly, R. A., Balligand, J.-L., and Smith, T. W. (1996) Circ. Res. 79, 363–380
6. Kaye, D. M., Wiviott, D. S., Balligand, J.-L., Simmons, W. W., Smith, T. W., and Kelly, R. A. (1996) J. Pharmacol. Exp. Ther. 272, 945–952
7. Finkel, M. S., Oddis, C. V., Mayer, O. H., Hattler, B. G., and Simmons, R. L. (1995) J. Pharmacol. Exp. Ther. 272, 945–952
8. Yoshizumi, M., Perella, M., Burnett, J. C., Jr., and Lee, M. E. (1993) Circ. Res. 73, 205–209
9. Belhassen, K., Kelly, R. A., Smith, T. W., and Balligand, J.-L. (1996) J. Clin. Invest. 97, 1908–1915
10. Brenman, J. E., Chao, D. S., Xin, H., Aldape, K., and Bredt, D. S. (1995) Cell 82, 743–752
11. Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Frochnner, S. C., and Bredt, D. S. (1996) Cell 84, 757–767
12. Vodovotz, Y., Russel, D., Xie, Q.-W., Rogdan, C., and Nathan, C. (1995) J. Immunol. 154, 2914–2925
13. Shaub, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhunnna, I. S., Ying, Y., Anderson, R. G. W., and Michel, T. (1996) J. Biol. Chem. 271, 6518–6522
14. Feron, O., Belhassen, L., Kobzik, L., Smith, T. W., Kelly, R. A., and Michel, T. (1996) J. Biol. Chem. 271, 22810–22814
15. Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J. E., and Sessa, W. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6448–6453
16. Anderson, R. G. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 90, 10909–10913
17. Parton, R. G., and Simons, K. (1995) Science 269, 1398–1399
18. Conrad, P. A., Smart, E. J., Ying, Y.-S., Anderson, R. G. W., and Bloom, G. S. (1995) J. Cell. Biol. 131, 1421–1433
19. Busconi, L., and Michel, T. (1993) J. Biol. Chem. 268, 8410–8413
20. Robinson, L. J., Busconi, L., and Michel, T. (1995) J. Biol. Chem. 270, 995–998
21. Robinson, K. J., and Michel, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11776–11780
22. Michel, T., Li, G. K., and Busconi, L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6252–6256
23. Liu, P., Ying, Y., Ko, Y.-G., and Anderson, R. G. W. (1996) J. Biol. Chem. 271, 10299–10303
24. Sevinsky, J. R., Rao, L. V. M., and Ruf, W. (1996) J. Cell. Biol. 133, 293–304
25. Li, S., Seitz, R., and Lisanti, M. P. (1996) J. Biol. Chem. 271, 3863–3868
26. Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sardiaco, M., and Lisanti, M. P. (1996) J. Biol. Chem. 271, 9690–9697
27. Berger, H.-J., Prasad, S. K., Davidoff, A. J., Pimental, D., Ellingsen, Ø., Marsh, J. D., Smith, T. W., and Kelly, R. A. (1994) Am. J. Physiol. 266, H341–H349
28. Ellingsen, Ø., Davidoff, A. J., Prasad, S. K., Berger, H.-J., Springhorn, J. P., Marsh, J. D., Kelly, R. A., and Smith, T. W. (1993) Am. J. Physiol. 265, H1747–H1754
29. Mery, P.-F., Pavoine, C., Belhassen, L., Pecker, F., and Fischmeister, R. (1993) J. Biol. Chem. 268, 26286–26295
30. Laemmli, U. K. (1970) Nature 227, 680–685
31. Sessa, W. C., Garcia-Cardena, G., Liu, J., Keb, A., Pollock, J. S., Bradley, J., Thiru, S., Braverman, J. M., and Desai, K. M. (1995) J. Biol. Chem. 270, 17641–17644
32. Parton, R. G., Jogerst, B., and Simons, K. (1994) J. Cell. Biol. 127, 1199–1215
33. Smart, E. J., Ying, Y., and Anderson, R. G. W. (1995) J. Cell. Biol. 131, 929–938
34. Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J., and Oh, P. (1995) Science 269, 1435–1439
35. Mineo, C., James, G. L., Smart, E. J., and Anderson, R. G. W. (1996) J. Biol. Chem. 271, 11930–11935