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Modulation of the Endothelial Nitric-oxide Synthase-Caveolin Interaction in Cardiac Myocytes

IMPLICATIONS FOR THE AUTONOMIC REGULATION OF HEART RATE*

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The endothelial isoform of nitric oxide synthase (eNOS) is dually acylated and thereby targeted to signal-transducing microdomains termed caveolae. In endothelial cells, eNOS interacts with caveolin-1, which represses eNOS enzyme activity. In cardiac myocytes, eNOS associates with the muscle-specific caveolin-3 isoform, but whether this interaction affects NO production and regulates myocyte function is unknown. We isolated neonatal cardiac myocytes from mutant mice with targeted disruption of the eNOS gene and transfected them with wild-type (WT) eNOS or myristoylation-deficient (myr$^-$) eNOS mutant cDNA. In myocytes expressing WT eNOS, the muscarinic cholinergic agonist carbachol completely abrogated the spontaneous beating rate and induced a 4-fold elevation of the cGMP level. By contrast, in the myr$^-$ eNOS myocytes, carbachol failed to exert its negative chronotropic effect and to increase cGMP levels. We then used a reversible permeabilization protocol to load intact neonatal rat myocytes with an oligopeptide corresponding to the caveolin-3 scaffolding domain. This peptide completely and specifically inhibited the carbachol-induced negative chronotropic effect and the accompanying cGMP elevation. Thus, our results suggest that acylated eNOS may couple muscarinic receptor activation to heart rate control and indicate a key role for eNOS/caveolin interactions in the cholinergic modulation of cardiac myocyte function.

The endothelial isoform of nitric-oxide synthase (eNOS)[1], originally identified in large vessel endothelium, is now known to be expressed in numerous cell types, including cardiac myocytes. The eNOS enzyme is dually acylated (for review, see Refs. 1 and 2) and is thereby specifically targeted to plasmalemmal signal-transducing microdomains termed caveolae (3, 4). In endothelial cells and cardiac myocytes, eNOS is quantitatively associated with caveolin (4), the structural protein of caveolae, and a stable protein-protein interaction takes place at consensus sequences present within both proteins and leads to the inhibition of the eNOS activity (5–8). More recently, we have also documented that, in endothelial cells, when intracellular Ca^{2+} concentration is increased by an agonist, a regulatory cycle is initiated (2, 9), wherein (i) Ca^{2+}/calmodulin activates eNOS by disrupting the heteromeric complex formed between eNOS and caveolin; (ii) activated, caveolin-free eNOS is translocated from caveolae, probably associated with enzyme desensitization; (iii) when Ca^{2+} returns to basal levels, eNOS reassociates with caveolin; and (iv) the inhibitory complex is restored to caveolae, a process facilitated by eNOS palmitoylation. The role (if any) of this eNOS-caveolin regulatory cycle in cardiac myocytes, however, remains to be established.

The location of eNOS in plasmalemmal caveolae and its interaction with caveolin may find several biological justifications. First, the compartmentation of eNOS with other signaling proteins may facilitate, or improve the efficacy of, the coupling between agonist stimulation and eNOS activation. We and others have, for instance, reported that G-protein-coupled receptors known to stimulate NO production, such as the muscarinic and bradykinin receptors, undergo rapid translocation and are targeted to caveolae upon agonist stimulation (10, 11). More recently, McDonald *et al.* (12) have reported the existence of a caveolar complex between the arginine transporter CAT1 and eNOS, thereby providing a mechanism for a highly efficient delivery of substrate to eNOS. Second, the close control of eNOS activity by caveolin is probably required to maintain a low basal production of NO and protect the cell from undesired, potentially cytotoxic bursts of NO in response to subtle increases in intracellular calcium. Finally, the targeting of eNOS in plasmalemmal caveolae probably facilitates paracrine signaling by NO, a pathway most clearly delineated in the vascular wall (13) and not yet clearly established in other tissues expressing eNOS.

In cardiac myocytes, eNOS is also located within plasmalemmal caveolae, but, to date, the NO generated has only been shown to act endogenously by modulating the myocyte responsiveness to neurohumoral or mechanical stimuli. Thus, for instance, activation of eNOS in cardiac myocytes by muscarinic

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[1] The abbreviations used are: eNOS, endothelial nitric oxide synthase; NO, nitric oxide; WT, wild-type; myr$^-$, myristoylation-deficient mutant; Cav-3, caveolin-3 scaffolding domain peptide; Cav-3X, Cav-3 scrambled peptide; L-NNA, N^{ω}-nitro-L-arginine; PBS, phosphate-buffered saline.

cholinergic agonist or by increased pacing frequencies leads to a net increase in cytosolic cGMP and subsequently to processes such as inactivation of L-type calcium current and alteration in myofilaments Ca^{2+} sensitivity (see Ref. 14 for review). eNOS targeting to plasmalemmal caveolae may serve to facilitate the enzyme's proximity to receptors and G-protein-dependent signaling cascades, as well as its interactions with regulatory proteins such as caveolin and calmodulin. The caveolin isoform expressed in myocytes (caveolin-3) is only found in muscle tissue (15, 16), and evidence for the involvement of caveolin-3 in the dynamic regulation of cardiac myocyte eNOS activity remains less well understood.

To explore the role of eNOS acylation and caveolar targeting in receptor-dependent responses in cardiac myocytes, we used the paradigm of the muscarinic cholinergic NO-mediated regulation of heart rate previously studied in our laboratory (17, 18). We isolated myocytes from neonatal mice with targeted disruption of the eNOS gene and transfected these cells with cDNA constructs encoding the wild-type eNOS or a myristoylation-deficient eNOS mutant. Thus, these transfections provided us with myocytes expressing eNOS protein in the caveolae or in the cytosolic compartment. We introduced a step of selection of transfected myocytes to isolate highly uniform cell populations to permit the study the physiological responses of myocytes, and we have characterized the responses of these selected cells to the muscarinic cholinergic agonist carbachol. This experimental approach has permitted us to reconstitute, within cells deficient only in the protein of interest, the differential coupling of muscarinic cholinergic responses mediated by wild-type *versus* acylation-deficient eNOS. The role of caveolin-3 in this signaling system was further explored in rat neonatal myocytes reversibly permeabilized and loaded with peptides corresponding to the caveolin scaffolding domain.

EXPERIMENTAL PROCEDURES

Myocyte Isolation and Transfection—Hearts were removed from 2-day-old mice with targeted disruption of eNOS ($eNOS^{null}$ mice (19) kindly provided by Paul Huang and Mark Fishman, Massachusetts General Hospital), were trimmed free of atrial tissues, and incubated with trypsin and collagenase, as described (20). Cell suspensions underwent two preplating steps to minimize contamination with nonmyocyte cells; in some experiments, cardiac myocytes were isolated from neonatal rats exactly as described (21). Dissociated cells were placed in culture medium consisting of Dulbecco's modified essential medium, 7.5% fetal calf serum, and 0.1% penicillin/streptomycin. Cells were plated on 10-mm coverslips or 35-mm dishes and incubated at 37 °C in 95% O_2, 5% CO_2. After 24 h of culture, myocytes from $eNOS^{null}$ neonatal mice were transfected using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's protocol. cDNA constructs encoding wild-type (WT) eNOS and the myristoylation-deficient (myr^-) eNOS mutant have been described previously (5, 22). It is important to note that this myr^- eNOS mutant, as a consequence of its myristoylation site's being inactivated, *undergoes neither myristoylation nor palmitoylation, and is therefore acylation-deficient*. Caveolin-3 cDNA in the eukaryotic expression vector pCB-7 was obtained from Michael Lisanti (Whitehead Institute, Cambridge, MA), and pHook-1 is from Invitrogen; an irrelevant plasmid encoding β-galactosidase (pcDNA3.1 vector, Invitrogen) was used as a control to maintain identical total amounts of DNA in each transfection. In addition, the β-galactosidase activity was monitored with a colorimetric assay (β-galactosidase staining kit, Invitrogen) to optimize and evaluate the transfection efficiency.

Transfected Myocyte Selection—To isolate transfected myocytes from untransfected cells, we have used the Capture-Tec kit (Invitrogen), which allows rapid selection and isolation of transiently transfected cells. This system utilizes the vector pHook-1 that expresses and displays a single-chain antibody (sFv) against the specific hapten phOX (4-ethoxy-methylene-2-phenyl-2-oxazolin-5-one) on the surface of transfected cells. The antibody is fused at the C terminus to the transmembrane domain from platelet-derived growth factor receptor allowing the antibody to be anchored and displayed on the extracellular side of the plasma membrane. Cells expressing the sFv can then be isolated from the culture by binding to hapten-coated magnetic beads and a strong magnet.

Briefly, the pHook-1 plasmid was added to the eNOS and/or caveolin-3 constructs in a 1:1 molar ratio, and myocytes were transfected with the plasmid mixture. 36 h after transfection, myocytes were detached from the 35-mm plates by using PBS containing 3 mM EDTA, pelleted, and resuspended in 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics. The cell suspension was added to a tube containing 2×10^6 Capture-Tec magnetic beads (Invitrogen) and incubated for 30 min at 37 °C on a slow rotor. To select cells, the tubes were placed in a magnetic stand and mixed for 1 min. The nonselected cells were removed with a pipette and the beads resuspended in 1 ml of medium. The steps of selection and washing were repeated twice, and the transfected myocytes were finally resuspended in 300 μl of medium and plated on 10-mm coverslips for 36 h before utilization in contractility experiments.

Myocyte Permeabilization—Myocytes were reversibly permeabilized by using a method modified from Morgan and Morgan (23) and Johnson *et al.* (24). Briefly, after culturing rat neonatal myocytes on 10-mm coverslips placed in 24-well plates for 96 h, media (referred to as conditioned media) were removed, saved, and placed at 37 °C. The cells were then slowly brought down in temperature by two sequential incubations with 0.5 ml of fresh PBS: the first one was carried out (with PBS prewarmed at 37 °C) for 10 min at room temperature and the second for 30 min on ice. The PBS was discarded, and freshly prepared permeabilization buffer (20 mM HEPES, pH 7.4, 10 mM EGTA, 140 mM KCl, 5 mM NaN3, and 5 mM oxalic acid dipotassium salt) was added to the cells with the desired peptides and 5 mM ATP for 15 min on ice. The cells were then washed five times with 0.5 ml of chilled PBS and incubated in the final PBS at room temperature. After 15-min recovery, the PBS was removed, replaced by 0.5 ml of PBS containing 1 mM Ca^{2+}, and incubated at 37 °C for 15 min. Finally, the PBS was gently aspirated, and the conditioned medium was added back to cells at 37 °C. The cells were used in contraction experiments after an additional 30-min incubation at 37 °C.

Myocyte Contractility—The spontaneous and carbachol-modulated beating rate of neonatal myocytes was determined on a temperature-controlled chamber on the stage of a microscope connected to a video-motion analyzer (17); light-dark contrast at the cell edge was used as a marker for the measurement of the frequency of cell shortening.

Peptide Synthesis—Peptides were synthesized corresponding to the amino acids 55–74 scaffolding domain of rat caveolin-3 (DGVWRVSYTTFTVSKYWCYR, termed Cav-3 herein) (25), to an rearranged version of the caveolin-3 peptide (WGVDRVFYTTSTVSYKWCRY, termed Cav-3X). These peptides were synthesized by the Biopolymers Facilities of the Howard Hughes Medical Institute at Harvard Medical School and purified by high pressure liquid chromatography.

cGMP Assay—The basal and agonist-stimulated intracellular cGMP production was determined by cGMP enzyme immunoassay. Briefly, neonatal myocytes were used 96 h after plating on 35-mm plates, *e.g.* 72 h after transfection for the $eNOS^{null}$ mice myocytes. After removing the medium, cells were washed twice with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5.6 mM glucose, and 10 mM HEPES, pH 7.4). Cells were then equilibrated at 37 °C for 30 min in Locke's buffer containing 1 mM 3-isobutyl-1-methylxanthine and 1 mM L-arginine or 1 mM L-NNA. Superoxide dismutase (100 units/ml) was added at 25 min of the equilibrium period. Myocytes were treated with 5 μM carbachol or 10 μM 3-morpholinosydnominine (SIN-1) for 3 min at 37 °C, the medium was rapidly removed, and the reaction was terminated with ice-cold 0.1 M HCl. Following 20-min incubation on ice, cells were scrapped, and the extracts were collected, neutralized by NaOH, and buffered with 2.5 M sodium acetate, pH 5.8. After centrifugation for 5 min at 14,000 × *g*, cGMP levels in the supernatant were determined by a specific enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). Experiments were performed in triplicate, and data were calibrated against standard curves. Note that the contractility experiments were performed on transfected myocytes selected with the Capture-Tec kit (Invitrogen), whereas the cGMP experiments were analyzed in transfected but unselected cells.

RESULTS

Compartmentation and Caveolin-3 Association of the Recombinant eNOS Protein in Transfected $eNOS^{null}$ Mice Myocytes—Isolated $eNOS^{null}$ mice myocytes were first cultured on 35-mm plates for 24 h and then transfected with a cDNA construct encoding either the WT eNOS or the acylation-deficient myr^- eNOS mutant. Myocytes were collected 72 h after transfection,

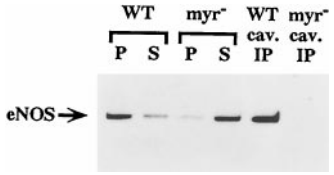

FIG. 1. **Subcellular location and caveolin-3 association of wild-type and acylation-deficient eNOS transfected into neonatal cardiac myocytes isolated from eNOS^{null} mice.** Shown are the eNOS immunoblots of the particulate (*P*) and soluble (*S*) subcellular fractions isolated from myocytes transfected with the WT eNOS and myr$^-$ eNOS constructs. The eNOS immunoblots of the immunoprecipitations (*IP*) performed from these myocytes with anti-caveolin-3 antibodies are also shown. These experiments were repeated three times with equivalent results.

and the subcellular distribution of eNOS as well as its association with caveolin-3 were analyzed. Differential centrifugation (100,000 × *g*, 1 h) revealed that WT eNOS was almost exclusively recovered in the particulate fraction in contrast to the myr$^-$ eNOS mutant, which was found in the supernatant fraction of transfected myocytes (Fig. 1). Furthermore, WT eNOS was nearly completely co-immunoprecipitated by its interaction with the endogenous caveolin-3 that is present in neonatal myocytes (Fig. 1). In contrast, the cytosolic myr$^-$ mutant was not immunoprecipitated by caveolin-3 antibodies.

Chronotropic Response to Carbachol of Cardiac Myocytes: Roles of eNOS Subcellular Compartmentation and Its Interaction with Caveolin—To enrich for transfected cells, and perform physiological analyses on a more uniform population of cells for contractility studies, the transfected eNOS^{null} mice myocytes were selected using magnetic beads as detailed under "Experimental Procedures." β-Galactosidase activity determination revealed that more than 70–85% of the cells selected by the Capture-Tec kit were transfected *versus* less than 10% in the unselected cells (not shown). The transfected neonatal myocytes usually started to beat spontaneously 24 h after selection, *e.g.* 3–4 days after their original isolation. The agonist-dependent regulation of the beating rate was studied 36 h after selection at which time the spontaneous beating rate had stabilized. After 15 min of equilibration in superfusion buffer, the spontaneous beating rate was not different in myocytes expressing WT eNOS or the myr$^-$ eNOS mutant (71 ± 7 beats/min *versus* 73 ± 5 beats/min, respectively; $n = 6$). Neonatal myocytes issued from wild-type backcrossed animals were used as a control phenotype of myocytes otherwise genetically identical but expressing native eNOS. Carbachol (10 μM) completely abrogated the beating rate of these control myocytes (Fig. 2*A*) as well as of 75% eNOS^{null} mice myocytes transfected with WT eNOS cDNA (Fig. 2*B*); the 15–30% proportion of nonresponding myocytes likely is accounted by the residual nontransfected myocytes remaining in this preparation. The effect persisted through the duration of superfusion and was slowly, but completely, reversible after drug washout (not shown); L-NNA was able to prevent the decrease in beating rate when added to the cells 30 min before stimulation (Fig. 2*C*). By contrast, in myocytes expressing the myr$^-$ eNOS protein, carbachol did not induce significant changes in the rate of contraction (Fig. 2*D*). Furthermore, to address the role of the caveolin-eNOS interaction in the muscarinic cholinergic regulation of cardiac function, we have next co-transfected neonatal myocytes from eNOS^{null} mice with constructs encoding for WT-eNOS and caveolin-3. The results presented in Fig. 2*E* show that when exogenous caveolin-3 was co-expressed in our system, carbachol did not decrease the beating rate of WT eNOS myocytes. In several experiments, a small positive chronotropic effect was even observed in the myocytes co-transfected with WT eNOS and caveolin-3 (Fig. 2*E*).

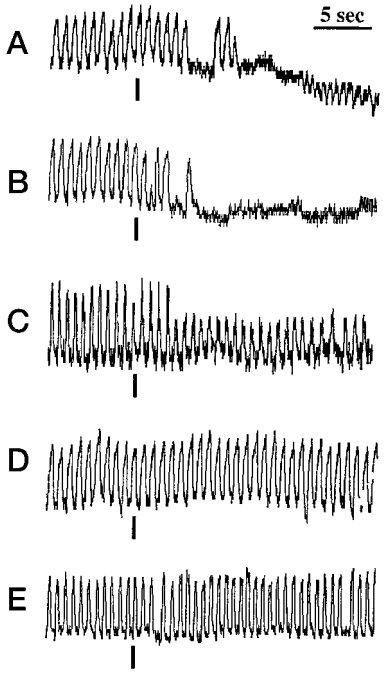

FIG. 2. **Differential coupling of the muscarinic cholinergic negative chronotropic response mediated by wild-type and acylation-deficient eNOS.** Representative tracings illustrating the spontaneous beating rate of neonatal myocytes from wild-type mice (*A*) and transfected neonatal eNOS^{null} mice myocytes (*B–E*), at base line and after adding the cholinergic agonist carbachol. The transfected eNOS^{null} myocytes were selected by the Capture-Tec method and expressed the following recombinant proteins: WT eNOS (B), WT eNOS (+ L-NNA treatment) (*C*), myr$^-$ eNOS (*D*) and WT eNOS and caveolin-3 (*E*). Cells were allowed to equilibrate for at least 15 min before determinations of basal rating rate and were then exposed to 10 μM carbachol. Note that L-NNA was applied 30 min before adding carbachol and that the ~5 s delay between carbachol application and its effect is due to the time required for the perfusion solution to reach the chamber.

Effect of Carbachol on the cGMP Production of Cardiac Myocytes: Roles of eNOS Subcellular Compartmentation and Its Interaction with Caveolin—Since the signal transduction cascade linking muscarinic cholinergic receptor stimulation, eNOS, and neonatal myocyte contraction appears to act via guanylyl cyclase activation, we next examined the cGMP levels in myocytes transfected with constructs encoding WT NOS or myr$^-$ eNOS. As shown in Fig. 3*A*, carbachol significantly increased cGMP levels in WT eNOS myocytes, an effect that could be blocked by the NOS inhibitor, L-NNA. In contrast, carbachol had no effect on cellular cGMP levels in myr$^-$ eNOS myocytes (Fig. 3*A*). To demonstrate that this absence of effect was due to the lack of the functional coupling between the mAchR and eNOS, the calcium ionophore A23187 was used to artificially increase the intracellular calcium concentration. As depicted in Fig. 3*A*, 25 μM A23187 markedly increase cGMP levels in myocytes, 5.9-fold in myr$^-$ eNOS myocytes *versus* 6.9-fold in WT-eNOS myocytes. Finally, to verify that cGMP levels in myocytes are elevated by NO and that guanylyl cyclase is similarly expressed in both types of transfected myocytes, the pharmacological NO donor SIN-1 was employed. Fig. 3*A* shows that SIN-1 increased, to the same extent, cGMP levels in myocytes transfected with the WT eNOS or the myr$^-$ eNOS constructs. Finally, we observed a dramatic decrease in the basal and carbachol-stimulated levels of cGMP in myocytes expressing WT-eNOS in association with recombinant caveolin-3, suggesting that the cellular caveolin pool is critical for regulating eNOS activation (Fig. 3*B*). By contrast, increased expression of caveolin-3 in the myr$^-$ eNOS myocytes did not change the cGMP amounts in resting and carbachol-stimulated

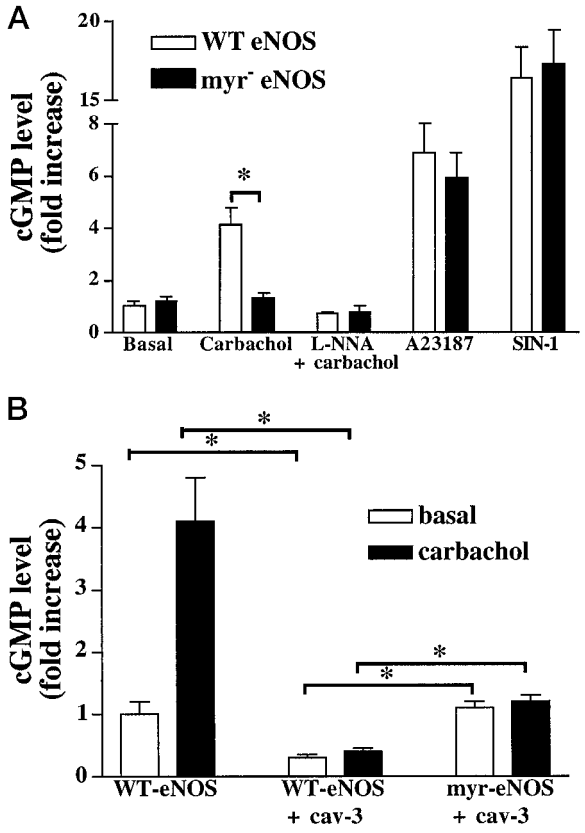

FIG. 3. **Effects of caveolin-3 expression on guanylate cyclase activation by carbachol: differential muscarinic cholinergic receptor coupling with wild-type *versus* acylation-deficient eNOS.** Myocytes expressing WT eNOS or myr$^-$ eNOS, or co-expressing recombinant eNOS and caveolin-3, were obtained by transfecting neonatal eNOS^{null} mice myocytes. *A*, carbachol significantly increased cGMP levels in WT eNOS myocytes but not in myr$^-$ eNOS myocytes. The NO donor SIN-1 (100 μM) and the calcium ionophore A23187 (25 μM) were used as controls (see "Results"). Note that the NO dependence of the carbachol-induced increase in cGMP is proven by the use of the NOS inhibitor L-NNA (1 mM). All the data are expressed as *X*-fold the absolute cGMP amounts detected at the basal level in WT eNOS myocytes. *B*, caveolin-3 co-expression decreased the basal and carbachol-stimulated cGMP levels in WT eNOS myocytes but did not significantly affect the cGMP levels in myr$^-$ eNOS myocytes.

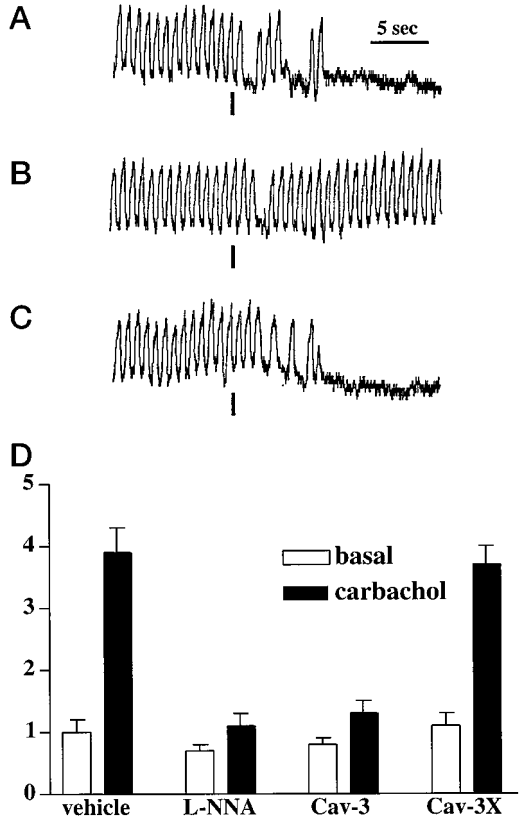

FIG. 4. **Effect of the caveolin-3 scaffolding domain peptide on carbachol-induced negative chronotropic responses and cGMP production in cardiac myocytes.** The rat myocytes were submitted to the reversible permeabilization protocol described under "Experimental Procedures," and the indicated peptides were loaded at an extracellular concentration of 10 μM. Cells were allowed to equilibrate for at least 15 min before determinations of basal beating rate and were then exposed to 10 μM carbachol. Shown are representative tracings illustrating the spontaneous beating rate of neonatal rat myocytes permeabilized as follows: *A*, vehicle-permeabilized (no peptide applied); *B*, Cav-3 peptide-loaded; *C*, control Cav-3X peptide-loaded. Note that the 5-s delay between carbachol application (*arrows*) and its effect is due to the time for the perfusion solution to reach the chamber. *D*, cGMP amounts determined in the peptide-loaded myocytes at base line and 5 min after adding the cholinergic agonist carbachol. All the data are expressed as *X*-fold the absolute cGMP amount detected at the basal level in sham-permeabilized myocytes. The NO dependence of the effect observed with the Cav-3 peptide was proven by the use of the NOS inhibitor L-NNA.

cells; this is entirely consistent with the lack of compartmentation of the myr$^-$ eNOS protein within caveolin-enriched plasmalemmal microdomains.

Modulation of Myocyte Beating Rate and cGMP Production by Specific Peptides Corresponding to the Caveolin-3 Scaffolding Domain—To address the specificity of the interaction between eNOS and caveolin-3, we have used an approach that allows specific peptides to enter cultured myocytes by reversible cell permeabilization. The method was developed by Morgan and Morgan (23) for loading aequorin in vascular smooth muscle cells; the reversible permeabilization is ensured by the exposure of myocytes to an ice-cold solution containing a Ca^{2+} chelator and the peptide, followed by the progressive rewarming of the medium bathing the myocytes and the restoration of physiological calcium concentration. In contrast to a more recent methodology specifically developed by Johnson *et al.* (24) to transiently permeabilize cardiac myocytes, our method does not require the use of detergents such as saponin but still allows the introduction of control polydextran (molecular mass: 5–100 kDa, Molecular Probe) into the cells.[2] In preliminary experiments, we observed that saponin did not improve the delivery of peptides to the myocytes but instead altered their viability and contractile properties.

We next studied neonatal rat myocytes, which endogenously express WT eNOS and caveolin-3; these cells were reversibly permeabilized to introduce synthetic oligopeptides corresponding to the scaffolding domain of caveolin-3. The caveolin scaffolding domain peptide corresponding to residues 55–74 of the rat caveolin-3 sequence is termed Cav-3. A scrambled peptide was designed wherein residues were exchanged pairwise at eight highly conserved positions within the 20 amino acid sequence of the Cav-3 peptide. This use of this conservatively designed peptide as control, which shares 100% identical amino acid composition and 60% amino acid sequence identity with the Cav-3 peptide, helps to provide confidence in the specificity of effects seen with the Cav-3 peptide. The rat neonatal myocytes usually started to beat spontaneously 3 days after isolation, and the rate of beating stabilized in the next 12–24 h. The permeabilization protocol did not significantly alter the beating rate which amounted 70 ± 3 and 72 ± 4 min^{-1} (n = 5), before and after the peptide loading. As shown in Fig. 4*A*, when

[2] K. G. Morgan, unpublished observations.

sham-permeabilized myocytes were exposed to 1 μM carbachol, the myocyte contractility was completely abrogated; several minutes of washout were required to restore the initial beating rate (not shown). By contrast, in neonatal myocytes loaded with the Cav-3 peptide, carbachol did not induce any change in beating rate (Fig. 4*B*). The specificity of this effect was demonstrated by using myocytes loaded with the control peptide Cav-3X (Fig. 4*C*). With this scrambled control peptide, we never observed a suppression of the inhibitory effect of carbachol on the rate of myocyte contraction. cGMP determinations were performed on parallel myocyte cultures and revealed that the Cav-3 peptide inhibits the carbachol-evoked increase in cGMP levels to the same extent as observed with the NOS inhibitor L-NNA applied on sham-permeabilized myocytes (Fig. 4*D*). Importantly, the Cav-3X peptide failed to reverse the ~4-fold increase in cGMP observed in control native myocytes (Fig. 4*D*).

DISCUSSION

In this study, we have used two complementary experimental approaches to address the functional role of the subcellular targeting and caveolin-3 interactions of eNOS in cardiac myocytes. First, we used myocytes isolated from eNOS^{null} mice and devised an efficient transfection and selection procedure that permitted us to study expression of recombinant eNOS cDNAs re-introduced into a normal physiological mileu. A second experimental approach used a reversible permeabilization protocol to introduce into intact rat myocytes a caveolin scaffolding peptide corresponding to the consensus sequence implicated in the protein-protein interactions of caveolin with eNOS. Collectively, our findings confirm the obligatory role of eNOS in coupling muscarinic receptor activation to the control of myocyte beating rate and indicate that the caveolar targeting of eNOS and its close regulation by caveolin-3 may serve to modulate the parasympathetic regulation of heart rate.

The native enzyme eNOS must undergo dual acylation by myristate and palmitate to be targeted to the specialized plasmalemmal microdomains termed caveolae (1, 3, 5). Inactivation of the eNOS myristoylation site (glycine 2 mutated to alanine) blocks not only myristoylation but also prevents subsequent enzyme palmitoylation yielding an acylation-deficient enzyme (5, 22). We found that in transfected neonatal eNOS^{null} mice myocytes, expression of the acylation-deficient myr$^-$ eNOS mutant is restricted to the cell cytosol (1, 5, 22), and the mutant enzyme is not co-immunoprecipitated by caveolin antibodies (Fig. 1). By contrast, the distribution of recombinant WT eNOS expressed in transfected neonatal eNOS^{null} mice myocytes is similar to that of native myocyte eNOS (4, 18) and is found almost exclusively located in the particulate fraction associated with caveolin (see Fig. 1). Using a powerful system of magnet-based cell selection, providing a uniform cell system suitable for physiological testing, we are able to rescue the phenotype of myocytes with targeted disruption of the eNOS gene by transfections with WT but *not* acylation-deficient myr$^-$ eNOS. Thus, in eNOS^{null} myocytes expressing recombinant WT eNOS, the muscarinic cholinergic agonist carbachol elicited a strong negative chronotropic response and markedly activated cGMP production, whereas in the myr$^-$ eNOS mutant transfected myocytes, there was no response whatsoever to carbachol stimulation (Figs. 2 and 3). Importantly, the calcium ionophore A23187, which increases intracellular Ca^{2+} in a receptor-independent fashion, increases cellular cGMP levels similarly in myocytes expressing the WT or myr$^-$ mutant. We may therefore postulate that the myr$^-$ eNOS mutant is uncoupled from the muscarinic receptor because of the mutant's aberrant subcellular localization and failure to target to caveolae. Besides these findings, the rescuing of eNOS^{null} mice myocytes by WT eNOS transfection provides an unequivocal demonstration that eNOS activation is essential for muscarinic cholinergic control of the heart rate. Together with our recent work (26) that explored muscarinic cholinergic control of ventricular L-type calcium current in adult eNOS^{null} and wild-type mice, eNOS activation within myocytes appears to be a critical mediator of the negative chronotropic and inotropic effects of parasympathetic stimulation in the mammalian heart.

In endothelial cells, we have documented that caveolin-1 interacts specifically with eNOS and maintains the enzyme in its inactivated state. The current study presents the first evidence that caveolin-3 regulates eNOS activity in intact cells. We report here that the expression of exogenous caveolin-3 in our system completely abrogated the cGMP-dependent negative chronotropic effect of carbachol observed in WT eNOS myocytes. This hypothesis is in agreement with the small increase in the spontaneous beating rate (*e.g.* positive chronotropic effect) observed in WT eNOS myocytes in which the cellular caveolin-3 pool is increased by co-expression of recombinant caveolin-3 (see Fig. 2*E*). Moreover, in myocytes co-transfected with the caveolin-3 construct, the cGMP amounts in the WT eNOS myocytes were significantly lower than in caveolin-3 transfected myr$^-$ eNOS myocytes, consistent with a model in which the cytosolic myr$^-$ eNOS is not tonically repressed by plasmalemmal caveolin.

Peptides derived from the scaffolding domain of caveolin-3 appear to inhibit the activity of purified eNOS and other caveolin-associated enzymes *in vitro* (see Ref. 27 for review). In the present studies, we have shown that neonatal rat myocytes can be loaded with peptides corresponding to the caveolin-3 scaffolding domain (Cav-3), leading to the blockade of both the negative chronotropic effect and the cGMP increase induced by carbachol. A highly similar control peptide (Cav-3X) has no effect on muscarinic coupling in this experimental system, providing confidence in the specificity of the effects seen with the Cav-3 peptide (see Fig. 4). Our results provide an interesting contrast to those of Venema *et al.* (28) who reported studies suggesting that an additional C-terminal sequence within caveolin, distinct from the caveolin scaffolding domain analyzed in this report, may be involved in interactions between caveolin-3 and the neuronal isoform of nitric-oxide synthase. However, the caveolin-3 scaffolding domain peptide used in these studies is sufficient to completely block the muscarinic response.

Mutations in the caveolin-3 gene, leading to alterations in caveolin-3 protein in muscle fibers, cause an autosomal dominant limb-girdle muscular dystrophy (29). It seems plausible that derangements in the eNOS/caveolin-3 association may directly affect cardiac myocyte physiological responses. The present studies have established in cardiac myocytes a key role for caveolin-3 both in the regulation of eNOS enzyme activity and the enzyme's subcellular targeting, thereby extending the paradigm of the caveolin-1/eNOS regulatory cycle previously established in endothelial cells (9). Furthermore, acylation of eNOS is required for coupling the enzyme's physiological stimulus (muscarinic cholinergic activation) to its physiological response (*e.g.* negative chronotropic effect), a signaling pathway closely regulated by caveolin-3. Thus, the caveolar compartmentation of eNOS plays an apparently paradoxical role, both tonically repressing basal eNOS activity by the enzyme's interactions with caveolin-3, but also ensuring the efficient activation of the enzyme upon agonist stimulation. Further studies are required to determine whether pathological conditions might lead to a modulation of caveolin expression or function and thereby modulate NO-dependent physiological processes both in cardiac myocytes and vascular endothelium.

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