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Nitric Oxide Inhibition of Adenylyl Cyclase Type 6 Activity Is Dependent upon Lipid Rafts and Caveolin Signaling Complexes*

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Several cell types, including cardiac myocytes and vascular endothelial cells, produce nitric oxide (NO) via both constitutive and inducible isoforms of NO synthase. NO attenuates cardiac contractility and contributes to contractile dysfunction in heart failure, although the precise molecular mechanisms for these effects are poorly defined. Adenylyl cyclase (AC) isoforms type 5 and 6, which are preferentially expressed in cardiac myocytes, may be inhibited via a direct nitrosylation by NO. Because endothelial NO synthase (eNOS and NOS3), β-adrenergic (βAR) receptors, and AC6 all can localize in lipid raft/caveolin-rich microdomains, we sought to understand the role of lipid rafts in organizing components of βAR-G_s-AC signal transduction together with eNOS. Using neonatal rat cardiac myocytes, we found that disruption of lipid rafts with β-cyclodextrin inhibited forskolin-stimulated AC activity and cAMP production, eliminated caveolin-3-eNOS interaction, and increased NO production. βAR- and G_s-mediated activation of AC activity were inhibited by β-cyclodextrin treatment, but prostanoid receptor-stimulated AC activity, which appears to occur outside caveolin-rich microdomains, was unaffected unless eNOS was overexpressed and lipid rafts were disrupted. An NO donor, SNAP, inhibited basal and forskolin-stimulated cAMP production in both native cardiac myocytes and cardiac myocytes and pulmonary artery endothelial cells engineered to overexpress AC6. These effects of SNAP were independent of guanylyl cyclase activity and were mimicked by overexpression of eNOS. The juxtaposition of eNOS with βAR and AC types 5 and 6 results in selective regulation of βAR by eNOS activity in lipid raft domains over other G_s-coupled receptors localized in nonraft domains. Thus co-localization of multiple signaling components in lipid rafts provides key spatial regulation of AC activity.

Seven membrane-spanning G protein-coupled receptors (GPCR)[1] signal via heterotrimeric G-proteins that regulate effector molecules that generate second messengers. Numerous GPCR, including β-adrenergic (βAR) and certain prostanoid receptors, couple to G_s to stimulate adenylyl cyclase (AC) activity and the generation of cyclic AMP (cAMP). In cardiac myocytes, increased cAMP levels change several aspects of cardiac function to enhance, for example, rate and force of contraction and the rate of relaxation. cAMP action primarily occurs via activation of protein kinase A, which alters intracellular Ca^{2+} dynamics and contractile function by phosphorylating calcium channels, troponin I and phospholamban (1, 2). βAR, activated by catecholamines, is the predominant G_s-coupled GPCR in cardiac myocytes, but other receptors are also capable of regulating AC activity in these cells (3). We and others have demonstrated that β_1AR are efficiently coupled to activation of AC in cardiac myocytes due to their high degree of co-localization in a membrane microdomain composed of lipid rafts or caveolae, where AC is preferentially expressed (3–6). β_2AR and prostanoid EP_2R and EP_4R couple with lower efficiency to AC due to either a transient localization in lipid rafts (β_2AR) or exclusion from these microdomains ($EP_{2/4}R$) (4). Thus, co-localization of components in a signal transduction cascade appears to be a critical determinant of signaling efficiency by receptors that stimulate AC.

Caveolae, detectable as plasma membrane invaginations enriched in the protein caveolin, are considered a subset of lipid rafts, membrane regions that are enriched in sphingolipids and cholesterol. The distinct lipid environment in lipid rafts and caveolae favors retention of certain plasma membrane proteins, creating a unique signaling microdomain (7–11). The fact that only certain GPCR, AC, and portions of cellular G_s reside in this microdomain challenges the traditional concept that membrane-associated signaling proteins are randomly distributed in the plasma membrane and interact via diffusion (9). Instead, many proteins involved in GPCR signal transduction are apparently restricted to lipid raft domains, perhaps in preformed signaling complexes that facilitate rapid and specific signal transduction (12, 13) and provide the close interaction needed for cross-talk between molecules of other signal transduction pathways (9, 14, 15).

One signaling molecule highly enriched in lipid rafts is endothelial nitric-oxide synthase (eNOS, NOS3) (16). NO is important for cardiovascular physiology (17, 18), but the role of NO in regulation of cardiac contractility has been controversial (19, 20). NO appears to have mixed effects; at certain concentrations and certain subcellular locations, NO enhances Ca^{2+} transients by activating ryanodine receptor Ca^{2+} release channels, whereas at other concentrations and locations NO can inhibit βAR-induced cardiac inotropy (21–23). The effect of NO

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[1] The abbreviations used are: GPCR, G protein-coupled receptor(s); AC, adenylyl cyclase(s); βAR, β-adrenergic receptor; βCD, β-cyclodextrin; cAMP, cyclic AMP; eNOS, endothelial nitric-oxide synthase; EP_2R, prostanoid EP_2 receptor; L-NMMA, *N*-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric-oxide synthase; PDE, phosphodiesterase; PGE_2, prostaglandin E_2, SNAP, (±)-*S*-nitroso-*N*-acetylpenicillamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; CPAE, calf pulmonary artery endothelial cell(s).

on βAR signaling is specific to eNOS relative to other NOS isoforms, perhaps because of the spatial co-localization of this isoform with βAR and AC in caveolae (24). NO regulation of βAR-mediated inotropy may contribute to blunted function in failing hearts perhaps because of an increased number of caveolae (25). The precise mechanism by which NO attenuates βAR signaling is not well characterized, but it has been suggested that NO can regulate AC types 5 and 6, the predominant AC isoforms expressed in the heart, via *S*-nitrosylation (26, 27).

The present study was designed to examine the role of lipid rafts/caveolin-rich domains in GPCR-G_s-AC signaling and to assess if NO is an important regulator of cAMP production stimulated by GPCR. By using an NO donor and by overexpressing eNOS and AC6 in cardiac myocytes and vascular endothelial cells, we conclude that NO inhibits AC activity, attenuating signaling via GPCR that are co-localized with eNOS and AC in lipid rafts. Thus, NO selectively regulates βAR-stimulated cAMP production by inhibiting raft-localized AC6. In contrast, AC activity stimulated by GPCR not localized in lipid raft domains is unaffected by NO or raft disruption unless eNOS is overexpressed and lipid rafts are disrupted. The data thus emphasize the key role of lipid rafts in maintaining the association between caveolin and eNOS so as to facilitate regulation of βAR-G_s-AC signaling.

EXPERIMENTAL PROCEDURES

Materials—Adenovirus expressing the murine AC6 cDNA was generated as described previously (28). Adenovirus expressing the bovine eNOS cDNA was kindly donated by Dr. Donald Heistad and the Vector Development Core at the University of Iowa (29). Primary antibodies for β_1AR, β_2AR, and AC5/6 and all secondary antibodies were obtained from Santa Cruz Biotechnology. Primary antibodies for caveolin-3 and eNOS were obtained from BD PharMingen. Primary antibody for EP_2R was obtained from Cayman Chemical. Radiolabeled chemicals were obtained from PerkinElmer Life Sciences. Forskolin, *N*-monomethyl-L-arginine (L-NMMA), and SNAP were obtained from Calbiochem. All other chemicals and reagents were obtained from Sigma.

Measurement of cAMP Production—Neonatal rat ventricular myocytes were prepared and maintained as described previously (3). One day after plating, cells were incubated with indicated adenoviral construct(s) for 20 h (10–100 multiplicity of infection/cell), following which cells were washed extensively and allowed to equilibrate for 24 h. Myocytes were washed three times with serum-free and $NaHCO_3$-free Dulbecco's modified Eagle's medium supplemented with 20 mM HEPES, pH 7.4 (DMEH). In some assays, cells were treated with 2% 2-hydroxypropyl-β-cyclodextrin (βCD) for 1 h at 37 °C to disrupt lipid rafts and washed with DMEH. Other cells were treated with βCD for 1 h, washed with DMEH, and then treated with βCD-cholesterol complexes (10 μg/ml cholesterol-βCD in a 1:6 molar ratio; Sigma catalog no. C4951) to deliver cholesterol back to the cells (30). Cells were equilibrated in DMEH for 30 min and then assayed for cAMP accumulation by adding drugs of interest in the presence of a cyclic nucleotide phosphodiesterase (PDE) inhibitor, 0.2 mM isobutylmethylxanthine for 10 min. When inhibitors were used, these agents were incubated with cells for 5 min before the addition of agonists. Assay medium was aspirated, and 250 μl of ice-cold trichloroacetic acid (7.5%, w/v) was immediately added to each well to terminate reactions. Trichloroacetic acid extracts were assayed for cAMP content by radioimmunoassay, as previously described (4).

Calf pulmonary artery endothelial cells (CPAE; ATCC number CCL-209) were cultured in minimum essential medium containing Earle's salt (Invitrogen) and 20% fetal bovine serum (Omega Scientific) at 37 °C in 95% humidified air, 5% CO_2. Cells were grown in 75-cm^2 flasks and passed once a week with a split ratio of 1:5. cAMP accumulation was measured as described above on passage 7–15 CPAE seeded in 24-well plates.

Measurement of NOS and AC Activity—AC and NOS activities were measured in membranes prepared from neonatal rat ventricular myocytes. Cells were rinsed twice in ice-cold PBS and then scraped into hypotonic homogenizing buffer (30 mM Na-HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol, pH 7.5) and homogenized with 20 strokes of a Dounce homogenizer. The homogenate was centrifuged at 300 × *g* for 5 min at 4 °C; the supernatant was then transferred to a centrifuge tube and centrifuged at 5,000 × *g* for 10 min. The pellet was suspended in

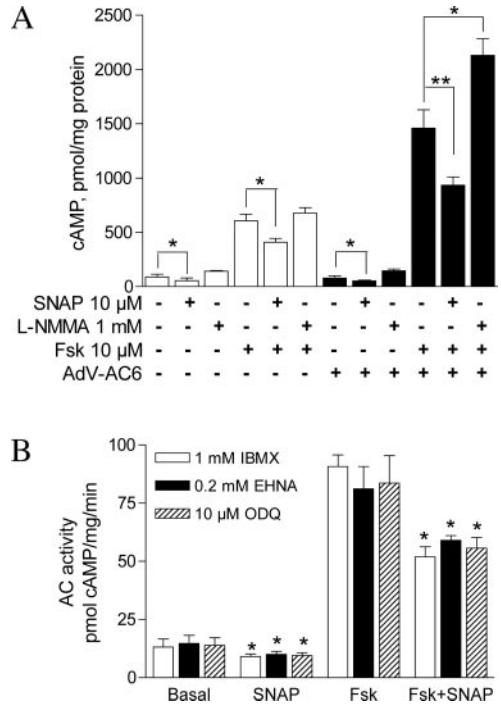

FIG. 1. **Nitric oxide inhibits cAMP production in control and AC6-overexpressing cardiac myocytes via a guanylyl cyclase-independent mechanism.** *A*, cAMP production in response to forskolin (*Fsk*; 3 μM) with and without SNAP (10 μM) or L-NMMA (1 mM). Significant difference is denoted as follows: *, $p \geq 0.05$; **, $p \geq 0.01$ by paired *t* test. *B*, adenylyl cyclase activity was measured in membranes prepared from AC6-overexpressing cardiac myocytes. Responses to SNAP (10 μM) and/or forskolin (3 μM) were measured in the presence of either a high concentration of a nonselective PDE inhibitor (1 mM isobutylmethylxanthine, *open bars*), a selective PDE2 inhibitor (0.2 mM erythro-9-(2-hydroxy-3-nonyl)adenine (*EHNA*), *filled bars*), or a guanylyl cyclase inhibitor (10 μM 1H-(1,2,4)-oxadiazole[4,3-a]quinoxalon-1-one (*ODQ*), *hatched bars*). *, $p \geq 0.05$ by paired *t* test as compared with the corresponding condition with no SNAP. All data are expressed as the mean ± S.E. of six experiments.

buffer (30 mM Na-HEPES, 5 mM $MgCl_2$, 2 mM dithiothreitol, pH 7.5) to attain ~1 mg/ml total protein concentration before being added into tubes containing drug and either AC assay buffer (30 mM Na-HEPES, 100 mM NaCl, 1 mM EGTA, 10 mM $MgCl_2$, 1 mM isobutylmethylxanthine, 1 mM ATP, 10 mM phosphocreatine, 5 μM GTP, 60 unit/ml creatine phosphokinase, and 0.1% bovine serum albumin, pH 7.5.) or NOS assay buffer (Stratagene). AC activity assays were terminated after 15 min by boiling, and cAMP content was determined by radioimmunoassay. NOS activity was assayed by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline using the NOSdetect™ assay kit (Stratagene).

Membrane Fractionation—Neonatal rat ventricular myocytes were fractionated using a detergent-free method adapted from Song *et al.* (31) and described previously (4). Briefly, cells were homogenized in 500 mM sodium carbonate plus mammalian protease inhibitor mixture (Sigma catalog no. P-8340) with 20 strokes in a Dounce homogenizer, three 10-s bursts in a tissue grinder, and then three 20-s bursts with a sonicator. The homogenate was brought to 45% sucrose by the addition of 90% sucrose in 25 mM MES, 150 mM NaCl, pH 6.5, and loaded in an ultracentrifuge tube. A discontinuous sucrose gradient was layered on top of the sample by placing 4 ml of 35% sucrose and then 4 ml of 5% sucrose. The gradient was centrifuged at 33,000 rpm on a SW41Ti rotor (Beckman Instruments) for 16–20 h at 4 °C. Fractions were collected in 1-ml aliquots from the top of the gradient.

Immunoprecipitation—Caveolin-3 was immunoprecipitated from isolated lipid raft fractions and from whole cell lysates using a protein A- or protein G-agarose method. For precipitations from lipid raft fractions, we pooled fractions 4 and 5 from the gradient after membrane fractionation and treated half the sample with 2% βCD and the other half with vehicle for 2 h on ice. For precipitations from whole cells, 10-cm plates of either control or βCD-treated neonatal rat ventricular myocytes were washed twice with cold PBS, scraped in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630 plus mammalian protease inhibitor mixture; Sigma catalog no. P-8340) and

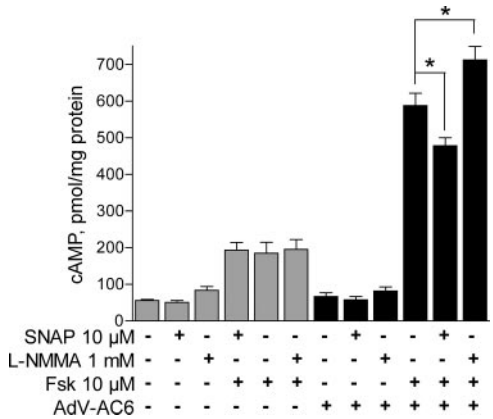

FIG. 2. **Nitric oxide inhibits cAMP production in AC6-overexpressing vascular endothelial cells.** cAMP production in response to forskolin (*Fsk*; 10 μM) with or without SNAP (10 μM) or L-NMMA (1 mM) was measured in control (*light bars*) or AC6-overexpressing (*dark bars*) calf pulmonary artery endothelial cells. *, $p \geq 0.05$ by paired t test.

homogenized on ice with 20 strokes in a Dounce homogenizer. Both types of samples were precleared with protein A-agarose or protein G-agarose (Roche Applied Science) and then incubated with primary antibody for 1–3 h on a rocking platform at 4 °C. Antibody conjugates were precipitated by incubating with protein A/G-agarose overnight on a rocking platform at 4 °C and centrifuging at 13,000 × g for 5 min. Protein A/G-agarose pellets were then washed once in lysis buffer followed by three washes each in wash buffer 2 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% Igepal CA-630) and wash buffer 3 (10 mM Tris-HCl, pH 7.5, 0.2% Igepal CA-630). Pellets and immunoprecipitation supernatants were suspended in sample buffer containing 20% β-mercaptoethanol and heated at 70 °C for 10 min. Proteins in both the immunoprecipitates and the supernatants were analyzed by immunoblot analysis.

Immunoblot Analysis—Proteins were separated by SDS-PAGE as described previously (32). Briefly, samples were loaded into polyacrylamide gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Millipore Corp.) by electroblotting. Membranes were blocked in 3% phosphate-buffered saline/milk, incubated with primary antibody (see "Materials") followed by secondary antibody with conjugated horseradish peroxidase (Santa Cruz Biotechnology). Blots were visualized using chemiluminescence and a digital imaging system (UVP, Inc.). All bands shown migrated at the expected size, as determined by comparison with molecular weight standards (Santa Cruz Biotechnology). The amount of protein per fraction was determined using a dye-binding protein assay (Bio-Rad).

Data Analysis and Statistics—Data are presented as the mean ± S.E. of at least three separate experiments or as representative images of at least three separate experiments. Statistical comparisons (t tests and one-way analysis of variance), nonlinear regression analysis, and graphics were performed using Graph Pad Prism 4.0 (GraphPad Software). For analysis of concentration-response curves, individual experiments were fitted by nonlinear regression, and paired t tests were performed comparing EC_{50} values and maximum responses between treatment conditions.

RESULTS

NO as a Regulator of cAMP Production in Cardiac Myocytes and Endothelial Cells—To determine if NO is a regulator of cardiac myocyte cAMP production, we used a NO donor, (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP) and measured both basal and forskolin-stimulated cAMP production. Inclusion of SNAP (10 μM) significantly reduced basal and forskolin-stimulated cAMP production in both control cardiac myocytes and myocytes incubated with an adenovirus to overexpress AC6 (Fig. 1*A*). The inhibition by SNAP of forskolin-stimulated cAMP was greater in AC6-overexpressing cells in terms of absolute amounts of cAMP (197 ± 36 pmol of cAMP in control cells and 525 ± 93 pmol of cAMP in AC6-overexpressing cells) but similar in proportional terms (38 ± 5.3% inhibition in control cells, 38 ± 2.2% inhibition in AC6-overexpressing cells). We also investigated whether a NOS inhibitor, L-NMMA, could

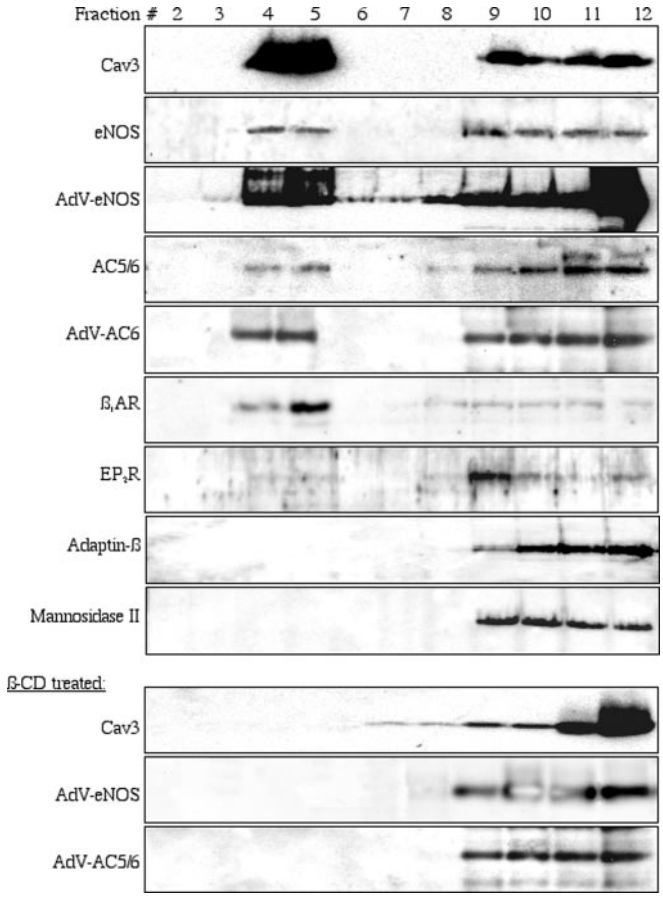

FIG. 3. **Caveolin-3, eNOS, AC6, and β-AR co-localize in buoyant lipid raft fractions that exclude EP_2R, and βCD disrupts these rafts.** Neonatal cardiac myocytes were fractionated using a non-detergent-based method followed by centrifugation in a discontinuous sucrose gradient (see "Experimental Procedures"). Fractions collected from the gradient were loaded in equal volume, separated by SDS-PAGE, and probed with antibodies specific for the indicated proteins. The bulk of caveolin-3 immunoreactivity localized in fractions 4 and 5 (*top image*), whereas mannosidase II and adaptin-β immunoreactivity (markers of Golgi and clathrin-coated pits, respectively) were excluded from these fractions. In *lower panels*, cells were treated with 2% βCD for 1 h before fractionation and centrifugation. Each *image* is representative of at least three experiments.

release a tonic inhibition of cAMP production. Inclusion of 1 mM L-NMMA did not alter basal or forskolin-stimulated cAMP production in control cardiac myocytes but significantly increased forskolin-stimulated cAMP production in AC6-overexpressing cells (Fig. 1*A*). These data imply that increases in NO can blunt formation of cAMP in neonatal cardiac myocytes but that NO derived from endogenous NOS activity does not tonically inhibit cAMP production in these cells unless adenylyl cyclase is expressed at greater than ambient levels.

In cardiac fibroblasts, NO production can act via soluble guanylyl cyclase to induce the expression of a phosphodiesterase, PDE2, which inhibits cAMP accumulation stimulated by forskolin or isoproterenol (33). Therefore, we tested a 5-fold higher concentration of isobutylmethylxanthine (1 mM), the nonspecific PDE inhibitor used in our other assays, a PDE2-specific inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine, and a guanylyl cyclase inhibitor, 1H-(1,2,4)-oxadiazole[4,3-a]quinoxalon-1-one, in measurements of AC activity in membranes from cardiac myocytes to determine if a similar mechanism exists in these cells. We found that neither high levels of isobutylmethylxanthine nor inclusion of erythro-9-(2-hydroxy-3-nonyl)adenine or 1H-(1,2,4)-oxadiazole[4,3-a]quinoxalon-1-one altered the ability of SNAP to inhibit forskolin-stimulated AC activity (Fig. 1*B*). These data are

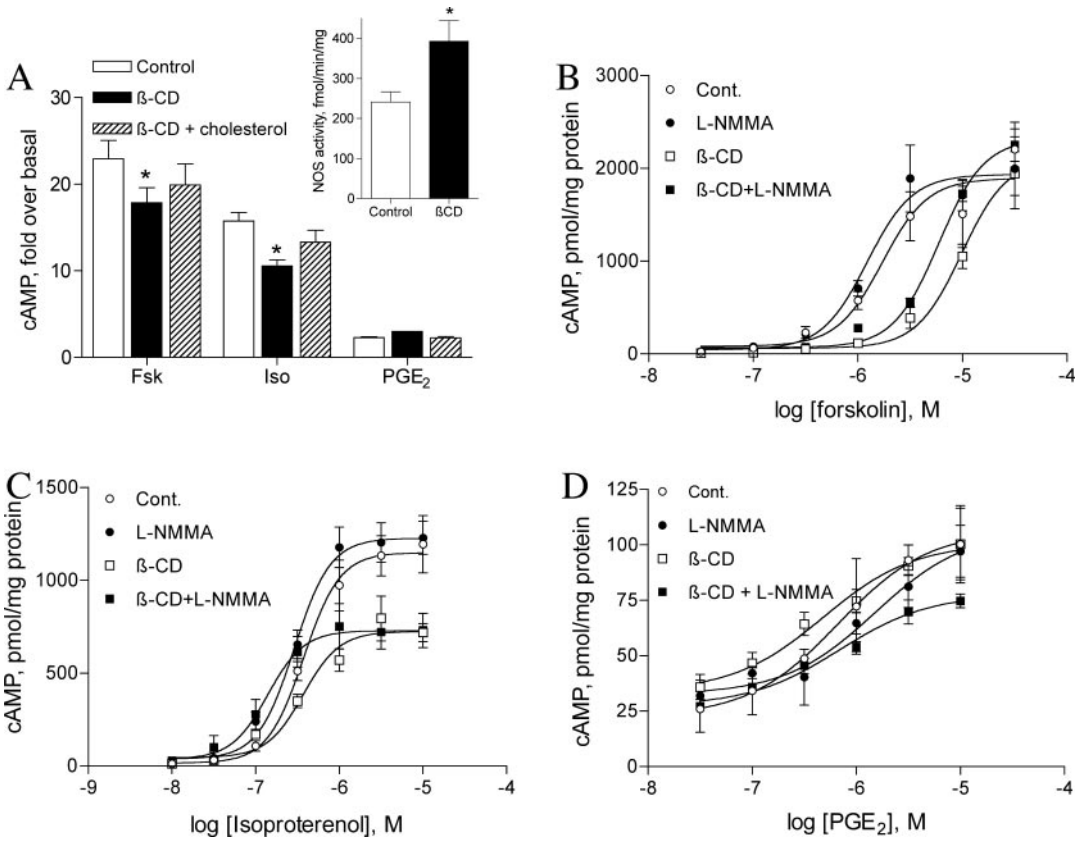

FIG. 4. **Disruption of lipid rafts inhibits cAMP production stimulated by forskolin and isoproterenol but not by PGE_2: Role of NOS activity.** *A*, cAMP production was measured in AC6-overexpressing cardiac myocytes that were either untreated (*open bars*), pretreated with 2% βCD for 1 h to disrupt lipid rafts (*closed bars*), or pretreated with 2% βCD followed by treatment with cholesterol-βCD complexes for 1 h (*hatched bars*). Responses to forskolin (*Fsk*; 10 μM), isoproterenol (*Iso*; 1 μM), or PGE_2 (10 μM) are shown, expressed as -fold over basal. NOS activity was measured in control and βCD-treated cardiac myocytes (*A*, *inset*). Significant difference from control is denoted as follows: *, $p \geq 0.05$ by paired *t* test. *B–D*, cAMP production was measured in AC6-overexpressing cardiac myocytes that were either untreated (*circles*) or pretreated with 2% βCD for 1 h (*squares*) and in the absence (*open symbols*) or presence of L-NMMA (1 mM; *closed symbols*). Responses to various concentrations of forskolin (*B*), isoproterenol (*C*), or PGE_2 (*D*) are shown as the mean ± S.E. of six experiments. Statistically significant changes in EC_{50} values or maximum effects between treatment conditions are reported under "Results." Maximum effect of PGE_2 in βCD plus L-NMMA-treated cells (*closed squares* in *D*) was not significantly different from control.

consistent with the idea that NO inhibits activity of AC5 and AC6 independent of guanylyl cyclase activation or induction of a cyclic nucleotide phosphodiesterase.

We hypothesized that direct NO regulation of AC activity would be evident in other cell types that expressed eNOS and AC6. To test this idea, we used CPAE and measured the effects of an NO donor on cAMP production. By contrast with the results that we observed in cardiac myocytes, SNAP (10 μM) did not reduce basal or forskolin-stimulated cAMP production in CPAE cells (Fig. 2). However, SNAP did reduce forskolin-stimulated cAMP levels in CPAE cells engineered to overexpress AC6. Although L-NMMA (1 mM) did not alter basal or forskolin-stimulated cAMP production in control CPAE cells, L-NMMA significantly ($p < 0.05$ by paired *t* test) increased forskolin-stimulated cAMP production in AC6-overexpressing CPAE cells. Thus, CPAE cells only demonstrate NO-inhibited cAMP production when engineered to overexpress AC6, a result consistent with the idea that eNOS-derived NO preferentially inhibits activity of AC6 (26, 27).

The Role of Intact Lipid Rafts in GPCR-G_s-AC Signal Transduction—Previous reports indicate that eNOS is highly localized in lipid rafts and caveolae in endothelial cells and cardiac myocytes and that its activity is inhibited by binding to the caveolin scaffold (18). Therefore, we hypothesized that intact lipid rafts or caveolae retain eNOS in the inactive state, thereby perhaps minimizing its role in regulating cAMP production under normal conditions (Fig. 1*B*). To test this hypothesis, we disrupted lipid rafts by treating cells with 2% βCD, a cholesterol binding agent, for 1 h and then measured cAMP production. These conditions lead to the disruption of morphologically identified caveolae (34) and to the loss of caveolin-3, eNOS, and AC5/6 immunoreactivity from buoyant fractions of a discontinuous sucrose gradient (Fig. 3) (6). We overexpressed AC6 in these studies in order to enhance the absolute extent of NO inhibition of cAMP accumulation (as shown in Fig. 1).

βCD treatment inhibited forskolin- and isoproterenol-stimulated cAMP production but did not inhibit PGE_2-stimulated cAMP production (Fig. 4*A*), consistent with the co-localization of βAR and AC6 in lipid raft/caveolin-rich domains and the exclusion of PGE_2 receptors from those domains (3, 6). Adding cholesterol back to the cells by treating with βCD-cholesterol complexes following βCD treatment largely reversed these effects, indicating the specificity and reversibility of βCD treatment. To investigate the impact of βCD treatment in more detail, we measured cAMP production in response to multiple concentrations of forskolin, isoproterenol, and PGE_2. βCD treatment caused a 5.8 ± 0.3-fold rightward shift ($p < 0.01$) in the forskolin concentration-response curve with no significant change in the maximal response (Fig. 4*B*). Although inclusion of L-NMMA (1 mM) did not alter the forskolin response in untreated cells (Fig. 1*B*), L-NMMA partially reversed the rightward shift induced by βCD treatment (a 2.3 ± 0.3-fold leftward shift as compared with the βCD response, $p < 0.05$) (Fig. 4*B*). NOS activity was increased in membranes prepared from car-

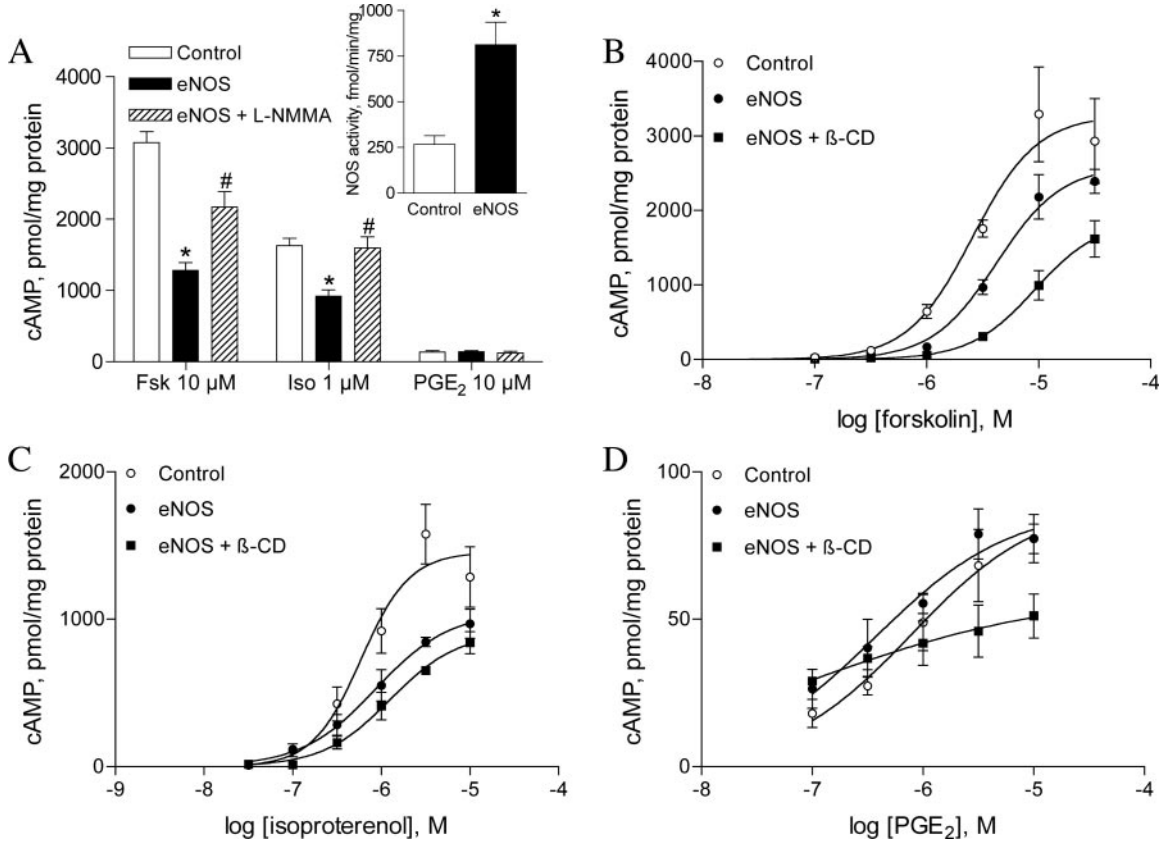

FIG. 5. **Overexpression of eNOS inhibits cAMP production stimulated by forskolin and isoproterenol but not by PGE_2 unless lipid rafts are disrupted.** *A*, cAMP production was measured in cardiac myocytes overexpressing AC6 (*open bars*), AC6 and eNOS (*closed bars*), or AC6 and eNOS but treated with L-NMMA (1 mM; *hatched bars*). NOS activity was measured in control and eNOS-overexpressing cardiac myocytes (*A*, *inset*). *B–D*, cAMP production was measured in untreated AC6-overexpressing cardiac myocytes (*open circles*), untreated AC6- and eNOS-overexpressing cardiac myocytes (*closed circles*), or AC6- and eNOS-overexpressing cardiac myocytes that had been treated with 2% βCD for 1 h (*closed squares*). Responses to various concentrations of forskolin (*A*), isoproterenol (*B*), or PGE_2 (*C*) are shown as the mean ± S.E. of five experiments. Statistically significant changes in EC_{50} values or maximum effects between treatment conditions are reported under "Results."

diac myocytes treated with βCD as compared with those prepared from vehicle-treated cells (Fig. 4*A*, *inset*). Therefore, lipid raft disruption appears to inhibit forskolin-stimulated cAMP production due, in part, to an increase in NOS activity.

By contrast with the impact of βCD treatment on forskolin response, we found different effects on the concentration-response curves for isoproterenol and PGE_2 (Fig. 4, *C* and *D*), the receptors for which show different patterns for localization in lipid raft, caveolin-rich domains (Fig. 3) (4). βCD treatment caused no shift in the isoproterenol concentration-response curve and reduced the maximal response by 37 ± 8% as compared with untreated cells ($p < 0.05$; Fig. 4*C*). Inclusion of the NOS inhibitor caused a leftward shift of the response curve (1.5 ± 0.2-fold as compared with control, 1.6 ± 0.4-fold *versus* βCD treated alone, both $p < 0.05$) but did not alter the βCD-induced reduction in maximal response. Thus, βCD treatment has qualitatively different effects on βAR-stimulated *versus* forskolin-stimulated cAMP production; for βAR, but not forskolin, we found an inhibition of maximal response, whereas for both types of agonists, we observe a NOS-dependent component that decreases potency (to a greater extent in the case of forskolin). In contrast to effects on forskolin- and isoproterenol-mediated responses, PGE_2 potency and maximal response were not statistically altered by βCD treatment and/or inclusion of L-NMMA (Fig. 4*D*). The minimal effect of disruption of lipid rafts on cAMP production stimulated by prostanoid receptors presumably results from exclusion of these receptors from those microdomains (4).

The Effects of eNOS Overexpression on cAMP Production—To directly examine the role of eNOS in the regulation of cAMP production, we used an eNOS-containing adenovirus to increase expression of the enzyme and then measured responses to forskolin, isoproterenol, and PGE_2 with and without βCD treatment. Overnight incubation of myocytes with the eNOS adenovirus dramatically increased eNOS immunoreactivity in a lipid raft *versus* nonraft pattern that matches the pattern of eNOS and caveolin-3 localization in native cells (Fig. 3). NOS activity was increased 3.1-fold in membranes prepared from cardiac myocytes overexpressing eNOS as compared with membranes prepared from control cells (Fig 5*A*, *inset*). The potency of forskolin-stimulated cAMP production was shifted 1.7 ± 0.1-fold rightward ($p < 0.05$), and the maximal response was inhibited 21 ± 7% ($p < 0.05$) in eNOS-overexpressing cells (Fig. 5*B*). This reduction in cAMP production in response to a maximal concentration of forskolin was blunted by inclusion of L-NMMA (Fig. 5*A*), reflecting the fact that membranes from eNOS-overexpressing cells had 3-fold higher basal NOS activity as compared with membranes from control cells. βCD treatment of eNOS-overexpressing myocytes caused an additional rightward shift in forskolin potency and further reduction in maximum response (3.6 ± 0.3-fold rightward shift and 43 ± 6% reduction in maximum as compared with control, both $p < 0.01$). Similar effects were observed when cAMP production was measured in response to isoproterenol; eNOS overexpression alone caused a 1.5-fold rightward shift in potency and a 28 ± 5% reduction in maximum response ($p < 0.05$), whereas βCD treatment of eNOS-overexpressing cells increased these effects to a 2.1 ± 0.2-fold shift and a 37 ± 4% reduction as

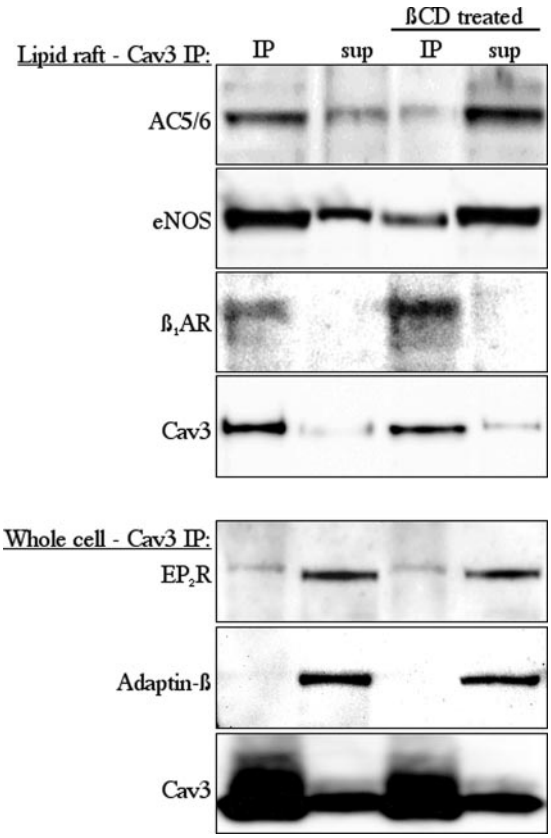

FIG. 6. **Disruption of lipid rafts causes AC6 and eNOS to dissociate from the caveolin complexes.** Immunoblot analyses were conducted for the indicated proteins on caveolin-3 immunoprecipitates (*IP*) and the resultant supernatants (*sup*). Caveolin-3 was immunoprecipitated from isolated lipid raft fractions treated with vehicle or βCD (*top*) or from either control or βCD-treated cardiac myocytes (see "Experimental Procedures"). Images are representative of at least three experiments.

compared with control ($p < 0.05$; Fig. 5*C*). In contrast, eNOS overexpression alone induced little change in the potency or maximum response of PGE_2-stimulated cAMP production (Fig. 5*D*). However, eNOS overexpression and βCD treatment together caused a reduction in the maximal cAMP production stimulated by PGE_2 (29 ± 9%; $p < 0.05$ as compared with control) that was similar in magnitude to the reduction in responses to forskolin and isoproterenol. Thus, eNOS overexpression inhibits cAMP production stimulated by forskolin and βAR activation, and these effects are enhanced when lipid rafts are disrupted. However, overexpression of eNOS has little effect on prostanoid receptor-mediated activation of cAMP production unless cells are also treated with βCD; raft disruption thus appears to uncover an eNOS-mediated inhibition of PGE_2 signaling, perhaps via an effect on AC.

Lipid raft disruption by βCD treatment alters the migration of proteins on a discontinuous sucrose gradient (Fig. 3) (6). However, little is known about how βCD treatment alters the complexes formed between caveolin, eNOS, and other signaling proteins such as AC and βAR. To investigate the effects of raft disruption on the stability of caveolin signaling complexes, we conducted caveolin-3 immunoprecipitations from the lipid raft fractions from AC6- and eNOS-overexpressing cardiac myocytes. We found that AC5/6 and β_1AR co-immunoprecipitate with caveolin-3 (Fig. 6) (4). eNOS immunoreactivity was also detected in caveolin-3 immunoprecipitations. AC5/6, eNOS, and β_1AR immunoreactivity was also detected in caveolin-3 immunoprecipitations from whole cells (data not shown) (4), whereas immunoreactivity for EP_2R and adaptin-β (a marker of clathrin-coated pits) was only detected in immunoprecipitation supernatants (Fig. 6). When either lipid raft fractions or intact cardiac myocytes were βCD-treated prior to immunoprecipitation, both AC5/6 and eNOS, but not β_1AR, immunoreactivity were reduced in the immunoprecipitates (Fig. 6). Incubation of cells with cholesterol-βCD complexes for 1 h following βCD treatment partially reversed the loss of AC5/6 and eNOS immunoreactivity in caveolin-3 immunoprecipitates in whole cells (data not shown). Therefore, AC and eNOS binding to caveolin-3 depends upon intact lipid rafts, whereas β_1AR appears not to require integrity of the lipid raft domains. βCD-induced loss of eNOS-caveolin-3 interactions probably leads to the increase in NOS activity and the resultant inhibition of cAMP production that we observe (Figs. 4 and 5).

DISCUSSION

The current study has uncovered new information regarding signaling microdomains, in particular in cardiac myocytes. The work attempts to synthesize previous results related to eNOS and its localization in lipid rafts/caveolin-rich microdomains (16, 35, 36) with findings regarding GPCR-G_s-AC signaling in such microdomains (3–6) and ability of eNOS-derived NO to regulate cAMP formation (26, 27).

The coupling of GPCR to overexpressed AC6 in cardiac myocytes depends upon co-localization of a sufficient quantity of receptor with AC6 in lipid rafts or caveolae (4). In the present studies, we extend the idea that those plasma membrane microdomains are important sites for organizing signaling proteins by demonstrating that such domains regulate NO-AC cross-talk. The current and previous data indicate that caveolae have dual roles in regulating eNOS signaling: 1) eNOS binds to the caveolin-3 scaffold, an interaction associated with decreased eNOS activity and NO generation (7, 16, 35, 37, 38), and 2) caveolae can co-localize certain GPCR, eNOS, and AC6 while excluding other GPCR, such that signaling by co-localized receptors is selectively regulated by cross-talk from NO. A schematic representation of this idea is shown in Fig. 7. Our evidence for this latter mechanism comes from studies of disruption of lipid rafts with βCD and with overexpression of eNOS. Overexpression of eNOS increases basal levels of NO and inhibits lipid raft-localized GPCR signaling. Treatment of cardiac myocytes with βCD leads to dissociation of eNOS from caveolin, thereby increasing NO production and, in turn, reducing cAMP production stimulated by βAR or forskolin. The effects of NO on cAMP production in cardiac myocytes are consistent with the evidence that AC5 and AC6 are preferentially expressed in these cells (3–6) and with the idea that NO selectively inhibits these AC isoforms via direct nitrosylation (26, 27).

Accordingly, this inhibitory effect of NO is more readily appreciated in cardiomyocytes that overexpress AC6 and only observed in endothelial cells (CPAE) that overexpress AC6 (but not in native cells), providing further evidence that NO regulation of AC activity is AC isoform-specific (27). The current findings thus identify a molecular mechanism of cross-talk between NO and βAR signaling and help explain the ability of NO to inhibit contractility stimulated by catecholamines (21, 24, 39).

The effects of cellular cholesterol depletion are complex and poorly understood (*e.g.* see Ref. 10). βCD treatment may have numerous consequences, not all of which are necessarily attributable to the disruption of lipid rafts. For this reason, we used an inhibitor of NOS in conjunction with βCD treatment in order to draw conclusions about the role of NOS activity in the observed effects. Our data are consistent with the idea that NOS activity increases following βCD treatment and that the resultant increase in NO inhibits the activity of AC, in partic-

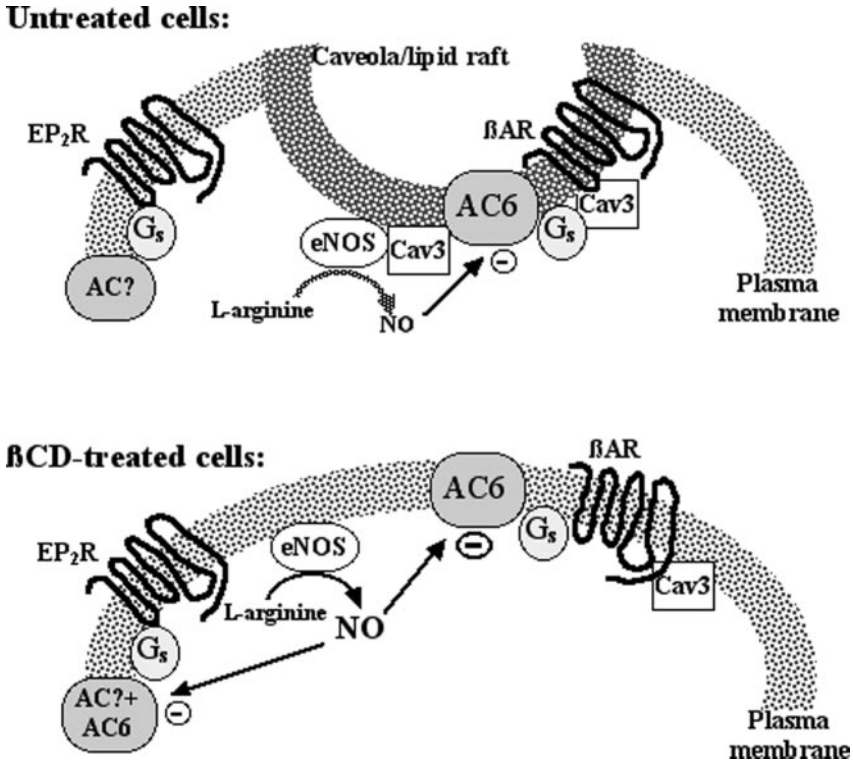

FIG. 7. **Schematic diagram of spatial organization and NO regulation of GPCR signaling in cardiac myocytes.** In untreated cardiac myocytes, caveolae/lipid rafts contain complexes of βAR, AC6, eNOS, and caveolin-3 but exclude EP_2R, such that NO selectively inhibits direct (forskolin) or βAR-mediated stimulation of AC6 activity. βCD treatment of cardiac myocytes disrupts caveolae/lipid rafts and disperses AC6 and eNOS. This disruption eliminates caveolin inhibition of eNOS activity, raising NO levels and inhibiting AC6 activity. If eNOS is overexpressed, βCD treatment also inhibits EP_2R-stimulated cAMP production, an effect probably attributable to this receptor being able to couple to NO-sensitive AC isoforms normally compartmentalized in caveolae/lipid rafts.

ular by lipid raft co-localized βAR and AC. By contrast, PGE_2-stimulated cAMP production (a non-raft-localized signal) is not significantly altered by βCD treatment (Fig. 4*B*). βCD treatment decreased caveolin-eNOS binding, implying that direct, molecular interaction between eNOS, βAR, and AC is lost following this treatment (Fig. 6). However, it is possible that βCD treatment only weakens the interaction, such that binding is not retained during the immunoprecipitation procedure but the complex is retained in intact cells (as in assays of cAMP production). Whereas effects of βCD treatment other than disruption of lipid rafts may contribute to the responses we observe (particularly the different effects of βCD treatment on βAR- and forskolin-stimulated cAMP production), the use of L-NMMA and the overexpression of eNOS specifically probe for the effects of this treatment on eNOS function.

The combination of eNOS overexpression and βCD treatment causes the greatest inhibition of βAR-AC signaling and is the only condition in which we observed a reduction of PGE_2-stimulated cAMP production. Thus, NO can regulate prostanoid receptor signaling if lipid raft organization is disrupted and sufficient capacity for NO generation exists. The fact that exogenous NO (via the addition of SNAP) could not replicate this effect implies that the spatial component provided by lipid raft organization prevents coupling of prostanoid receptors to NO-inhibited isoforms of AC (Fig. 7). The importance of lipid raft domains (which may exist in different forms) *versus* caveolae (one subset of lipid rafts) in compartmentalizing signaling is not known (40). Lipid rafts and caveolae appear to have different influences on eNOS, implying that signaling in these two domains can be qualitatively different (36).

NO is a highly diffusible messenger, begging the question of "why does localization of NOS matter?" Studies by Barouch *et al.* (24), who examined the effects of NO generated by either overexpressed eNOS (which localizes in caveolae) or overexpressed neuronal NOS (NOS1, which localizes in cardiac sarcoplasmic reticulum), indicate that the subcellular locale of NO generation is a critical determinant of its effects. Thus, whereas NO may freely diffuse, its short half-life probably makes for a sharp concentration gradient. Because high concentrations of NO are required for the *S*-nitrosylation of AC (26), this effect may necessitate co-localization of the substrate (AC) and the site of NO generation, whereas the stimulation of cyclic GMP production by guanylyl cyclase can occur over diffusible distances (41).

The current data contrast somewhat with findings reported by Rybin *et al.* (6), who observed that disruption of cardiac myocyte lipid rafts with 2% βCD led to an increase in AC activity stimulated by βAR agonists or forskolin. We believe that experimental differences involved in preparation and growth of cells, especially as related to expression of caveolins (data not shown), may be responsible for these differences.

We conclude that NO is an important regulator of AC activity in cardiac myocytes and probably in other cells that express predominantly AC5 and/or AC6. Since AC6 appears to be a predominant isoform in several cell types (42–44), this effect of NO may be quite common. Lipid rafts appear critical for establishing the close proximity of eNOS and AC required for this cross-talk and, by excluding other GPCR coupled to G_s and the stimulation of AC activity, create a selective inhibition of signaling by certain GPCR, in particular βAR.

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Nitric Oxide Inhibition of Adenylyl Cyclase Type 6 Activity Is Dependent upon Lipid Rafts and Caveolin Signaling Complexes

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