Differential Targeting of β-Adrenergic Receptor Subtypes and Adenylyl Cyclase to Cardiomyocyte Caveolae

A MECHANISM TO FUNCTIONALLY REGULATE THE cAMP SIGNALING PATHWAY*

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Differential modes for β1- and β2-adrenergic receptor (AR) regulation of adenylyl cyclase in cardiomyocytes is most consistent with spatial regulation in microdomains of the plasma membrane. This study examines whether caveolae represent specialized subdomains that concentrate and organize these moieties in cardiomyocytes. Caveolae from quiescent rat ventricular cardiomyocytes are highly enriched in β2-AR, Goβ, protein kinase A RIα subunits, caveolin-3, and flocculin (caveolin functional homologues); β1-AR, m2-muscarinic cholinergic receptors, Goα, and cardiac types VVI adenylyl cyclase distribute between caveolae and other cell fractions, whereas protein kinase A RIα subunits, G protein-coupled receptor kinase-2, and clathrin are largely excluded from caveolae. Cell surface β2-ARs localize to caveolae in cardiomyocytes and cardiac fibroblasts (with markedly different β2-AR expression levels), indicating that the fidelity of β2-AR targeting to caveolae is maintained over a physiologic range of β2-AR expression. In cardiomyocytes, agonist stimulation leads to a marked decline in the abundance of β2-ARs (but not β1-ARs) in caveolae. Other studies show coinmunoprecipitation of cardiomyocytes adenylyl cyclase VVI and caveolin-3, suggesting their in vivo association. However, caveolin is not required for adenylyl cyclase targeting to low density membranes, since adenylyl cyclase targets to low buoyant density membrane fractions of HEK cells that lack prototypical caveolae. Nevertheless, cholesterol depletion with cycloextrin augments agonist-stimulated cAMP accumulation, indicating that caveolae function as negative regulators of cAMP accumulation. The inhibitory interaction between caveolae and the cAMP signaling pathway as well as domain-specific differences in the stoichiometry of individual elements in the β-AR signaling cascade represent important modifiers of cAMP-dependent signaling in the heart.

Catecholamines act through cardiac β-adrenergic receptors (β-ARs)1 to influence the contractile state of the heart. The direct inotropic and chronotropic support provided by cardiac β-ARs represents a critical compensatory mechanism to preserve cardiac function during stress and/or states associated with circulatory compromise. In the hearts of most mammalian species, the physiologic effects of catecholamines are mediated by the predominant β1-AR subtype (75–80% of the total β-ARs), which activates a signaling pathway involving the Gβγ-dependent stimulation of adenylyl cyclase leading to the accumulation of cAMP and protein kinase A-dependent phosphorylation of key target proteins. Cardiomyocytes also express β2-ARs that support contractile function. Until quite recently, most studies of β2-AR signaling in cardiomyocytes were wedded to the concept that β2-ARs signal to the Gβγ/cAMP pathway in a manner that is essentially equivalent to the pathway activated by β1-ARs. However, there is evidence that β2-ARs are not functionally redundant, including the findings that β2-ARs couple to the Gβγ/cAMP pathway more efficiently than β1-ARs and that the pathway for β1-AR activation of adenylyl cyclase is susceptible to inhibitory modulation by m2-muscarinic cholinergic receptors (m2-mAChRs), whereas the pathway for β2-AR activation of adenylyl cyclase is not (1, 2). This suggests that individual β-AR subtypes might fulfill distinct roles in transmembrane signaling due to spatially or developmentally regulated patterns of expression (3), but a specific molecular mechanism to adequately explain all of the distinct signaling properties of individual β-AR subtypes has not been identified.

Until quite recently, the prevailing concept was that components of the β-AR complex are freely mobile in the plasma membrane and that the specificity of molecular interactions is dictated entirely by information encoded in the three-dimensional structures and recognition surfaces of individual moieties. However, a simple “random collision coupling” model is inadequate to explain all of the experimental data, including the aforementioned evidence that β1- and β2-ARs display distinct susceptibility to inhibitory modulation by the m2-mAChR (2–4). One potential molecular mechanism that could impart this type of specificity to β-AR subtype signaling is compartmentalization to membrane subdomains such as caveolae. Caveolae were first identified as flask-shaped uncoated invaginations on the surface of highly differentiated cells. Caveolae

1 The abbreviations used are: AR, adrenergic receptor; GRK, G protein-coupled receptor kinase; PKA, protein kinase A; EDA, epidermal surface antigen; PNG-F, peptide N-glycosidase F; NCM, noncaveolae membrane; m2-mAChR, m2-muscarinic cholinergic receptor; Mes, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; Tricine, N-β-hydroxy-1,1-bis(hydroxymethyl)ethylglycine.
are now recognized to be plasma membrane compartments with distinct lipid and protein composition that sequester and regulate the function of cytoplasmically oriented signal transduction molecules (5). In particular, there is evidence that β2-ARs and m3 mAChRs (6–10), their associated G protein α and β subunits (11–14), certain adenylyl cyclase isoforms (15–18), one member of the G protein-coupled receptor kinase family (GRK2, which phosphorylates agonist-activated β-ARs (19)), and the catalytic subunit of protein kinase A (PKA) (20) accumulate in caveolae at steady state and/or following ligand-induced activation. Localization of these diverse signaling molecules to caveolae suggests that this structure can serve as a scaffold to preassemble membrane-bound oligomeric complexes and thereby facilitate efficient and rapid coupling of agonist-occupied receptors to effectors. Such a mechanism could be particularly pertinent for sympathetic regulation of cardiomyocyte function, where rapid and productive signaling from receptor-activated G protein subunits to the adenyl cyclase enzyme could be restricted by low (potentially limiting) levels of the adenyl cyclase enzyme. Caveolae also may act to dampen signaling as a result of the properties of their principle structural protein, caveolin. The mammalian caveolin gene family consists of caveolin-1, caveolin-2, and the muscle-specific caveolin-3 (5). Domain-mapping studies identify a cytosolic membrane-proximal region (designated the “caveolin scaffolding domain”) in caveolin-1 (as well as the structurally homologous caveolin-3) that interacts with putative caveolin-binding motifs in a wide range of signaling molecules (including G protein α subunits and the catalytic domains of certain adenyl cyclase isoforms, GRK2, and the catalytic subunit of PKA (5, 19–21)). In vitro studies suggest that caveolin negatively regulates the activation state of heterotrimeric G proteins and functions as a “general kinase inhibitor” for many signaling enzymes. Accordingly, the goal of the present study was to determine whether caveolae form a signaling module for components of the β-AR complex in cardiomyocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies and their sources were as follows: anti-β2-AR, anti-β2-AR, anti-Goαs, anti-type V/VI adenylyl cyclase, anti-GRK2, anti-PKA Iα regulatory subunit, anti-PKA Iα regulatory subunit, anti-PKAα catalytic subunit, and anti-clathrin affinity-purified polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-Goαi/αs and anti-Goαi/αs antisera (PerkinElmer Life Sciences); anti-Gi3 subunit antisera (Upstate Biotechnology, Inc., Lake Placid, NY); anti-caveolin-1, anti-caveolin-3, anti-flotillin, and anti-epidermal surface antigen (anti-ESA) monoclonal antibodies (Transduction Laboratories); anti-caveolin-1, anti-caveolin-3, anti-flotillin, and anti-epidermal surface antigen (anti-ESA) monoclonal antibodies (Transduction Laboratories); anti-m3 mAChR affinity-purified polyclonal antibodies (Alomone Labs, Israel); anti-goat IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology); anti-mouse IgG-horseradish peroxidase conjugate (Bio-Rad); anti-rabbit IgG-horseradish peroxidase conjugate; and the enhanced chemiluminescence kit (Amer sham Pharmacia Biotech). Other reagents were purchased commercially from the following sources: Percoll, OptiPrep, Nonidet P-40, octyl glucoside, protein G-Sepharose, 2-hydroxypropyl-β-cyclodextrin (Sigma) and peptide N-glycosidase F (PNG-F) (Roche Molecular Biochemicals).

**Cell Culture**—Cardiac myocytes were isolated from hearts of 2-day-old Wistar rats by a trypsin dispersion procedure according to a protocol previously described (21). Experiments were performed following 5–6 days of culture in minimal essential medium (Life Technologies, Inc.) with 0% fetal calf serum, 5 × 10⁻⁶ M hypoxanthine, and 12 mM NaHCO₃. HEK293 cells were obtained from Dr. Jonathan Javitich and were propagated in Dulbecco’s modified Eagle’s medium supplemented with Geneticin and 10% fetal bovine serum. Cardiac fibroblast cultures were obtained from cells adherent to culture dishes during preplating and cultured according to standard methods (23).

**Purification of Caveolin-rich Membrane Fractions**—Fractions enriched in the muscle-specific caveolin-3 isoform were prepared by two methods. Most of the isolations were performed according to the protocol of Song et al. (24) essentially as described previously. All steps were carried out at 4 °C. Briefly, cells from five 100-mm diameter dishes were washed twice with ice-cold phosphate-buffered saline and then scraped into 0.5 M sodium carbonate, pH 11.0 (0.5 ml/dish; total volume ∼2.5 ml for each preparation). To disrupt cellular membranes, homogenization was carried out sequentially with a Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts), and a tip sonicator (three 20-s bursts). The homogenate was then adjusted to 40% sucrose by adding an equal volume of 80% sucrose prepared in Mes-buffered saline (25 mM Mes, pH 6.5, 0.15 M NaCl), placed on the bottom of an ultracentrifuge tube, overlaid with a 5–35% discontinuous sucrose gradient (4 m3l of 5% sucrose, 3 ml of 35% sucrose; both in Mes-buffered saline containing 250 mM sodium carbonate), and centrifuged at 38,000 rpm for 16–18 h in an SW40 rotor (Beckman). After centrifugation, 12 ml gradient fractions were concentrated by precipitation with trichloroacetic acid as follows. Fractions were mixed with 7.92% trichloroacetic acid (1.10; v/v) and incubated for 30 min on ice. Precipitated proteins were pelleted by centrifugation at 3700 × g for 15 min at 4 °C (IEC; Centra-8R centrifuge). The resulting pellet was washed once with 5 ml of ethyl ether and dissolved in SDS-PAGE sample buffer (generally without boiling, to prevent aberrant migration during immunodetection of receptors and adenyl cyclase). In some experiments, the entire 40% lower sucrose layer (fractions 8–12) was pooled for immunoblot analysis.

Some studies also were performed with caveolin-3-enriched fractions prepared according to the method of Smart et al. (13). In this case, plasma membranes were isolated by scraping cells from 10 100-mm plates into 30 ml of ice-cold buffer A (0.25 M sucrose, 1 mM EDTA, 20 mM Tricine, pH 7.8) followed by centrifugation at 1500 × g for 5 min. Cells were then resuspended in 1 ml of buffer A, placed in a 2 ml Potter-Elvehjem tissue grinder (Wheaton; catalog no. 358003), and homogenized (20 strokes). The suspension was centrifuged at 1000 × g for 10 min to yield a postnuclear supernatant fraction, which was removed and stored on ice. The nuclear pellet was resuspended in 1 ml of buffer A, homogenized, and centrifuged at 1000 × g for 10 min. The supernatant was combined with the previous postnuclear supernatant, layered on top of 23 ml of 30% Percoll in buffer A, and centrifuged at 65,000 × g for 30 min (Beckman Ti60 rotor). The plasma membrane fraction, a visible band ~5.7 cm from the bottom of the centrifuge tube, was collected, washed with buffer A, and adjusted to a concentration of 100–300 mg protein/ml.

**Intracellular Membranes (IM)** were obtained by centrifuging fractions below the plasma membrane at 360,000 × g for 1 h. The IM fraction was pelleted with 4 × SDS-PAGE sample buffer (3:1, v/v). A 0.2 ml aliquot of plasma membrane sonicate was diluted in 60 times with buffer A (1 ml EDTA, 20 mM Tricine, pH 7.8, 150 mM NaCl), centrifuged at 360,000 × g for 1 h, and then dissolved in SDS-PAGE sample buffer. This supernatant was concentrated (1.7 ml) to 0.2 ml with a 30% (v/v) 35% OptiPrep sample buffer (0.5 M sucrose, 6 mM EDTA, 2 mM Tricine, pH 7.8) plus 1.01 ml of buffer A to make a 23% OptiPrep solution. This was placed on the bottom of an ultracentrifuge tube, a linear 20 to 10% OptiPrep gradient (prepared by diluting 50% OptiPrep in buffer C with buffer A) was layered on top, and the sample was centrifuged at 57,600 × g for 90 min in an SW40 rotor. The bottom fractions were collected and designated noncaveolar membrane (NMC). The top 6.5 ml of the gradient was collected and mixed with 5.38 ml of 50% OptiPrep in buffer C plus 0.12 ml of buffer A to make an 30% OptiPrep solution. This was overlaid with 0.5 ml of 15% OptiPrep and then with 0.5 ml of 5% OptiPrep (both in buffer A) and centrifuged at 57,600 × g for 90 min. An opaque band located at the 5/15% OptiPrep interface was collected and designated caveolae fraction. Cavela fraction was diluted 10–13 times with buffer B followed by centrifugation at 360,000 × g for 1 h. The resulting caveolae (yield typically 12 μg of protein) was dissolved in SDS-PAGE sample buffer. NMC was diluted four times with buffer B followed by centrifugation at 360,000 g for 1 h and solubilization in SDS-PAGE sample buffer.

**Immunoblotting and Immunoprecipitation**—Samples were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose, which, in general, was cut into longitudinal strips for incubation with various primary antibodies. Sample boiling was avoided to prevent aberrant migration during immunodetection of receptors and adenyl cyclase. Immunoblotting was performed with antibodies against the β2-AR, β2-AR, Goαi, type V/VI adenylyl cyclase, GRK2, RIIa, RIIβ, α-catalytic subunit of protein kinase A, clathrin, flotillin, and ESA (diluted in 50
mm Tris, pH 7.5, 0.2 M NaCl containing 5% non-fat dry milk, 0.1% Tween 20, and 0.02% NaN₃; anti-caveolin-1 and caveolin-3 antibodies (diluted in 50 mM Tris, pH 7.5, 0.2 M NaCl containing 1% nonfat dry milk, 0.5% Tween 20, and 0.02% NaN₃; anti-Gα₁/₂, Gα₁₅, and -G protein β₃γ₃ subunit antibodies (diluted in 50 mM Tris, pH 7.5, 0.2 M NaCl containing 5% bovine serum albumin, 0.05% Tween 20, and 0.02% NaN₃); and anti-m₁-,m₃-MAChR-antibodies (diluted in 50 mM Tris, pH 7.5, 0.2 M NaCl containing 1% nonfat dry milk, 3% bovine serum albumin, and 0.02% NaN₃). Primary antibodies were used at final dilutions of 1:5000 (ESA), 1:1000 (caveolin-3, Gα₁/₂, Gα₁₅), 1:500 (Gα₁₅, GRK2, G protein β₃γ₃ subunit), 1:250 (Rln, RIIα, α-catalytic subunit of protein kinase A, flotillin, m₁-,m₃-MAChR), 1:100 (clathrin), 1:200 (clathrin), 1:200 (clathrin), 1:250 (Rln, RIIα, α-catalytic subunit of PKA, flotillin, m₁-,m₃-MAChR), and 1:100 (clathrin, type VVI adenylyl cyclase), and bound primary antibodies were visualized with enhanced chemiluminescence according to the manufacturer’s instructions. Each of the antibodies was initially screened with total cell lysates to ensure that it reacted with a band (or bands) of the appropriate molecular weight in cardiomyocytes. To establish immunospecificity, polyclonal antibodies were also subjected to preblocking with their respective immunogen peptide as follows: β₁-AR antibody and a peptide corresponding to the C terminus of the mouse β₁-AR; β₂-AR antibody and a peptide corresponding to the C terminus of the mouse β₂-AR, Gα₃γ₃ antibody and a peptide corresponding to the C terminus of rat Gα₃; type VVI adenylyl cyclase antibody and a peptide corresponding to the C terminus of human adenylyl cyclase V; GRK2 antibody and a peptide corresponding to the C terminus of human GRK2 (amino acids 675–689); Rln α antibody and a peptide corresponding to the C terminus of human Rln (amino acids 343–361); RIIα antibody and a peptide corresponding to the C terminus of human RIIα (amino acids 385–404); α-catalytic subunit of PKA antibody and a peptide corresponding to the C terminus of human PKAα; clathrin antibody and a peptide corresponding to the N terminus of human clathrin heavy chain; m₁-,m₃-MAChR antibody and a fusion protein of glutathione S-transferase and amino acids 225–356 of the i3 intracellular loop of the human m₁-,m₃-MAChR. For all but the anti-m₁-,m₃-MAChR antibody, 20 μg of antibody was preincubated with 100 μg of peptide in a final volume of 0.6 ml for 2 h at room temperature. 6 μg of the anti-m₁-,m₃-MAChR antibody was preincubated with 30 μg of the respective protein in a final volume of 80 μl for 1 h at room temperature. After preincubation, antibodies were brought to working concentration (see above).

In some experiments, samples (5–20 μg) were diglycosylated by preincubation with PNG-F in 60 mM NaCl, 1.25 mM EDTA, 143 mM β-mercaptoethanol, 5 mM sodium phosphate, 15 mM Tris-Cl (pH 7.5), 0.1% SDS, and 0.5% Nonidet P-40 for 2 h at 37°C. The reaction was terminated with 0.33 volumes of 4× SDS-PAGE sample buffer.

Immunoprecipitation—Cardiomyocytes from two 100-mm diameter dishes (~2 mg of protein) were rinsed with ice-cold phosphate-buffered saline and harvested by the addition of 1.8 ml of extraction buffer (10 mM Tris-Cl, pH 8, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 60 mM octyl glucoside). The cells were scraped and sonicated. Lysates were centrifuged at 4°C for 15 min at maximal speed in a microcentrifuge, and the supernatant was removed. For immunoprecipitation, supernatant was incubated with anti-caveolin-3 antibodies or irrelevant mouse IgG1 for 1 h at 4°C followed by the addition of 120 μl of a 1:1 slurry of protein G-Sepharose beads (Sigma) and incubation overnight at 4°C. The beads were washed three times with extraction buffer, and bound proteins were eluted with 140 μl of SDS-PAGE sample buffer and boiled for 5 min. Samples were subjected to SDS-PAGE and immunoblotting with caveolin-3 and adenylyl cyclase type VVI-specific antibodies.

(32 μg of protein or a volume equal to that of the fraction with the least amount of detectable protein for “protein-free” fractions 1–3) were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with the indicated antibodies. Fractions 4 and 5 represent the 5–35% sucrose interface, while the fractions at the bottom of the gradient are the 40% sucrose cushion, and P represents the insoluble pellet. This scheme typically accomplishes an ~500-fold purification of caveolin-3 relative to total cellular protein; ~15 μg of caveolin-3-enriched domains are purified from ~8 mg of total cellular protein. Only sections of the Western blots corresponding to the proper molecular mass of the identified proteins are shown. Molecular weight markers are on the right. B, aliquots of protein (20 μg/lane) pooled from fractions 4 and 5 (left), 8–12 (middle), or the insoluble pellet (right) were probed for clathrin, flotillin-1, flotillin-2/ESA, or caveolin-3. Only the regions of the gel where the indicated proteins migrate are shown. For each immunoblot, results are representative of three separate experiments.

**FIG. 1.** Subcellular distribution of components of the β-AR signaling complex in cardiomyocytes. A, cardiomyocyte cultures were lysed in sodium carbonate followed by subcellular fractionation using a 5–35% discontinuous sucrose gradient as described under “Experimental Procedures.” 1-ml fractions collected from the top of the gradient were concentrated (see “Experimental Procedures”). Aliquots

- Adenyl Cyclase
- Type VVI
- GRK2
- Protein Kinase A
- Regulatory Subunits:
  - α-Catalytic Subunit
- Caveolin-3

**B**

| CAV | Fab12 | P |
|-----|-------|---|
| 200 | 116   |   |
| 48  | 45    |   |
| 48  | 45    |   |
| 21.5|       |   |

Caveolin-3

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RESULTS

Selective Association of Individual β-AR Subtypes with Caveolae—The cAMP pathway in cardiac cells was among the first and most rigorously studied signal transduction pathways, but there has been surprisingly little scrutiny of the subcellular localization of individual β-ARs and their downstream effector molecules. Studies of β-AR targeting in particular have been confined to β-ARs overexpressed at high levels in heterologous expression systems (10, 17). There is no information on the targeting of native β-ARs expressed at physiologic concentrations in cardiomyocytes. Neonatal rat ventricular myocytes represent an optimal model to compare the subcellular targeting of individual β-AR subtypes and their downstream effector components as they co-express β1- and β2-ARs; both β1- and β2-ARs couple to an increase in intracellular calcium and enhanced contractility via a cAMP-dependent pathway in this cell type (3).

Caveolae were prepared according to two methods for these studies. First, membranes enriched in caveolin were separated from the bulk of cellular membranes and soluble proteins by extraction in detergent-free alkaline sodium carbonate buffer followed by isopycnic centrifugation on a sucrose gradient. We previously demonstrated that the light scattering band that forms at the 53/55% sucrose interface (fractions 4 and 5) of bottom-loaded discontinuous sucrose gradients contains ~50–100-nm vesicular structures by transmission electron microscopy and the bulk of the cellular caveolin-3 (but excludes >99% of other cellular proteins including markers of the Golgi (25)). The results of immunoblot analyses examining the partitioning of selected components of the β-AR signaling cascade across these gradient fractions are shown in Fig. 1. This isolation method identifies resident caveolae proteins, but it provides no information as to whether proteins that are excluded from caveolae reside on the remainder of the cell surface plasma membrane or on intracellular membranes. Therefore, the partitioning of selected components of the β-AR signaling complex between caveolae, the remainder of the cell surface plasma membrane, and intracellular membranes was compared according to a second method (13). Here, plasma membranes were purified from the remainder of the intracellular proteins by Percoll gradient centrifugation. Membranes were then subjected to two OptiPrep density gradient centrifugations to separate and concentrate light caveolae vesicles from noncaveolae plasma membranes. Immunoblot analysis on these fractions is shown in Fig. 2.

The predicted molecular mass of the β1-AR is ~51 kDa (26). However, an antibody directed against the C-terminal domain of the β1-AR specifically recognizes multiple protein species, including bands that migrate at approximately 45, 51, 65, 96, and 150 kDa (shown in Fig. 1 and in more detail in Fig. 3). Immunoreactivity for all of these species is blocked by preincubation of antiserum with peptide antigen (Fig. 3A). Similar molecular heterogeneity of β1-ARs was detected previously in the hearts of wild type mice but not β1-AR null mutants (27), suggesting that each of these immunoreactive species represents a bona fide β1-AR gene product. Fig. 3B shows that the 96- and 65-kDa species undergo appreciable mobility shifts upon treatment with PNG-F (to remove glycosyl moieties). Hence, even the 96-kDa species, which migrates as a relatively sharp band, is a glycosylated protein. Surprisingly, the diffuse migration of the smaller 51-kDa species is not appreciably increased by PNG-F. Of note, treatment with PNG-F does not eliminate the molecular heterogeneity of the β1-AR (even when incubations are extended for longer intervals and samples are treated with more enzyme). These results indicate that factors other than protein N-glycosylation contribute to the differences in β1-AR migration. Although caveolae are isolated in buffers that do not contain protease inhibitors, the multiple bands are not likely to result from proteolytic degradation during sample preparation, since identical results are obtained in particulate fractions prepared according to a protocol that uses protease inhibitors liberally (Fig. 3A). Although the 96- and 150-kDa species are detected in reducing SDS-PAGE sample buffer (in some experiments even with 10% β-mercaptoethanol, data not
maleimide or iodoacetamide prior to cell lysis and then maintained in buffers supplemented with these SH group blocking agents throughout membrane isolation. Fig. 3A shows that by preventing disulfide bond exchange reactions during the preparation of samples for SDS-PAGE, the 96- and 150-kDa species are converted to 65- and 51-kDa forms of the $\beta_1$-ARs. These smaller immunoreactive species represent bona fide $\beta_1$-AR gene products; their mobility precisely matches the mobility of the species expressed by cardiomyocytes transfected with a $\beta_1$-AR receptor expression plasmid (using the adenoviral component system described by Kohout et al.; Fig. 3C (30)). Hence, the 96-kDa immunoreactive species is a $\beta_1$-AR, formed as a result of dimerization or nonspecific aggregation of 65- and 51-kDa species of $\beta_1$-AR. Of importance to the studies at hand, Fig. 1 shows that $\beta_2$-ARs are detected across the sucrose gradient; they reside in caveolae but also are abundant in heavy fractions. The OptiPrep gradient fractionation method shows that $\beta_1$-ARs distribute between caveolae, noncaveolae cell surface plasma membrane fractions, and internal membranes (Fig. 2). Caveolae prepared according to both methods appear to be enriched in the most rapidly migrating $\beta_1$-AR species, but the interpretation of this finding is uncertain. It is possible that incomplete glycosylation or other factors contributes to a true preferential targeting of the 51-kDa species to caveolae. Alternatively, an apparent increase in the abundance of the 51-kDa $\beta_1$-AR species in caveolae could be an artifact, due to a diminished propensity of free thiol groups on this form of the native $\beta_1$-AR to undergo disulfide bond exchange reactions and form larger species in the local caveolae microenvironment.

Immunoblot analysis of cardiomyocyte $\beta_2$-ARs was more straightforward. Although $\beta_2$-ARs are the minor $\beta$-AR subtype in cardiomyocytes (representing only approximately 16% of total $\beta$-ARs), they are readily detected as a broad $\approx$66-kDa species in caveolin-enriched fractions of resting cardiomyocytes (obtained either by the alkaline sodium carbonate extraction and sucrose gradient flotation scheme (Fig. 1) or sonication and flotation on OptiPrep gradients (Fig. 2)). Specific $\beta_2$-AR immunoreactivity (blocked by competing antigen peptide) is detected in caveolae and not in any other fractions (even with 10-fold heavier protein loading and long exposures of the gel; Figs. 1, 2, and 4A). Indeed, $\beta_2$-ARs are enriched in caveolae relative to the total plasma membrane fraction, consistent with the conclusion that cell surface $\beta_2$-ARs are confined to caveolae (Fig. 2). The diffuse appearance of $\beta_2$-ARs in caveolae can be attributed to glycosylation, since this receptor migrates as a distinct $\approx$47-kDa band (in close agreement with its calculated molecular mass deduced from the gene sequence) following treatment with PNG-F (data not shown). Collectively, these studies indicate that in resting cardiomyocytes the cell surface distribution of $\beta_1$- and $\beta_2$-ARs is quite different. Although $\beta_1$-ARs are readily detected in all membrane fractions, the vast majority of the $\beta_1$-AR subtype is recovered entirely from caveolae along with $\approx$99% of total cell protein. In contrast, $\beta_2$-ARs are confined to caveolae.

To address the possibility that $\beta_2$-ARs localize to caveolae as a result of the low $\beta_2$-AR expression level in cardiomyocytes, the analysis was extended to cardiac fibroblasts. Fig. 5A shows that $\beta_2$-ARs migrate as an approximately 66-kDa band in cardiac fibroblasts. $\beta_2$-ARs are abundant in the caveolar fraction, but they also are detected in the heavy sucrose gradient fractions (F8–12). To determine whether the $\beta_2$-ARs in the heavy fraction reside in noncaveolae plasma membranes versus internal membrane, caveolae were purified from the remainder of the plasma membrane by flotation of sonicated plasma membranes on OptiPrep gradients. Fig. 5B shows that fibroblast $\beta_2$-ARs are recovered entirely from caveolae and internal mem-

![Fig. 3. Immunoblot analysis of $\beta_2$-ARs in cardiomyocytes. A, effects of chemical reducing agents. Particulate fractions were prepared from cardiomyocyte cultures according to standard methods described previously (Ref. 53; lanes 1 and 4) or following exposure of the cultures to 1 mM DTT for 30 min followed by 5 mM N-ethylmaleimide (NEM, lane 2) or iodoacetamide (IAM, lane 3) for 30 min prior to the preparation of the particulate fractions. 100 mM N-ethylmaleimide or 100 mM iodoacetamide was included in the homogenization buffer, and all subsequent sample-processing buffers to prevent spurious formation of receptor aggregates. Samples (60 mg/lane) were then subjected to SDS-PAGE. Immunoblot analysis was with an antibody that recognizes the C terminus of the $\beta_1$-AR (lanes 1–3) or following antibody preblocking with an antigen peptide (lane 4). Epitope-specific $\beta_1$-AR immunoreactivity is denoted by the arrowheads. B, the $\beta_1$-AR is expressed as multiple glycosylated forms in cardiomyocytes. Caveolae (lanes 1, 2, and 7), heavy sucrose fractions (lanes 3, 4, and 8), and the insoluble pellet fraction (lanes 5, 6, and 9) were prepared, and aliquots of protein pooled from these fractions were treated with PNG-F as described under "Experimental Procedures." Samples (25 mg/lane) were then subjected to SDS-PAGE and immunoblot analysis with the $\beta_1$-AR antibody (left) or following preblocking of this antibody with antigen peptide (right). Panel C, detection of heterologously expressed $\beta_2$-ARs in cardiomyocytes. Samples were prepared at 24, 48, or 96 h after transfection with a $\beta_2$-AR expression vector or 24 h following transfection with the vector control (+) using the "adenoviral component system" described previously (30). Immunoblot analysis was with the $\beta_1$-AR antibody and 10 mg of particulate protein fraction.](image-url)
branes; $\beta_2$-AR are excluded from noncaveolae cell surface plasma membranes. This result suggests that the strict localization of cell surface $\beta_2$-ARs to caveolae is a generalized phenomenon and not a unique property of cardiomyocytes.

A previous report indicated that $m_2$-mAChRs are excluded from cardiomyocyte caveolae under basal conditions but traffic to caveolae following stimulation by agonist (9). $m_2$-mAChR localization was identified by receptor binding techniques in that previous study. When the subcellular localization of $m_2$-mAChRs was examined by immunoblot analysis with a highly sensitive/specific $m_2$-mAChR antibody (and sensitive enhanced chemiluminescence detection), $m_2$-mAChRs are readily detected as a broad 66–67-kDa band in caveolae as well as in heavy sucrose gradient fractions (obtained by alkaline sodium carbonate extraction and sucrose gradient flotation; Fig. 4A). $m_2$-mAChRs are detected in caveolae, noncaveolae cell surface plasma membranes, and internal membranes when the OptiPrep gradient fractionation method is used (Fig. 4B). Although these sensitive techniques identify $m_2$-mAChRs in caveolae, the vast majority of total $m_2$-mAChRs are excluded from caveolae (along with >99% of total cell protein). Hence, the data essentially concur with the result published previously (9). Of note, these studies identify similar membrane distributions for $m_2$-mAChRs and $\beta_1$-ARs in resting cardiomyocytes. The spatial co-localization of these receptors would be permissive for interactions at the level of cAMP formation. In contrast, $\beta_2$-ARs and $m_2$-mAChRs largely segregate to separate membrane subdomains, providing a potential explanation for the previous observation that $\beta_2$-AR-dependent cAMP formation is refractory to inhibitory modulation by mAChRs.

Recent studies identify $\beta_2$-AR trafficking to clathrin-coated vesicles as part of a dual process to terminate activation of the $G_s$-adenyl cyclase pathway as well as to initiate mitogenic signaling (31, 32). To determine whether agonist stimulation promotes the egress of $\beta_2$-ARs from caveolae to permit their trafficking to other cellular compartments, caveolae were prepared by sequential OptiPrep gradient centrifugation from quiescent cells and following stimulation with $10^{-7}$ M isoproterenol for 30 min; this concentration of agonist maximally activates both $\beta_1$- and $\beta_2$-ARs. Fig. 6 shows that agonist stimulation leads to a dramatic decrease in the abundance of $\beta_2$-ARs in caveolae, with no change in the recovery of caveolin-3 (or caveolae protein). This is associated with at best a trivial
in the abundance of β2-AR in the noncaveolae surface plasma membrane fraction. Based upon models of agonist-induced trafficking of β2-ARs to clathrin-coated vesicles, a commensurate increase in β2-ARs in the noncaveolae cell surface membrane would not be expected, since the bulk of the cellular clathrin is excluded from this fraction. Agonist-induced changes in β2-AR abundance in caveolae are specific (blocked by preincubation with propranolol to prevent receptor activation; data not shown). Agonist-stimulated trafficking is confined to the β2-AR; agonist-dependent changes in abundance (or mobility) of any molecular form of the β2-AR was not detected under these assay conditions.

Selective Association of Downstream Components of the Cardiomyocyte cAMP Signaling Pathway with Caveolae—G protein subunit partitioning between caveolae and the remainder of the cell is shown in Fig. 1. Individual G protein subunits distributed quite differently across the sucrose gradient. Neonatal cardiomyocytes express both short and long splice variants of Gaα (33); these proteins, as well as β subunits are recovered in both caveolae and the heavy sucrose fractions. Cultured neonatal rat ventricular myocytes also express three pertussis toxin-sensitive Ga subunits: Gaαq, Gaαi, and Gaαo. Fig. 1 shows that Gaαq is highly localized to caveolae. Similar results were obtained with an antibody that identifies Gaαq/Gaαo (without discriminating between these proteins). Fig. 2 shows that when cell surface plasma membranes are partitioned into caveolae and noncaveolae fractions by the OptiPrep-based cell fractionation method, the caveolae are particularly enriched in Gaαq, whereas long and short splice variants of Gaαi distribute between caveolae and the remainder of the cell surface membrane. Gaαi is detectable in internal membranes, whereas Gaαo is not. Quantitative analysis of a series of immunoblots (adjusted to linear range) reveals that the vast majority of Gaαi subunits, but only approximately ~50% of Gaαo and β subunits (expressed relative to total immunoreactivity in the cell), are recovered in the caveolin-enriched fraction.

The cardiac adenylyl cyclase enzymes (types V and VI) are the predominant adenylyl cyclase isoforms detected in cardiomyocytes (34). Nevertheless, they are scarce membrane proteins and generally difficult to detect by immunoblot analysis. Fig. 1 shows that caveolae are markedly enriched in cardiac adenylyl cyclase isoforms (detected with an antibody that does not discriminate between the two cardiac adenylyl cyclase isoforms), with as much as 50% of the total enzyme in this fraction. Although the mobility of adenylyl cyclase under reducing conditions in SDS-PAGE is considerably slower than would be expected based upon the calculated molecular mass of this protein, this is due to glycosylation of the protein; adenylyl cyclase migrates as a 120-kDa protein following treatment with PNG-F (Fig. 7). It also is pertinent to note that adenylyl cyclase is detected as a diffuse band with a tail of immunoreactivity only in caveolae preparations; in all other preparations, adenylyl cyclase runs as a single tight immunoreactive band. This provided the first clue that adenylyl cyclase might interact with caveolin-3 oligomeric complexes that are easily detected when this region of the gel is stripped and reprobed with the caveolin-3 antibody (data not shown).

cAMP actions in the heart largely result from cAMP binding to the regulatory subunits of the dormant PKA heterotetrameric complex, causing the release of the catalytic subunits and the subsequent phosphorylation of target proteins. PKA enzymes are classified according to their regulatory subunit (RI or RII), which display known differences in molecular weight, affinity for cAMP, phosphorylation state, and subcellular localization (35). Fig. 1 shows that RI and RII display totally different distribution patterns. RI is detected in only trace amounts in caveolae; it is primarily recovered in the heavy fractions. Similarly, the α-catalytic subunit of PKA (whose signaling function is subject to inhibitory modulation through an interaction with caveolin (20)) is largely excluded from the caveolin-enriched fraction of cardiomyocytes. In contrast, caveolae are highly enriched in RII.

GRKs phosphorylate agonist-activated G protein-coupled receptors. This generally is viewed as a mechanism to promote high affinity binding of arrestins, which acts to uncouple the receptor from G proteins and target the receptor for internalization via clathrin-coated pits. Nevertheless, there is recent evidence that the catalytic activity of GRK is inhibited through an interaction with caveolin (19). In resting cells, GRK2 primarily fractionates as a cytoplasmic protein. Nevertheless, a small fraction of GRK2 (5–15%) is reported to associate with the membrane fraction; in A431 cells, a large portion of this membrane-associated GRK2 is in the caveolin-enriched fraction (19). Fig. 1 shows that GRK2 is primarily a soluble protein in cardiomyocytes (recovered in the heavy sucrose fractions), but a minor component of GRK2 co-fractionates with caveolin-3.

Finally, flotillin-1 and flotillin-2/ESA are two newly described caveola-associated proteins that are believed to act as functional homologues of caveolins (36, 37). Although both were detected previously in murine heart muscle (37), immu-
Caveolae Regulate β-Adrenergic Receptor Signaling to cAMP

noreactivity in intact heart tissue preparations could result from flotillin expression in cardiomyocytes or noncardiomyocyte-containing cellular elements, since these proteins (particularly flotillin-2/ESA) display a rather wide tissue distribution. Fig. 1B shows that flotillins are expressed by cardiomyocytes and that they co-fractionate with caveolae. Caveolae prepared according to the OptiPrep method also are enriched in flotillin-1 relative to the remainder of the plasma membrane (data not shown). Importantly, clathrin is not detected in the caveolin-3-enriched fractions (i.e. these fractionation methods exclude clathrin-coated vesicles).

Caveolin-3 Interacts in Vivo with Adenyl cyclase, but This Is Not Required for the Targeting of Adenyl cyclase to Caveolae—The diffuse mobility of the adenyl cyclase enzyme in the caveole fraction suggested an interaction with homooligomers of caveolin-3. To determine whether there is an in vivo binding interaction between type V/VI adenyl cyclase and caveolin-3, cells were solubilized in Triton X-100- and octyl glucoside-containing buffer. Cleared lysates were subjected to immunoprecipitation with caveolin-3 or an irrelevant monoclonal antibody; bound proteins were eluted with SDS-PAGE sample buffer and subjected to electrophoresis and immunoblotting with adenyl cyclase- and caveolin-3-specific antibodies. Fig. 8 shows that adenyl cyclase co-immunoprecipitates with caveolin-3 antibodies; caveolin-3, but not adenyl cyclase V/VI, is completely cleared from the postimmune supernatant (data not shown). Adenyl cyclase V/VI does not co-immunoprecipitate with irrelevant antibody. These results indicate that the adenyl cyclase V/VI enzyme specifically associates with caveolin-3 in intact cardiomyocytes.

Further studies indicate that an interaction with caveolin is not required for adenyl cyclase V/VI (or β-AR subtype) localization to light vesicular membranes. These studies were performed in HEK293 cells, which lack caveolin-1 or -3 proteins but endogenously express type V adenyl cyclase. We reasoned that HEK293 cells might represent an informative model to examine the caveolin-3 requirement for adenyl cyclase targeting to low buoyant density membrane subdomains, since such fractions are readily isolated by equilibrium sucrose density gradient centrifugation from these cell extracts. Fig. 9A shows that a vesicular preparation that is highly enriched in the caveolae functional homologues, flotillin-1 and flotillin-2/ESA, can be floated from HEK293 cell extracts; flotillins are completely excluded from the heavy fractions. Flotillin purification is over 1000-fold relative to total cell lysates (10 μg of caveolin, which contain greater than 99% of total cell flotillins, was isolated from 10 mg of starting cell protein). Immunoreactivity for caveolin-1 and caveolin-3 is not detected in HEK293 cells, and clathrin is excluded from the light vesicular fraction prepared by this method. Consistent with the notion that flotillins drive formation of a low density vesicular structure, this preparation appears as 50–200-nm vesicles as well as curved membrane fragments by transmission EM (Fig. 9B). Using the position of flotillins as a marker to track the “caveolae-related membrane” domains, Fig. 9A shows the co-fractionation of the endogenous type V/VI adenyl cyclase enzyme and both β-AR subtypes. These results argue that neither caveolin-1 nor caveolin-3 is required for cardiac adenyl cyclase or β-AR

![Fig. 9. β1-ARs, β2-ARs, and adenyl cyclase V/VI are detectable in light membrane vesicles from HEK293 cells that lack caveolin expression. A, HEK293 cell derivatives that stably overexpress the β1-AR were lysed in sodium carbonate followed by subcellular fractionation using a 5-35% discontinuous sucrose gradient as in Fig. 1. Aliquots of protein (10 μg/lane) pooled from fractions 4 and 5 (F4–5, lane 1), 8–12 (F8–12, lane 2), or the insoluble pellet (P, lane 3) from HEK293 were probed for immunoreactivity for β1-ARs, the endogenous adenyl cyclase V/VI enzyme, caveolin-1, flotillin-1, flotillin-2/ESA, and clathrin. Localization of the β1-AR was examined in a similar manner in HEK293 cells that stably overexpress the β1-AR. Five times longer exposures of immunoblot analyses of β1- and β2-ARs are shown on the right, to show that both receptors are detectable in the heavy fractions and pellet of HEK293 cells. Caveolae (Cav) and heavy fractions (F8–12) from cardiac fibroblasts prepared in an identical manner are shown as controls for caveolin-1 immunoblotting. Only the regions of the gel where the indicated proteins migrate are shown. For each immunoblot, results are representative of two or three separate experiments. B, comparison of the electron micrographic appearance of the caveolin-3- and flotillin-enriched fractions isolated from cardiomyocytes and HEK293 cells, respectively. Each preparation shows vesicular structures and curved membrane sheets. Bar, 0.2 μm.](image)
localization to low buoyant density membrane domains.

Functional Significance of Caveolin-3/Adenylyl Cyclase Interactions—To determine whether localization to caveolae constitutes a mechanism to regulate the functional activity of the adenylyl cyclase enzyme, cardiomyocytes were treated with cholesterol-binding drugs to disrupt the functional integrity of the very cholesterol-enriched caveolae membranes. Initial experiments indicated that invasive/membrane-permeable cholesterol-binding agents such as filipin and nystatin induce gross cardiomyocyte toxicity (at 5 μg/ml for 20–30 min) before any changes in cellular cholesterol (or cAMP) can be detected. This precluded their use in these studies. In contrast, 2% cyclodextrin (a membrane-impermeable cholesterol-binding drug) for 1 h extracts 2/3 of total cell cholesterol, without inducing any gross morphological toxicity or major changes in spontaneous automaticity (i.e., both cholesterol depletion and the cyclodextrin treatment appear to be well tolerated). Fig. 10 shows that treatment with cyclodextrin results in an obvious redistribution of caveolin-3 and adenylyl cyclase from the caveolae fraction to the heavy sucrose layer; there also is some shift in β1- and β2-ARs, whereas the movement of Gαs is inconsistent. The functional consequences of cyclodextrin treatment were assessed by comparing the time course of cAMP accumulation in response to 10–5 M isoproterenol (a low concentration, which primarily activates the predominant β1-AR subtype) and 10–7 M zinterol (a β2-AR-selective agonist). However, given recent evidence that cholesterol depletion with cyclodextrin can interfere with clathrin-coated pit internalization (38, 39) (which in theory could provide an alternate explanation for changes in receptor-dependent cAMP accumulation), cells also were challenged with forskolin, a direct activator of adenylyl cyclase. Fig. 11 shows that cAMP accumulation is markedly increased in cells that are cholesterol-depleted with cyclodextrin. In separate experiments, cholesterol depletion with cyclodextrin was followed by an additional incubation for 1 h with cyclodextrin-cholesterol complexes to replete cholesterol levels. This returned cAMP responses back to base-line values (data not shown), indicating that changes in cAMP accumulation result from a loss of cholesterol (and diminished caveolae integrity) rather than any direct effect of cyclodextrin.

FIG. 10. Cholesterol depletion with cyclodextrin promotes the egress of adenylyl cyclase V/VI and caveolin-3 from caveolae. Cardiomyocytes were incubated with vehicle (−) or 2% cyclodextrin (+) for 1 h at 37 °C and then subjected to lysis in sodium carbonate and subcellular fractionation using a 5–35% discontinuous sucrose gradient as in Fig. 1. Aliquots of protein (30 μg/lane) pooled from fractions 4 and 5 (Caveolae) or 8–12 (Heavy Fractions) were probed for immunoreactivity for β1-ARs, β2-ARs, Gαs, adenylyl cyclase V/VI, or caveolin-3. Results are representative of the results obtained in three separate preparations.

FIG. 11. Cholesterol depletion with cyclodextrin augments isoproterenol-, zinterol-, and forskolin-dependent accumulation of cAMP in cardiomyocytes. Following preincubation with 10 mM theophylline plus vehicle (open symbols) or 2% cyclodextrin (filled symbols) for 1 h at 37 °C, cardiomyocytes were challenged for the indicated intervals with 10–5 M isoproterenol, 10–7 M zinterol, or 10–5 M forskolin, and cAMP accumulation was measured as described under "Experimental Procedures." Cyclodextrin induced a trivial increase in basal cAMP accumulation over 30 min, but this was not statistically significant (control: 22 ± 4.5 pmol/dish; cyclodextrin 32 ± 4.8 pmol/dish; mean ± S.E. of triplicate determinations from three separate experiments).

DISCUSSION

This study provides direct evidence that conventional paradigms for β1-AR-dependent cAMP signaling in cardiomyocytes must be extended to incorporate the concept of localization to membrane subdomains. The major findings of this study are that individual β-AR subtypes display markedly distinct subcellular targeting (both between intracellular and surface membrane compartments and between caveolae and noncaveolae compartments on the surface membrane), that β2-ARs are confined to caveolae in the basal state and egress from caveolae upon activation, and that adenylyl cyclase V/VI is highly localized to cardiomyocyte caveolae, where it interacts with caveolin-3. The experiments in HEK cells establish that the prototypical caveolins are not required for β-AR and adenylyl cyclase V/VI targeting to low buoyant density membranes. Nevertheless, the experiments on cyclodextrin-treated (cholesterol-depleted) cardiomyocytes indicate that targeting to caveolae represents a mechanism to negatively regulate cAMP accumulation.

There is a substantial body of literature describing the properties of β2-ARs in heterologous expression systems. Here, most studies describe β2-AR redistribution to clathrin-coated vesicles following agonist stimulation (although two laboratories have presented immunocytochemical evidence that β2-ARs segregate to non-clathrin-coated invaginations following incubation with a monoclonal antibody to the receptor and antimouse IgG-gold in epidermoid A431 cells (8, 40)). There is little to no information on the subcellular distribution of native β-ARs in a physiologically relevant cell type such as a cardiomyocyte. In particular, few studies have examined the subcellular localization of β2-ARs in quiescent cells or extended the analysis to consider β1-ARs. While Ostrom et al. recently reported that epitope-tagged β2-ARs heterologously expressed in cardiomyocytes are detected as a single 72-kDa band in caveolae (17), the interpretation of this finding is uncertain in the context of results reported herein (and in a previous study (27)), which identify the endogenous cardiomyocyte β1-AR as multiple prominent immunoreactive species. This study identifies the endogenous cardiomyocyte β1-AR as multiple molecular species that distribute between caveolae, noncaveolae cell surface plasma membranes, and internal membranes. It is
important to recognize that <1% of total cellular protein is recovered in caveolae. Hence, the vast majority of total cellular β2-ARs are excluded from caveolae in resting cardiomyocytes. The subcellular distribution of β2-ARs is not grossly altered by ligand binding. In contrast, β2-ARs partition exclusively to caveolae in resting cardiomyocytes and egress from caveolae upon ligand binding, a conclusion that fully accommodates the prevailing paradigm for clathrin-dependent endocytosis of β2-AR following activation by agonist. β2-AR localization in caveolae was detected with two distinct purification techniques (flotation of sodium carbonate-insoluble membranes on sucrose gradients and flotation of sonicated plasma membranes on OptiPrep gradients), lessening the likelihood that β2-ARs are in contaminating noncaveolae membrane fragments with sufficiently similar physical properties that they are not resolved by centrifugation in density gradients. The high degree of localization of cell surface β2-AR to caveolae is not unique to cardiomyocytes (which contain only a minor population of β2-ARs), since cell surface β2-ARs also are confined to caveolae in quiescent cardiac fibroblasts. These results suggest that changes in β2-AR expression over the physiologic range do not alter the fidelity of β2-AR targeting to this structure. Whether targeting fidelity is retained in the context of very high levels of (epitope-tagged) β2-AR overexpression must be determined in future studies, since prevailing concepts regarding β-AR function rely heavily on the results of studies in overexpression systems.

Differences in the cell surface partitioning of β1-AR versus β2-AR provide a rational explanation for previously identified differences in m3-mAChR-β-AR subtype interactions at the level of cAMP accumulation (2). The similar cell surface distribution for m3-mAChRs and β1-ARs would be permissive for interactions on the cell surface. In contrast, the targeting of β2-AR to caveolae, a compartment that is distant from most of the cell surface m3-mAChRs, represents a very plausible mechanism to explain the absence of any β2-AR-mAChR interaction at the level of cAMP formation. However, targeting to caveolae also may constitute a mechanism to facilitate adenyl cyclase activation by the more minor β2-AR population. The current dogma is that β2-ARs inherently couple to the activation of adenyl cyclase better than β1-ARs (1, 41, 42). While this has been attributed to structural differences between β1- and β2-ARs (43), the results of this study suggest that the spatial proximity of β2-ARs with the adenyl cyclase enzyme in caveolae may selectively enhance the efficiency of signal transduction from this β-AR subtype to the adenyl cyclase enzyme. In this regard, the drastic differences in the relative density of β1- versus β2-ARs in caveolae (which contain all of the β2-ARs and only a fraction of β1-ARs) and the remainder of the plasma membrane (which only has β1-ARs) also are worth emphasis. Domain-specific differences in the stoichiometry of various elements in this signaling cascade are predicted to impact significantly on the efficiency of signal transduction.

In addition to β-AR subtypes, this study describes marked differences in the extent to which other components of the signaling machinery required to generate, propagate, or down-regulate the cAMP signal target to caveolae. For example, Gαs and GoQ differ markedly in the extent to which they localize to cardiomyocyte caveolae. This could provide a mechanism to generate a gradient in cAMP levels in cells exposed to catecholamines and is of interest, given the early evidence that local pools of cAMP may differentially activate PKA-dependent functions (44, 45). Similarly, the functional consequences of the cAMP signal may be modulated by local differences in the PKA enzyme. This study demonstrates that the RII subunit is abundant in caveolae; high concentrations of RII at this site would effectively increase local concentrations of the PKA II holoenzyme and could serve to promote the phosphorylation of proteins in the vicinity of this structure. In contrast, RI is excluded from this site, supporting the provocative (but as yet unproved) hypothesis that PKAI can phosphorylate a distinct spectrum of target proteins and subserve functions that are distinct from PKAII in the heart. Finally, this study demonstrates that GRK2 can be detected in caveolae. While GRK2 is reported to be negatively regulated by caveolin (19), the functional significance of this process in cardiomyocytes is uncertain, since only a minor component of GRK2 is detected in caveolae (at least under resting conditions).

On the basis of the numerous regulatory functions that have been attributed to caveolae, the integrated functional consequences of targeting to this microdomain are not entirely predictable. If one assumes that caveolin oligomers form a scaffold on the cytoplasmic surface of caveolae to sequester and organize signaling molecules, it might be predicted that disruption of this microdomain would impair cAMP-dependent signaling. Alternatively, the functional activity of multiple components of the β-AR complex (including such as G protein α subunits and the adenylyl cyclase enzyme) are reported to be dampened through interactions with caveolin. According to this formulation, signaling would be enhanced by the removal of this negative regulatory function for caveolae. Results reported herein show that disassembly of caveolae with cyclodextrin (and dispersion of caveolae proteins) leads to enhanced cAMP accumulation in response to β1-AR agonists, β2-AR agonists, and forskolin. This favors a formulation in which caveolae act to repress cAMP formation, with the inhibitory control at the level of the adenylyl cyclase enzyme. There are at least two potential mechanisms that could account for local negative regulation of adenyl cyclase in caveolae. First, based upon previous evidence that the activity of cardiac adenylyl cyclase isoforms is suppressed by caveolin-3-based scaffolding domain peptides (16) and the evidence reported herein that adenylyl cyclase and caveolin-3 co-immunoprecipitate, it is possible that in vivo adenylyl cyclase-caveolin-3 interactions tonically inhibit enzyme activity. Alternatively, there is recent evidence that the calcium-sensitive adenylyl cyclase VI isoform is susceptible to inhibitory regulation by calcium signals generated by capacitative calcium entry channels that co-localize to caveolae (47). A potential role for capacitative calcium entry channels in the regulation of adenylyl cyclase catalytic activity in electrically excitable cardiomyocytes, with spontaneous calcium cycling function, requires further study.

The identification of caveolae in mice to assemble functionally active β-AR complexes in cardiomyocytes is likely to be associated with important clinical implications. For example, there are almost decade-old reports that treatment of cardiomyocytes with cholesterol synthesis inhibitors in the presence of lipoprotein-depleted serum (which lowers plasma membrane cholesterol content and presumably disrupts the normal caveolar structure) leads to profound changes in β-AR responses in cardiomyocytes (48, 49). These early studies suggest that caveolae play a critically important role in calibrating signaling to adenylyl cyclase. There also is recent evidence that the activity of cardiac adenylyl cyclase-3 expression is regulated by the cAMP pathway and in response to β1-AR agonists and forskolin (48, 49). These results suggest that the spatial proximity of β2-ARs with the adenyl cyclase enzyme in caveolae may selectively enhance the efficiency of signal transduction from this β-AR subtype to the adenyl cyclase enzyme. In this regard, the drastic differences in the relative density of β1- versus β2-ARs in caveolae (which contain all of the β2-ARs and only a fraction of β1-ARs) and the remainder of the plasma membrane (which only has β1-ARs) also are worth emphasis. Domain-specific differences in the stoichiometry of various elements in this signaling cascade are predicted to impact significantly on the efficiency of signal transduction.

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