G-protein-coupled Receptor Signaling Components Localize in Both Sarcolemmal and Intracellular Caveolin-3-associated Microdomains in Adult Cardiac Myocytes*

This study tests the hypothesis that G-protein-coupled receptor (GPCR) signaling components involved in the regulation of adenylyl cyclase (AC) localize with caveolin (Cav), a protein marker for caveolae, in both cell-surface and intracellular membrane regions. Using sucrose density fractionation of adult cardiac myocytes, we detected Cav-3 in both buoyant membrane fractions (BF) and heavy/non-buoyant fractions (HF); β2-adrenergic receptors (AR) in BF; and AC5/6, βi₂-AR, M2-muscarinic acetylcholine receptors (mAChR), µ-opioid receptors, and Goαi in both BF and HF. In contrast, M3-mAChR, Goα3, and Goα12 were found only in HF. Immunofluorescence microscopy showed co-localization of Cav-3 with AC5/6, Goαi, β2-AR, and µ-opioid receptors in both sarcolemmal and intracellular membranes, whereas M2-mAChR were detected only intracellularly. Immunofluorescence of adult heart revealed a distribution of Cav-3 identical to that in isolated adult cardiac myocytes. Upon immunoelectron microscopy, Cav-3 co-localized with AC5/6 and Goαi in sarcolemmal and intracellular vesicles, the latter closely allied with T-tubules. Cav-3 immunoprecipitates possessed components that were necessary and sufficient for GPCR agonist-promoted stimulation and inhibition of cAMP formation. The distribution of GPCR, G-proteins, and AC with Cav-3 in both sarcolemmal and intracellular T-tubule-associated regions indicates the existence of multiple Cav-3-localized cellular microdomains for signaling by hormones and drugs in the heart.

Caveolar microdomains have been proposed as sites that concentrate G-protein-coupled receptors (GPCR),1 heterotrimeric G-proteins, and GPCR/G-protein-regulated effector molecules in a confined region so as to facilitate coordinated and kinetically favorable second messenger formation (1, 2). Caveolae (“little caves”), cholesterol- and sphingolipid-enriched 50–100-nm invaginations of the plasma membrane (3, 4), are considered a subset of lipid rafts (5). Caveolae (Cav), a protein marker for caveolae, is present in three isoforms (Cav-1, Cav-2, and -3); Cav-3 is preferentially expressed in skeletal and cardiac muscle. Caveolae help regulate a variety of cell functions, including endocytosis, calcium homeostasis, skeletal muscle T-tubule formation, and GPCR compartmentation (6, 7). Components involved in GPCR signaling, i.e. certain receptors, G-proteins, and G-protein-regulated effectors, localize with Cav in the plasma membrane (5, 8–14).

The caveolin/lipid raft signaling hypothesis proposes that compartmentation of signaling molecules in caveolae provides a mechanism for temporal and spatial signal transduction and cross-talk among signaling pathways (15). Despite substantial data supporting this notion (16, 17), little is known regarding the expression of caveolae in the plasma membrane versus non-plasma membrane locations. One cell type in which GPCR compartmentation has been studied is the cardiac myocyte (CM), but virtually all previous studies related to caveole have involved the use of neonatal CM (9, 18). Adult CM differ from neonatal CM in numerous ways, including being more extensively differentiated, multinucleated, and larger in volume and possessing a more developed T-tubule network. In this study, we hypothesized that Cav-3 co-localizes with GPCR signaling components in both sarcolemmal and intracellular membranes and used adult CM to determine whether (a) Cav distributes differently in adult CM versus other cardiac cell types; (b) GPCR (e.g., β-adrenergic, muscarinic, and µ-opioid receptors) and key post-receptor signaling components, including heterotrimeric G-proteins and adenylyl cyclase, co-localize with Cav-3 in intracellular domains (i.e. T-tubules) in addition to the sarcolemma; and (c) Cav-3 scaffolds GPCR signaling components capable of stimulating and inhibiting cAMP synthesis.

EXPERIMENTAL PROCEDURES

Materials—Antibodies for adenylyl cyclase (AC) type 5/6, βi and β2-adrenergic receptors (AR), Goαi, Goα3, Goαq, M2- and M3-muscarinic acetylcholine receptors (mAChR), µ-opioid receptors (µ-OR), and β-adaptin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies for Cav-3 and Cav-1 were from BD Biosciences. Antibodies for dihydropridine receptors (DHPR), vinucil, and ryanodine receptors (RyR) were purchased from Sigma. Anti-Cav-3 polyclonal antibodies were from Abcam (Cambridge, MA). Fluorescein isothiocyanate- and Alexa-conjugated secondary antibodies were obtained from Invitrogen. Other secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. All other chemicals and reagents were obtained from Sigma unless stated otherwise.

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* This work was supported by National Institutes of Health Grants HL66941, HL53773, HL63885, HL074625, and RGS C1A. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: GPCR, G-protein-coupled receptor(s); Cav, caveolin; CM, cardiac myocyte(s); AC, adenylyl cyclase; AR, adrenergic receptor(s); mAChR, muscarinic acetylcholine receptor(s); µ-OR, µ-opioid receptor(s); DHPR, dihydropridine receptor(s); RyR, ryanodine receptor(s); PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; BF, buoyant fraction(s); HF, heavy fraction(s); DAMGO, [5-αA,5-αM-Phe,6-αGly,1-ol]enkephalin; hCASMC, human coronary artery smooth muscle cells; ACF, adult rat cardiac fibroblasts; SR, sarcoplasmic reticulum.
CM Preparation—Adult male Sprague-Dawley rats (250–300 g) were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and hearts were excised and retrograde-perfused with medium containing collagenase (Roche) as described previously (19). Animals were heparinized (1000–2000 units intraperitoneal) 5 min prior to administration of anesthesia. Hearts were removed and placed in ice-cold cardioplegic solution (112 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 9 mM NaH₂PO₄, and 11.1 mM d-glucose supplemented with 10 mM HEPES, 30 mM taurocholine, 2 mM tri-carnitine, and 2 mM creatine, pH 7.4). The hearts were retrograde-perfused on a Langendorf apparatus at a rate of 5 ml/min for 5 min at 37 °C, followed by perfusion with medium containing collagenase II (250 units/ml) for 20 min. Following perfusion, both ventricles were isolated, minced in collagenase II-containing medium for 10–15 min, washed several times, and re-acclimated to 1.2 mM Ca²⁺ over 25 min to produce calcium-tolerant CM. To remove all non-myocytes, myocytes were plated in 4% fetal bovine serum on laminin (2 μg/cm²)-coated plates for 1 h, followed by serum-free medium (1% bovine serum albumin). CM were incubated at 37 °C in 5% CO₂ for 24 h prior to experiments.

Membrane Fractionation—CM were fractionated using both detergent-free and detergent-containing (1% Triton X-100) methods (20, 21). Buffer containing 10 mM KH₂PO₄, 5 mM MgCl₂, 5 mM EDTA, and 1 mM EGTA was used to extract the contractile myofilaments as described previously (21). A 15-cm plate was washed twice with ice-cold phosphate-buffered saline (PBS) and scraped in 3 ml of either 500 mM sodium carbonate, pH 11.0, to extract peripheral membrane proteins or TNE buffer (25 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA) containing 1% Triton X-100. For detergent-free extraction, cells were homogenized by three 10-s bursts of a tissue grinder and then sonicated by three cycles of 20-s bursts of sonication and 1 min of incubation on ice. Approximately 2 ml of homogenate were mixed with 2 ml of 90% sucrose in MES-buffered saline (25 mM MES and 150 mM NaCl, pH 6.5) to form 45% sucrose and loaded at the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient was generated by layering 4 ml of 35% sucrose prepared in MES-buffered saline and 250 mM Na₂CO₃, followed by 4 ml of 5% sucrose also in MES-buffered saline/Na₂CO₃. Gradients were centrifuged at 290,000 × g using a Beckman SW 41Ti rotor for 16–20 h at 4 °C. Centrifugation of homogenate were mixed with 2 ml of 90% sucrose in TNE buffer. A discontinuous sucrose gradient was generated by layering 4 ml of 30% sucrose in TNE buffer, followed by 4 ml of 5% sucrose in TNE buffer and centrifugation at 190,000 × g using the SW 41Ti rotor for 16–20 h at 4 °C. Samples were removed in 1-ml aliquots to form 12 fractions.

Immunoprecipitation of Buoyant Fractions (BF) and Heavy Fractions (HF)—Immunoprecipitations were performed using either protein G-agarose (Roche Applied Science). BF and HF (in which the pH was neutralized with HCl) or Triton X-100 fractions were incubated with primary antibody for 3 h at 4 °C, immunoprecipitated overnight with protein-agarose at 4 °C, and then centrifuged at 13,000 × g for 5 min. Protein-agarose pellets were washed once with lysis buffer (50 mM Tris-HCl, 10 mM NaCl, 1 mM EGTA, 2 mM dithiothreitol, and 0.5% Igepal CA-630 plus mammalian protease inhibitors mixture (Sigma)), followed by subsequent washes with wash buffer A (50 mM Tris- HCl, pH 7.5, 500 mM NaCl, and 0.2% Igepal CA-630) and wash buffer B (10 mM Tris-HCl, pH 7.5, and 0.2% Igepal CA-630).

Immunoblot Analysis—Proteins in fractions and cell lysates were separated by SDS-PAGE using 10 or 12% acrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore Corp.) by electrotransfer. Membranes were blocked with 20 mM PBS and 1% Tween containing 1.5% nonfat dry milk and incubated overnight with primary antibody at 4 °C. Primary antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) and ECL reagent (Amersham Biosciences). All displayed bands migrated at the appropriate size as determined by comparison with molecular weight standards (Santa Cruz Biotechnology, Inc.). The amount of protein per fraction was determined using the SW 41Ti rotor for 16–20 h at 4 °C. Centrifugation of homogenate were mixed with 2 ml of 90% sucrose in TNE buffer. A discontinuous sucrose gradient was generated by layering 4 ml of 35% sucrose in MES-buffered saline and 250 mM Na₂CO₃, followed by 4 ml of 5% sucrose also in MES-buffered saline/Na₂CO₃. Gradients were centrifuged at 290,000 × g using a Beckman SW 41Ti rotor for 16–20 h at 4 °C. Centrifugation of homogenate were mixed with 2 ml of 90% sucrose in TNE buffer. A discontinuous sucrose gradient was generated by layering 4 ml of 30% sucrose in TNE buffer, followed by 4 ml of 5% sucrose in TNE buffer and centrifugation at 190,000 × g using the SW 41Ti rotor for 16–20 h at 4 °C. Samples were removed in 1-ml aliquots to form 12 fractions.

Measurement of AC Activity—AC activity was measured in Cav-3 immunoprecipitates using a modification of a previously described method (10). A 15-cm plate of adult CM was homogenized on ice in lysis buffer, preclarified with protein A-agarose for 1 h, and incubated with primary antibody for 1 h at 4 °C. Antibody conjugates were immunoprecipitated with protein G-agarose for 1 h at 4 °C and centrifuged at 13,000 × g for 5 min. Agarose pellets were washed once with lysis buffer and subsequently with wash buffers A and B and then resuspended in 30 mM Na-HEPES, 5 mM MgCl₂, and 2 mM dithiothreitol, pH 7.5. Protein (30 μl of immunoprecipitate) was added to tubes containing 30 mM Na-HEPES, pH 7.5, 100 mM NaCl, 1 mM EGTA, 10 mM MgCl₂, 1 mM isobutylmethylxanthine (a cyclic nucleotide phosphodiesterase inhibitor), 10 mM phosphocreatine, 5 μM calcium, 1 mM EGTA, 2 mM dithiothreitol, and 0.1% bovine serum albumin. After 5 min, 0.1 μM noloxane (an opioid receptor antagonist) or vehicle was added, followed by 5 min later by addition of 1 μM [α-Ala², N-MePhe³, Gly⁵-OH]enkephalin (DAMGO; a selective μ-OR agonist) or vehicle. After 5 min, 10 μM forskolin was added, and samples were incubated for an additional 15 min. The reaction was stopped by boiling for 5 min, and cAMP content was assayed as described previously (27).

RESULTS

Cavosin Distributes Differently in Adult CM Compared with Other Cardiovascular Cells—The distribution of Cav was investigated in human coronary artery smooth muscle cells (hCASMC) and adult rat cardiac fibroblasts (ACF) and CM following sucrose density fractionation. We detected the major-Cav isoform at 100 kDa in Cav-3 (Fig. 1A). In adult CM, Cav-3 was detected in both BF 4 and 5 and HF (non-BF) fractions (Fig. 1B). Following equal protein loading of sucrose density fractions of adult CM (Fig. 1B), BF were enriched in Cav-3; HF had a lower proportion of Cav-3 (Fig. 1A, lower panels). BF contained <5% of total cellular protein (Fig. 1B). Adult CM fractions in Fig. 1A (from equal volume-loaded gels) that were immunoblotted for the T-tubule markers vinculin and DHPR (a marker for voltage-sensitive calcium chan-
nels) showed a distribution pattern similar to that with Cav-3 (Fig. 1C). RyR (a marker of the sarcoplasmic reticulum (SR)) and β-adaptin (a marker of clathrin-coated pits) were detected only in HF (Fig. 1C). To test whether the Cav-3 distribution pattern observed upon Na2CO3/sucrose density fractionation of adult CM was unique to this method of cellular disruption and fractionation, we fractionated adult CM in alternative lysis buffers, one containing 1% Triton X-100 and the other containing a high salt buffer to extract contractile myofibrils. Immunoblotting of equal volume-loaded fractions from the Triton X-100 fractionation revealed a broad distribution of Cav-3 (Fig. 1D). Following extraction of the contractile myofibrils using high salt buffer, we detected buoyant and heavy pools of Cav-3, albeit with a substantially greater amount of Cav-3 in the heavier fractions (Fig. 1D). Overall, these results show that Cav-3 was present in both BF and HF following sucrose density fractionation, indicating a different cellular distribution compared with Cav-1 in two other cardiovascular cell types, hCASMC and ACF.

**Fig. 1. Distribution of Cav-3 protein and markers in CM, ACF, and hCASMC.** A, Na2CO3 extraction and sucrose density fractionation were undertaken with adult CM and ACF and hCASMC as described under “Experimental Procedures.” IB, immunoblot. B, protein concentrations are shown for fractions 4–12 and are representative of caveolar fractionation experiments with adult CM (ACM) (n = 6). C, equal volume-loaded samples following sucrose density fractionation were assessed for localization of RyR (an SR marker), vinculin (a T-tubule marker), DHPR (a voltage-sensitive Ca2+ channel and T-tubule marker), and β-adaptin (a clathrin-coated pit marker) in adult CM. D, shown are the results from equal volume loading of adult CM subjected to Triton X-100 (TX) and high salt extraction, followed by subcellular fractionation.

**GPCR Signaling Components Distribute Non-uniformly with Cav-3 in Adult CM**—Using immunoblot analyses, we investigated co-localization of Gi and Gs protein-coupled receptor signaling components with Cav-3 in adult CM. AC5/6 and β1- and β2-AR were detected in BF, whereas a portion of β1-AR was also detected in HF (Fig. 2A). Gαs was detected as two bands (which represent the short and long splice variants of Gαs) in BF and
Because the β2-AR and mAChR pathways act antagonistically in the regulation of cardiac rate and force of contraction, we investigated whether M2- and M4-mAChR localize to BF. We found that M2-mAChR were excluded from BF, as were G\textsubscript{i3} and G\textsubscript{i2}, G-proteins through which both M2- and M4-mAChR signal, whereas we detected M4-mAChR in both BF and HF (Fig. 2B and C). We also detected µ-OR, another G\textsubscript{i}-coupled receptor, in adult CM by PCR (data not shown) and immunoblotting of both BF and HF (Fig. 2D). As an alternative means to test for interaction of the signaling components with Cav-3, we assessed BF and HF following immunoprecipitation with an anti-Cav-3 antibody (Fig. 2D). AC\textsubscript{5/6}, β\textsubscript{2}- and β\textsubscript{1}-AR, G\textsubscript{ai3}, G\textsubscript{ai2}, M\textsubscript{2}-mAChR, G\textsubscript{ai3}, G\textsubscript{ai2}, and Cav-3 were all detected in Cav-3 immunoprecipitates from both BF and HF. By contrast, M\textsubscript{4}-mAChR and G\textsubscript{ai3} (Fig. 2D) were detected only in Cav-3 immunoprecipitates from HF, whereas G\textsubscript{ai2} and β-adaptin were not detected in immunoprecipitates from either BF or HF. Thus, the results with the Cav-3 immunoprecipitates from BF and HF confirm the immunoblot findings with sucrose density fractions and demonstrate that GPCR signaling components vary in their cellular distribution with Cav-3.

**Immunofluorescence Microscopy Shows Co-localization of Cav-3 with AC\textsubscript{5/6}, G\textsubscript{ai3}, protein, β\textsubscript{2}-AR, µ-OR, and M\textsubscript{4}-mAChR in Sarcolemmal and Intracellular Regions in Adult CM**—We used immunofluorescence microscopy as an additional means to assess GPCR co-localization with Cav-3 in CM. Cav-3 was detected in the sarcolemma as a punctate pattern and in striations running transversely across the interior of the cell (Figs. 3–8). To assess co-localization of Cav-3 and AC\textsubscript{6}, we used an adenoviral vector to overexpress AC\textsubscript{6} because available antibodies do not readily detect endogenous AC\textsubscript{6} in adult CM. Individual components showed different extents of co-localization with Cav-3 in the sarcolemmal and intracellular regions (data expressed relative to Cav-3 expression) (Figs. 3–5): AC\textsubscript{5/6}, 27 and 11%, respectively; G\textsubscript{ai3}, 32 and 7%, respectively; β\textsubscript{2}-AR, 43 and 59%, re-
spectively; µ-OR, 39 and 32%, respectively; M₂-mAChR, 21 and 34%, respectively; and M₄-mAChR, 12 and 45%, respectively (Figs. 3–5). As a negative control, incubation with secondary antibodies revealed minimal background staining (Fig. 4, lower panel). AC6, Ga₃, β₂-AR, M₄-mAChR, and µ-OR displayed greater co-localization with Cav-3 in the sarcolemma than did M₂-mAChR. The distribution of intracellular β₂-AR was predominantly in subsarcolemmal locations (Fig. 3), whereas M₂-mAChR displayed a more transverse intracellular distribution (Fig. 5A, upper panels). Treatment with the agonist carbachol (100 µM) for 10 min disrupted the transverse staining pattern of M₂-mAChR, with redistribution to the sarcolemma and nucleus (Fig. 5A, lower panels). Quantitation of Cav-3 immunofluorescence revealed that ~25% of total cellular Cav-3 was sarcolemmal (Fig. 5B, left panel), a value similar to that for Cav-3 detected in BF (right panel) from sucrose density fractionation (Fig. 1C, third panel). Thus, Cav-3 appears to be present in both intracellular and sarcolemmal regions, and different GPCR differ in their co-localization with Cav-3.

Immunofluorescence Microscopy of Adult Heart Shows Co-localization of Cav-3 with T-tubule (DHPR and Vinculin) and Sarcoplasmic Reticulum (RyR) Markers in Both Adult CM and Heart—Immunostaining of adult CM revealed that Cav-3 co-localized with two different T-tubule markers (vinculin and DHPR) in a transverse pattern and along the sarcolemma (Fig. 7A). Tissue sections for Cav-3 and DHPR revealed a pattern similar to that found in isolated CM, with Cav-3 and DHPR co-localizing in intracellular regions running transversely across the cell, along the sarcolemma, and in intercalated discs (Fig. 7B, right panel, arrow). Staining for Cav-3 and RyR (an SR marker) revealed co-localization predominantly in intracellular regions running transversely across the cell in both isolated CM and heart (Fig. 8A) and in

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**FIG. 5.** Immunofluorescence and deconvolution analysis of the co-localization of Cav-3 and M₂-mAChR following treatment with carbachol in adult CM. A, cells were co-stained with antibodies for Cav-3 and M₂-mAChR under basal conditions and following treatment with 100 µM carbachol (Carb) for 10 min. Under basal conditions, M₂-mAChR were found predominantly in a discrete transverse pattern across the interior of the cell. After treatment with carbachol, M₂-mAChR were more sparsely distributed within the interior of the cell and redistributed to sarcolemmal and nuclear regions of the cell. B, quantitation of Cav-3 immunofluorescence and Cav-3 immunoblot from sucrose density fractionation.
intercalated discs in sections of heart (Fig. 8B, right panel, arrows). We detected minimal co-localization of Cav-3 and α-actinin, a Z-disc marker (data not shown). As an alternative means to test for Cav-3/RyR interaction, we assessed lysates from CM immunoprecipitated with anti-Cav-3 and anti-RyR antibodies (Fig. 8C) and observed Cav-3 and RyR in both Cav-3 and RyR immunoprecipitates, thus confirming results from immunofluorescence microscopy showing co-localization of Cav-3 and the SR marker. Thus, Cav-3 localizes not only along the sarcolemma, but also in T-tubule/SR regions in both adult CM and heart.

**Immunoelectron Microscopy Detects Cav-3 in Sarcolemmal Invaginations and Intracellular Domains in Adult CM**—We used immunogold labeling as an additional means to assess the distribution of Cav-3 in adult CM. Immunoelectron microscopy demonstrated abundant sarcolemmal caveolae, present as invaginations that labeled with antibodies directed against Cav-3 (Fig. 9A and B). We also detected Cav-3 in membranes flanked by Z-discs, which correspond to the T-tubule network within the cell interior (Fig. 9C). These immunoelectron microscopic data confirmed and extended results from immunofluorescence microscopy demonstrating Cav-3 in both sarcolemmal and intracellular regions and are consistent with the detection of Cav-3 in HF following sucrose density fractionation (Fig. 1).

**Immunoelectron Microscopy Shows Co-localization of Cav-3 with AC6 and Gαi3**—Using immunogold labeling, we detected Cav-3 (10-nm gold) with AC6 (5-nm gold) in invaginations of the sarcolemma (Fig. 9D) and in intracellular membranes (Fig. 9E) located between adjacent Z-discs. We also detected Cav-3 (10-nm gold) with Gαi3 (5-nm gold) in vesicles near myofibrils (Fig. 9, F and G). These immunoelectron microscopic findings are consistent with the results of Cav-3 immunoprecipitation studies showing multiprotein interaction between Cav-3 and AC6 and Gαi3 in adult CM (Fig. 2C).

**AC6 Overexpression Enhances GPCR-stimulated cAMP Production in Adult CM**—To confirm that the overexpressed AC6 that we analyzed microscopically (Figs. 3 and 9) was functional, we assessed cAMP production in adult CM. Overexpression of AC6 increased the levels of cAMP produced in response to isoproterenol (a β-AR agonist) without an increase in agonist potency (Fig. 10A). Thus, the overexpressed AC6 that co-localized with Cav-3 in adult CM (as shown by both immunofluorescence and immunoelectron microscopy) was enzymatically active. Moreover, by assaying forskolin-stimulated cAMP accumulation, we obtained functional evidence for the presence of μ-OR in adult CM: the μ-OR agonist DAMGO significantly reduced forskolin-stimulated cAMP production ($p < 0.05$, $n = 5$) (Fig. 10B).

**Cav-3 Immunoprecipitates Multiprotein Complexes Capable of Producing and Inhibiting cAMP**—To demonstrate that Cav-3 interacts with and organizes components that mediate stimulation and inhibition of cAMP formation, we assessed AC activity in Cav-3 immunoprecipitates of adult CM lysates. Forskolin increased cAMP production (Fig. 10C); this stimulation was significantly ($p < 0.01$) inhibited by DAMGO, a response that was inhibited by the opioid receptor antagonist naloxone. Immunoblot analysis detected μ-OR and Gαi3 in the Cav-3 immunoprecipitates used in the AC activity experiments (data not shown). These data show functional evidence for the existence of μ-OR and support the conclusion that Cav-3 interacts with multiprotein complexes that can both stimulate and inhibit cAMP production in adult CM.
DISCUSSION

Caveolae-localized signaling microdomains have been proposed as sites that concentrate GPCR, heterotrimeric G-proteins, and G-protein-regulated effector molecules so as to facilitate coordinated, precise, and rapid regulation of cell function (5, 8–14). Cav-rich domains thus may serve as spatial organizers of GPCR signaling, although not all data have supported this conclusion (13, 28). Using multiple experimental approaches (subcellular fractionation, immunoprecipitation, immunofluorescence, immunoelectron microscopy, and functional assays of cAMP formation), we have provided new evidence that extends the notion of Cav-rich domains as organizers of GPCR signaling components and in support of the novel conclusion that Cav-3 organizes GPCR signaling components in both sarcolemmal and intracellular regions (e.g. T-tubules in cardiac myocytes) in adult heart.

Most previous studies on compartmentation of GPCR signaling components in CM have utilized embryonic and neonatal cells (9, 18, 27, 29). However, it is preferable to study adult CM, which are more akin to the in vivo setting than are neonatal CM and other non-striated cardiovascular cells (30). Because of development-related changes in ion channels and contractile proteins, it can be difficult to extrapolate results from the neonatal to the adult heart. The present results regarding Cav-3 distribution in adult CM differ from findings reported for neonatal CM and certain non-cardiac cell types in which Cav distributed only to BF (9, 18, 27, 29, 31). Our demonstration that M₂-mAChR were excluded from BF and expressed in intracellular sites confirms and extends previous results for adult CM (32) and contrasts with findings obtained with neonatal CM in which M₂-mAChR were detected in both BF and HF (9). Such differences imply developmental changes related to caveolar compartmentation of the β-AR and mAChR signaling cascades, which perhaps contribute to differences during development in response to physiologic stimuli. Compartmentation of GPCR signaling components in adult CM also contrasts with findings obtained with adult rat aortic smooth muscle cells in which β₁- and β₂-AR were found only in HF, implying that localization of particular GPCR to Cav-rich fractions is cell type-dependent (2, 10).

Previous workers have observed intracellular Cav-3 in skeletal myocytes (6, 33, 34). A possible role for intracellular Cav in adult CM may be as a regulator of calcium homeostasis (35): T-tubules in adult CM are continuous with the sarcolemma and are essential for the influx of calcium via L-type Ca²⁺ channels or DHPR and regulation of myocyte contractility (36–39). DHPR are located primarily at the T-tubule/SR junction proximal to where the SR Ca²⁺ release channels or RyR are found

![Immunofluorescence and deconvolution analysis of the co-localization of Cav-3 and the SR marker RyR in adult CM and heart. A, cells co-stained with antibodies for Cav-3 and RyR revealed co-localization of Cav-3 and the SR marker in a transverse pattern within the cell interior. B, semithin sections (5 μm) of adult heart co-stained with antibodies for Cav-3 and RyR displayed co-localization in transverse striations within the cell and in intercalated discs (arrows) between adjacent CM. Images were deconvolved and are shown as single-stained or overlaid to show co-localization. Scale bar = 30 μm in B. C, RyR and Cav-3 immunoprecipitates (IP) were probed for RyR and Cav-3. IB, immunoblot.](http://www.jbc.org/)

![Immunoelectron microscopic localization of Cav-3 with AC6 and Go₆ in the sarcolemma and in intracellular membranes morphologically corresponding to T-tubules in adult CM. Immunogold labeling detected Cav-3 (10-nm gold) in the sarcolemmal membrane (A and B) and in intracellular membranes anchored to Z-discs (Z) (C). Additional immunogold labeling detected Cav-3 (10-nm gold) and AC6 (5-nm gold) in sarcolemmal invaginations (D) and in intracellular membranes near Z-discs (E). Similarly, Cav-3 (10-nm gold) and Go₆ (5-nm gold) were detected in intracellular membranes corresponding to T-tubules (t) (F and G). Scale bars = 0.05 μm.](http://www.jbc.org/)
Although caveolae are morphologic entities, virtually all previous work has utilized subcellular fractionation or immunoprecipitation to infer co-localization of GPCR signaling components and caveolins. Plating of adult CM on laminin-coated surfaces allowed us to utilize microscopic techniques (both light and electron) to show Cav-3 in a punctate pattern along the sarcolemma and in intracellular transverse striations. This study thus provides the first microscopic evidence of Cav-3 co-localization with GPCR and GPCR signaling components in both sarcolemma and intracellular membranes (in particular, T-tubule-associated membrane regions) in adult CM (Fig. 9, D–G). The results from immunofluorescence microscopy of adult myocardium showing a Cav-3 distribution similar to that seen in isolated adult CM (Figs. 6, 7B, and 8B) support the use of isolated adult CM as an in vitro model (30).

Opioid receptors have been shown to play an important role in protecting the heart from ischemic injury (43, 44) and arrhythmias (45). However, ambiguity exists regarding the receptor subtypes expressed and activated by agonists in the myocardium (46); in particular, the presence of μ-OR has been disputed (47). Past studies that utilized radioligand binding experiments were performed on membranes prepared from whole hearts, making it difficult to distinguish sarcolemmal from intracellular CM membranes and membranes contributed by other cell types. Our results obtained using four different techniques (PCR, Western blotting, immunofluorescence microscopy, and assay of cAMP generation) provide evidence consistent with the idea that functional μ-OR are expressed in CM of adult heart.

In conclusion, in this study, we used multiple complementary techniques (subcellular fractionation, immunocytochemistry, morphology, and functional assays) to document a role for cardiac Cav-3 as an organizer of signaling components that regulate cAMP production for multiple classes of GPCR (i.e. β-AR, mAChR, and μ-OR). The results imply that spatial organization of GPCR signaling components occurs in microdomains in both sarcolemmal and intracellular membrane regions in the heart.

Acknowledgments—We thank Nicole D. Chin for technical assistance and Jim Feramisco, Julie Sherman, Steve McMullen, and others at the University of California, San Diego, Cancer Center Digital Imaging Shared Resource. In some cases, the three-dimensional perspective views were made at the VisLab in the San Diego Supercomputer Center using National Partnership for Advanced Computational Infrastructure Scalable Visualization Tools.

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J. Biol. Chem. 2005, 280:31036-31044.
doi: 10.1074/jbc.M502540200 originally published online June 16, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M502540200

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