

Caveolar Localization Dictates Physiologic Signaling of β_2 -Adrenoceptors in Neonatal Cardiac Myocytes*

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Yang Xiang^{‡§}, Vitalyi O. Rybin[¶], Susan F. Steinberg[¶], Brian Kobilka^{‡||}

From the [‡]Department of Molecular and Cellular Physiology, Howard Hughes Medical Institute, Stanford Medical Center, Palo Alto, California 94305 and the [¶]Department of Pharmacology, Columbia University, New York, New York 10032

There is a growing body of evidence that G protein-coupled receptors function in the context of plasma membrane signaling compartments. These compartments may facilitate interaction between receptors and specific downstream signaling components while restricting access to other signaling molecules. We recently reported that β_1 - and β_2 -adrenergic receptors (AR) regulate the intrinsic contraction rate in neonatal mouse myocytes through distinct signaling pathways. By studying neonatal myocytes isolated from β_1 AR and β_2 AR knockout mice, we found that stimulation of the β_1 AR leads to a protein kinase A-dependent increase in the contraction rate. In contrast, stimulation of the β_2 AR has a biphasic effect on the contraction rate. The biphasic effect includes an initial protein kinase A-independent increase in the contraction rate followed by a sustained decrease in the contraction rate that can be blocked by pertussis toxin. Here we present evidence that caveolar localization is required for physiologic signaling by the β_2 AR but not the β_1 AR in neonatal cardiac myocytes. Evidence for β_2 AR localization to caveolae includes co-localization by confocal imaging, co-immunoprecipitation of the β_2 AR and caveolin 3, and co-migration of the β_2 AR with a caveolin-3-enriched membrane fraction. The β_2 AR-stimulated increase in the myocyte contraction rate is increased by ~2-fold and markedly prolonged by filipin, an agent that disrupts lipid rafts such as caveolae and significantly reduces co-immunoprecipitation of β_2 AR and caveolin 3 and co-migration of β_2 AR and caveolin-3 enriched membranes. In contrast, filipin has no effect on β_1 AR signaling. These observations suggest that β_2 ARs are normally restricted to caveolae in myocyte membranes and that this localization is essential for physiologic signaling of this receptor subtype.

Catecholamines act through cardiac β -adrenergic receptors (β ARs)¹ to modulate heart rate and contractility. Three β AR subtypes have been cloned (β_1 AR, β_2 AR, and β_3 AR). β_1 AR and β_2 AR are the primary subtypes responsible for cardiac response to catecholamines. β_1 AR and β_2 AR are also pharmaco-

logically more similar to each other than they are to the β_3 AR. The close structural and functional properties of β_1 AR and β_2 AR are paradigmatic of many other G protein-coupled receptor families in which two or more receptor subtypes respond to the same hormone or neurotransmitter and couple to the same effector systems. Although β_1 AR and β_2 AR have very similar signaling properties when expressed in undifferentiated cell lines (1), there is a growing body of experimental evidence that suggests that they have different signaling properties in regulating cardiac function. The β_1 AR knockout (β_1 AR-KO) mice lack the normal chronotropic and inotropic responses to the non-selective agonist isoproterenol (2). Thus, in the murine heart, β_2 ARs play no significant role in controlling heart rate and contractility. β_2 AR knockout (β_2 AR-KO) mice have normal inotropic and chronotropic responses to isoproterenol, confirming that β_1 AR is the subtype primarily responsible for regulating cardiac output in mice (3).

In vitro studies have also shown functional differences between β_1 AR and β_2 AR in cardiac myocytes. In rat neonatal myocytes, both β_1 AR and β_2 AR couple to the G_s /cAMP pathway to stimulate inotropic and chronotropic responses. However, the pathway for β_1 AR activation of adenylyl cyclase is susceptible to inhibitory modulation by m2 muscarinic cholinergic receptors, whereas the pathway for β_2 AR activation of adenylyl cyclase is not (4). Using neonatal myocytes from genetically modified β AR gene knockout mice, we observed that β_1 AR is the primary adrenergic subtype for controlling the myocyte contraction rate through activation of G_s and PKA. Stimulation by β_2 AR has a rather small effect on the myocyte contraction rate with a small stimulation of the rate followed by prolonged inhibition. The biphasic response by β_2 AR is mediated through the sequential coupling to G_s and G_i (5). Recent studies have also provided evidence on the functional difference between β_1 AR and β_2 AR in modulating myocyte growth and apoptosis (6–9). The stimulation of β_1 AR promotes myocyte apoptosis through a mechanism involving G_i /PKA (10). The stimulation of β_2 AR has a protective effect that involves the activation of G_i (7).

These observations suggest that the spatial segregation of β_1 AR and β_2 AR signaling pathways may be responsible for the observed subtype-specific differences in regulating myocyte function (11). Recent biochemical studies provide evidence for the differential distribution of β_1 AR and β_2 AR on the cell surface of cardiac myocytes. Membrane fractionation shows that β_2 ARs are found predominantly in a caveolin-enriched membrane fraction from rat neonatal myocytes. Moreover, the apparent association of the β_2 AR with caveolin-enriched membranes is disrupted following agonist-induced internalization (12). The distribution of β_1 AR relative to caveolin-enriched membranes remains controversial (12–14). Nevertheless, these observations suggest that caveolar localization may play a role in the differences in signaling by β_1 AR and β_2 AR that we

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§ Recipient of a postdoctoral fellowship from the American Heart Association.

|| To whom correspondence should be addressed. Tel.: 650-723-7069; Fax: 650-498-5092; E-mail: kobilka@stanford.edu.

¹ The abbreviations used are: β AR, β adrenergic receptor; KO, knockout; PKA, protein kinase A; HA, hemagglutinin; m.o.i., multiplicities of infection; PTX, pertussis toxin; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; IBMX, isobutylmethylxanthine.

observe in neonatal mouse myocytes (5). We therefore investigated the effects of filipin, a reagent that disrupts caveolae, on subtype specific signaling by the β_1 AR and β_2 AR in neonatal cardiac myocytes.

EXPERIMENTAL PROCEDURES

Culturing and Adenovirus Infection of Neonatal Mouse Ventricular Myocytes—Spontaneously beating neonatal cardiac myocytes were prepared from hearts of 1–2-day-old mouse pups (from wild-type, β_1 AR-KO, β_2 AR-KO, and $\beta_1\beta_2$ AR-KO mice) as described previously (5) with some modification. Briefly, hearts were excised, the atria were removed, and the ventricles were minced and digested at 37 °C for 3 × 15 min in calcium-free HEPES-buffered Hanks' solution, pH7.4, plus 300 units/ml collagenase type II (Invitrogen). The isolated myocytes were collected from the second and third digestion. To reduce the contribution of non-myocardial cells, the isolated cells were preplated for 1 h at 37 °C. The myocyte-enriched cells remaining in suspension were plated in 35-mm dishes for contraction rate studies, 12-well plates with coverslips for immunocytochemistry, or 10-cm dishes for membrane fractionation and immunoprecipitation experiments. Culture dishes or coverslips were precoated with 10 μ g/ml mouse laminin at 37 °C for 24 h. Myocyte cultures were maintained in Dulbecco's modified Eagle's media containing 10% horse serum, 5% bovine fetal serum, and 1× penicillin/streptomycin. The culture media were changed every 24 h. To further reduce fibroblast contamination, myocytes were cultured in the presence of 10 μ M cytosine- β -D-arabinofuranoside (Sigma) during the first 24 h to block fibroblast proliferation.

A recombinant adenovirus encoding amino-terminal FLAG-tagged mouse β_2 AR or HA-tagged mouse β_1 AR was generated with the pAdEasy system (Qbiogene, Carlsbad, CA). The virus titer was obtained by optical density as recommended by the manufacturer (Qbiogene). The β_2 AR virus was used to infect neonatal myocytes with different multiplicities of infection (m.o.i.) after the myocytes were cultured for 24 h. The cells were incubated with virus-containing media for 2 h at 37 °C, then the media were removed and replaced with fresh media. The infected myocyte culture was maintained for 2 days before experiments.

Measurement of Myocyte Contraction Rate—Measurement of the spontaneous contraction rate was carried out as described previously (5) with modifications. Briefly, myocytes were cultured in 35-mm dishes to obtain a uniformly beating syncytium. After 72 h in culture, the media were replaced with fresh media buffered with 20 mM HEPES pH 7.4. The culture was incubated at 37 °C for at least 1 h before contraction rate studies. To measure the myocyte contraction rate, culture dishes were placed in a temperature regulation apparatus positioned on the stage of an inverted microscope; the culture was equilibrated for 10 min before the addition of the nonselective β AR agonist isoproterenol (10 μ M final concentration). A video camera connected the microscope to a computer with MetaMorph image software (Universal Imaging). Stacks of myocyte images were stream acquired with MetaMorph Flashbus at 2 min intervals for 10 min before and 30 min after adrenergic ligand stimulation. The images were analyzed with MetaMorph Object-Track, and the resulting contraction rate data were plotted using Microsoft Excel. All assays were also recorded on videotape.

For time course experiments, the statistical significance between groups was analyzed with two-way analysis of variance corrected for repeated measures. If the analysis of variance was significant, a *t* test with Bonferroni's method was used to compare responses at multiple time points of interest where the maximal effects of treatment were observed. Analysis was done using Prism (GraphPad Software, San Diego, CA).

Drug Treatment—Neonatal myocytes were preincubated with filipin (2 μ g/ml; Sigma) at 37 °C for 30 min before isoproterenol (10 μ M; Sigma) or forskolin (10 μ M; Sigma) exposure. For some contraction assays, pertussis toxin (PTX) (0.75 μ g/ml; Sigma) was used together with filipin. PTX treatment was carried out as described previously (5).

Immunofluorescence Microscopy—Myocytes were cultured in 12-well plates with coverslips and infected with the FLAG- β_2 AR adenovirus as described above. The cells were fixed with 1× PBS containing 5% paraformaldehyde and stained with the anti-FLAG M1 antibody (mouse monoclonal IgG_{2b}, 1:600 dilution; Sigma). The myocytes were then permeabilized with 1× PBS containing 0.2% of Nonidet P-40 and stained with anti-caveolin-3 antibody (mouse monoclonal IgG₁, 1:600 dilution; BD PharMingen). The primary antibodies were detected with fluorescein isothiocyanate-conjugated Goat anti-mouse IgG_{2b} (1:200 dilution; Fisher) and Texas red-conjugated Goat anti-mouse IgG₁ antibodies (1:400 dilution; Fisher), respectively. The images were acquired with an Olympus IX70 confocal microscope.

Immunoprecipitation—Adenovirus-infected myocytes were treated with filipin or control media before harvesting. Cells were solubilized in immunoprecipitation buffer (1× PBS, 5 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, pepstatin, leupeptin, benzamide, phenylmethylsulfonyl fluoride) on ice for 30 min and clarified by centrifugation (5,000 rpm in a microcentrifuge at 4 °C). The supernatant was incubated with anti-FLAG M1 antibody for 1.5 h at 4 °C. The immune complexes were precipitated with protein G beads followed by five washes with immunoprecipitation buffer. The bound proteins were separated on 12% SDS-PAGE gel and Western blotted with anti- β_2 AR antibody (rabbit polyclonal, dilution 1:200; Santa Cruz Biotechnology), anti- β_1 AR antibody (rabbit polyclonal, dilution 1:200; Santa Cruz Biotechnology), and anti-caveolin-3 antibodies (mouse monoclonal, 1:1000 dilution; Transduction Signals). After staining with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG antibodies (1:1000 dilution; Bio-Rad, Hercules, CA), the Western blot was developed using an ECL kit (Amersham Biosciences).

Ligand Binding Assay—Membrane proteins were prepared from β_1 AR-KO myocytes and adenovirus-infected β_1/β_2 AR-KO myocytes cultured as described above. The myocytes were rinsed with ice-cold 1× PBS before harvesting with 20 mM HEPES (pH7.4) buffer containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 10 μ g/ml benzamide. The collected cells were disrupted by passing the suspension through a 25-gauge needle. Nuclei and unbroken cells were removed from the suspension by centrifugation (1,000 rpm in microcentrifuge at 4 °C). Membranes were obtained from the supernatant by centrifugation in a Beckman TLA-100.2 desktop centrifuge (50,000 rpm at 4 °C) and resuspended in 1× binding buffer (75 mM Tris-HCl, pH7.4, 12.5 mM MgCl₂, 1 mM EDTA). Saturation binding was carried out with the non-selective β AR antagonist [¹²⁵I]iodocyanopindolol (PerkinElmer Life Sciences) as described previously (15).

Purification of Caveolin-rich Membrane Fraction—Adenovirus-infected myocytes were harvested in 1× PBS buffer followed by centrifugation, and suspended in 2 ml of ice-cold 0.5 M sodium carbonate (pH ~11). The cells were then homogenized with a loose-fitting Dounce homogenizer followed by a Polytron tissue grinder at 4 °C. The cell lysates were loaded on the bottom of an ultracentrifuge tube, the volume was adjusted to 2.5 ml with equal volumes of sodium carbonate and MBS buffer (25 mM MES, pH6.5, 0.15 M NaCl) with 80% sucrose for a final concentration of 40% sucrose. A two-step gradient was loaded on the top of the sample with 3.5 ml of MBS and 0.25 M sodium carbonate with 35% sucrose and 4 ml of MBS and 0.25 M sodium carbonate with 5% sucrose. The samples were spun in an SW40 rotor at 38,000 rpm at 4 °C for 16–18 h. 1-ml fractions were collected from top to bottom, and the heavy pellet was also saved. Protein from each fraction was precipitated with trichloroacetic acid (final concentration 7.2%) and solubilized in 1× SDS-PAGE sample buffer. The pellets were directly dissolved in 1× SDS-PAGE sample buffer. The precipitated proteins in the fraction were separated on 12% SDS-PAGE gel and Western blotted with anti-FLAG M1 antibody (mouse monoclonal, dilution 1:350; Sigma) and anti-caveolin-3 antibodies (mouse monoclonal, 1:1000 dilution; Transduction Signals). After staining with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:1000 dilution, Bio-Rad), the Western blot was developed with an ECL kit (Amersham Biosciences).

Measurement of cAMP Accumulation—To measure intracellular cAMP, myocytes were cultured in 12-well plates (2.5 × 10⁶ cells per well). Cells were incubated for 30 min at 37 °C with 1 μ M isobutylmethylxanthine (IBMX; Sigma) immediately before the addition of the agonist isoproterenol (10 μ M) for 5 min at room temperature. Cells without the addition of isoproterenol were used as control. In some experiments, 2 μ g/ml filipin was added with 1 μ M IBMX before the addition of isoproterenol. The assay was terminated by the aspiration of the incubation buffer and the addition of 0.5 ml of 100% ice-cold ethanol to each well. The cell lysates were then collected. Aliquots were dried in a spin vacuum, and cAMP in the residue was determined using a radioimmunoassay (Amersham Biosciences).

RESULTS

Subcellular Localization of β_2 AR in Neonatal Mouse Cardiac Myocytes—We have reported previously (5) that the activated β_2 AR undergoes sequential coupling to G_s and G_i in mouse neonatal myocytes. In density gradient fractionation studies on membranes from neonatal rat cardiac myocytes (12), β_2 ARs are found primarily in membrane fractions that contain caveolin. This observation suggests that β_2 AR may be preferentially

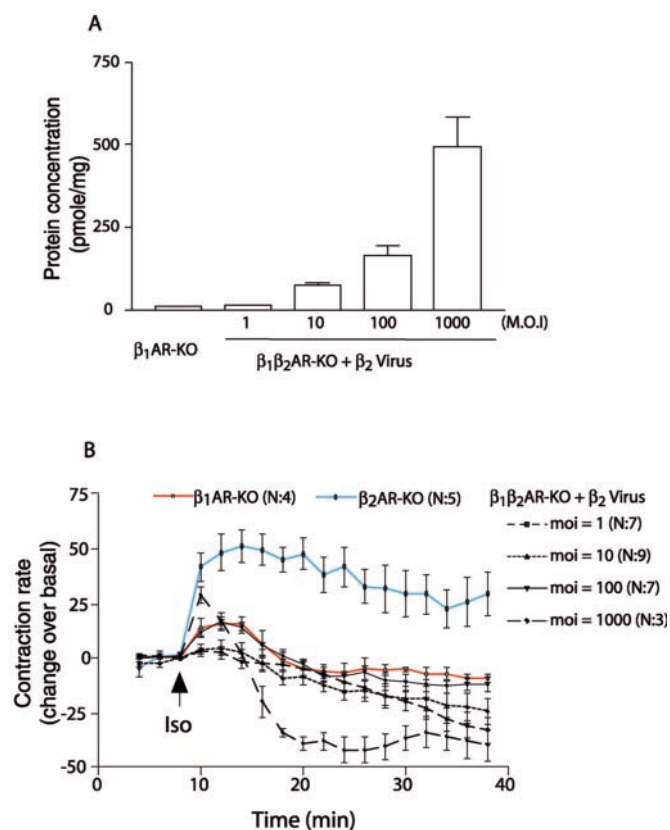


FIG. 1. Expression and function FLAG- β_2 AR in β_1/β_2 AR-KO myocytes. Neonatal myocytes were cultured and infected with different concentrations of the recombinant adenovirus expressing FLAG- β_2 AR as described under "Experimental Procedures." *A*, the expression of FLAG- β_2 AR was determined by saturation binding to obtain B_{max} . Cells infected at multiplicities of infection (M.O.I.) of 1, 10, 100, and 1,000 resulted in FLAG- β_2 AR expression of 1.3-, 7-, 17-, and 45-fold relative to the endogenous β_2 AR protein level in β_1 AR-KO myocytes. *B*, the effect of 10 μ M isoproterenol on the contraction rate of $\beta_1\beta_2$ AR-KO myocytes infected at different multiplicities of infection (m.o.i.) with an adenovirus expressing FLAG- β_2 AR. Also, the contraction rate responses in β_1 AR-KO myocytes (red trace) and β_2 AR-KO myocytes (blue trace) are shown for comparison. The data represent the mean \pm S.E. of *N* experiments from at least three different myocyte preparations.

located in lipid rafts, possibly caveolae. This localization may be important for the specific signaling properties observed for the β_2 AR in mouse neonatal cardiac myocytes.

To facilitate immunocytochemical studies of the cellular distribution of β_2 AR in mouse neonatal cardiac myocytes, we expressed β_2 AR with an amino-terminal FLAG epitope (FLAG- β_2 AR) in neonatal cardiac myocytes using a recombinant adenovirus. To ensure that FLAG- β_2 AR exhibited the same functional properties as the wild-type β_2 AR endogenously expressed in cardiac myocytes, we expressed different levels of FLAG- β_2 AR in myocytes prepared from β_1/β_2 AR-KO mice. β_1/β_2 AR-KO myocytes lack endogenous β_1 AR and β_2 AR and serve as a differentiated primary cell culture system to study the functional properties of wild-type and mutant β_1 ARs and β_2 ARs. Fig. 1*A* shows the level of β_2 AR binding in β_1 AR-KO myocytes and β_1/β_2 AR-KO neonatal myocytes infected with different concentrations of the recombinant adenovirus for FLAG- β_2 AR. β_1 AR-KO myocytes express low levels of endogenous β_2 AR (10 fmol/mg of membrane protein); this is comparable with the level of expression reported in intact β_1 AR-KO mouse hearts (2). The expression of FLAG- β_2 AR in β_1/β_2 AR-KO myocytes with the recombinant adenovirus ranged from 1.3- to 45-fold above that observed in β_1 AR-KO myocytes, depending on the virus inoculum (Fig. 1*A*). Fig. 1*B* shows the

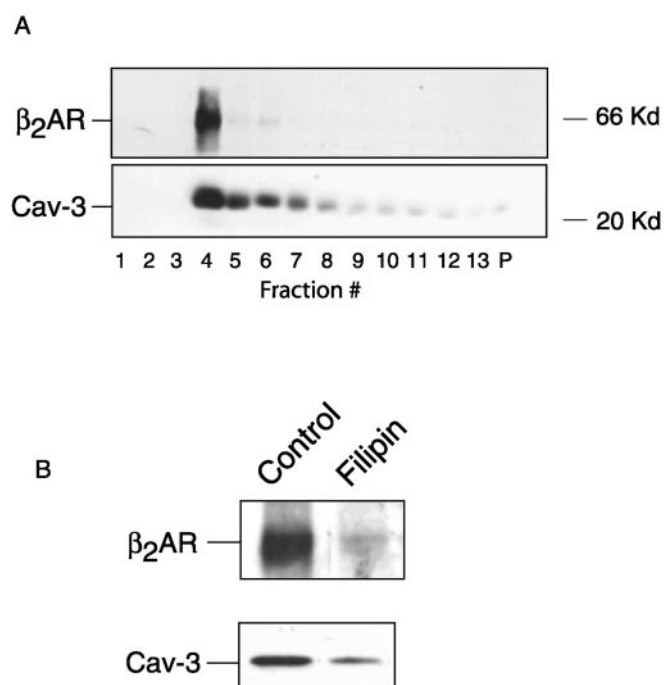


FIG. 2. Distribution of FLAG- β_2 AR in caveolin-enriched membranes from neonatal myocytes. Neonatal myocytes were cultured and infected with 100 m.o.i. of FLAG- β_2 AR adenovirus as described under "Experimental Procedures." 48 h after infection, myocytes were treated with filipin (2 μ g/ml) containing media or control media for 30 min before membrane isolation and sucrose gradient fractionation as described under "Experimental Procedures." *A*, Western blot of membranes from gradient fractions stained for FLAG-tagged β_2 AR and caveolin-3 (Cav-3). *B*, the effect of filipin on the localization of FLAG- β_2 AR in the caveolin-enriched membranes. For control cells, fraction 4 (Fraction #4) contained 55 μ g of protein (1.2% of total cellular protein). For filipin-treated cells, fraction 4 contained 46 μ g of protein (1.1% of total cellular protein). 7 μ g of protein from each caveolin-3 enriched fraction 4 was resolved by 10% SDS-PAGE protein gel, and the resulting Western blots were immunostained with anti-FLAG M1 and anti-caveolin-3 monoclonal antibodies. The Western blot was analyzed with NIH Image; the image intensities from the FLAG- β_2 AR blot were normalized by the intensities of the caveolin-3 blot for each sample. The FLAG- β_2 AR signal intensity was reduced by \sim 44% in membranes from filipin-treated cells.

effect of isoproterenol on the contraction rate of β_1/β_2 AR-KO myocytes expressing different levels of FLAG- β_2 AR. The maximum contraction rate increase by isoproterenol was dependent on the receptor expression level. All virus-infected β_1/β_2 AR-KO myocytes displayed a biphasic contraction rate response to isoproterenol, a feature consistent with the sequential coupling of β_2 AR to G_s and G_i . At the highest virus inoculum (m.o.i., 1:1000), the biphasic response was exaggerated with a greater effect on both the stimulation and the inhibition of the contraction rate than that observed in β_1 AR-KO myocytes (red trace, Fig. 1*B*). Interestingly, even at this high level of β_2 AR expression the stimulation of the contraction rate was small relative to that observed with endogenous β_1 AR in β_2 AR-KO myocytes (blue trace, Fig. 1*B*). At an inoculum of 1:100, the β_2 AR adenovirus gave a response comparable with that observed from endogenous β_2 AR in β_1 AR-KO myocytes (red trace, Fig. 1*B*).

Membrane fractionation studies were carried out to access the cell surface distribution of FLAG- β_2 AR expressed in wild-type mouse myocytes with a recombinant adenovirus (m.o.i., 1:100). Total cell lysates were loaded on a sucrose gradient to separate the caveolin-3-enriched fraction from other cytosolic and membrane fractions. The membrane fractions were subjected to Western blot analysis. We observed that FLAG- β_2 AR was found almost exclusively in membrane fractions enriched

with caveolin-3 (Fig. 2A). The pretreatment of myocytes with filipin significantly reduced the amount of FLAG- β_2 AR protein in caveolin-3 enriched membranes (Fig. 2B). Filipin is a cholesterol-binding reagent that can disrupt the cholesterol-enriched caveolae pit structures on plasma membranes (16).

The targeting of FLAG- β_2 AR to caveolae in cardiac myocytes was further examined using co-immunoprecipitation and colocalization by confocal microscopy. Fig. 3 shows that the caveolin-3 co-immunoprecipitated with FLAG- β_2 AR but not with the HA epitope-tagged β_1 AR (Fig. 3A). The co-immunoprecipitation of caveolin-3 with FLAG- β_2 AR was reduced to about 50% in cells treated with filipin (Fig. 3B). As shown in Fig. 4, there is a significant overlap in the immunostaining of FLAG- β_2 AR (green) and caveolin-3 (red).

Filipin Selectively Affects β_2 AR Signaling in Neonatal Myocytes—The studies shown in Figs. 2 and 3 demonstrate that β_2 AR targeting to caveolin-3-enriched caveolae can be partially disrupted by filipin. We therefore examined the effect of filipin on β_2 AR regulation of the cardiac myocyte contraction rate. Neonatal myocytes were harvested and maintained in culture for 3 days before the contraction rate studies were conducted. As previously reported, the effect of isoproterenol on the contraction rate of β_1 AR-KO myocytes was relatively small and biphasic with an initial increase that peaked at 5 min followed by a sustained decrease (Fig. 5A). This biphasic response reflects the sequential coupling of activated β_2 AR to G_s and G_i in neonatal myocytes. We incubated β_1 AR-KO myocytes with filipin for 30 min before isoproterenol stimulation. Treatment with filipin did not change the basal level of the myocyte contraction rate. However, compared with non-treated controls, we observed a greater and more prolonged increase in the contraction rate in the filipin-treated myocytes, and no decrease below base line was observed (Fig. 5A). We observed a similar effect of filipin on β_1/β_2 AR-KO myocytes expressing recombinant FLAG- β_2 AR (Fig. 5B).

Filipin can affect membrane protein mobility and membrane permeability, which might cause a nonspecific effect on β AR signaling and the myocyte contraction rate. Therefore, we examined the effect of filipin on signaling in wild-type and β_2 AR-KO myocytes. As shown in Fig. 6, after isoproterenol stimulation both β_2 AR-KO and wild-type myocytes displayed a robust contraction rate increase that peaked at 10 min followed by a gradual decline (Fig. 6, A and B). Filipin did not have a significant effect on the contraction rate increase in β_2 AR-KO myocytes, indicating that filipin does not alter β_1 AR signaling (Fig. 6A). Filipin did not have a significant effect on the initial increase in the contraction rate induced by isoproterenol in wild-type myocytes (Fig. 6B). However, compared with control myocytes, filipin-treated wild-type myocytes had a more sustained contraction rate response to isoproterenol (Fig. 6B). To test for the nonspecific effects of filipin on downstream signaling components, we studied the contraction rate response to forskolin in β_1/β_2 AR-KO myocytes. Stimulation with forskolin induced a robust contraction rate increase on β_1/β_2 AR-KO myocytes, and the increase was not significantly changed by filipin treatment (Fig. 6C). Together, these data indicate that filipin selectively alters regulation of the myocyte contraction rate by the β_2 AR.

We examined whole-cell cAMP accumulation at base line and in response to isoproterenol and forskolin stimulation in myocytes from various knockout mice (Fig. 7). The base-line cAMP levels range between 2 and 8 pmol per well among wild-type, β_1 AR-KO, β_2 AR-KO, and β_1/β_2 AR-KO myocytes. After isoproterenol stimulation, the whole-cell cAMP levels were greatly increased to about 50 pmol per well in both wild-type and β_2 AR-KO myocytes. The isoproterenol-stimulated increase in

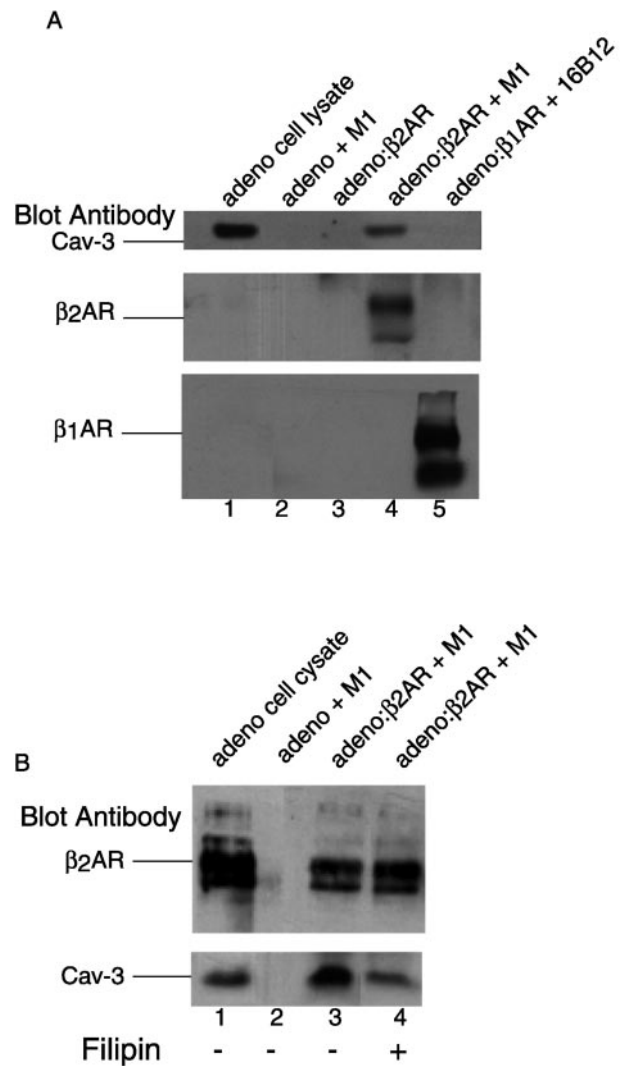


FIG. 3. β_2 ARs associate with caveolin-3 in cardiac myocytes. Neonatal myocytes infected with the wild-type adenovirus or the FLAG- β_2 AR or HA- β_1 AR adenovirus (at m.o.i. of 100) were cultured and treated with either filipin (2 μ g/ml) or control media before immunoprecipitation. Lysates from cardiac myocytes were incubated without or with anti-FLAG M1 antibody or with the anti-HA 16B12 antibody before precipitation with protein G beads. Immunoprecipitates were analyzed by Western blots stained for β_2 AR, β_1 AR, and caveolin-3 as described under "Experimental Procedures." **A**, FLAG- β_2 ARs but not HA- β_1 ARs associate with caveolin-3 (Cav-3) in cardiac myocytes. Lane 1, cell lysate from the myocytes infected with wild-type adenovirus; lane 2, lysates from myocytes infected with the wild-type adenovirus immunoprecipitated with anti-FLAG M1 antibody; lane 3, lysates from myocytes infected with the FLAG- β_2 AR adenovirus immunoprecipitated without anti-FLAG M1 antibody; lane 4, lysates from myocytes infected with the FLAG- β_2 AR adenovirus immunoprecipitated with anti-FLAG M1 antibody; and lane 5, lysates from myocytes infected with the HA- β_1 AR adenovirus immunoprecipitated with the anti-HA 16B12 antibody. **B**, Filipin treatment reduces the β_2 AR association with caveolae. Lane 1, cell lysate from the myocytes infected with the FLAG- β_2 AR adenovirus; lane 2, lysates from myocytes infected with the wild-type adenovirus immunoprecipitated with the anti-FLAG M1 antibody; lane 3, lysates from myocytes infected with the FLAG- β_2 AR adenovirus immunoprecipitated with the anti-FLAG M1 antibody; and lane 4, lysates from filipin-treated myocytes infected with the FLAG- β_2 AR adenovirus immunoprecipitated with the anti-FLAG M1 antibody. The signal intensities of caveolin-3 from Western blot were analyzed with NIH image. In comparison with the control, the signal intensity of caveolin-3 that co-immunoprecipitated with FLAG- β_2 AR was reduced by ~50% after treating cells with filipin.

cAMP was smaller in β_1 AR-KO myocytes (17 pmol per well), and isoproterenol had no effect on the cAMP level in β_1/β_2 AR-KO myocytes. Forskolin stimulated an increase in whole-

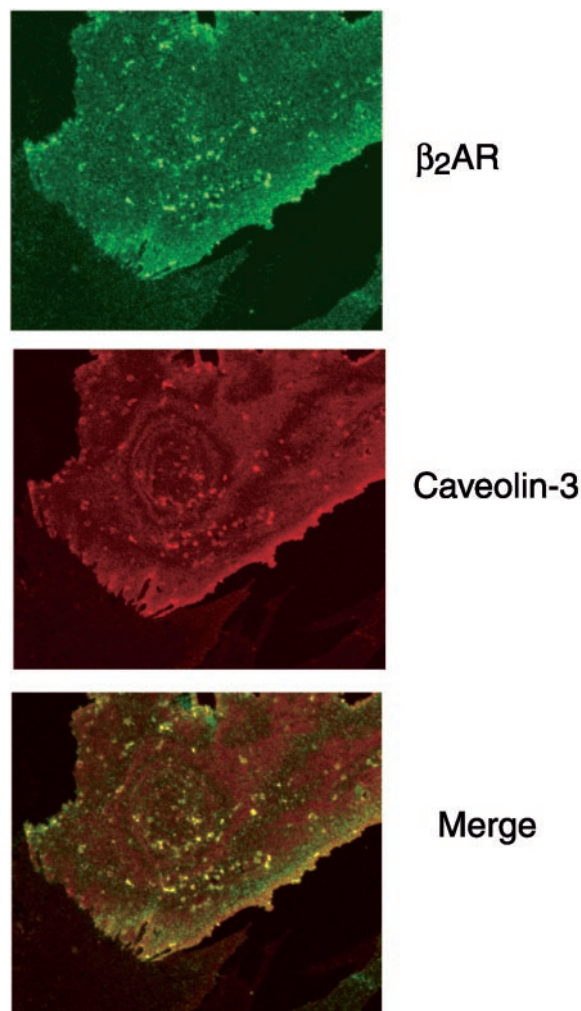


FIG. 4. Co-localization of FLAG- β_2 AR and caveolin-3 in neonatal cardiac myocytes. Neonatal myocytes infected with the β_2 AR adenovirus (at m.o.i. of 100) were cultured and fixed for immunostaining and analysis by confocal microscopy as described under "Experimental Procedures." The cell surface staining of FLAG- β_2 AR (green) shows significant overlap with the staining of caveolin-3 (red).

cell cAMP levels to a similar extent (~ 60 pmol per well) in myocytes from wild-type, β_1 AR-KO, β_2 AR-KO, and β_1/β_2 AR-KO mice. In contrast to the effect of filipin on the isoproterenol-stimulated contraction rate, filipin treatment did not have a significant effect on isoproterenol-stimulated cAMP levels in β_1 AR-KO myocytes (Fig. 7).

Filipin Does Not Prevent β_2 AR Coupling to G_i —As shown in Fig. 5, the contraction rate stimulated by β_2 AR in β_1 AR-KO myocytes did not drop below the basal level after filipin treatment. This could be due to enhanced coupling to G_s and/or the inhibition of coupling to G_i . To examine the effect of filipin on the coupling of the β_2 AR to G_i , myocytes were treated with PTX prior to contraction rate studies in filipin-treated cells. The isoproterenol-stimulated increase in the contraction rate was significantly greater in cells treated with both PTX and filipin than in cells treated with filipin alone (Fig. 8). Thus, filipin treatment does not block coupling of the β_2 AR to G_i .

DISCUSSION

There is a growing body of evidence that subtype-specific signaling of G protein-coupled receptors involves the organization of receptors, G proteins, effectors, and regulatory proteins in discrete signaling complexes in the plasma membrane. These complexes may be localized within specialized mem-

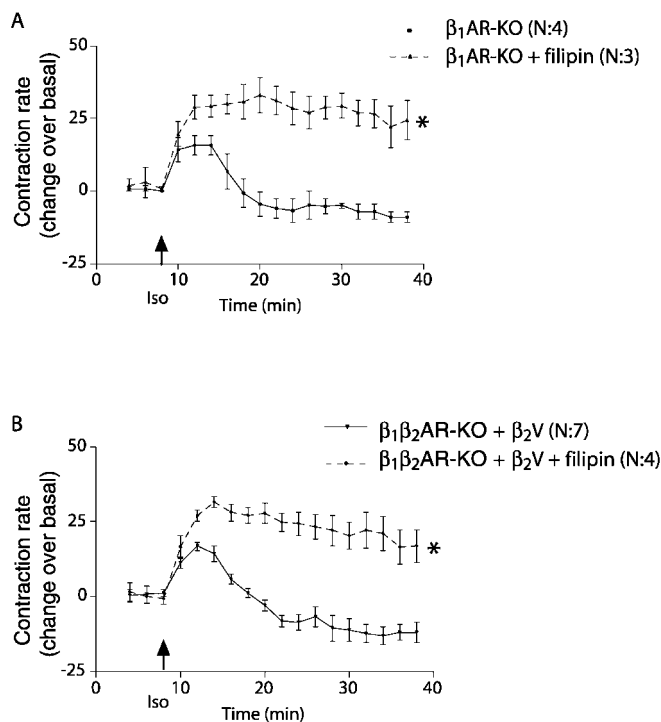


FIG. 5. The effect of filipin treatment on the isoproterenol-stimulated contraction rate of β_1 AR-KO neonatal myocytes and FLAG- β_2 AR expressed in β_1/β_2 AR-KO myocytes. Neonatal myocytes were cultured and treated with either filipin ($2 \mu\text{g/ml}$) or control media 30 min before contraction rate experiments as described under "Experimental Procedures." The basal contraction rate of myocytes was not altered significantly by filipin treatment. However, filipin treatment significantly increased the contraction rate response to isoproterenol (Iso) in β_1 AR-KO neonatal myocytes (A) and in β_1/β_2 AR-KO myocytes expressing FLAG- β_2 AR (B). The data represent the mean \pm S.E. of N experiments from at least three different myocyte preparations. * $p < 0.05$; a Bonferroni's t test was performed on curves that were found to be significantly different by two-way analysis of variance corrected for repeated measures.

brane compartments such as lipid rafts. Lipid rafts are enriched in cholesterol and sphingolipids and often contain associated proteins such as caveolins, flotillins, and stomatins, which may serve as scaffolds for signaling complexes (17). Caveolae are perhaps the best characterized of these lipid rafts. A number of studies have demonstrated that caveolae are enriched in components of signal transduction cascades, including G proteins, G protein-coupled receptors, and effector molecules (18, 19). Thus, caveolae may act as a scaffold promoting the interaction of specific signaling molecules. However, there is also evidence that caveolins may also act to inhibit the function of several signaling molecules, including G proteins and kinases (18, 20).

Recent studies provide evidence that caveolae may play a role in β AR signaling in cardiac myocytes (12, 14). In rat neonatal myocytes, the β_2 AR, $G_{i2\alpha}$, $G_{i3\alpha}$, adenylyl cyclase 5/6, and the PKA regulatory subunit II are all preferentially localized to caveolin-enriched membrane fractions from sucrose density gradients (12, 14). Approximately 50% of G_s was found in caveolin-enriched membranes, whereas β_1 AR was found predominantly in non-caveolar membranes (12). Disruption of caveolae with cyclodextran resulted in enhanced cAMP accumulation by β_1 AR agonists, β_2 AR agonists, and forskolin (12).

In agreement with these studies, we found that β_2 ARs co-migrate with caveolin-enriched membranes in mouse neonatal cardiac myocytes (Fig. 2). We also provide more direct evidence that β_2 ARs reside in caveolae by co-localization of β_2 AR with caveolin-3 in intact myocytes (Fig. 4) and co-immunoprecipita-

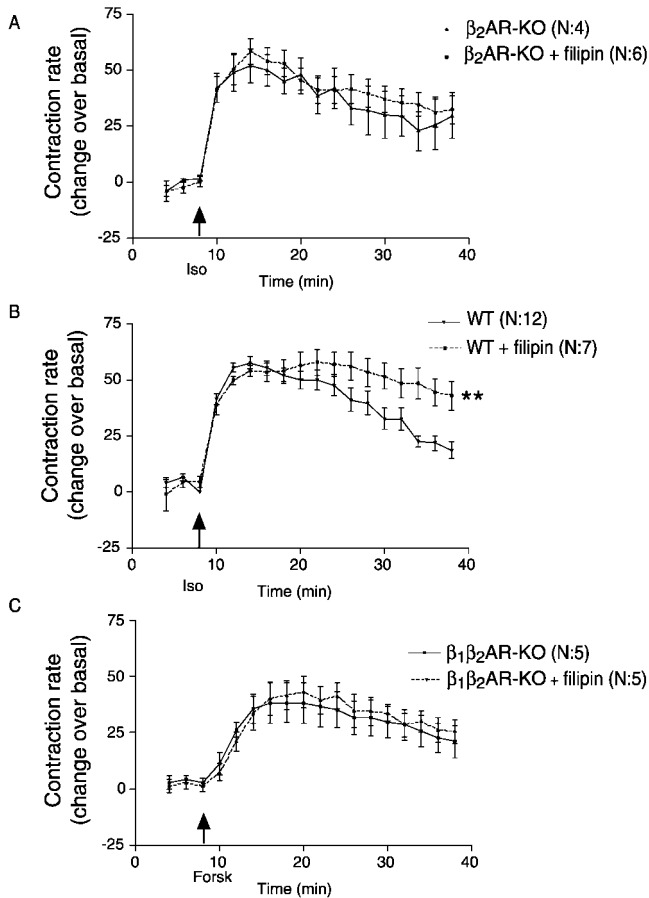


FIG. 6. The effect of filipin treatment on the contraction rate of wild-type, β_2 AR-KO, and β_1/β_2 AR-KO myocytes. Neonatal myocytes were cultured and treated with either filipin (2 μ g/ml) or control media for 30 min before contraction rate experiments as described under "Experimental Procedures." The basal contraction rate of myocytes was not altered significantly by filipin treatment. A, filipin treatment did not change the isoproterenol-stimulated contraction rate increase in β_2 AR-KO myocytes. $p > 0.05$ by two-way analysis of variance corrected for repeated measures. B, filipin treatment did not significantly change the early phase of the isoproterenol-stimulated contraction rate increase in wild-type myocytes. However, in the late period of stimulation the contraction rate increase in the filipin-treated myocytes was higher than that of control myocytes. **, $p < 0.05$; a Bonferroni's t test was performed on the end of curves that were found to be significantly different by two-way analysis of variance corrected for repeated measures. C, the filipin treatment did not change the forskolin-stimulated contraction rate increase in β_1/β_2 AR-KO myocytes. The data represent the mean \pm S.E. of N experiments from at least three different myocyte preparations. $p > 0.05$ by two-way analysis of variance corrected for repeated measures. Iso, isoproterenol; Forsk, forskolin.

tion of caveolin-3 with the FLAG-tagged β_2 AR from myocyte membranes (Fig. 3). The treatment of cells with filipin altered the distribution of β_2 AR relative to caveolin-3 and dramatically increased the contraction rate response to β_2 AR activation by the non-selective β AR agonist isoproterenol (Fig. 5). Filipin is a cholesterol-binding reagent that disrupts lipid rafts but also increases membrane protein mobility as well as membrane permeability (16). The latter property may nonspecifically alter the contraction rate. However, the effect of filipin was observed only with β_2 AR signaling; no effect was observed on β_1 AR signaling or the activation of the contraction rate by forskolin. The fact that the contraction rate response to β_2 AR stimulation is enhanced by filipin suggests that localization in caveolae may limit access of the β_2 AR to signaling pathways that modulate the contraction rate more efficiently. Once freed from the constraints of the lipid raft, the β_2 AR may have greater access to signaling molecules that stimulate the contraction rate.

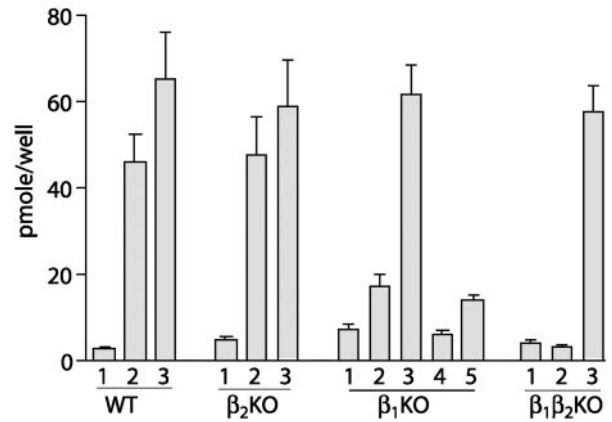


FIG. 7. The global cAMP level at base line and in response to isoproterenol and forskolin in myocytes from different knockout strains. Bar 1, base-line cAMP level; bar 2, cAMP level after isoproterenol stimulation; bar 3, cAMP level after forskolin stimulation; bar 4, base-line cAMP level after filipin treatment; and bar 5, cAMP level after isoproterenol stimulation on filipin-treated myocytes. The data represent the mean \pm S.E. of experiments from three to eight different myocyte preparations on each mouse strain.

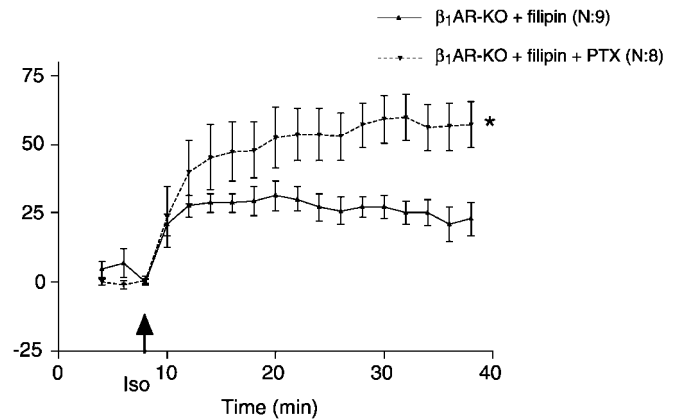


FIG. 8. The effect of filipin treatment on β^2 AR- G_s signaling in myocytes. Neonatal myocytes were cultured and treated with PTX (0.75 μ g/ml) or control media for 2.5 h before the addition of filipin (2 μ g/ml). Myocytes were incubated for an additional 30 min before contraction rate experiments were performed as described under "Experimental Procedures." The data represent the mean \pm S.E. of N experiments from at least three different myocyte preparations. *, $p < 0.05$; a Bonferroni's t test was performed on curves that were found to be significantly different by two-way analysis of variance corrected for repeated measures. Iso, isoproterenol.

Although cAMP plays a critical role in regulating myocytes function, our previous study did not demonstrate a strong correlation between whole-cell cAMP accumulation and the myocyte contraction rate in response to isoproterenol (5). In the present study, the disruption of caveolae with filipin resulted in an enhanced contraction rate response to isoproterenol (Fig. 5) but had no effect on cAMP accumulation stimulated by isoproterenol (10 μ M) in neonatal β_1 AR-KO myocytes (Fig. 7). However, at a much lower concentration (1 pM) of isoproterenol it has been shown that cAMP accumulation in wild-type rat myocytes can be enhanced by the disruption of caveolae with cyclodextran (12). The difference between these two studies may be due to differences in the assay conditions, the species studied, or the fact that our studies were done on myocytes from β_1 AR-KO mice. The discrepancy in the effect of filipin on the contraction rate assay and the cAMP accumulation assay may be due to technical differences in the assays. To detect cAMP accumulation in myocytes, cultures must be preincubated with a phosphodiesterase inhibitor (IBMX) to prevent hydrolysis of cAMP.

Thus, the cAMP assay conditions will not fully replicate the more physiologic conditions of the myocyte contraction rate assay. Moreover, a recent report shows that the stimulation of adrenergic receptors generates focal increases in the concentration of cAMP that localizes to the region of the transverse tubule/junctional sarcoplasmic reticulum membrane (21). The restricted pools of cAMP show a range of action as small as $\sim 1 \mu\text{m}$, and free diffusion of the second messenger is limited by the activity of phosphodiesterases (21). Therefore, it is conceivable that the whole-cell cAMP accumulation assay may not be sensitive enough to detect small but functionally significant focal increases in cAMP adjacent to effector molecules.

We observe a biphasic response to stimulation of the β_2 AR in β_1 AR-KO myocytes (Fig. 5). The inhibition of the contraction rate observed ~ 15 min after β_2 AR stimulation is mediated by G_i and can be inhibited by pertussis toxin (5). Previous studies have shown that caveolae are enriched in G_i proteins (12, 14). Therefore, one might predict that the enhanced stimulation of the contraction rate observed in the presence of filipin could be attributed to the dissociation of β_2 AR from caveolae and the associated G_i proteins. However, we observed an even greater increase in the contraction rate in myocytes treated with both filipin and PTX than in myocytes treated with filipin alone (Fig. 8). This suggests that the enhanced stimulation of the contraction rate mediated by β_2 AR in the presence of filipin is not due to the disruption of the coupling of the β_2 AR to G_i but to the more efficient coupling of the β_2 AR to the stimulation of the contraction rate, most likely through G_s . By disrupting lipid rafts, filipin may enhance the diffusion of the β_2 AR within the plasma membrane, thereby increasing its access to downstream effectors (such as PKA or specific channels) that influence the contraction rate.

In conclusion, we present evidence that β_2 ARs are localized within caveolae and that this localization is essential for the physiologic signaling of this receptor subtype. In contrast, filipin, a reagent that disrupts caveolae, has no effect on the functional properties of the β_1 AR in cardiac myocytes. These results provide support for the hypothesis that β_1 AR and β_2 AR

are segregated into discrete signaling compartments in neonatal myocytes. We also show that neonatal cardiac myocytes from β_1/β_2 AR-KO mice can be used as a differentiated expression system to study the function and cell biology of wild-type, epitope-tagged, and mutant receptors. Using this system, it should be possible to identify the structural domains that dictate the subtype-specific localization and function of β_1 AR and β_2 AR in cardiac myocytes.

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