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 β1- and β2-adrenergic receptors (AR) regulate the intrinsic contraction rate in neonatal mouse myocytes through distinct signaling pathways. By studying neonatal myocytes isolated from β1AR and β2AR knockout mice, we found that stimulation of the β1AR leads to a protein kinase A-dependent increase in the contraction rate. In contrast, stimulation of the β2AR 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 β2AR but not the β1AR in neonatal cardiac myocytes. Evidence for β2AR localization to caveolae includes co-localization by confocal imaging, co-immunoprecipitation of the β2AR and caveolin 3, and co-migration of the β2AR with a caveolin-3-enriched membrane fraction. The β2AR-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 β2AR and caveolin 3 and co-migration of β2AR and caveolin-3 enriched membranes. In contrast, filipin has no effect on β1AR signaling. These observations suggest that β2ARs 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 (βARs) to modulate heart rate and contractility. Three βAR subtypes have been cloned (β1AR, β2AR, and β3AR). β1AR and β2AR are the primary subtypes responsible for cardiac response to catecholamines. β1AR and β2AR are also pharmacologically more similar to each other than they are to the β3AR. The close structural and functional properties of β1AR and β2AR 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 β1AR and β2AR 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 β1AR knockout (β1AR-KO) mice lack the normal chronotropic and inotropic responses to the non-selective agonist isoproterenol (2). Thus, in the murine heart, β2ARs play no significant role in controlling heart rate and contractility. β2AR knockout (β2AR-KO) mice have normal inotropic and chronotropic responses to isoproterenol, confirming that β1AR is the subtype primarily responsible for regulating cardiac output in mice (3).

In vitro studies have also shown functional differences between β1AR and β2AR in cardiac myocytes. In rat neonatal myocytes, both β1AR and β2AR couple to the Gs/cAMP pathway to stimulate inotropic and chronotropic responses. However, the pathway for β2AR activation of adenylyl cyclase is susceptible to inhibitory modulation by m2 muscarinic cholinergic receptors, whereas the pathway for β1AR activation of adenylyl cyclase is not (4). Using neonatal myocytes from genetically modified βAR gene knockout mice, we observed that β1AR is the primary adrenergic subtype for controlling the myocyte contraction rate through activation of Gs and PKA. Stimulation by β2AR 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 β2AR is mediated through the sequential coupling to Gs and Gi (5). Recent studies have also provided evidence on the functional difference between β1AR and β2AR in modulating myocyte growth and apoptosis (6–9). The stimulation of β1AR promotes myocyte apoptosis through a mechanism involving Gi/PKA (10). The stimulation of β2AR has a protective effect that involves the activation of Gi (7).

These observations suggest that the spatial segregation of β1AR and β2AR 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 β1AR and β2AR on the cell surface of cardiac myocytes. Membrane fractionation shows that β1ARs are found predominantly in a caveolin-enriched membrane fraction from rat neonatal myocytes. Membrane fractionation also showed that β2ARs are found predominantly in a caveolin-enriched membrane fraction from rat neonatal cardiac myocytes. Membrane fractionation shows that β1ARs are found predominantly in a caveolin-enriched membrane fraction from rat neonatal myocytes. Membrane fractionation also showed that β2ARs are found predominantly in a caveolin-enriched membrane fraction from rat neonatal cardiac myocytes. Membrane fractionation also showed that β2ARs are found predominantly in a caveolin-enriched membrane fraction from rat neonatal cardiac myocytes. Membrane fractionation also showed that β2ARs are found predominantly in a caveolin-enriched membrane fraction from rat neonatal cardiac myocytes.
observe in neonatal mouse myocytes (5). We therefore investigated the effects of filipin, a reagent that disrupts caveolae, on subtype specific signaling by the βAR and β2AR 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, βAR-KO, β2AR-KO, and β2AR/βAR-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’s solution, pH 7.4, 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 μg/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 μg cytokine-βα-araabinofuranoside (Sigma) during the first 24 h to block fibroblast proliferation.

A recombinant adenovirus encoding amino-terminal FLAG-tagged mouse βAR or HA-tagged mouse β2AR was generated with the pAdEasy system (Qiogene, Carlsbad, CA). The virus titer was obtained by optical density as recommended by the manufacturer (Qiogene). The βAR 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 on a temperature regulation apparatus positioned on a computer with MetaMorph image software (Universal Imaging). Stacks of myocyte images were stream acquired with MetaMorph Flahsbus at 2 min intervals for 10 min before and 30 min after adrenergic 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 μg/ml; Sigma) at 37°C for 30 min before isoproterenol (10 μM; Sigma) or forskolin (10 μM; Sigma) exposure. For some contraction assays, pertussis toxin (PTX) (0.75 μg/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-βAR adenovirus as described above. The cells were fixed with 1× PBS containing 5% paraformaldehyde and stained with the anti-FLAG M1 antibody (mouse monoclonal IgG2a, 1:1000 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 IgG2a (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, peptatin, leupeptin, benzamidine, phenylmethylsulfonyl fluoride) on ice for 30 min and clarified by centrifugation (5,000 rpm, 4°C, 10 min). Supernatants were 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-βAR antibody (rabbit polyclonal, dilution 1:200; Santa Cruz Biotechnology), anti-β2AR antibody (rabbit polyclonal, dilution 1:200; Santa Cruz Biotechnology), and anti-FLAG antibody (rabbit polyclonal, 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 βAR-KO myocytes and adenovirus-infected β2AR-KO myocytes cultured as described above. The myocytes were rinsed with ice-cold 1× PBS before harvesting with 20 mm HEPES (pH 7.4) buffer containing 1 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml benzamidine. 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 a Sorvall RC-2B centrifuge at 4°C) for 10 min. Membranes were obtained 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, pH 7.4, 12.5 mm MgCl2, 1 mm EDTA). Saturation binding was carried out with the non-selective βAR 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 Polytorn 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 MBS, pH 6.5, 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 × 10^6 cells per well). Cells were incubated for 30 min at 37°C with 1 μM isobutylmethylxanthine (IBMX; Sigma) immediately before the addition of the agonist isoproterenol (10 μM) for 5 min at room temperature. Cells without the addition of isoproterenol were used as control. In some experiments, 2 μg/ml filipin was added with 1 μM 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 βAR in Neonatal Mouse Cardiac Myocytes**—We have reported previously (5) that the activated βAR undergoes sequential coupling to Gs and Gi in mouse neonatal myocytes. In density gradient fractionation studies on membranes from neonatal rat cardiac myocytes (12), β2ARs are found primarily in membrane fractions that contain caveolin. This observation suggests that β2AR may be preferentially
located in lipid rafts, possibly caveolae. This localization may be important for the specific signaling properties observed for the β2AR in mouse neonatal cardiac myocytes.

To facilitate immunocytochemical studies of the cellular distribution of β2AR in mouse neonatal cardiac myocytes, we expressed β2AR with an amino-terminal FLAG epitope (FLAG-β2AR) in neonatal cardiac myocytes using a recombinant adenovirus. To ensure that FLAG-β2AR exhibited the same functional properties as the wild-type β2AR endogenously expressed in cardiac myocytes, we expressed different levels of FLAG-β2AR in myocytes prepared from β1/β2AR-KO mice. β1/β2AR-KO myocytes lack endogenous β1AR and β2AR and serve as a differentiated primary cell culture system to study the functional properties of wild-type and mutant β1ARs and β2ARs. Fig. 1A shows the level of β2AR binding in β1AR-KO neonatal myocytes and β1/β2AR-KO neonatal myocytes infected with different concentrations of the recombinant adenovirus for FLAG-β2AR. β2AR-KO myocytes express low levels of endogenous β2AR (10 fmol/mg of membrane protein); this is comparable with the level of expression reported in intact β2AR-KO mouse hearts (2). The expression of FLAG-β2AR in β1/β2AR-KO myocytes was monitored by Western blot analysis using anti-FLAG M1 and anti-caveolin-3 monoclonal antibodies. The Western blot was analyzed with NIH Image; the image intensities from the FLAG-β2AR blot were normalized by the intensities of the caveolin-3 blot for each sample. The FLAG-β2AR signal intensity was reduced by ~44% in membranes from filipin-treated cells.

Fig. 1. Expression and function FLAG-β2AR in β1/β2AR-KO myocytes. Neonatal myocytes were cultured and infected with different concentrations of the recombinant adenovirus expressing FLAG-β2AR as described under “Experimental Procedures.” A, the expression of FLAG-β2AR was determined by saturation binding to obtain Bmax. Cells infected at multiplicities of infection (M.O.I.) of 1, 10, 100, and 1,000 resulted in FLAG-β2AR expression of 1.3-, 7-, 17-, and 45-fold relative to the endogenous β2AR protein level in β2AR-KO myocytes. B, the effect of 10 μM isoproterenol on the contraction rate of β2AR-KO myocytes infected at different multiplicities of infection (moi) with an adenovirus expressing FLAG-β2AR. Also, the contraction rate responses in β2AR-KO myocytes (red trace) and β2AR-KO myocytes (blue trace) are shown for comparison. The data represent the mean ± S.E. of N experiments from at least three different myocyte preparations.

Fig. 2. Distribution of FLAG-β2AR in caveolin-enriched membranes from neonatal myocytes. Neonatal myocytes were cultured and infected with 100 m.o.i. of FLAG-β2AR adenovirus as described under “Experimental Procedures.” A, Western blot of membranes from gradient fractions stained for FLAG-tagged β2AR and caveolin-3 (Cav-3). B, the effect of filipin on the localization of FLAG-β2AR in the caveolin-enriched membranes. For control cells, fraction 4 (Fraction #4) contained 55 μg of protein (1.2% of total cellular protein). For filipin-treated cells, fraction 4 contained 46 μg of protein (1.1% of total cellular protein). 7 μg 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-β2AR blot were normalized by the intensities of the caveolin-3 blot for each sample. The FLAG-β2AR signal intensity was reduced by ~44% in membranes from filipin-treated cells.

The expression of β2AR in mouse hearts (2). The expression of FLAG-β2AR in β1/β2AR-KO myocytes with the recombinant adenovirus ranged from 1.3- to 45-fold above that observed in β1/β2AR-KO myocytes, depending on the virus inoculum (Fig. 1A). Fig. 1B shows the effect of isoproterenol on the contraction rate of β1/β2AR-KO myocytes expressing different levels of FLAG-β2AR. The maximum contraction rate increase by isoproterenol was dependent on the receptor expression level. All virus-infected β1/β2AR-KO myocytes displayed a biphasic contraction rate response to isoproterenol, a feature consistent with the sequential coupling of β2AR to Gs and Gi. At an inoculum of 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 β2AR-KO myocytes (red trace, Fig. 1B). Interestingly, even at this high level of β2AR expression the stimulation of the contraction rate was small relative to that observed with endogenous β2AR in β2AR-KO myocytes (blue trace, Fig 1B). At an inoculum of 1:100, the β2AR adenovirus gave a response comparable with that observed from endogenous β2AR in β2AR-KO myocytes (red trace, Fig 1B).
increased to about 50 pmol per well in both wild-type and 

terenol stimulation, the whole-cell cAMP levels were greatly 

biphasic with an initial increase that peaked at 5 min followed 

by a sustained decrease (Fig. 5b). Filipin did not have a significant effect on the initial 

contraction rate increase that peaked at 10 min followed by a gradual decline (Fig. 6, 

contraction rate increase that peaked at 10 min followed by a decrease below base line was observed (Fig. 5a). We observed a similar effect of filipin on β2/β2AR-KO myocytes expressing recombinant FLAG-β2AR (Fig. 5b).

Filipin can affect membrane protein mobility and membrane permeability, which might cause a nonspecific effect on βAR signaling and the myocyte contraction rate. Therefore, we examined the effect of filipin on signaling in wild-type and β2AR-KO myocytes. As shown in Fig. 6, after isoproterenol stimulation both β2AR-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 β2AR-KO myocytes, indicating that filipin does not alter β2AR 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 β2AR-KO myocytes. Stimulation with forskolin induced a robust contraction rate increase on β2AR-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 β2AR.

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 baseline cAMP levels range between 2 and 8 pmol per well among wild-type, β1AR-KO, β2AR-KO, and β2AR/β2AR-KO myocytes. After isoproterenol stimulation, the whole-cell cAMP levels were greatly increased to about 50 pmol per well in both wild-type and β2AR-KO myocytes. The isoproterenol-stimulated increase in cAMP was smaller in β2AR-KO myocytes (17 pmol per well), and isoproterenol had no effect on the cAMP level in β1/ 

β2AR-KO myocytes. Forskolin stimulated an increase in whole
inhibition of coupling to G_i. To examine the effect of filipin on 
myocytes. This could be due to enhanced coupling to G_s and/or the 
myocytes did not drop below the basal level after filipin treat-
ment. The cell surface staining of FLAG-β2AR (green) shows significant overlap with the staining of caveolin-3 (red).

**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-
brane compartments such as lipid rafts. Lipid rafts are en-
riched in cholesterol and sphingolipids and often contain asso-
ciated proteins such as caveolins, flotillins, and stomatinas, 
which may serve as scaffolds for signaling complexes (17). 
Caveolae may be the best characterized of these lipid rafts. 
Recent studies provide evidence that caveolae may play a 
role in βAR signaling in cardiac myocytes (12, 14). In rat 
non-caveolar membranes, whereas β2AR was found pre-
dominantly in non-caveolar membranes (12). Disruption of 
caveolae with cyclodextran resulted in enhanced cAMP accum-
ulation by β1AR agonists, β2AR agonists, and forskolin (12).

In agreement with these studies, we found that β2ARs co-
migrate with caveolin-enriched membranes in mouse neonatal 
cardiac myocytes (Fig. 2). We also provide more direct evidence 
that β2ARs reside in caveolae by co-localization of β2AR with 
caveolin-3 in intact myocytes (Fig. 4) and co-immunoprecipita-

**FIG. 4. Co-localization of FLAG-βAR and caveolin-3 in neonatal cardiac myocytes.** Neonatal myocytes infected with the βAR adenovirus (at m.o.i. of 100) were cultured and fixed for immuno-

![β2AR](Image 308x458 to 552x728)

![Caveolin-3](Image 66x351 to 280x728)

![Merge](Image 308x458 to 552x728)

![Fig. 5. The effect of filipin treatment on the isoproterenol-stimulated contraction rate of β1AR-KO neonatal myocytes and FLAG-β2AR expressed in β1/β2AR-KO myocytes.](Image 66x351 to 280x728)

![A](Image 66x351 to 280x728)

![B](Image 66x351 to 280x728)
The distribution of membranes (Fig. 3). The treatment of cells with filipin altered increased the contraction rate response to was higher than that of control myocytes. **, stimulation the contraction rate increase in the filipin-treated myocytes tion 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. **, 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 β2/β3AR-KO myocytes. The data represent the mean ± S.E. of 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.

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 μM) in neonatal β2AR-KO myocytes (Fig. 7). However, at a much lower concentration (1 μM) of isoproterenol it has been shown that cAMP accumulation in wild-type rat myocytes can be enhanced by the disruption of caveolae with cyclohextrin (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 β2AR-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/functional sarcoplasmic reticulum membrane (21). The restricted pools of cAMP show a range of action as small as ~1 μm, 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 β2AR in β2AR-KO myocytes (Fig. 5). The inhibition of the contraction rate observed ~15 min after β2AR stimulation is mediated by Gi and can be inhibited by pertussis toxin (5). Previous studies have shown that caveolae are enriched in Gi 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 β2AR from caveolae and the associated Gi 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 β2AR in the presence of filipin is not due to the disruption of the coupling of the β2AR to Gi but to the more efficient coupling of the β2AR to the stimulation of the contraction rate, most likely through Gs. By disrupting lipid rafts, filipin may enhance the diffusion of the β2AR 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 β2ARs 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 β1AR in cardiac myocytes. These results provide support for the hypothesis that β1AR and β2AR are segregated into discrete signaling compartments in neonatal myocytes. We also show that neonatal cardiac myocytes from β1/β2AR-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 β1AR and β2AR in cardiac myocytes.

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