

The PDZ Binding Motif of the β_1 Adrenergic Receptor Modulates Receptor Trafficking and Signaling in Cardiac Myocytes*

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β_1 and β_2 adrenergic receptors (AR) regulate the intrinsic contraction rate in neonatal mouse cardiac myocytes through distinct signaling pathways. It has been shown that stimulation of β_1 ARs leads to a protein kinase A-dependent increase in contraction rate. In contrast, stimulation of β_2 ARs has a biphasic effect on contraction rate, with an initial protein kinase A-independent increase followed by a sustained decrease that is blocked by pertussis toxin. The β_2 AR undergoes agonist-induced endocytosis in cardiac myocytes while the β_1 AR remains on the cell surface. It has been shown that a PDZ domain binding motif at the carboxyl terminus of β_1 AR interacts with the postsynaptic density protein PSD-95 when both are expressed in HEK293 cells. We found that mutation of this PDZ binding motif in the β_1 AR (β_1 AR-PDZ) enabled agonist-induced internalization in cardiac myocytes. Moreover, stimulation of β_1 AR-PDZ had a biphasic effect on the myocyte contraction rate similar to that observed following stimulation of the β_2 AR. The secondary decrease in the contraction rate was mediated by G_i and could be blocked by pertussis toxin. Furthermore, a non-selective endocytosis inhibitor, concanavalin A, inhibited the internalization of wild type β_2 AR and the mutated β_1 AR-PDZ, and blocked the coupling of both receptors to G_i . Finally, treating myocytes with a membrane-permeable peptide representing β_1 AR PDZ motif caused the endogenous β_1 AR to behave like β_1 AR-PDZ. These studies suggest that association of the β_1 AR with PSD-95 or a related protein dictates signaling specificity by retaining the receptor at the cell surface and preventing interaction with G_i .

function in animals (1). *In vitro* studies show that β_1 and β_2 ARs have very similar signaling properties when expressed in undifferentiated cell lines (2). However, evidence also suggests that they have different signaling properties in differentiated cells *in vivo* (3, 4). Moreover, these receptors may differ in other functional parameters such as desensitization (5). β_1 AR knockout mice lack the normal inotropic and chronotropic response to the adrenergic agonist, isoproterenol (6), whereas these responses are preserved in β_2 AR knockout mice (7).

We recently reported that β_1 and β_2 ARs regulate contraction rate in mouse neonatal myocytes through different signal transduction pathways (8). Activated β_1 AR couples only to G_s and leads to a PKA-dependent increase in contraction rate. Activated β_2 AR undergoes sequential coupling to G_s and G_i and has a biphasic effect on contraction rate, with an initial PKA-independent increase followed by a sustained decrease that can be blocked by pertussis toxin (8). Recent biochemical evidence suggests that the functional differences between β_1 and β_2 AR in cardiac myocytes may be mediated in part by subtype-specific targeting in the myocyte plasma membrane. Fractionation studies show that β_2 ARs are concentrated in caveolae structures, whereas β_1 ARs are mainly distributed in non-caveolar membrane on the cardiac myocyte plasma membrane (9, 10). Moreover, studies show that β_2 AR signaling can modulate L-type Ca^{2+} channel activity in distinct subcellular microdomains in hippocampal neurons and cardiac myocytes (11–13). These observations suggest that distinct signaling complexes may exist in cardiac myocytes to conduct β_1 and β_2 AR signaling.

Evidence also shows that GPCRs can form complexes with downstream effectors to facilitate signaling. β_2 AR has been shown to associate with AKAP79 and Gravin, which are scaffolding proteins that connect β_2 AR to PKA, PKC, PP2A, and/or L-type Ca^{2+} channels (11, 14–16). GPCRs can also be recruited into the signaling complexes through the receptor carboxyl-terminal PDZ domain binding motifs, which directly interact with PDZ domain-containing scaffolding proteins. It has been shown that the β_2 AR carboxyl-terminal PDZ motif interacts with the Na^+/H^+ exchanger regulatory factor (NHERF, also known as EBP50) (17). This interaction is critical for the receptor recycling after sequestration from the cell surface, and affects Na^+/H^+ exchange in HEK293 cells (17, 18). Recent reports show that the β_1 AR PDZ motif can interact with postsynaptic scaffolding proteins PSD-95 (SAP90) and MAGI-2 in HEK293 cells (19, 20). A homologue of PSD-95, SAP97, is expressed in the heart and associates with potassium channels in cardiac myocytes (21, 22); however, the functional role of an interaction between the β_1 AR and a PDZ domain-containing scaffold proteins in cardiac myocytes has not been reported.

To investigate the functional role of the PDZ binding motif in subtype-specific signaling by the β_1 AR, we expressed wild type and a PDZ mutant (β_1 AR-PDZ) in neonatal myocytes from

β adrenergic receptors (β_1 AR, β_2 AR, and β_3 AR)¹ are heptahelical G protein-coupled receptors (GPCRs) that mediate physiological responses to the hormone epinephrine and the neurotransmitter norepinephrine. Both β_1 and β_2 ARs are expressed in the heart and play critical roles in regulating cardiac

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¹ The abbreviations used are: AR, adrenergic receptor; GPCRs, G protein-coupled receptors; PKA, protein kinase A; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; KO, knockout; PTX, pertussis toxin; MAPK, mitogen-activated protein kinase; conA, concanavalin A; SAP, synaptic-associated protein; PSD, postsynaptic density; MAGI, membrane-associated guanylate inverted.

β_1/β_2 AR knockout mice. In comparison to wild type β_1 AR, β_1 AR-PDZ undergoes significant internalization upon agonist stimulation. Moreover, like the β_2 AR, the β_1 AR-PDZ couples to both G_s and G_i in cardiac myocytes. Coupling of both β_2 AR and β_1 AR-PDZ to G_i is dependent on receptor internalization and can be blocked by concanavalin A. These results demonstrate that association of β_1 AR with PSD-95 or a related protein dictates signaling specificity by retaining the receptor at the cell surface and preventing interaction with G_i .

EXPERIMENTAL PROCEDURES

Culture and Adenovirus Infection of Neonatal Mouse Ventricular Myocytes—Spontaneously beating neonatal cardiac myocytes were prepared from hearts of 1- to 2-day-old mouse pups (from wild type, β_1 AR-KO, β_2 AR-KO, and β_1/β_2 AR-KO mice) as previously described (8) 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, pH 7.4, plus 300 unit/ml collagenase type II (Invitrogen, Carlsbad, CA). The isolated myocytes were collected from the second and third digestions. 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, in 12-well plates for ELISA, in 12-well plates with coverslips for immunocytochemistry, or in 10-cm dishes for Western blot and ligand binding assays. 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 was changed every 24 h. To further reduce fibroblast contamination, myocytes were cultured in the presence of 10 μ M cytosine- β -D-arabino-furanoside (Sigma) during the first 24 h to block fibroblast proliferation.

Recombinant adenoviruses encoding amino-terminal FLAG-tagged mouse β_2 AR or HA-tagged mouse β_1 AR or β_1 AR-PDZ (the β_1 AR carboxyl-terminal PDZ motif ESKV was mutated into EAAA) were generated with the pAdEasy system (Q.biogen, Carlsbad, CA). The virus titer was obtained by optical density as recommended by the manufacturer (Q.biogen). HA- β_1 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 myocytes were incubated with virus-containing media for 2 h at 37 °C. The virus media was then removed and replaced with fresh media. The infected myocyte culture was maintained for 2 days before being used for subsequent experiments.

Measurement of Myocyte Contraction Rate—Measurement of the spontaneous contraction rate was carried out as described previously (8) with modifications. Briefly, myocytes were cultured in 35-mm dishes to obtain a uniformly beating syncytium. After 72 h in culture, the culture media was replaced with fresh media buffered with 20 mM HEPES, pH 7.4, at 37 °C for at least 1 h before contraction rate studies. To measure 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 connecting the microscope to a computer with Metamorph image software was used to acquire images (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 the time course experiments, statistical significance between groups was analyzed with two-way analysis of variance. Analysis was done with Prism software (Graph Pad Software, Inc., San Diego, CA).

Measurement of cAMP Accumulation—To measure intracellular cAMP, myocytes were cultured in 12-well plates (2.5×10^5 cells per well). Cells were incubated for 30 min at 37 °C with 1 mM isobutylmethylxanthine (Sigma) immediately before the addition of the agonist isoproterenol (10 μ M) for 10 or 30 min at room temperature. 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)

Drugs and Drug Treatment—Tat and Tat-Scr- ψ eR peptides were generous gifts from Daria Mochly-Rosen (23). Tat-PDZ consisting of Tat

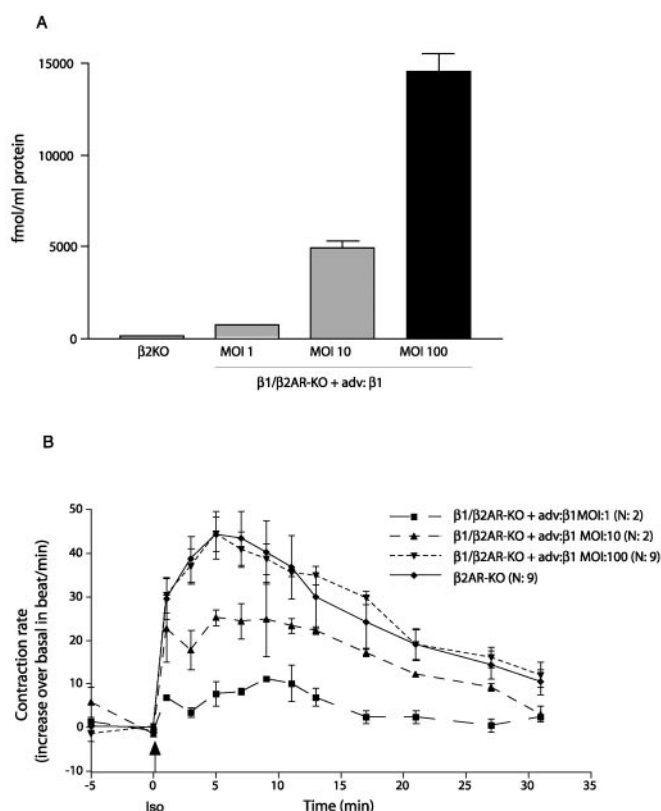


FIG. 1. Functional Expression of HA- β_1 AR in β_1/β_2 AR-KO myocytes. Neonatal myocytes were cultured and infected with different concentrations of recombinant adenovirus expressing HA- β_1 AR (adv: β_1) as described under "Experimental Procedures." A, expression of HA- β_1 AR was determined by saturation binding to obtain B_{max} . Cells infected at m.o.i. of 1, 10, and 100 resulted in HA- β_1 AR expression of 5, 35, and 98-fold relative to the endogenous β_1 AR protein level in β_2 AR-KO myocytes. B, the effect of 10 μ M isoproterenol on the contraction rate of β_1/β_2 AR-KO myocytes infected at different m.o.i. with adenovirus-expressing HA- β_1 AR. Also the contraction rate responses in β_2 AR-KO myocytes are shown for comparison. The data represent the mean \pm S.E. of n experiments from different myocyte preparations.

and amino-terminal cysteine-bridge linked 466 GRQGFSSSESKV $^{COO-}$ of β_1 AR was synthesized in the Stanford Core facility. Neonatal myocytes were preincubated at 37 °C with concanavalin A (conA, 400 μ g/ml, Sigma, MO) for 30 min. or peptides (Tat, Tat-Scr- ψ eR or Tat-PDZ, 1 μ M) for 25 min. before isoproterenol (10 μ M, sigma, MO) or forskolin (10 μ M, sigma, MO) exposure. For some contraction assays, PTX (0.75 μ g/ml, Sigma, MO) was used together with conA or the peptides. PTX treatment was carried out as described previously (8).

Immunofluorescence Microscopy—Myocytes were cultured in 12-well plates with coverslips and infected with the FLAG- β_2 AR, HA- β_1 AR, or HA- β_1 AR-PDZ adenoviruses as described above. Myocytes were fixed with 1× PBS containing 5% paraformaldehyde and permeabilized with 1× PBS containing 1% Nonidet P-40. Cells were then stained with anti-FLAG M1 antibody (mouse monoclonal IgG $_{2b}$, 1:600 dilution, Sigma) or anti-HA 16b12 antibody (mouse monoclonal IgG $_{1}$, 1:600 dilution, Covance, Berkeley, CA). The primary antibodies were detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:200 Fisher, Pittsburgh, PA). The images were acquired with a Ziss Axioplan 2 Imaging microscope (Thornwood, NY).

Ligand Binding Assay—Membrane proteins were prepared from β_2 AR-KO myocytes and adenovirus-infected β_1/β_2 AR-KO myocytes as described above. The myocyte cultures 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 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 (1000 rpm at 4 °C). Membranes were obtained from the supernatant by centrifugation in a Beckman TLA100.2 desktop centrifuge (50,000 rpm at 4 °C) and resuspended in 1× binding buffer (75 mM Tris-HCl, pH 7.4, 12.5 mM MgCl $_2$, 1 mM EDTA). Saturation binding was carried out with the

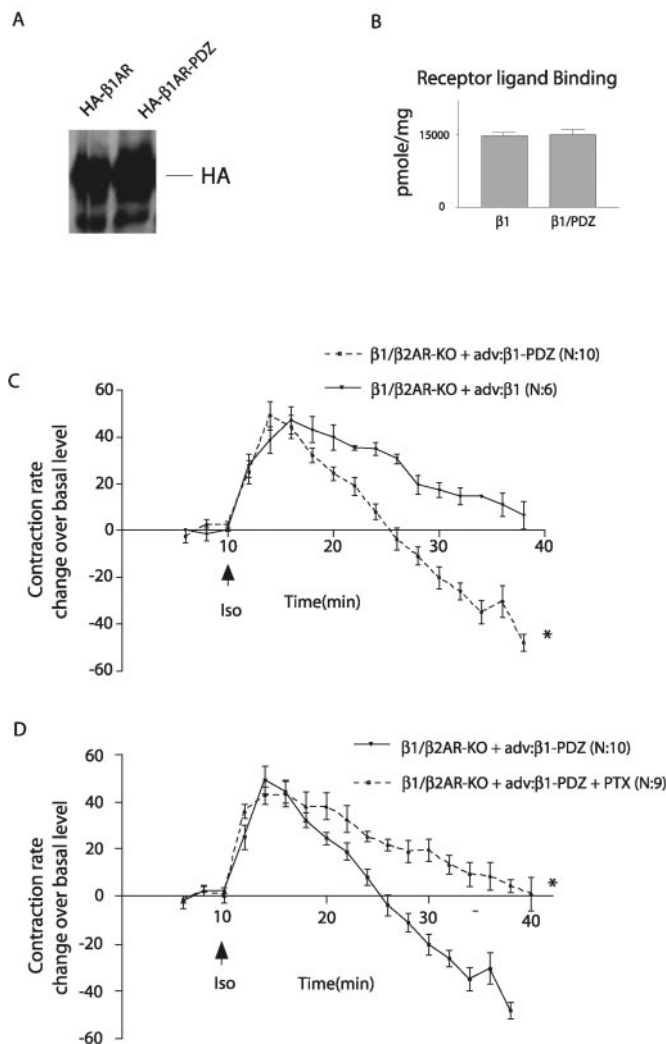


FIG. 2. Disruption of the β_1 AR PDZ motif enables the receptor to couple to G_i signaling pathways. Neonatal myocytes were cultured and infected with recombinant adenovirus-expressing HA- β_1 AR (adv: β_1 AR) or HA- β_1 AR-PDZ (adv: β_1 -PDZ) as described under "Experimental Procedures." **A**, the protein expression of HA- β_1 AR or HA- β_1 AR-PDZ in β_1/β_2 AR-KO myocytes. The protein expression was examined by Western blot using antibodies against the HA epitope. **B**, protein expression of HA- β_1 AR or HA- β_1 AR-PDZ was determined by saturation binding to obtain B_{max} . **C**, the effect of 10 μ M isoproterenol on the contraction rate of β_1/β_2 AR-KO myocytes infected at 100 m.o.i. with adenovirus-expressing HA- β_1 AR or HA- β_1 AR-PDZ. **D**, HA- β_1 AR-PDZ couple to G_i in neonatal myocytes. Neonatal myocytes expressing HA- β_1 AR-PDZ were cultured and treated with PTX (0.75 μ g/ml) or control media for 3 h 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. *, $p < 0.05$. Two time-course curves were found to be significantly different by two-way analysis of variance.

non-selective β AR antagonist [125 I]iodocyanopindolol (PerkinElmer Life Sciences) as described previously (6).

Western Blot—Membrane proteins were prepared from β_2 AR-KO myocytes and adenovirus-infected β_1/β_2 AR-KO myocytes. The myocytes were rinsed with ice-cold 1 \times PBS before harvesting with 20 mM HEPES, pH 7.4, buffer containing 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml benzamide. The collected cells were lysed on ice for 30 min, and the unbroken cells and nuclei were cleared by centrifugation at 1000 rpm at 4 $^{\circ}$ C for 5 min. The proteins were separated on 10% SDS-PAGE gels and Western blotted with anti-HA antibody (mouse monoclonal IgG₁, 1:600 dilution, Covance). After staining with a horseradish peroxidase-conjugated goat-anti mouse IgG antibody (1:1000 dilution, Bio-Rad, Hercules, CA), the Western blot was developed with the ECL kit (Amersham Biosciences).

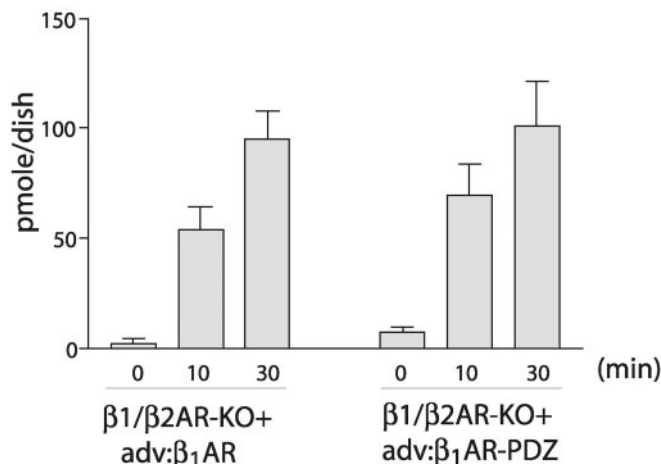


FIG. 3. The effect of 10 μ M isoproterenol on the whole-cell cAMP accumulation of β_1/β_2 AR-KO myocytes infected at 100 m.o.i. with adenovirus-expressing HA- β_1 AR or HA- β_1 AR-PDZ. The data represent the mean \pm S.E. of four experiments from at least three different myocyte preparations.

ELISA Assay—Myocytes were cultured in 12-well plates and infected with the FLAG- β_2 AR, HA- β_1 AR, or HA- β_1 AR-PDZ adenoviruses as described above. Myocytes were incubated in media containing 400 μ g/ml concanavalin A or control media for 30 min, and then the cells were treated with 10 μ M isoproterenol for an additional 30 min. Myocytes without isoproterenol stimulation were used as controls. All of the myocytes were fixed with 1 \times PBS containing 5% paraformaldehyde and stained with anti-FLAG M1 antibody (mouse monoclonal IgG_{2b}, 1:600 dilution, Sigma) or anti-HA 16b12 antibody (mouse monoclonal IgG₁, 1:600 dilution, Covance). The myocytes were then stained with horseradish peroxidase-conjugated goat-anti mouse antibody (1:1000, Bio-Rad) and processed for the 1-Step ABTS reaction (Pierce). The reaction was stopped by addition of 2% SDS, and the color density was measured with Softmax (Molecular Devices, Sunnyvale, CA). The data obtained from the isoproterenol-treated myocytes were normalized to that of the controls.

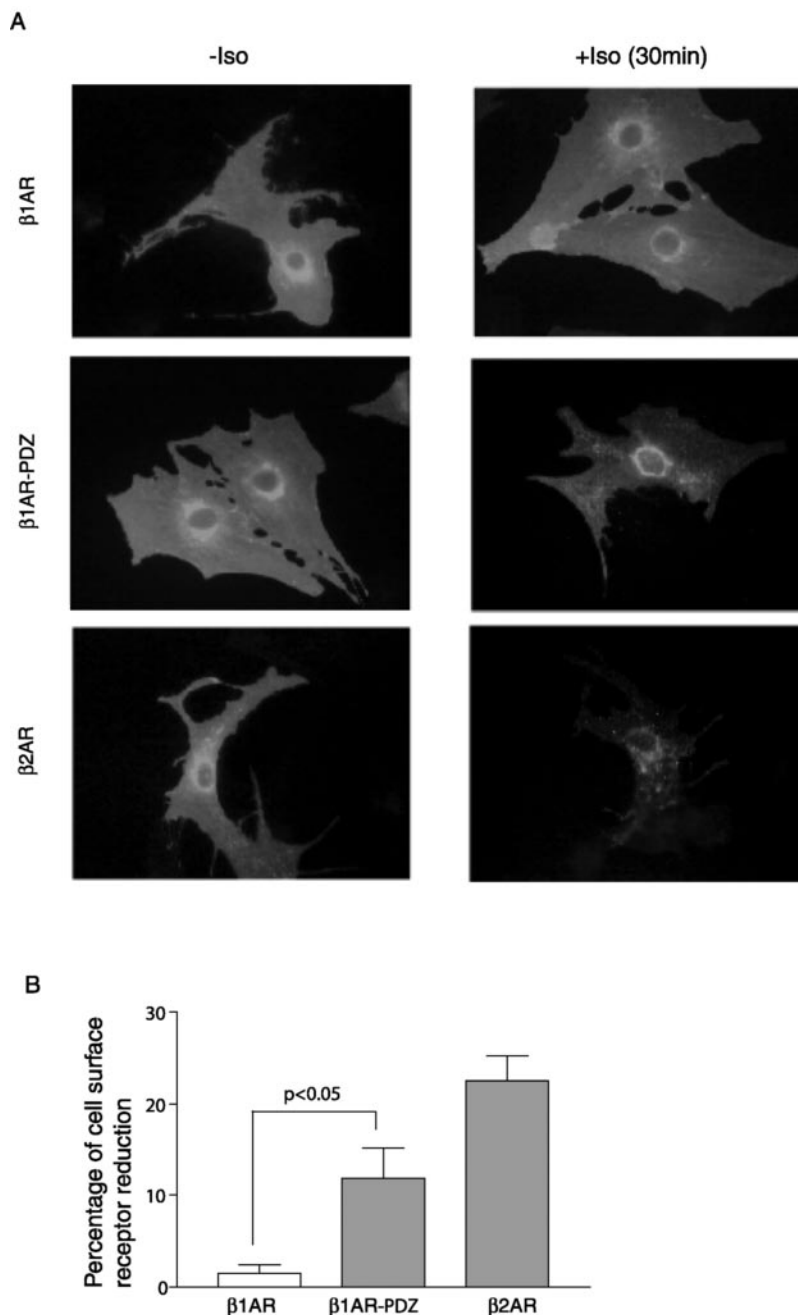
RESULTS

Disruption of the β_1 AR PDZ Motif Leads to Promiscuous G Protein Coupling—To perform structure-function studies on the β_1 AR, we used recombinant adenovirus to deliver wild type or mutant β_1 ARs into β_1/β_2 AR-knockout (β_1/β_2 AR-KO) neonatal cardiac myocytes. An amino-terminal HA epitope was inserted into the mouse β_1 AR (HA- β_1 AR) to facilitate immunocytochemical and biochemical studies. To ensure that HA- β_1 AR exhibited functional properties similar to those of endogenous β_1 AR in neonatal cardiac myocytes, we infected β_1/β_2 AR-KO myocytes with different titers of recombinant adenovirus expressing HA- β_1 AR. Endogenous β_1 AR expression in β_2 AR-KO myocytes is relatively low (148 fmol/mg of membrane protein), but is comparable with that of β_1 AR in the intact β_2 AR-KO mouse heart (Fig. 1A) (6). The expression of HA- β_1 AR in β_1/β_2 AR-KO myocytes ranged from 5- to 98-fold of that of endogenous β_1 AR, depending on the virus inoculum (Fig. 1A). Fig. 1B shows the effect of isoproterenol on the contraction rate of β_1/β_2 AR-KO myocytes expressing different levels of HA- β_1 AR. The maximum contraction rate increase induced by isoproterenol was dependent on the level of HA- β_1 AR expressed (Fig. 1B). In cells infected with 100 virus particles per cell (m.o.i. of 100), HA- β_1 AR gave a response comparable with that observed from endogenous β_1 AR in β_2 AR-KO myocytes (Fig. 1B).

To study the role of the PDZ motif in β_1 AR function in neonatal cardiac myocytes, we generated a recombinant adenovirus expressing a mutant β_1 AR that lacks a functional PDZ motif (HA- β_1 AR-PDZ). HA- β_1 AR and HA- β_1 AR-PDZ were expressed in β_1/β_2 AR-KO neonatal by infecting myocytes with recombinant adenovirus at a m.o.i. of 100. Both receptors could be efficiently expressed in neonatal myocytes (Fig. 2A), and the

FIG. 4. β_1 and β_2 ARs display distinct trafficking properties in neonatal cardiac myocytes.

Neonatal myocytes infected with HA- β_1 AR, HA- β_1 AR-PDZ, or FLAG- β_2 AR adenovirus (at m.o.i. of 100) were cultured and fixed for immunofluorescence microscopy and ELISA as described under "Experimental Procedures." A, the effect of isoproterenol on the distribution of HA- β_1 AR, HA- β_1 AR-PDZ, and FLAG- β_2 AR in neonatal myocytes. HA- β_1 AR, HA- β_1 AR-PDZ, and FLAG- β_2 AR display cell surface distribution on neonatal myocytes at steady-state. No significant internalization of HA- β_1 AR is observed following agonist stimulation, whereas punctate intracellular staining is observed following agonist stimulation of HA- β_1 AR-PDZ and FLAG- β_2 AR. B, the cell surface receptor loss after agonist stimulation. The cell surface receptor was measured by ELISA assay. Minimal loss of cell surface HA- β_1 AR was detected (1.7%), while there was a significant decrease in cell surface HA- β_1 AR-PDZ (11%) and FLAG- β_2 AR (22%) after isoproterenol stimulation. *, $p < 0.05$; t test significant different between HA- β_1 AR and HA- β_1 AR-PDZ.



level of receptor expression was comparable (Fig. 2B). Figure 2C shows the effect of isoproterenol on the contraction rate of β_1/β_2 AR-KO myocytes expressing either HA- β_1 AR or HA- β_1 AR-PDZ. The initial maximum contraction rate increase induced by HA- β_1 AR-PDZ was similar to that induced by HA- β_1 AR. However, after 15 min of stimulation, the contraction rate of HA- β_1 AR-expressing myocytes remained above the basal level, while the contraction rate of HA- β_1 AR-PDZ-expressing myocytes decreased to below the basal level (Fig. 2C). The biphasic contraction rate response resulting from stimulation of HA- β_1 AR-PDZ is similar to that of endogenous β_2 AR in β_1 AR-KO myocytes (see Fig. 5B and Ref. 8). We also examined cAMP accumulation in cultures of β_1/β_2 AR-KO myocytes expressing either HA- β_1 AR or HA- β_1 AR-PDZ. In contrast to different contraction rate responses to isoproterenol, the cAMP accumulation was not significantly different between myocytes expressing HA- β_1 AR and myocytes expressing HA- β_1 AR-PDZ (Fig. 3). Our previous studies have shown that the biphasic

contraction rate response to stimulation of the β_2 AR is due to sequential coupling of the receptor to G_s and G_i . Therefore, we used pertussis toxin (PTX), a G_i protein inhibitor, to examine the role of G_i in the HA- β_1 AR-PDZ-mediated inhibition of contraction rate in β_1/β_2 AR-KO neonatal myocytes. PTX treatment prevented the secondary decrease in contraction rate mediated by HA- β_1 AR-PDZ (Fig. 2D), and the resulting contraction rate profile was similar to that of cells expressing HA- β_1 AR in the absence of PTX (Fig. 2C). Thus, like the β_2 AR in β_1 AR-KO myocytes, HA- β_1 AR-PDZ undergoes sequential coupling to G_s and G_i in β_1/β_2 AR-KO myocytes.

The β_1 AR PDZ Motif Is Required to Retain the Activated Receptor on the Surface of Neonatal Myocytes—The β_2 AR undergoes rapid and robust agonist-induced internalization in HEK293 cells, whereas agonist-induced internalization of the β_1 AR is more limited (2, 24). Biochemical studies suggest that receptor endocytosis is necessary for the activation of the G_i -dependent MAPK pathway (25). Thus, the trafficking of β ARs

may play a critical role in their specific signaling behavior in mouse neonatal cardiac myocytes. Immunocytochemistry revealed that both HA- β_1 ARs and FLAG-tagged β_2 ARs (FLAG- β_2 AR) are localized predominantly on the cell surface of neonatal myocytes at steady-state (Fig. 4A). After isoproterenol stimulation, HA- β_1 ARs remained predominantly on the cell surface, whereas significant internalization of the β_2 AR was detected as punctate intracellular staining. Quantitative ELISA assays confirmed that isoproterenol stimulation caused a 22% decrease in cell surface FLAG- β_2 ARs in comparison to a 1.7% decrease in cell surface HA- β_1 ARs (Fig. 4B). HA- β_1 AR-PDZ also displayed cell surface staining on neonatal myocytes at steady state. However, in contrast to HA- β_1 AR, HA- β_1 AR-PDZ underwent significant internalization after isoproterenol stimulation (Fig. 4A), associated with a 11% loss of cell surface receptor by ELISA (Fig. 4B). These results suggest that the PDZ motif on β_1 AR is required to retain the activated receptor on the cell surface of neonatal myocytes.

Blocking β AR Internalization Inhibits the Receptor/ G_i Coupling in Neonatal Cardiac Myocytes—To examine the role of agonist-induced internalization in β_2 AR function, a nonselective endocytosis inhibitor, concanavalin A (conA), was used to block the receptor internalization in cardiac myocytes. conA treatment reduced β_2 AR internalization by 45% (Fig. 5A and data not shown). Fig. 5B shows the effect of conA on the contraction rate response to isoproterenol mediated by endogenous β_2 AR in β_1 AR-KO myocytes. As previously reported, β_1 AR-KO myocytes display a biphasic contraction rate response after isoproterenol stimulation due to sequential coupling of the β_2 AR to G_s and G_i (8). However, the conA-treated β_1 AR-KO myocytes displayed a larger maximum contraction rate response to isoproterenol, and the contraction rate did not drop below basal level (Fig. 5B). These results suggest that conA treatment enhances coupling to G_s and/or inhibits coupling to G_i . The role of G_i in the conA-treated β_1 AR-KO myocytes was examined with PTX. Additional treatment with PTX did not further augment the maximum contraction rate increase by isoproterenol, nor did it change the contraction rate response profile of the conA-treated β_1 AR-KO myocytes (Fig. 5C). In contrast, conA did not significantly alter the β_1 AR-mediated contraction rate response to isoproterenol in β_2 AR-KO myocytes (Fig. 6A). Moreover, conA did not significantly affect the contraction rate response to isoproterenol in wild type myocytes (Fig. 6B).

We then examined the effect of conA on the contraction rate by isoproterenol in β_1/β_2 AR-KO myocytes expressing β_1 AR-PDZ. In comparison to untreated controls, conA prevented the contraction rate from decreasing below basal level (Fig. 7A). Moreover, PTX treatment did not alter the contraction rate response to isoproterenol in the conA-treated myocytes (Fig. 7B). Thus, our observations suggest that conA treatment can block the internalization of β_1 AR-PDZ and β_2 AR and inhibit these receptors from coupling to G_i in neonatal cardiac myocytes. Together, these data suggest that receptor internalization may be required for the sequential coupling observed in β_2 AR and β_1 AR-PDZ.

A Membrane-permeable Peptide Containing the β_1 AR-PDZ Motif Affects β_1 AR Signaling in Cardiac Myocytes—To confirm our observations on β_1 AR-PDZ in β_1/β_2 AR-KO myocytes, we used a membrane-permeable peptide (Tat- β_1 PDZ) to interfere with the interaction between endogenous β_1 AR and its binding partners in β_2 AR-KO myocytes. Fig. 8A shows the effect of Tat- β_1 PDZ on the contraction rate by isoproterenol in β_2 AR-KO myocytes. In comparison to non-treated cells, the Tat- β_1 PDZ-treated myocytes showed a smaller maximum contraction rate increase, and the contraction rate decreased below basal levels. In contrast, both the control peptides, Tat and Tat-Scr- ψ ER, did

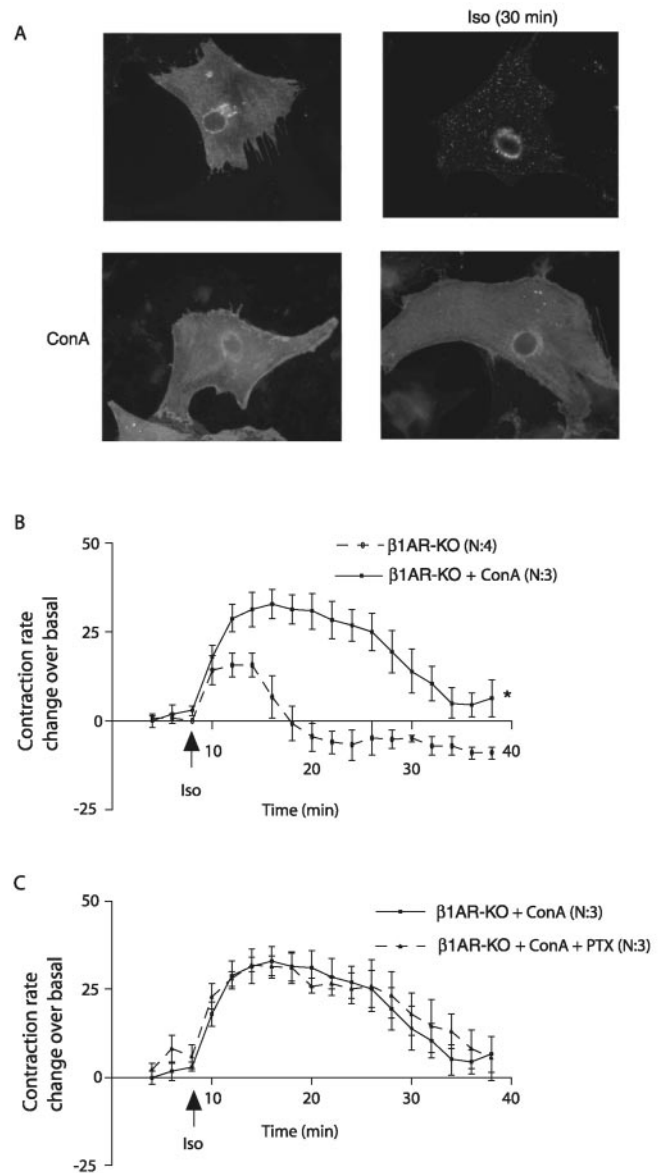


FIG. 5. The effect of conA treatment on β_2 AR trafficking and signaling in neonatal cardiac myocytes. A, conA blocks FLAG- β_2 AR internalization in neonatal cardiac myocytes. β_1/β_2 AR-KO myocytes expressing FLAG- β_2 AR (adv: β_2) were treated with conA (400 μ g/ml) or control media for 30 min before isoproterenol stimulation. The cells were fixed and stained for immunofluorescence microscopy. B, the effect of conA on β_2 AR-mediated myocyte contraction rate. β_1 AR-KO neonatal myocytes were cultured and treated with either conA (400 μ g/ml) or control media 30 min before contraction rate experiments as described under "Experimental Procedures." The basal contraction rate of β_1 AR-KO myocytes was not altered significantly by conA treatment. C, the effect of conA treatment on β_2 AR- G_i coupling in myocytes. β_1 AR-KO neonatal myocytes were cultured and treated with PTX (0.75 μ g/ml) or control media for 2.5 h before the addition of conA (400 μ g/ml). Myocytes were incubated for an additional 30 min before contraction rate experiments were performed as described under "Experimental Procedures." The data in B and C represent the mean \pm S.E. of n experiments from at least three different myocyte preparations. *, $p < 0.05$; two time-course curves were found to be significantly different by two-way analysis of variance.

not significantly change the contraction rate (data not shown and Fig. 8B). The biphasic contraction rate response in Tat- β_1 PDZ-treated β_2 AR-KO myocytes suggests a sequential coupling of β_1 AR-PDZ to both G_s and G_i . Thus, the role of G_i in the Tat- β_1 PDZ-treated β_2 AR-KO myocytes was evaluated. PTX treatment caused a greater maximum contraction rate increase in response to isoproterenol and prevented the contraction rate

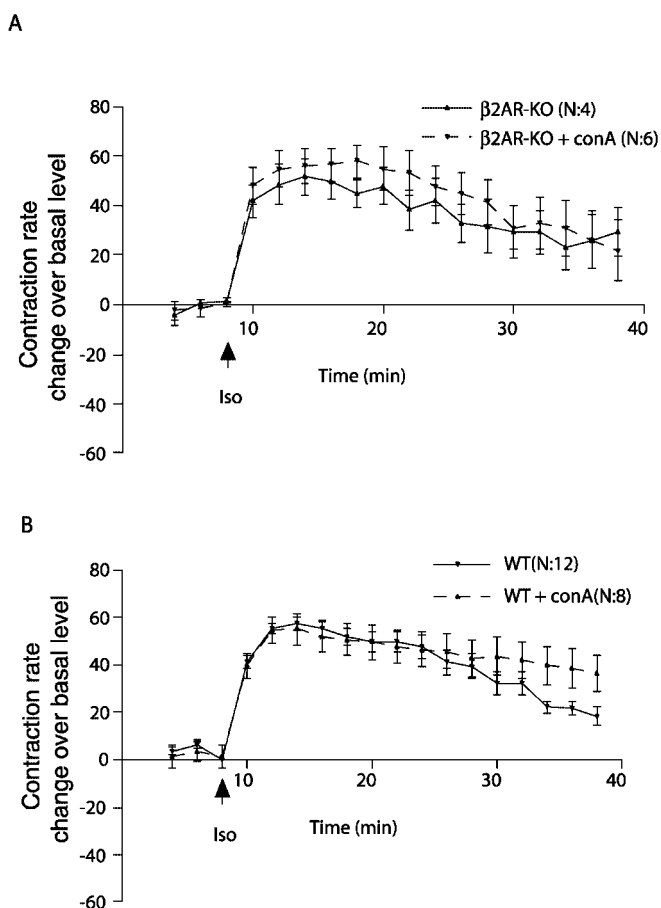


FIG. 6. The effect of conA treatment on β signaling in neonatal cardiac myocytes from β_2 AR-KO and wild type myocytes. *A*, the effect of conA on β_1 AR-mediated myocyte contraction rate. β_2 AR-KO neonatal myocytes were cultured and treated with either conA (400 μ g/ml) or control media 30 min before contraction rate experiments as described under "Experimental Procedures." Contraction rate of β_2 AR-KO myocytes was not altered significantly by conA treatment. *B*, the effect of conA treatment on contraction rate in wild type myocytes. Wild type neonatal myocytes were cultured and treated with either conA (400 μ g/ml) or control media 30 min before contraction rate experiments as described under "Experimental Procedures." The data represent the mean \pm S.E. of *n* experiments from at least three different myocyte preparations.

from decreasing below basal level in β_2 AR-KO myocytes treated with TAT- β_1 PDZ (Fig. 8C). In contrast, the Tat- β_1 PDZ peptide had no significant effect on the contraction rate of β_2 AR-KO myocytes stimulated by forskolin (Fig. 8D). Thus, our results suggest that the peptide Tat- β_1 PDZ can selectively modulate the β_1 AR-mediated signaling in cardiac myocytes by competing with the binding of endogenous β_1 ARs to PDZ domain-containing proteins.

DISCUSSION

β adrenergic receptors play critical roles in mediating physiologic responses to the hormone epinephrine and the neurotransmitter norepinephrine in animal hearts. Although both β_1 and β_2 ARs are expressed in the hearts and respond to the same physiologic agonists, they show distinct functions *in vivo*. We have previously shown that β_1 and β_2 ARs display distinct signaling properties in controlling neonatal myocyte contraction rate. Activated β_1 ARs couple to G_s , leading to a large increase in contraction rate (8). In this study, we have found that the PDZ motif on β_1 AR is required to restrict coupling of this receptor to G_s in mouse neonatal cardiac myocytes (Figs. 2, 7, and 8). Mutation of the PDZ motif on β_1 AR or inhibiting the

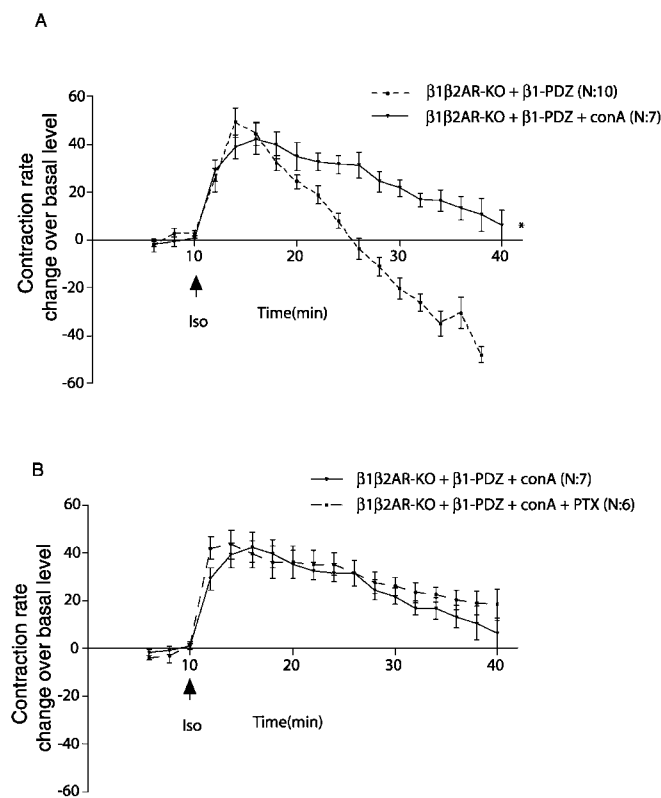


FIG. 7. The effect of conA treatment on the isoproterenol-stimulated contraction rate of β_1/β_2 AR-KO myocytes expressing HA- β_1 AR-PDZ. Neonatal myocytes from β_1/β_2 AR-KO mice were cultured and infected with recombinant adenovirus expressing HA- β_1 AR-PDZ as described under "Experimental Procedures." *A*, the effect of conA on β_1 AR-PDZ signaling in myocytes. Neonatal myocytes were treated with either conA (400 μ 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 conA treatment. *B*, the effect of conA on β_1 AR-PDZ coupling to G_i 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 conA (400 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; two time-course curves were found to be significantly different by two-way analysis of variance.

interaction between the β_1 AR and the PDZ domain protein with a cell-permeable peptide enables the receptor to couple to both G_s and G_i , resulting in a biphasic effect on myocyte contraction rate (Figs. 2 and 8). Thus, β_1 AR appears to have the structural motifs necessary for efficient coupling to G_i *in vitro*; however, this coupling is inhibited in cardiac myocytes by the association between the β_1 AR PDZ motif and its binding partner. Based on the observed effect of the PDZ mutation on contraction rate *in vitro* (Fig. 2), we might expect that a similar mutant expressed *in vivo* would have a significant and possibly detrimental effect on cardiac function. Therefore, preventing β_1 ARs from coupling to G_i may be essential for the physiologic function of this receptor.

Of interest, the effect of the PDZ mutation on contraction rate is not associated with detectable changes in whole cell cAMP accumulation (Fig. 3). The apparent discrepancy between the results of the contraction rate and the cAMP accumulation assays may be due to technical differences in the assays. To detect cAMP accumulation in myocytes, cultures must be preincubated with a phosphodiesterase inhibitor (isobutylmethylxanthine) to prevent hydrolysis of cAMP. Thus, the cAMP assay conditions will not fully replicate the more

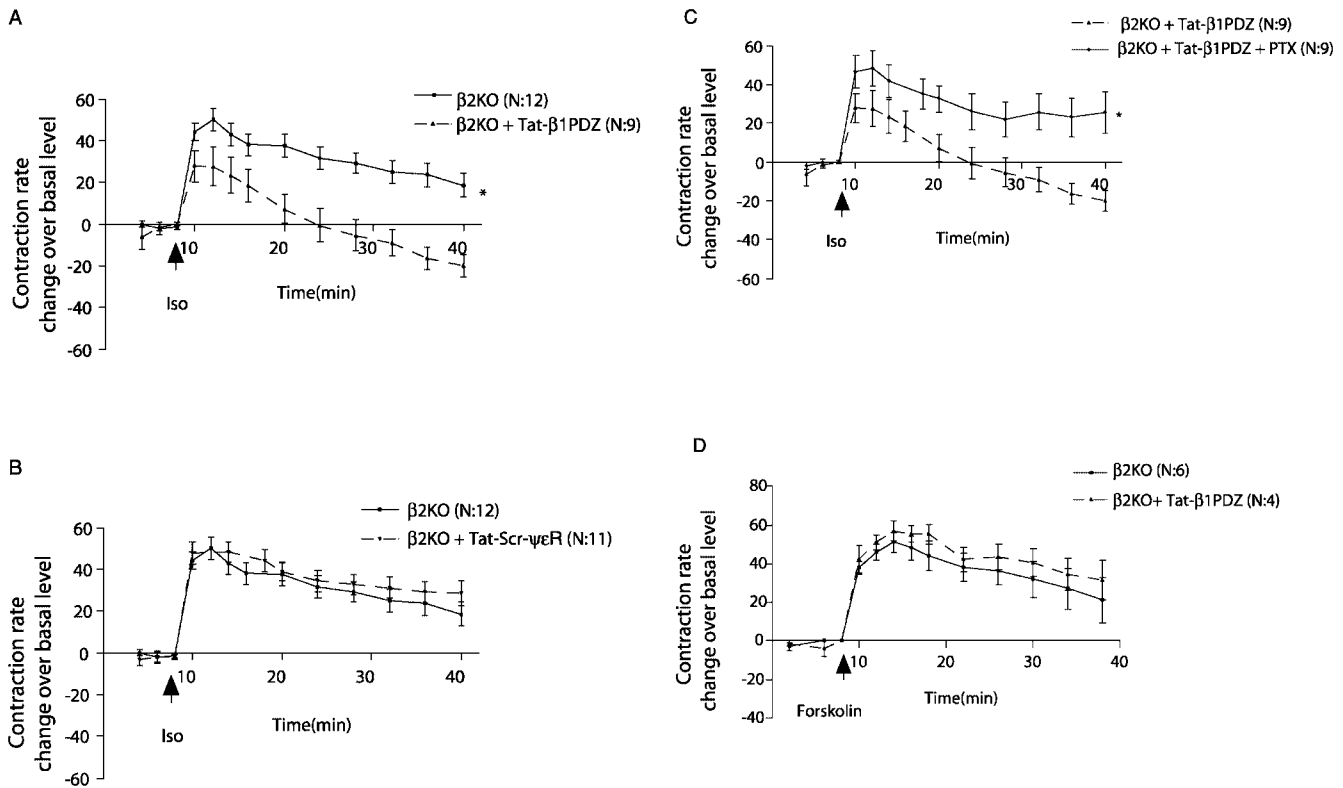


FIG. 8. A β_1 AR PDZ peptide affects contraction rate of β_2 AR-KO myocytes. β_2 AR-KO neonatal myocytes were cultured and treated with either peptide Tat- β_1 PDZ (1 μ g/ml) or control Tat-Scr- ψ eR peptide (1 μ g/ml) for 25 min before contraction rate experiments as described under "Experimental Procedures." The basal contraction rate of myocytes was not altered significantly by peptide treatment. Tat- β_1 PDZ (A) but not Tat-Scr- ψ eR (B) treatment significantly changed the isoproterenol-stimulated contraction rate increase in β_2 AR-KO myocytes. C, Tat- β_1 PDZ treatment promotes coupling of the β_1 AR to G_i . β_2 AR-KO neonatal myocytes were cultured and treated with PTX (0.75 μ g/ml) or control media for 2.5 h before the addition of conA (400 μ g/ml). Myocytes were incubated for an additional 30 min before contraction rate experiments were performed as described under "Experimental Procedures." D, the effect of the Tat- β_1 PDZ treatment on the contraction rate of β_2 AR-KO myocytes stimulated by forskolin. The data represent the mean \pm S.E. of n experiments from at least three different myocyte preparations. *, $p < 0.05$; two time-course curves were found to be significantly different by two-way analysis of variance.

physiologic conditions of the myocyte contraction rate assay. Moreover, a recent report demonstrates that discrete microdomains of cAMP accumulation can be detected in neonatal myocytes following adrenergic stimulation (26). Thus, 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.

In contrast to β_1 ARs, native β_2 ARs undergo sequential coupling to both G_s and G_i , resulting in a biphasic effect on contraction rate with an initial small increase followed by a sustained decrease. Notably, activated β_2 AR has only a moderate effect on myocyte contraction rate in comparison to activated β_1 AR (Figs. 1 and 5, and Ref. 8). It has been recently reported that G_s activity may be involved in myocyte apoptosis, whereas activated G_i confers a protective effect against myocyte apoptosis (27, 28). Therefore, although β_2 AR/ G_i coupling only generates a small negative effect on heart rate (8), its main function may be to activate the anti-apoptosis pathway to protect against the damage induced by chronic β_1 AR activation during prolonged periods of stress. Indeed, moderate overexpression of β_2 AR in the heart of transgenic mice leads to an enhanced cardiac contractility without developing cardiomyopathy, suggesting that β_2 AR can provide contractile support without significant a cardiotoxic consequence (29). The dual regulation mediated by both β_1 and β_2 ARs in cardiac myocytes represents a sophisticated mechanism critical for the ability of the hearts to respond to, but not be damaged by catecholamines.

There is a growing body of evidence that subtype-specific signaling of GPCRs involves organization of membrane recep-

tors, G proteins, effectors, and regulatory proteins in discrete signaling complexes in the plasma membrane. These complexes are organized by scaffold proteins such as AKAPs (30) and MAGUKs (31, 32), which contain multiple protein-protein interaction domains that can bind to GPCRs, PKA, protein kinase C, PP2A/PP1, and/or ion channels (11). Recent studies show that the PDZ motif on β_1 AR can directly interact with two postsynaptic MAGUK proteins, PSD-95 (SAP90) (19) and MAGI-2 (20). Overexpressing PSD-95 inhibits β_1 AR internalization and promotes the receptor clustering with *N*-methyl-D-aspartate receptors in HEK293 cells (19). However, overexpressing MAGI-2 enhances β_1 AR internalization and promotes association of the receptor with a cytoskeleton component β -catenin but not receptor oligomerization in HEK293 cells (20). Therefore, the β_1 AR PDZ motif can bind to proteins within distinct signaling complexes that determine the functional properties of the receptor in different cells. Interestingly, β_2 AR also has a carboxyl-terminal PDZ motif that does not interact with SAP or MAGI proteins. Instead, it selectively associates with the Na^+/H^+ exchanger regulatory factor NHERF/EBP50, a distinct PDZ domain-containing protein (17). This interaction is critical for the receptor recycling after internalization from the cell surface and affects Na^+/H^+ exchange in HEK293 cells (17, 18). The observed differences in the β_1 AR and β_2 AR PDZ domain-binding proteins may play a role in the functional differences these receptors exhibit in neonatal cardiac myocytes. In agreement with this hypothesis, the β_2 AR undergoes significant internalization while the β_1 AR remains on the cell surface following agonist stimulation in cardiac myocytes.

Moreover, the β_1 AR-PDZ, like β_2 AR, undergoes significant internalization after agonist stimulation in cardiac myocytes. Therefore, the β_1 AR PDZ motif is critical in determining both the trafficking and the signaling properties of β_1 AR in neonatal cardiac myocytes (Figs. 2 and 4).

SAP proteins include SAP90 (PSD-95), SAP93, SAP97, and SAP102, whereas MAGI proteins consist of three close relatives, MAGI-1, MAGI-2, and MAGI-3. Sequence analysis predicts that all SAPs and MAGIs can act as binding partners for β_1 AR (19, 20). Interestingly, MAGI-1 and SAP97 are also expressed in the hearts, and SAP97 associates with cardiac sodium (33) and potassium (21, 22) channels. Studies show that both MAGI-1 and SAP97 also associate with cytoskeleton proteins including β -catenin, α -actinin-2 and E-cadherin (20, 34, 35). Thus, SAP97, MAGI-1, or similar proteins may recruit β_1 ARs and ion channels into cytoskeleton-associated signaling complexes in cardiac myocytes. These complexes may not only facilitate the signaling from the bound receptors to downstream ion channels but also tether the receptors on the cell surface by associating with cytoskeleton. Moreover, the cell surface-tethered β_1 ARs signaling complexes could also prevent the receptor from coupling to G_i .

Membrane-permeable peptides have recently been used in cell culture and animals to interfere with protein-protein interactions (36). A membrane-permeable peptide containing the β_1 AR PDZ motif facilitated coupling of endogenous β_1 AR to G_i in cardiac myocytes (Fig. 8) confirming the conclusions based on studies of the β_1 AR-PDZ mutant expressed by recombinant adenovirus in β_1/β_2 AR-KO myocytes. This data suggests that the peptide can compete with the binding of endogenous β_1 AR to other cellular components. As a result, β_1 AR is free to couple to more abundant G_s in addition to G_i in cardiac myocytes. These peptide studies raise the possibility of pharmacologic modulation of GPCR-mediated signaling by disrupting interactions between receptors and scaffolding proteins.

In conclusion, we present evidence that the β_1 AR PDZ motif is essential for the physiologic signaling of this receptor subtype in cardiac myocytes. Disruption of the β_1 AR PDZ motif enabled the receptor to couple to both G_s and G_i and to enhance receptor internalization after isoproterenol stimulation in neonatal cardiac myocytes. Our observation provides the first clue to the structural basis for the functional differences between β_1 AR and β_2 ARs in neonatal cardiac myocytes.

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