

The PDZ-binding motif of the β_2 -adrenoceptor is essential for physiologic signaling and trafficking in cardiac myocytes

Yang Xiang and Brian Kobilka*

Department of Molecular and Cellular Physiology, Stanford University, Palo Alto, CA 94305

Edited by Robert J. Lefkowitz, Duke University Medical Center, Durham, NC, and approved July 15, 2003 (received for review March 25, 2003)

β_1 - and β_2 -adrenergic receptors (AR) regulate cardiac myocyte function through distinct signaling pathways. In addition to regulating cardiac rate and contractility, β_1 AR and β_2 AR may play different roles in the pathogenesis of heart failure. Studies on neonatal cardiac myocytes from β_1 AR and β_2 AR knockout mice suggest that subtype-specific signaling is determined by subtype-specific membrane targeting and trafficking. Stimulation of β_2 ARs has a biphasic effect on contraction rate, with an initial increase followed by a sustained G_i -dependent decrease. Recent studies show that a PDZ domain-binding motif at the carboxyl terminus of human β_2 AR interacts with ezrin-binding protein 50/sodium-hydrogen exchanger regulatory factor, a PDZ-domain-containing protein. The human β_2 AR carboxyl terminus also binds to *N*-ethylmaleimide-sensitive factor, which does not contain a PDZ domain. We found that mutation of the three carboxyl-terminal amino acids in the mouse β_2 AR (β_2 AR-AAA) disrupts recycling of the receptor after agonist-induced internalization in cardiac myocytes. Nevertheless, stimulation of the β_2 AR-AAA produced a greater contraction rate increase than that of the wild-type β_2 AR. This enhanced stimulation of contraction rate can be attributed in part to the failure of the β_2 AR-AAA to couple to G_i . We also observed that coupling of endogenous, wild-type β_2 AR to G_i in β_1 AR knockout myocytes is inhibited by treatment with a membrane-permeable peptide representing the β_2 AR carboxyl terminus. These studies demonstrate that association of the carboxyl terminus of the β_2 AR with ezrin-binding protein 50/sodium-hydrogen exchanger regulatory factor, *N*-ethylmaleimide-sensitive factor, or some related proteins dictates physiologic signaling specificity and trafficking in cardiac myocytes.

The β -adrenergic receptors (ARs) are essential for the physiologic regulation of cardiac function by the sympathetic nervous system (1), and may play important roles in the pathogenesis of heart failure (2). The three known subtypes of β ARs (β_1 AR, β_2 AR, and β_3 AR) belong to the large family of G protein-coupled receptors. Although all three subtypes have been detected in mammalian hearts (3), most of the functional responses to β -agonists can be attributed to β_1 AR and β_2 AR (3, 4). Although β_1 AR and β_2 AR respond to the same physiologic ligands (the hormone epinephrine and the neurotransmitter norepinephrine), they have distinct functional properties *in vivo* (5, 6). Specifically, 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 (5).

When expressed in undifferentiated fibroblast cells, β_1 AR and β_2 AR exhibit similar signaling properties (7). However, recent studies suggest that these subtypes activate different signaling pathways in differentiated cells *in vivo* (8, 9). We have recently reported that β_1 AR and β_2 AR regulate the intrinsic contraction rate in mouse neonatal myocytes through different signal-transduction pathways (4). Activation of β_1 ARs leads to a PKA-dependent increase in contraction rate. In contrast, activated β_2 ARs undergo sequential coupling to G_s and G_i , which 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 (PTX) (4). The functional differences between β_1 AR and β_2 AR in cardiac myocytes are mediated in part by subtype-specific targeting of the receptors on the myocyte plasma membrane (10, 11). Immunofluorescence and membrane fractionation studies show that the β_2 ARs are concentrated in caveolar structures, whereas the β_1 ARs are mainly distributed in noncaveolar membrane in cardiac myocytes (11, 12). Disruption of caveolar structures in cardiac myocytes selectively enhances and prolongs the increase in myocyte contraction rate mediated by β_2 AR activation, but has no effect on signaling by the β_1 AR (12). 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 (13–15). These observations suggest that subtype-specific signaling complexes conduct β_1 AR and β_2 AR signaling in cardiac myocytes.

A growing body of evidence from *in vitro* studies supports the notion that G protein-coupled receptors can form complexes with downstream effectors to facilitate signaling. Activated β_2 AR recruits β -arrestin that scaffolds many trafficking molecules such as clathrin, AP-2, and ARF, and signaling molecules including the tyrosine kinase Src and mitogen-activated kinases c-Jun amino-terminal kinase and extracellular signal-regulate kinase 1/2 (16). β_2 ARs can also associate with AKAP79 and Gravin, which are scaffolding proteins that connect β_2 AR to PKA, PKC, PP2A, and L-type Ca^{2+} channels (17–19). Moreover, interactions mediated by carboxyl-terminal PDZ-domain-binding motifs serve as another general mechanism to recruit G protein-coupled receptors into signaling complexes. Studies show that the β_1 AR PDZ motif can interact with postsynaptic scaffolding proteins PSD-95 (synaptic associated protein 90) and membrane-associated guanylate-inverted 2 in HEK293 cells (20, 21). In cardiac myocytes, the interaction between β_1 AR PDZ motif with PSD-95 or related proteins dictates signaling specificity by retaining the receptor at the cell surface and preventing interaction with G_i proteins (22).

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 ezrin-binding protein 50 (EBP50) (23, 24)], which affects Na^+/H^+ exchange in HEK293 cells (23). The β_2 AR carboxyl terminus also binds to *N*-ethylmaleimide-sensitive factor (NSF) (25), which does not contain a PDZ domain. This interaction is critical for receptor recycling to the plasma membrane after agonist-induced internalization. However, the functional role of an interaction between the β_2 AR carboxyl terminus and its associated protein(s) in cardiac

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AR, adrenergic receptor; NHERF, sodium-hydrogen exchanger regulatory factor; EBP50, ezrin-binding protein 50; PTX, pertussis toxin; AKAP, A-kinase associate protein; KO, knockout; NSF, *N*-ethylmaleimide-sensitive factor.

*To whom correspondence should be addressed. E-mail: kobilka@stanford.edu.

© 2003 by The National Academy of Sciences of the USA

myocytes has not been reported. To examine the role of the β_2 AR carboxyl terminus on receptor trafficking and signaling in neonatal cardiac myocytes, we generated recombinant adenovirus expressing a mutant β_2 AR in which the three carboxyl-terminal amino acids are mutated to alanine (β_2 AR-AAA). This mutation is predicted to disrupt interactions with NSF- and PDZ-domain-containing proteins. We compared the functional properties of wild-type β_2 AR and β_2 AR-AAA in neonatal myocytes from β_1/β_2 AR knockout (KO) mice. Our results demonstrate that the carboxyl terminus of the β_2 AR is essential for physiologic signaling and trafficking in cardiac myocytes.

Materials and Methods

Culture and Adenovirus Infection of Neonatal Mouse Ventricular Myocytes. Spontaneously beating neonatal cardiac myocytes were prepared from hearts of 1-day-old mouse pups (from wild-type, β_1 AR-KO, β_2 AR-KO, and β_1/β_2 AR-KO mice) as described (4). The myocyte-enriched cells remaining in suspension after preplating 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 as described (22).

Recombinant adenovirus encoding amino-terminal Flag-tagged mouse β_2 AR-AAA (the β_2 AR carboxyl terminus DSPL was mutated into DAAA) was generated with the pAdEasy system (Q.biogen, Carlsbad, CA); adenoviruses encoding HA- β_1 AR-PDZmut and Flag- β_2 AR were described (12, 22). Neonatal myocytes were infected with viruses at a multiplicity of infection of 100 after being cultured for 24 h as described (12).

Measurement of Myocyte Contraction Rate and cAMP Accumulation. Measurement of spontaneous contraction rate was carried out as described (4). In the time-course experiments, statistical significance between groups was analyzed with two-way analysis of variance with PRISM software (GraphPad, San Diego). Myocyte cAMP accumulation was determined by using a RIA as described (12).

Tat peptide, Tat- β_2 -DSPL consisting of Tat linked to GRQG-FSSDSPL of β_2 AR, Tat- β_2 -DAAA consisting of Tat linked to GRQGFSSDAAA, Tat- β_2 -DSAL consisting of Tat linked to GRQGFSSDSAL, and Tat- β_2 -ASPL consisting of Tat linked to GRQGFSSASPL through a cysteine bridge were synthesized in the Stanford Core facility. Neonatal myocytes were preincubated at 37°C with 1 μ M peptide (Tat, Tat- β_2 -DSPL, Tat- β_2 -DAAA, Tat- β_2 -DSAL, or Tat- β_2 -ASPL) for 25 min, or filipin (2 μ g/ml; Sigma) for 30 min before isoproterenol (10 μ M; Sigma) exposure. In some assays, PTX (0.75 μ g/ml; Sigma) or PKI (20 μ M; Calbiochem) was used as described (12) before isoproterenol stimulation.

Immunofluorescence Microscopy and ELISA. Myocytes cultured on coverslips were infected with the Flag- β_2 AR, Flag- β_2 AR-AAA, or HA- β_1 AR-PDZmut adenoviruses as described above. Myocytes were pulsed with 10 μ M isoproterenol for 10 min. The cells were either fixed with 1 \times PBS containing 5% paraformaldehyde or washed three times before being chased for 15, 30, or 60 min with medium containing 1 μ M alprenolol. The fixed cells were permeabilized with 1 \times PBS containing 1% Nonidet P-40, 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, Berkeley, CA). The primary antibodies were detected with FITC-conjugated goat anti-mouse IgG_{2b} (1:200) and Texas red-conjugated goat anti-mouse IgG₁ (1:400; Fisher). The images were acquired with a Zeiss Axioplan 2 microscope. Quantitative analysis of myocyte cell-surface receptors was carried out with ELISA as described (22).

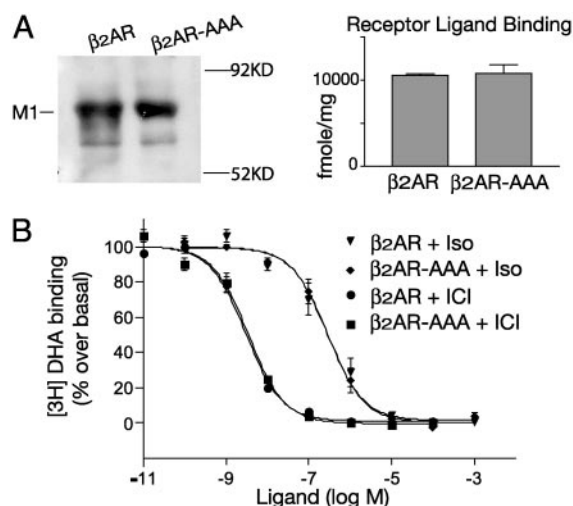


Fig. 1. Functional expression of Flag- β_2 AR or Flag- β_2 AR-AAA in β_1/β_2 AR-KO myocytes. (A) The expression of Flag- β_2 AR and Flag- β_2 AR-AAA protein in the β_1/β_2 AR-KO myocytes was examined by Western blot analysis (Left) and saturation binding (Right) to determine B_{max} . (B) The expression of Flag- β_2 AR and Flag- β_2 AR-AAA was characterized by competition binding assays with β_2 AR agonist isoproterenol (IC_{50} = 284 and 294 nM, respectively) and the selective antagonist ICI118551 (IC_{50} = 3 and 3.5 nM, respectively). The data represent the mean \pm SE of five experiments (triplicates) from different myocyte preparations. [³H]DHA, [³H]dihydroalprenolol; Iso, isoproterenol; ICI, ICI118551.

Ligand Binding and Western Blot Analysis. Membrane proteins were prepared from adenovirus-infected β_1/β_2 AR-KO myocytes as described (22). Saturation binding was carried out with 20 nM nonselective β AR antagonist [³H]dihydroalprenolol (NEN). Alprenolol (1 μ M) was used to define nonspecific binding. In competition binding experiments, assay tubes contained 5 μ g of membrane protein, 2 nM [³H]dihydroalprenolol, and different concentrations of the β_2 AR antagonist ICI118551 or the β AR agonist isoproterenol. Membrane proteins were subjected to Western blot analysis with anti-Flag M1 antibody as described (22).

Results

Mutation of the Carboxyl-Terminal Three Amino Acids of the β_2 AR Inhibits Receptor Coupling to G_i . To study the role of the β_2 AR PDZ motif in receptor function in neonatal cardiac myocytes, we generated a recombinant adenovirus expressing a mutant β_2 AR that lacks a functional PDZ motif (Flag- β_2 AR-AAA). This mutation would also be predicted to disrupt interactions between the β_2 AR and NSF (25). Flag- β_2 AR and Flag- β_2 AR-AAA were expressed in β_1/β_2 AR-KO neonatal myocytes by recombinant adenovirus infection at a multiplicity of infection of 100. Both receptors could be efficiently expressed in neonatal myocytes, and the levels of the functional receptor expression were similar (Fig. 1A). Moreover, β_2 AR-AAA and the wild-type β_2 AR displayed similar binding affinities to the agonist isoproterenol and to the β_2 AR-selective antagonist ICI118551 (Fig. 1B).

Fig. 2 shows the effect of isoproterenol on the contraction rate of β_1 AR-KO myocytes or β_1/β_2 AR-KO myocytes expressing either Flag- β_2 AR or Flag- β_2 AR-AAA. The activated Flag- β_2 ARs induce a contraction rate response in the β_1/β_2 AR-KO myocytes similar to that induced by the activated endogenous β_2 AR in the β_1 AR-KO myocytes. The response displayed an initial increase followed by a decrease to below the basal level (Fig. 2A) (4). In comparison with the wild-type β_2 AR, the activated β_2 AR-AAA induced a much more robust contraction-rate increase, and the contraction rate did not

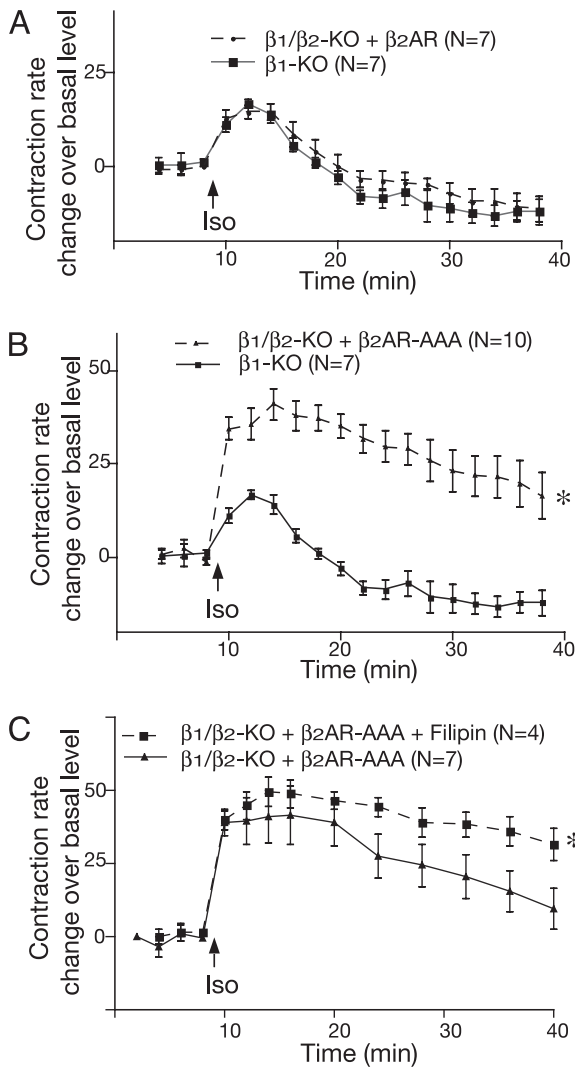


Fig. 2. The β_2 AR PDZ mutation alters receptor regulation of contraction rate in cardiac myocytes. (A) The effect of 10 μ M isoproterenol on the contraction rate of the β_1/β_2 AR-KO myocytes expressing Flag- β_2 AR or of the β_1 -AR-KO myocytes with endogenous β_2 AR. (B) The effect of 10 μ M isoproterenol on the contraction rate of β_1/β_2 AR-KO myocytes expressing Flag- β_2 AR-AAA or of the β_1 -AR-KO myocytes with endogenous β_2 AR. (C) Disruption of caveolae with filipin enhances the increase in contraction rate after β_2 AR-AAA stimulation. The data represent the mean \pm SE of *N* experiments from at least three different myocyte preparations. *, *P* < 0.05; time-course curves were found to be significantly different by two-way analysis of variance.

drop below the basal level (Fig. 2B). We have previously shown that β_2 ARs are enriched in the caveolar structures on myocyte plasma membrane. Filipin, a reagent that disrupts caveolar structure by binding cholesterol, selectively enhances and prolongs the contraction-rate increase mediated by β_2 ARs, but not by β_1 ARs (4). Disruption of caveolar structures with filipin also enhanced the contraction rate increase induced by the activated β_2 AR-AAA (Fig. 2C). We also examined cAMP accumulation in the cultures of β_1/β_2 AR-KO myocytes expressing either the Flag- β_2 AR or the Flag- β_2 AR-AAA. In contrast to the different contraction-rate responses, the cAMP accumulations by the activated receptors were not significantly different in the myocytes expressing either β_2 ARs (basal, 10.6 ± 2.9 pmol per dish; isoproterenol, 35.6 ± 5.5 pmol per dish) or β_2 AR-AAA (basal, 10 ± 3.8 pmol per dish; isoproterenol, 37.7 ± 5.8 pmol per dish).

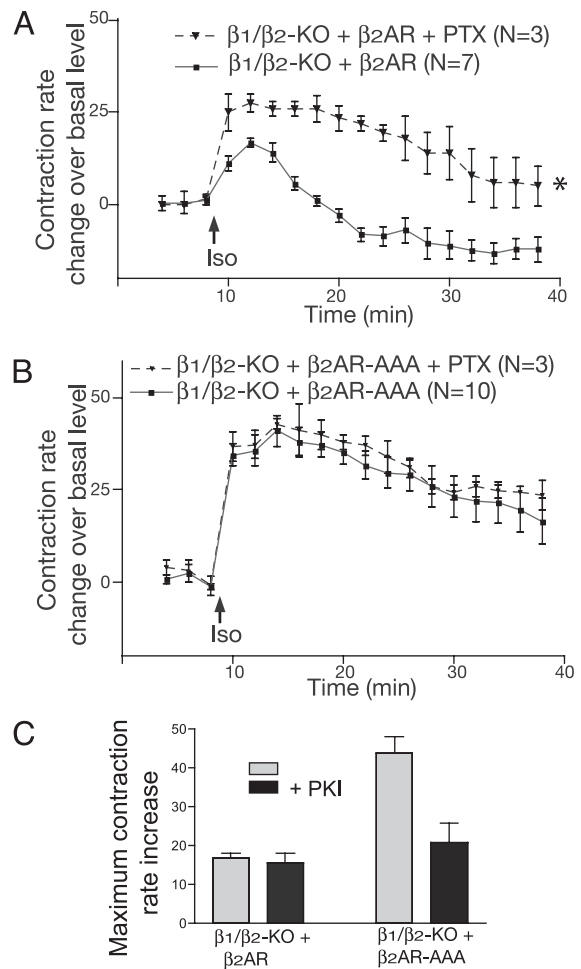


Fig. 3. Disruption of the β_2 AR PDZ motif inhibits receptor coupling to G_i . PTX (0.75 μ g/ml) treatment selectively affected the contraction rate of the β_1/β_2 AR-KO myocytes expressing Flag- β_2 AR (A) but not Flag- β_2 AR-AAA (B). (C) PKI partially inhibits the myocyte contraction-rate increase mediated by Flag- β_2 AR-AAA, but not by Flag- β_2 AR. The data represent the mean \pm SE of four experiments from at least three different myocyte preparations. *, *P* < 0.05; time-course curves were found to be significantly different by two-way analysis of variance.

The biphasic contraction-rate response mediated by the activated β_2 AR is due to sequential coupling of the receptor to G_s and G_i (4). PTX, a G_i protein inhibitor, efficiently prevents the secondary decrease in contraction rate induced by activation of the β_2 AR (Fig. 3A). The larger, monophasic contraction-rate response to stimulation of the β_2 AR-AAA suggests a limited role of G_i signaling. Furthermore, pretreatment of myocytes with PTX had no significant effect on the contraction rate induced by the β_2 AR-AAA (Fig. 3B). Thus, the β_2 AR-AAA appears to couple only to G_s in β_1/β_2 AR-KO neonatal myocytes. We also examined the role of PKA in the contraction-rate response induced by the wild-type β_2 AR and the β_2 AR-AAA. We have previously shown that PKI, a selective PKA inhibitor, has no significant effect on the β_2 AR-mediated contraction-rate increase at concentrations that markedly inhibit β_1 AR signaling (4). Thus, the β_2 AR normally stimulates contraction rate through a PKA-insensitive mechanism, possibly through activation of the cAMP-gated, nonselective cation channel. PKI partially inhibits the maximum contraction-rate increase after β_2 AR-AAA stimulation (Fig. 3C).

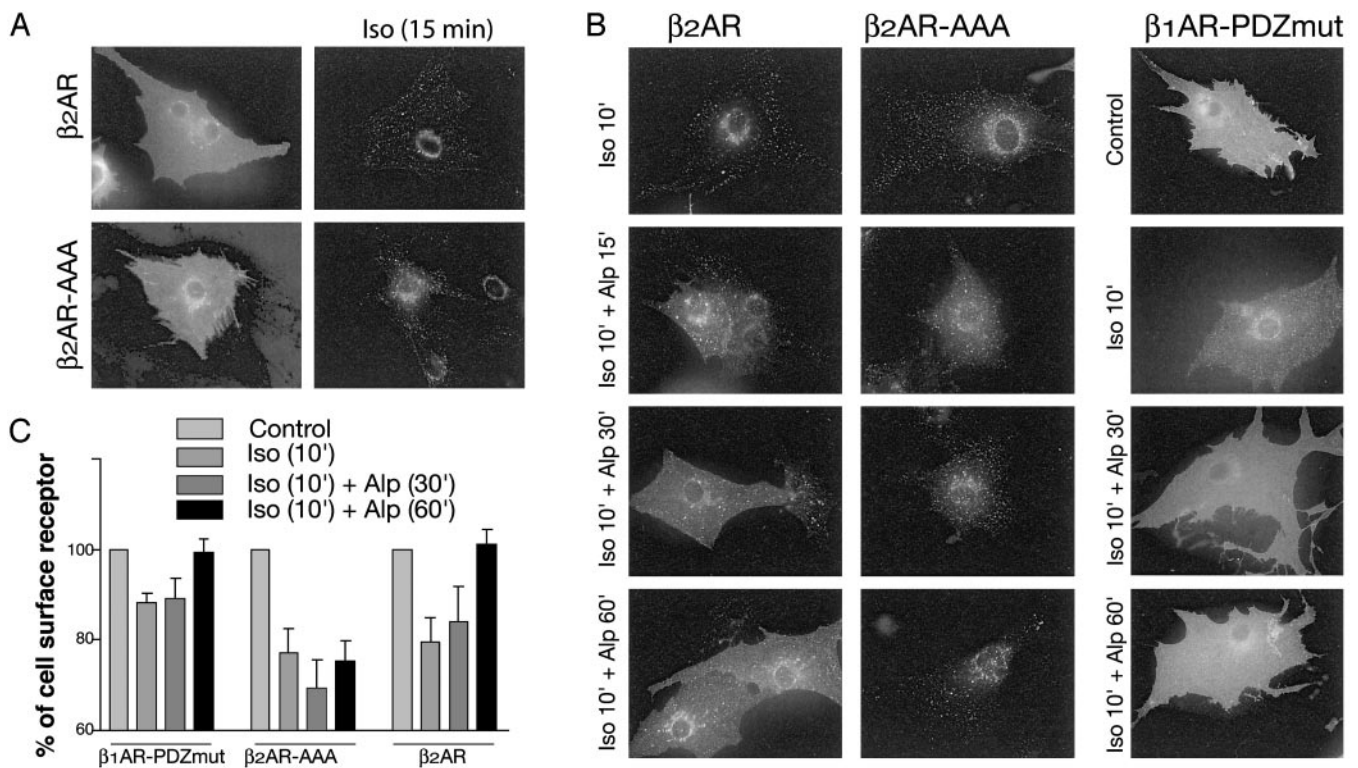


Fig. 4. Agonist-induced internalization of Flag- β_2 AR, Flag- β_2 AR-AAA, and HA- β_1 AR-PDZmut in neonatal cardiac myocytes. (A) Flag- β_2 AR and Flag- β_2 AR-AAA are localized on the cell surface in neonatal myocytes at steady state. Punctate intracellular staining is observed after agonist stimulation of both receptors. (B) Flag- β_2 AR and HA- β_1 AR-PDZmut efficiently recycle back to the myocyte cell surface after removal of isoproterenol, while the Flag- β_2 AR-AAA remains inside the cell. (C) The cell-surface receptor level was measured by ELISAs after agonist-induced internalization and recycling. Cell-surface HA- β_1 AR-PDZmut (11%), Flag- β_2 AR (22%), and Flag- β_2 AR-AAA (23%) decreased significantly after isoproterenol stimulation. Although the surface density of HA- β_1 AR-PDZmut and Flag- β_2 AR was restored by 60 min after removal of isoproterenol, the surface density of Flag- β_2 AR-AAA remained low. Iso, isoproterenol; Alp, alprenolol.

Mutation of the Carboxyl-Terminal Three Amino Acids of the β_2 AR Disrupts Receptor Recycling After Endocytosis in Neonatal Myocytes.

Immunostaining shows that both Flag- β_2 AR and Flag- β_2 AR-AAA are localized predominantly on the cell surface of neonatal myocytes at steady state (Fig. 4A). On isoproterenol stimulation, both receptors undergo significant internalization, as indicated by punctate intracellular staining (Fig. 4A). Quantitative ELISAs confirm that isoproterenol stimulation causes an equivalent (≈ 21 – 24%) decrease in cell-surface receptor density in the myocytes expressing either the Flag- β_2 AR or the Flag- β_2 AR-AAA (Fig. 4C). However, whereas the wild-type β_2 AR efficiently recycles back to the cell surface, the mutant Flag- β_2 AR-AAA fails to do so (Fig. 4B and C). These results are consistent with previously published studies (24) and suggest that the β_2 AR PDZ motif is required for the receptor recycling to the cell surface and for the receptor coupling to G_i in the myocytes. In contrast, mutation of the PDZ-binding motif in the β_1 AR has very different consequences. Disruption of the PDZ motif in the HA- β_1 AR (HA- β_1 AR-PDZmut) dramatically enhances agonist-induced internalization and promotes coupling to G_i in the neonatal myocytes. In contrast to the β_2 AR-AAA, the β_1 AR-PDZmut efficiently recycles back to the cell surface after isoproterenol-induced internalization in myocytes (Fig. 4B and C).

A Membrane-Permeable Peptide Containing the β_2 AR Carboxyl Terminus Alters β_2 AR Signaling in Cardiac Myocytes. The experiments using adenovirus to express β_2 AR-AAA in β_1/β_2 AR-KO myocytes provide strong evidence for a functionally important interaction between the β_2 AR and a PDZ-domain-containing protein, such as EBP50/NHERF, or another protein that binds

to the β_2 AR carboxyl terminus, such as NSF. To confirm these observations with endogenously expressed β_2 AR, we used a membrane-permeable peptide representing the wild-type β_2 AR carboxyl terminus (Tat- β_2 -DSPL) to interfere with the interaction between the endogenous β_2 ARs and their binding partners in β_1 AR-KO myocytes. In comparison with the control cells, the Tat- β_2 -DSPL-treated myocytes showed a greater maximum contraction-rate increase, and the contraction rate did not decrease below the basal level (Fig. 5A). In contrast, the control peptide, Tat-Flag- β_2 -DAAA, had no significant effect on the contraction rate (Fig. 5B). Previous studies have shown that disruption of the carboxyl-terminal PDZ motif of the human β_2 AR could alter binding to NHERF/EBP50, a PDZ-domain-containing protein, and NSF, a protein that lacks a PDZ domain (25). Interactions between these proteins could be distinguished by more selective mutations of the carboxyl terminus (25). We therefore examined the effect of two additional Tat-peptides, Tat- β_2 -ASPL and Tat- β_2 -DSAL. Based on the D410A mutation in the human β_2 AR (25), Tat- β_2 -ASPL would not be expected to bind NHERF/EBP50. We found that, like Tat- β_2 -DAAA, Tat- β_2 -ASPL had no effect on myocyte contraction rates (Fig. 5C). Based on the L412A mutation in the human β_2 AR (25), Tat- β_2 -DSAL would be expected to bind NHERF/EBP50, but not NSF. The carboxyl terminus of the mouse β_2 AR (-DSPL) differs from the human (-DSLL), and it is not known whether the wild-type mouse β_2 AR binds to NSF. Myocytes treated with Tat- β_2 -DSAL exhibited the same behavior as myocytes treated with Tat- β_2 -DSPL, showing a greater agonist-stimulated contraction rate (Fig. 5D). Thus, our results suggest that peptides that can bind NHERF/EBP50 can selectively modulate β_2 AR-mediated signaling in cardiac myocytes by competing with the

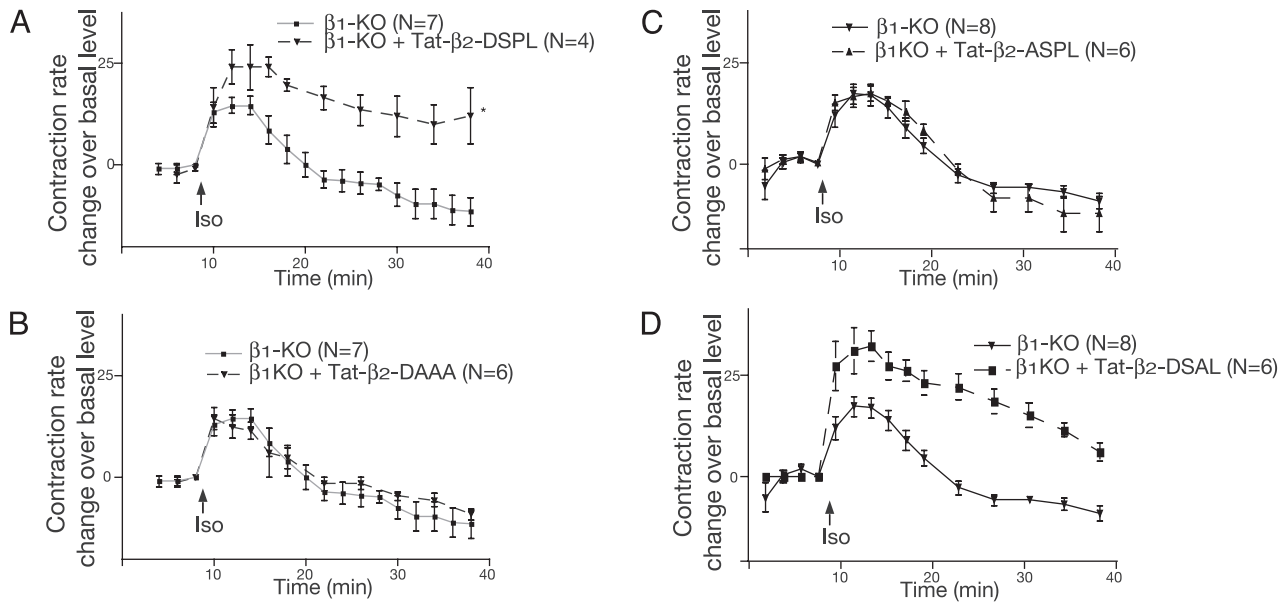


Fig. 5. Cell-permeable β_2 AR carboxyl-terminal peptides affect the contraction rate in β_1 AR-KO myocytes. β_1 AR-KO neonatal myocytes were cultured and treated with peptide (1 μ M) for 25 min before contraction-rate experiments. The basal contraction rate of myocytes was not altered significantly by peptide treatment. Pretreatment with Tat- β_2 -DSPL (A) and Tat- β_2 -DSAL (D), but not Tat- β_2 -DAAA (B) or Tat- β_2 -ASPL (C), significantly changed the isoproterenol (Iso)-stimulated contraction-rate increase in β_1 AR-KO myocytes. *, $P < 0.05$; time-course curves were found to be significantly different by two-way analysis of variance.

endogenous β_2 ARs for binding to one or more 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. Both β_1 AR and β_2 AR are expressed in cardiac myocytes and respond to the same stimuli, but they possess distinct functions *in vivo*. We have previously shown that β_1 AR and β_2 AR display different trafficking and signaling properties in neonatal myocytes. Activated β_1 ARs remain on the cell surface and couple to G_s (22). In contrast, activated β_2 ARs undergo robust endocytosis, and the receptors couple sequentially to G_s and G_i in cardiac myocytes (4). A mutation disrupting the interaction between the β_1 AR PDZ motif and its binding partners enables agonist-induced receptor internalization (22). The same mutation also enables the receptor to couple sequentially to G_s and G_i after activation (22).

In this study, we examined the effect of mutating the carboxyl-terminal amino acids of the mouse β_2 AR. Based on previous studies (25), β_2 AR-AAA should not bind to NSF or PDZ domain proteins such as NHERF/EBP50. Stimulation of wild-type β_2 AR induces a biphasic contraction-rate response in neonatal myocytes, with an initial G_s -dependent increase in contraction rate and a secondary G_i -dependent decrease in contraction rate below baseline. In contrast, stimulation of β_2 AR-AAA leads to a larger, more sustained increase in contraction rate that does not decrease below baseline and is not altered by pretreatment of cells with PTX. These results are most consistent with the failure of β_2 AR-AAA to couple to G_i . One must also consider the possibility that G_i coupling is masked by much more efficient coupling of β_2 AR-AAA to G_s , thereby exceeding the cAMP levels needed for maximum contraction-rate response. However, we found that it is possible to further stimulate contraction rate with forskolin after isoproterenol stimulation of β_2 AR-AAA (data not shown). Thus, we feel our results are most consistent with a failure of β_2 AR-AAA to

couple to G_i . Moreover, the results of the Tat-peptide studies (Fig. 5) suggest that the failure of β_2 AR-AAA to couple to G_i is caused by disruption of an interaction with a PDZ-domain-containing protein, such as NHERF/EBP50, and not by disruption of an interaction with NSF.

It has recently been reported that activation of G_s by β ARs is involved in inducing myocyte apoptosis, whereas activation of G_i confers a protective effect against myocyte apoptosis (26, 27). The dual signaling mediated by β_2 ARs in cardiac myocytes may represent a sophisticated mechanism enabling enhanced cardiac function in response to acute increases in sympathetic tone, but protecting myocytes from the detrimental effects of chronic catecholamine stimulation. Indeed, moderate overexpression of β_2 AR in the heart of transgenic mice leads to an enhanced cardiac contractility without developing cardiomyopathy (28). In contrast, overexpression of the β_1 AR leads to heart failure (29). Because the coupling of β_2 ARs to G_i is inhibited by mutation of the PDZ binding motif, we might expect that a similar mutant expressed *in vivo* would have a significant and possibly detrimental effect in animal hearts under chronic stress.

Filipin is a reagent that disrupts caveolar structures in cardiac myocytes. Filipin does not affect the basal contraction rate and has no effect on β_1 AR stimulation of contraction rate; however, it selectively enhances the contraction-rate increase after stimulation of β_2 AR (12). In this study, the β_2 AR-AAA-induced contraction-rate increase under isoproterenol stimulation is further enhanced by filipin. Thus, the PDZ motif may not be required for the targeting of the β_2 AR to caveolae in cardiac myocytes. In contrast to the wild-type β_2 AR, the contraction-rate increase induced by β_2 AR-AAA is partially inhibited by the PKA inhibitor PKI (Fig. 3C). This inhibition suggests that the enhanced coupling of the β_2 AR-AAA to an increase in contraction rate is due in part to activation of a PKA-dependent pathway that is not activated by the wild-type β_2 AR. This altered signaling may be explained by the inability of the β_2 AR-AAA to associate with regulatory components of a signaling complex. β_2 ARs associate with different scaffold proteins, including arrestin, A-kinase associate protein (AKAP), NSF, and the PDZ-

domain-containing protein NHERF/EBP50 (16). EBP50 associates with an A-kinase-anchoring protein (AKAP) ezrin (23). A muscle-specific mA-KAP can scaffold both PKA and a phosphodiesterase PDE4D in cardiac myocytes (30). Therefore, the signaling complexes containing the β_2 ARs can orchestrate tight regulation of the second messenger cAMP within a plasma membrane microdomain (31). For example, disruption of the PDZ-mediated interaction between β_2 ARs and PDE4 may allow cAMP to diffuse over a larger subcellular volume, thereby activating signaling pathways not normally activated by the wild-type β_2 AR. We did not observe detectable differences in the whole-cell cAMP accumulation stimulated by the wild-type β_2 AR and β_2 AR-AAA. However, the cAMP studies were performed in myocytes that are preincubated with a phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine).

In contrast to the inhibitory effect of mutating the β_2 AR PDZ-binding motif on receptor coupling to G_i , mutation of the PDZ-binding motif in the β_1 AR promotes coupling to G_i in the neonatal myocytes (12). The ability of these receptors to couple to G_i correlates with their propensity to undergo agonist-induced internalization and recycle back to the plasma membrane. The wild-type β_1 AR does not undergo agonist-induced internalization, and the β_2 AR-AAA cannot recycle back to the cell surface after internalization. In contrast, both the wild-type β_2 AR and β_1 AR-PDZmut undergo agonist-induced internalization and efficient recycling to the plasma membrane (Fig. 4C). The role of the PDZ-binding motif in subtype-specific trafficking has previously been demonstrated in HEK293 cells. The β_1 AR PDZ motif has been shown to interact with two postsynaptic scaffold proteins, PSD-95 (synaptic associated protein 90) (20) and

membrane-associated guanylate-inverted 2 (21), in HEK293 cells. In contrast, the β_2 AR PDZ motif selectively associates with EBP50/NHERF (23, 24) and NSF (25). Although overexpression of PSD-95 in HEK293 cells inhibits agonist-induced internalization of the β_1 AR (20), mutations disrupting of the interaction between the human β_2 AR and NSF inhibit receptor recycling (25). However, it is noteworthy that a key residue (leucine 410) in human β_2 AR necessary for NSF binding is not conserved in the mouse β_2 AR used in this study. Therefore, we cannot be certain that disruption of interactions between NSF and the mouse β_2 AR account for the failure of β_2 AR-AAA to efficiently recycle after agonist-induced internalization (Fig. 4).

In conclusion, we present evidence that the β_2 AR PDZ motif is essential for the physiologic signaling of this receptor subtype in neonatal mouse cardiac myocytes. Disruption of the β_2 AR PDZ motif inhibited receptor recycling after isoproterenol-induced internalization and inhibited the receptor coupling to G_i . These results, together with our previous studies on the β_1 AR, demonstrate that interactions between β -adrenergic receptors and specific PDZ-domain-containing proteins play an essential role in subtype-specific signaling of β -adrenergic receptors in cardiac myocytes. These interactions may serve to organize receptors, G proteins, effectors, and regulatory proteins into discrete signaling complexes in the plasma membrane (13). The formation of signaling complexes facilitates local, rapid, and highly specific cellular responses *in vivo*.

Y.X. is the recipient of a postdoctoral fellowship from the American Heart Association. This study was supported by National Institutes of Health Grant 1R01 HL71078-01 (to B.K.).

- Koch, W. J., Milano, C. A. & Lefkowitz, R. J. (1996) *Circ. Res.* **78**, 511–516.
- Lefkowitz, R. J., Rockman, H. A. & Koch, W. J. (2000) *Circulation* **101**, 1634–1637.
- Gauthier, C., Langin, D. & Balligand, J. L. (2000) *Trends Pharmacol. Sci.* **21**, 426–431.
- Devic, E., Xiang, Y., Gould, D. & Kobilka, B. (2001) *Mol. Pharmacol.* **60**, 577–583.
- Chruscinski, A. J., Rohrer, D. K., Schauble, E., Desai, K. H., Bernstein, D. & Kobilka, B. K. (1999) *J. Biol. Chem.* **274**, 16694–16700.
- Rohrer, D. K., Desai, K. H., Jasper, J. R., Stevens, M. E., Regula, D. P., Jr., Barsh, G. S., Bernstein, D. & Kobilka, B. K. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 7375–7380.
- Green, S. A., Holt, B. D. & Liggett, S. B. (1992) *Mol. Pharmacol.* **41**, 889–893.
- Aprigliano, O., Rybin, V. O., Pak, E., Robinson, R. B. & Steinberg, S. F. (1997) *Am. J. Physiol.* **272**, H2726–H2735.
- Zhou, Y. Y., Cheng, H., Bogdanov, K. Y., Hohl, C., Altschuld, R., Lakatta, E. G. & Xiao, R. P. (1997) *Am. J. Physiol.* **273**, H1611–H1618.
- Ostrom, R. S., Gregorian, C., Drenan, R. M., Xiang, Y., Regan, J. W. & Insel, P. A. (2001) *J. Biol. Chem.* **276**, 42063–42069.
- Rybin, V. O., Xu, X., Lisanti, M. P. & Steinberg, S. F. (2000) *J. Biol. Chem.* **275**, 41447–41457.
- Xiang, Y., Rybin, V. O., Steinberg, S. F. & Kobilka, B. (2002) *J. Biol. Chem.* **277**, 34280–34286.
- Davare, M. A., Avdonin, V., Hall, D. D., Peden, E. M., Burette, A., Weinberg, R. J., Horne, M. C., Hoshi, T. & Hell, J. W. (2001) *Science* **293**, 98–101.
- Chen-Izu, Y., Xiao, R. P., Izu, L. T., Cheng, H., Kuschel, M., Spurgeon, H. & Lakatta, E. G. (2000) *Biophys. J.* **79**, 2547–2556.
- Kuschel, M., Zhou, Y. Y., Cheng, H., Zhang, S. J., Chen, Y., Lakatta, E. G. & Xiao, R. P. (1999) *J. Biol. Chem.* **274**, 22048–22052.
- Hall, R. A. & Lefkowitz, R. J. (2002) *Circ. Res.* **91**, 672–680.
- Shih, M., Lin, F., Scott, J. D., Wang, H. Y. & Malbon, C. C. (1999) *J. Biol. Chem.* **274**, 1588–1595.
- Cong, M., Perry, S. J., Lin, F. T., Fraser, I. D., Hu, L. A., Chen, W., Pitcher, J. A., Scott, J. D. & Lefkowitz, R. J. (2001) *J. Biol. Chem.* **276**, 15192–15199.
- Fraser, I. D., Cong, M., Kim, J., Rollins, E. N., Daaka, Y., Lefkowitz, R. J. & Scott, J. D. (2000) *Curr. Biol.* **10**, 409–412.
- Hu, L. A., Tang, Y., Miller, W. E., Cong, M., Lau, A. G., Lefkowitz, R. J. & Hall, R. A. (2000) *J. Biol. Chem.* **275**, 38659–38666.
- Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A. & Hall, R. A. (2001) *J. Biol. Chem.* **276**, 41310–41317.
- Xiang, Y., Devic, E. & Kobilka, B. (2002) *J. Biol. Chem.* **277**, 33783–33790.
- Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J., *et al.* (1998) *Nature* **392**, 626–630.
- Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A. & von Zastrow, M. (1999) *Nature* **401**, 286–290.
- Cong, M., Perry, S. J., Hu, L. A., Hanson, P. I., Claing, A. & Lefkowitz, R. J. (2001) *J. Biol. Chem.* **276**, 45145–45152.
- Zhu, W. Z., Zheng, M., Koch, W. J., Lefkowitz, R. J., Kobilka, B. K. & Xiao, R. P. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 1607–1612.
- Xiao, R. P. (2000) *Circ. Res.* **87**, 635–637.
- Dorn, G. W., II, Tepe, N. M., Lorenz, J. N., Koch, W. J. & Liggett, S. B. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 6400–6405.
- Engelhardt, S., Hein, L., Wiesmann, F. & Lohse, M. J. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 7059–7064.
- Dodge, K. L., Khouangsathiene, S., Kapiloff, M. S., Mouton, R., Hill, E. V., Houslay, M. D., Langeberg, L. K. & Scott, J. D. (2001) *EMBO J.* **20**, 1921–1930.
- Zaccolo, M. & Pozzan, T. (2002) *Science* **295**, 1711–1715.