0026-895X/07/7202-429–439$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 72:429–439, 2007

Vol. 72, No. 2
37747/3234545
Printed in U.S.A.

N-Ethylmaleimide-Sensitive Factor Regulates β_2 Adrenoceptor Trafficking and Signaling in Cardiomyocytes

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Received May 3, 2007; accepted May 16, 2007

ABSTRACT

Recycling of G protein-coupled receptors determines the functional resensitization of receptors and is implicated in switching β_2 adrenoceptor (β_2AR) G protein specificity in cardiomyocytes. The human β_2AR carboxyl end binds to the *N*-ethylmaleimide-sensitive factor (NSF), an ATPase integral to membrane trafficking machinery. It is interesting that the human β_2AR (hβ_2AR) carboxyl end pulled down NSF from mouse heart lysates, whereas the murine one did not. Despite this difference, both β_2ARs exhibited substantial agonist-induced internalization, recycling, and G_i coupling in cardiomyocytes. The hβ_2AR, however, displayed faster rates of agonist-induced internalization and recycling compared with the murine β_2AR (mβ_2AR) and a more profound G_i component in its contraction response. Replacing the mβ_2AR proline (−1) with a leucine generated a gain-of-function mutation, mβ_2AR-P417L, with a rescued ability to bind NSF, faster internalization and recycling than the mβ_2AR, and a significant enhancement in G_i signaling, which mimics the hβ_2AR. Selective disruption of the mβ_2AR-P417L binding to NSF inhibited the receptor coupling to G_i. Meanwhile, inhibiting NSF with *N*-ethylmaleimide blocked the mβ_2AR recycling after agonist-induced endocytosis. Expressing the NSF-E329Q mutant lacking ATPase activity inhibited the mβ_2AR coupling to G_i in cardiomyocytes. Our results revealed a dual regulation on hβ_2AR trafficking and signaling by NSF through direct binding to cargo receptor and its ATPase activity and uncovered an unprecedented role for the receptor binding to NSF in regulating G protein specificity that has diverged between mouse and human β_2ARs.

β-Adrenoceptors play a pivotal role in regulating cardiomyocyte contraction through distinct signaling pathways. The β_1AR couples to G_s protein(s), which increases cAMP/protein kinase A activity and the contraction rate, whereas the activated β_2AR sequentially couples to both G_s and G_i in neonatal cardiomyocytes, creating a biphasic change in contraction. β_2AR G_i coupling seems to be dependent on receptor trafficking, which includes both endocytosis and recycling. Inhibiting either process blocks receptor coupling to G_i in cardiomyocytes (Xiang et al., 2002; Xiang and Kobilka, 2003).

Many G protein-coupled receptors (GPCRs) undergo endocytosis in response to activation, yet their subsequent sorting in endosomes is variable, creating variable regulation of their activity during prolonged or repeated stimulation. Some receptors are targeted to lysosomes to down-regulate cellular responses mediated by the receptor, whereas many GPCRs possess the ability to efficiently return to the cell surface. This recycling of receptors underlies the resensitization of corresponding cellular responses (von Zastrow, 2003). Many GPCRs depend on sequences residing in their intracellular domains for recycling. A well-defined class of recycling sequences are PSD-95/Discs-large/ZO-1 (PDZ) domain binding motifs (also called PDZ ligands) that are usually located at the carboxyl-terminal end of different GPCR tails (Bockaert et al., 2004; Gage et al., 2005). The β_2AR has a type I PDZ ligand at its carboxyl-terminal end that is necessary for recycling and sufficient to reroute the δ-opioid receptor from a degradative to a recycling pathway (Cao et al., 1999; Gage et

These studies were supported by grants 1R01-HL71078 (to B.K.K.), R01-DA12864 (to M.V.Z.), and 1R01- HL82846 (to Y.X.) from the National Institutes of Health.
Y.W. and B.L. contributed to this study equally.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.037747.

ABBREVIATIONS: β_2AR, β_2-adrenergic receptor; β_1AR, β_1-adrenergic receptor; GPCR, G protein-coupled receptor; KO, knockout; NSF, *N*-ethylmaleimide-sensitive factor; PDZ, PSD-95/Discs-large/ZO-1; NHERF/EBP50, Na^+/H^+ exchanger regulatory factor/ezrin/radixin/moesin-binding phosphoprotein of 50 kDa; GST, glutathione; PTX, pertussis toxin; NEM, *N*-ethylmaleimide; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; ANOVA, analysis of variance; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

al., 2001). In cultured neonatal mouse cardiomyocytes, this sequence is also required for the temporal switch from G_s to G_i-mediated signal transduction observed in the contraction-rate response to the agonist isoproterenol (Xiang and Kobilka, 2003). Several lines of evidence now indicate that membrane trafficking of this receptor dictates not only cellular resensitization but also signal transduction specificity. Despite progress in understanding the β_2AR recycling process, numerous questions concerning the core mechanism and physiological variations remain.

Although the recycling sequence at the β_2AR C terminus has been shown to bind PDZ domains in NHERF family proteins (NHERF-1/EBP50 and NHERF-2/E3KARP) (Hall et al., 1998; Cao et al., 1999), it also binds at least one protein with no identifiable PDZ domain: the *N*-ethylmaleimide sensitive factor (NSF) (Cong et al., 2001). NSF has been identified as an ATPase that binds SNAP receptor (SNARE) complexes in an ATP-dependent fashion to separate them during ATP hydrolysis; this and a wealth of other evidence has demonstrated its general role in vesicle fusion between various membrane compartments (Morgan and Burgoyne, 2004; Whiteheart and Matveeva, 2004). Moreover, NSF has been shown to bind to β-arrestin, an adaptor protein involved in GPCR desensitization and endocytosis upon agonist stimulation. β-Arrestin preferentially interacts with the ATP-bound form of NSF, and this NSF binding facilitates clathrin coat-mediated GPCR internalization (McDonald et al., 1999). In heterologous HEK293 cells, selective ablation of NSF binding to the β_2AR was inferred to inhibit recycling of receptors, whereas imparting NSF binding on the δ-opioid receptor slightly enhanced its ability to recycle (Cong et al., 2001; Gage et al., 2005). Although there is also evidence to show that PDZ interactions promote receptor recycling (Cao et al., 1997) and are functionally important for G_i coupling in cardiomyocytes (Xiang and Kobilka, 2003), it is not clear how NSF may affect β_2AR trafficking and signaling in these cells.

Here, we used neonatal mouse cardiomyocytes as a model system to address these questions. It is interesting that the NSF binding sites on the β_2AR were not conserved among mammalian species, providing a naturally occurring divergence in NSF binding to exploit. The -1 position of the β_2AR carboxyl terminus is proline in mβ_2AR and leucine in hβ_2AR. Because of this single amino acid difference, mβ_2AR binding to NSF was not detectable. Nevertheless, despite the lack of detectable binding of the mβ_2AR carboxyl terminus to NSF in biochemical assays, we found that inhibition of NSF activity with *N*-ethylmaleimide (NEM) inhibited murine β_2AR (mβ_2AR) recycling despite this poor affinity. In addition, both human and murine β_2ARs sufficiently recycled after endocytosis and coupled to G_i pathways in cardiomyocytes. The different affinities for NSF seemed to have a minimum role on receptor trafficking and signaling. In contrast, inactivation of NSF ATPase activity with a point mutation was sufficient to block both human and murine β_2AR recycling and coupling to G_i in cardiomyocytes, indicating that NSF is required for proper trafficking and signaling of β_2ARs in cardiomyocytes independent of a high-affinity interaction with the receptor. This study strengthens the relationship between β_2AR recycling and signaling specificity and demonstrates an unprecedented role for NSF in regulating physiologically relevant signal transduction.

Materials and Methods

cDNA Constructs and Mutagenesis. Constructs containing the cloned human and murine β_2AR in pcDNA3 (Invitrogen, Carlsbad, CA) with a FLAG epitope attached at the N terminus were used for these studies and have been described before (Cao et al., 1999; Swaminath et al., 2004). Constructs encoding for GST-β_2AR and GST-β_2AR-alanine proteins (encompassing amino acids 328 to 413 of the human β_2AR, and the latter with an additional alanine added to the C terminus) have also been reported (Cao et al., 1999). A comparable murine GST-β_2AR construct was created by insertion of a polymerase chain reaction product of the region encoding amino acids 328 to 418 of the FLAG-mβ_2AR construct using primers containing EcoRI and HindIII appendages and performing the appropriate digestion and ligation into pGEX-KG (Pfizer, New York, NY). The human NSF coding sequence was similarly ligated into pEGFP-N1 (Clontech, Mountain View, CA) after SacI digestion of the vector and a polymerase chain reaction product containing a 3′-SacI appendage and including the 5′-SacI restriction site from the source vector, NSF in pBluescriptR (American Type Culture Collection, Manassas, VA). The P417L mutation was introduced into the FLAG-mβ_2AR and GST-mβ_2AR constructs via the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), as was the E329Q NSF mutation into the pEGFP-N1 construct. Plasmid amplification was done in DH5α *Escherichia coli,* and all sequences were verified by dideoxynucleotide sequencing (University of California San Francisco Biomolecular Resource Center, San Francisco, CA).

Cell Culture and Transfection. Spontaneously beating neonatal cardiomyocytes were prepared from hearts of 1-day-old β_1/β_2AR-KO mouse pups as before (Devic et al., 2001). The myocyte-enriched cells remaining in suspension after preplating were plated in 35-mm dishes for contraction-rate studies and in 12-well plates for immunological assays (with coverslips for immunofluorescent microscopy). Recombinant adenovirus encoding FLAG-mβ_2AR has been described previously (Xiang et al., 2002), and the FLAG-mβ_2AR/P417L, FLAG-hβ_2AR, GFP-NSF, and GFP-NSF-E329Q adenoviral vectors were generated with the same pAdEasy system (Qbiogene Inc., Irvine, CA). Neonatal myocytes were infected with viruses at a multiplicity of infection of 100 after being cultured for 24 h. The receptor expression levels were determined by ligand binding assays as described previously (Xiang et al., 2002). They were expressed at equivalent levels in cardiac myocytes (FLAG-mβ_2AR, 147.3 $\pm$ 22 fmol/mg; FLAG-mβ_2AR/P417L, 171.6 $\pm$ 9.1 fmol/mg; and FLAG-hβ_2AR, 160.3 $\pm$ 21.8 fmol/mg membrane).

GST Pulldown Assays. The various GST-β_2AR fusion proteins were produced in BL21 *E. coli* and bound to glutathione-Sepharose agarose beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Beads containing 10 μg of the full-length fusion protein (assessed by densitometry of Coomassie-stained protein resolved by SDS-polyacrylamide gel electrophoresis) were incubated for 4 h at 4°C in 0.5 ml of clarified extracts from frozen mouse hearts with atria removed (Pel-Freez Biologicals, Rogers, AR), prepared to ~10 mg/ml. Beads were washed four times in 1 ml of extract buffer [0.1% (v/v) Triton X-100, 150 mM NaCl, 25 mM KCl, and 10 mM Tris, pH 7.4, complete Roche protease inhibitor cocktail], and protein was eluted in lithium dodecyl sulfate sample buffer (Invitrogen) with dithiothreitol added to 20 mM. Samples were divided in two for SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose, and Western blotting using rabbit anti-EBP50 antibodies (courtesy of Dr. Anthony Bretscher, Cornell University, Ithaca, NY) or the mouse 2E5 anti-NSF antibody (courtesy of Dr. Sidney W. Whiteheart, University of Kentucky, Lexington, KY).

Immunofluorescence Microscopy. Myocyte images were obtained using a similar setup on a Zeiss Axioplan 2 microscope (Carl Zeiss Inc., Thornwood, NY). Fluorescent measurements of the myocyte receptor trafficking were made by a ratiometric normalization of fluorescent intensities measured using Metamorph software (Molecular Devices, Sunnyvale, CA). Epitope-tagged receptors were de-

tected using M1 anti-FLAG antibody (Sigma, St. Louis, MO). Selective detection of surface relative to total pools of receptor and its use to estimate receptor recycling have been described previously (Tanowitz and von Zastrow, 2003). The recycling estimates were conducted without the EDTA strip. The primary antibody used in these experiments was M1 conjugated to Alexa Fluor 488 (Invitrogen) using standard procedures as described previously (Tanowitz and von Zastrow, 2003). Secondary staining was performed using a commercial goat antimouse IgG Alexa Fluor 594 conjugate (Invitrogen). Experiments were performed at least in triplicate, and representative results are shown.

Immunofluorescence Spectroscopy. Surface receptor levels were determined as before (Swaminath et al., 2004) in the indicated cell type expressing the indicated FLAG-β_2AR. Media were refreshed 1 h before 10 μM isoproterenol (Sigma) stimulation for 10 or 30 min. Periods of agonist washout after 30-min isoproterenol stimulations were also performed for an additional 30 or 60 min as indicated.

Myocyte Contraction Rate Assay. Measurement of spontaneous contraction rates from myocytes expressing either the endogenous or the indicated FLAG-β_2AR were carried out with and without the use of PTX as described previously (Devic et al., 2001). In some assays, NEM was applied 30 min before the addition of isoproterenol. Tat peptide, Tat-β_2-DSAL consisting of Tat linked to GRQGFSSDSAL of β_2AR, and Tat-β_2-ASLL consisting of Tat linked to GRQGFSSASLL of β_2AR through a cysteine bridge were synthesized in the Stanford Core facility and EZ-Biolab (Indianapolis, IN). Neonatal myocytes were preincubated at 37°C with 10 μM peptide for 25 min before isoproterenol (10 μM; Sigma) exposure.

Statistical Analysis. Curve-fitting and statistical analyses were performed using Prism (GraphPad Software, Inc., San Diego, CA).

Results

NSF Had Higher Binding Affinity to Human β_2AR than Murine β_2AR. To understand the molecular mechanism of the NSF effect on β_2AR signaling in cardiomyocytes, the interaction between β_2AR and NSF from heart lysate was examined. NSF, a hexameric ATPase involved in membrane fusion, can bind to the carboxyl terminus of the hβ_2AR. The protein-binding region on this receptor involves a four-residue stretch at the distal C terminus of the receptor (Cong et al., 2001). In addition, NHERF-1/EBP50, a cytoskeleton-associated protein, can also bind to the same stretch of residues on the carboxyl terminus of the hβ_2AR. It is interesting that several rodent β_2ARs, including the mβ_2AR, are identical with the hβ_2AR in the carboxyl-binding region except at one residue (leucine -1 of the hβ_2AR, DSLL) that is required for binding to NSF but not to NHERF-1/EBP50 (Cong et al., 2001). Rather, the mβ_2AR has a proline at the -1 position (DSPL). To determine whether both the human and murine β_2ARs have a similar capacity to bind NSF, GST-fusion proteins, including various β_2AR carboxyl-terminal tail sequences, were prepared. Protein binding was evaluated with a pull-down assay using the GST-fusion proteins coupled to glutathione-agarose beads.

GST fusion proteins were incubated with tissue lysate prepared from mouse hearts. NSF only bound to the cytoplasmic tail of the hβ_2AR but not the mβ_2AR (Fig. 1A). In contrast, NHERF-1/EBP50 bound to the cytoplasmic tail of both the hβ_2AR and mβ_2AR under these conditions (Fig. 1B). As a negative control for nonspecific binding, an addition of a single alanine residue to the hβ_2AR carboxyl terminus (GST-hβ_2AR-Ala) was tested as well. As reported previously, this mutant failed to exhibit the PDZ domain-mediated and NSF protein binding (Fig. 1, A and B; Cao et al., 1999). In addition, we attempted to "rescue" an NSF interaction with the mβ_2AR tail by substitution of the mβ_2AR proline 417 with a leucine residue (mβ_2AR-P417L). The mβ_2AR-P417L pulled down similar amounts of NSF from lysates compared with the hβ_2AR, indicating that the mβ_2AR-P417L cytoplasmic tail fully rescued binding to NSF (Fig. 1A). Likewise, this mutant mβ_2AR-P417L pulled down NHERF-1/EBP50 from mouse heart lysates (Fig. 1B).

The Binding of NSF and the NSF ATPase Activity Had Distinct Effects on β_2AR Trafficking in Cardiomyocytes. To examine whether NSF plays any role in β2AR trafficking in cardiomyocytes, we analyzed the localization of flag-tagged β_2ARs in cardiac myocytes. The mβ_2AR, mβ_2AR-P417L, and hβ_2AR were transiently expressed in cardiac myocytes using recombinant adenovirus. Immunofluorescence studies showed that all three receptors had a cell-surface staining during a nonstimulated state (Fig. 2A). Upon isoproterenol stimulation, all three receptors had reduced cell-surface staining together with increased punctate intracellular staining, suggesting a significant internalization of the receptors in cardiac myocytes. These observations were confirmed quantitatively using an ELISA-based method for assaying surface receptor levels (Swaminath et al., 2004) in a large number of cells and a ratiometric method for analysis of fluorescence micrographs (Tanowitz and von Zastrow, 2003) (Fig. 2B; data not shown). It is interesting that when we measured the short-term decrease in cell-surface receptors after agonist stimulation in cardiac myocytes, we found that the mβ_2AR-P417L had a faster rate ($t_{1/2}$ = 2.63 ± 0.05 min) of cell surface-receptor decrease than the mβ_2AR ($t_{1/2}$ = 10.93 ± 0.01 min; Fig. 3). Because the receptor level change in the short time points after

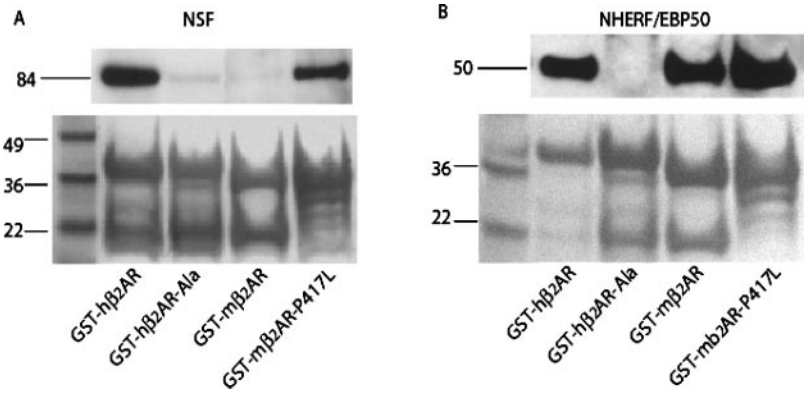

Fig. 1. The binding of mβ_2AR and hβ_2AR to NSF and NHERF-1/EBP50. The binding of mβ_2AR and hβ_2AR to NSF and NHERF-1/EBP50 from mouse heart extracts. GST pull-downs were performed as described under *Materials and Methods*. Western detection of NHERF-1/EBP50 pulled down by the indicated GST-β_2AR fusion protein from mouse heart extract, and the corresponding detection of NSF is shown in A, whereas the detection of NHERF-1/EBP50 from this extract is shown in B. Ponceau-stained GST fusion proteins are shown under each blot. Images are representative of three or more experiments.

agonist stimulation is primarily determined by agonist-induced endocytosis, these data suggested a faster rate of endocytosis for the mβ_2AR-P417L than for the mβ_2AR in cardiac myocytes. The hβ_2AR had a similar rate ($t_{1/2}$ = 3.4 ± 0.03 min) of cell surface-receptor decrease compared with the mβ_2AR-P417L (Fig. 3). However, after 30 min of agonist stimulation, we observed a similar amount of surface receptor decreases with the mβ_2AR (30.54 ± 2.90%), the mβ_2AR-P417L (28.43 ± 1.69%), and the hβ_2AR (24.94 ± 2.74%). The observed decrease of receptor density at 30 min of stimulation should have been a composite of receptor endocytosis and recycling. The equivalent decreases of receptors at cell surface are usually due to much slower recycling process than endocytosis in cells.

When isoproterenol was removed, both the hβ_2AR and mβ_2AR-P417L recovered cell-surface staining almost completely after a 60-min incubation (Fig. 2). In contrast, the mβ_2AR did not show a fully recovered cell-surface staining pattern, and some residual intracellular staining was observed in these cells (Fig. 2), even though the majority of the internalized receptors seemed to return to the surface within 60 min. When we examined the cell surface-receptor density at different time points with the fluorescent ELISA assay, the mβ_2AR exhibited a lower recovery of cell surface-receptors after recycling for 60 min than the mβ_2AR-P417L mutant and the hβ_2AR (Fig. 2B; *, $p < 0.05$). A significant difference in surface recovery was also observed using ratiometric image measurements 60 min after agonist washout (data not shown). These data indicate that the mβ_2AR, although capable of undergoing agonist-induced internalization and recycling in cardiomyocytes, differs in rates of recycling compared with the hβ_2AR and mβ_2AR-P417L.

It has been well-established that NSF ATPase activity plays an important role in membrane cargo trafficking. We then tested whether NSF activity was necessary for the endocytic recycling of the receptor by using NEM to inhibit NSF activity in myocytes. In the presence of NEM, endocytosis of the receptor was preserved; however, a return of the receptor to the cell surface after removal of agonist for 60 min was not (Fig. 4A). This observation was confirmed with measurements of surface receptor levels by a fluorescent ELISA assay. The cell surface receptor levels dropped after agonist addition and only recovered with agonist withdrawal in the absence of NEM (Fig. 4B). These data suggested that NEM treatment can block the receptor from recycling after endocytosis.

Dominant-Negative NSF Lacking ATPase Activity Inhibited Endogenous mβ_2AR Coupling to G_i Pathway in Cardiomyocytes. Our finding that NEM inhibits β_2AR recycling in cardiac myocytes suggested that NSF function is required for this process. To further probe whether NSF enzymatic activity can affect the receptor signaling independent from the direct NSF-receptor interaction, we examined the signaling mediated by the endogenous mβ_2AR when overexpressing an inactivated NSF, the E329Q mutant (Whiteheart et al., 1994). This mutation abolishes ATPase activity and has been shown to block AMPA receptor trafficking (Whiteheart et al., 1994; Whiteheart and Matveeva, 2004).

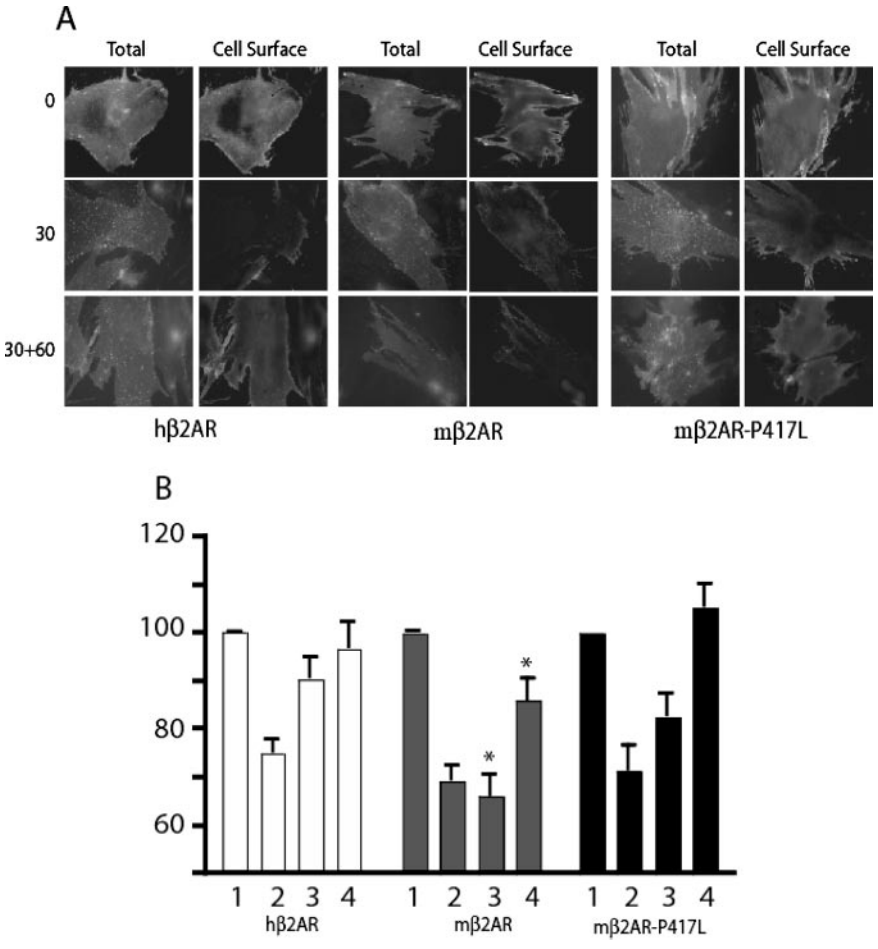

Fig. 2. NSF binding enhances recycling of FLAG-β_2ARs in neonatal cardiac myocytes from β_1/β_2AR-KO mice. A, human and murine β_2ARs internalize and recycle in cardiac myocytes. Cardiac myocytes expressing a FLAG-tagged hβ_2AR, mβ_2AR, or mβ_2AR-P417L were stained with M1 primary antibody conjugated to the Alexa-488 fluorophore to observe a starting "total" receptor population. After no treatment (0), 30-min 10 μM isoproterenol treatment (30), or 30-min isoproterenol treatment followed by a surface antibody strip and 60 min of agonist removal (30 + 60), cells were stained under nonpermeable conditions with a goat anti-mouse-IgG secondary antibody conjugated to the Alexa-594 fluorophore to observe the relative complement of "surface" receptor. Images are representative of three experiments. B, NSF binding β_2ARs recycle faster. Surface levels of the three β_2ARs were quantified by fluorescence spectroscopy measurements of M1-Alexa 488 associated with the cell surface receptors after the indicated periods of drug administration and removal (1, control; 2, 30 min of isoproterenol stimulation; 3, 30 min of isoproterenol followed by 30 min of drug removal; and 4, 30 min of isoproterenol followed by 60 min of drug removal). Surface levels are normalized as a percentage of untreated cell surface fluorescence, and error bars reflect standard deviations over three experiments. *, $p < 0.05$, significantly different between mβ_2AR and hβ_2AR or mβ_2AR-P417L by t test.

When the endogenous mβ_2AR in the β_1AR-KO myocyte was stimulated by isoproterenol, the activated receptor induced a biphasic contraction-rate response with an initial increase mediated by G_s coupling followed by a sustained G_i-dependent decrease to reduce the contraction rate below basal level (Fig. 5A; Xiang et al., 2002). When wild-type NSF was expressed in β_1AR-KO cardiac myocytes, we did not observe any significant change in the endogenous mβ_2AR-mediated contraction-rate response (Fig. 5A). In contrast, when the NSF-E329Q mutant was overexpressed in cardiomyocytes, the contraction rate mediated by the mβ_2AR was significantly higher than the control and did not display a decrease lower than the basal level (Fig. 5B). This response profile was similar to that observed with an inhibition of G_i by PTX (Fig. 5C). Indeed, additional treatment of PTX did not generate any further increases in contraction rates (Fig. 5D). Therefore, the NSF-E329Q behaved as a dominant-negative to block the receptor coupling to G_i in cardiomyocytes. In addition, when myocytes are pretreated with NEM to inhibit the NSF ATPase activity, we also observed effects similar to those by NSF-E329Q mutant on mβ_2AR signaling mediated contraction-rate response (data not shown).

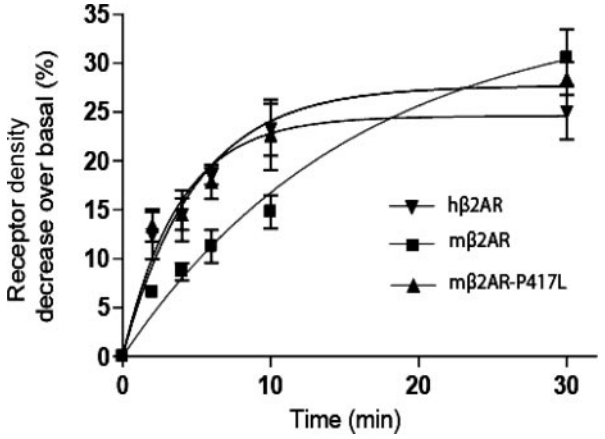

Fig. 3. NSF binding enhances the internalization kinetics of FLAG-β_2ARs expressed in neonatal cardiac myocytes from β_1/β_2AR KO mice. Surface levels of the three β_2ARs were quantified by fluorescent measurement of M1-Alexa 488 associated with the cell surface receptors after the indicated periods of 10 μM isoproterenol administration. Data were normalized as a percentage decrease of untreated cell surface fluorescence, and error bars reflect standard deviations over three experiments. The data represent the mean ± S.E. of experiments from at least three different myocyte preparations.

The Divergent C Termini of the Human and Murine β_2AR Had Different Effects on Contraction Rate Responses in Neonatal Cardiomyocytes. Our previous studies have shown that the localization and trafficking of the mβ_2AR is important for the receptor's G protein signaling specificity and subsequent regulation of the myocyte contraction rate. In the course of this study, we found that the divergent PDZ ligand of the human and murine β_2AR affected the receptor trafficking rates after agonist stimulation

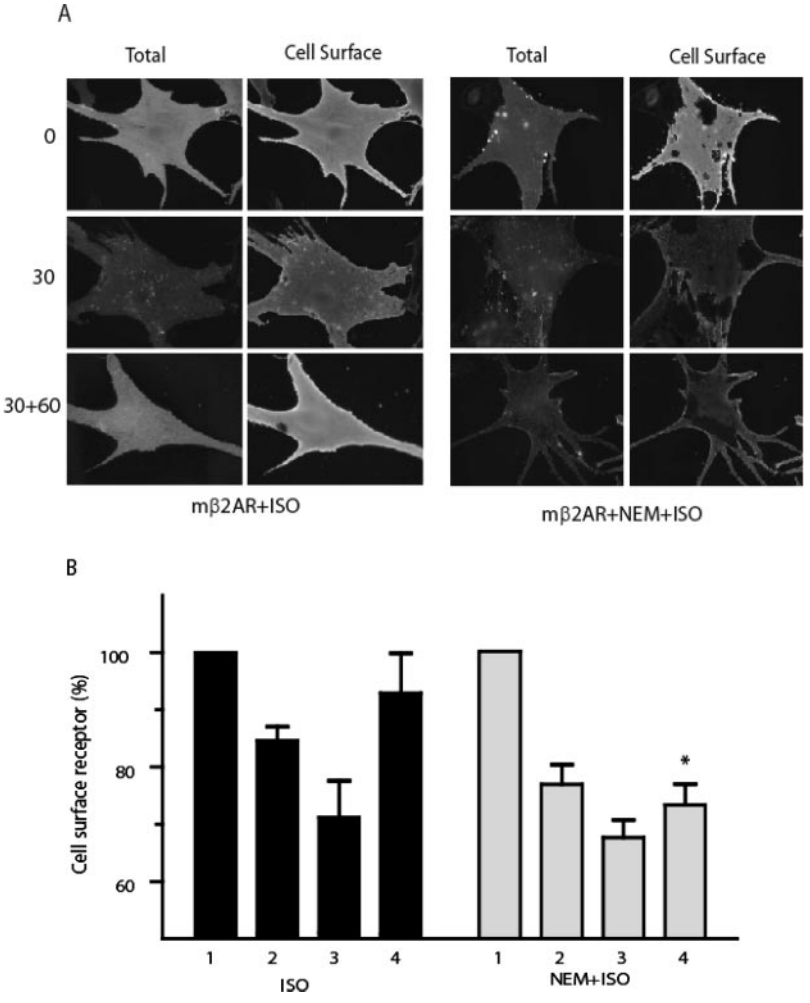

Fig. 4. Inhibiting NSF with NEM blocks the FLAG-β_2AR recycling after agonist-induced endocytosis in cardiomyocytes. A, murine β_2ARs internalize and recycle in cardiac myocytes. Cardiac myocytes expressing a FLAG-tagged mβ_2AR were treated as described under *Materials and Methods* and Fig. 3. Images are representative of three experiments. Inhibiting NSF with NEM blocks the FLAG-β_2AR recycling after agonist-induced endocytosis in cardiomyocytes. B, surface levels of the mβ_2ARs were quantified by fluorescent measurement of M1-Alexa 488 associated with the cell surface receptors after the indicated periods of drug administration and removal (1, control; 2, 30 min of isoproterenol stimulation; 3, 30 min of isoproterenol followed by 30 min of drug removal; and 4, 30 min of isoproterenol followed by 60 min of drug removal). Surface levels were normalized as a percentage of untreated cell surface fluorescence, and error bars reflect standard deviations over three experiments. *, $p < 0.05$, significantly different between cells with and without NEM treatment by t test.

in cardiac myocytes. Thus, we wanted to examine whether differences in NSF binding and/or altered trafficking rates could modulate the receptor signaling in cardiac myocytes. When the mβ_2AR was expressed in β_1/β_2AR-KO myocytes and stimulated by isoproterenol, the activated receptor induced a biphasic contraction-rate response with an initial increase followed by a sustained decrease to reduce the contraction rate lower than basal level (Fig. 6A; Xiang et al., 2002). This contraction-rate change is equivalent to that induced by the endogenous mβ_2AR in β_1AR-KO myocytes (Fig. 6A). The mβ_2AR-P417L induced a similar contraction-rate response profile and initial increase compared with the mβ_2AR in β_1/β_2AR-KO myocytes (Figs. 6C and 7D). However, the contraction rate decreased faster, and the contraction rate was lower than that induced by the mβ_2AR during late stimulation in cardiac myocytes (Figs. 6C and 7E). In addition, when stimulating the hβ_2AR expressed in β_1/β_2AR-KO myocytes with isoproterenol, the activated receptor also induced a biphasic, contraction-rate change with an initial increase followed by a sustained decrease (Fig. 6B). Although it is interesting that the initial contraction-rate increase was smaller than that induced by the activated mβ_2AR and mβ_2AR-P417L (Figs. 6B and 7D), it is more surprising that the late decrease in contraction rate induced by the exogenous hβ_2AR was greater than that induced by the mβ_2AR and mβ_2AR-P417L (Figs. 6B and 7E).

The profound contraction-rate decrease induced by the mβ_2AR-P417L and the hβ_2AR suggests that these receptors may have enhanced coupling to G_i and/or reduced coupling to G_s compared with the mβ_2AR. We therefore examined the G_i signaling induced by the activated receptors in cardiac myocytes. PTX was used to block G_i signaling in cardiac myocytes expressing the different β_2ARs before isoproterenol stimulation. Upon inhibiting G_i with PTX, the isoproterenol-stimulated mβ_2AR induced a slightly greater but not significant contraction-rate increase in myocytes compared with the control and prevented the late G_i-dependent contraction rate decrease (Fig. 7A; Xiang et al., 2002). PTX treatment also inhibited the contraction rate decrease mediated by the mβ_2AR-P417L or the hβ_2AR during the late phase of stimulation (Fig. 7, B and C). These data suggest that compared with the activated mβ_2AR, the mβ_2AR-P417L had an enhanced G_i coupling upon isoproterenol stimulation, and the activated hβ_2AR coupled to G_i more efficiently in neonatal cardiac myocytes (Fig. 7E). This indicates that the divergent receptor C termini can induce different changes in contraction-rate responses that correlate with subtle changes in receptor transportation rates.

The Binding of NSF and PDZ Had Distinct Effects on β_2AR Activation-Induced Contraction Rates in Cardiomyocyte. To further probe the effect of the β_2AR binding to NSF and PDZ on receptor signaling in cardiomyocytes, we took advantage of the different binding affinities between receptor and proteins by using peptides to selectively disrupt the interactions. We expressed either mβ_2AR or mβ_2AR-P417L (the hβ_2AR mimic) in β_1/β_2AR-KO cardiomyocyte for the contraction rate assay. Membrane-permeable peptides containing ASLL sequence and DSAL sequence were used to selectively disrupt NSF and NHERF/EBP50 binding, respectively. When mβ_2AR-expressing myocytes were treated with NSF (ASLL) peptide, the activated receptor induced a slightly bigger but not significant initial increase than the cells without pretreatment (Fig. 8, A and C). The increase was sustained during stimulation and lacked a late decrease mediated by receptor/G_i coupling in control cells (Fig. 8, A and D). When mβ_2AR-expressing myocytes were treated with PDZ (DSAL) peptide, the activated receptor induced a significantly greater initial increase than the control (Fig. 8, B and C), and the increase was sustained and lacked a late G_i-dependent decrease (Fig. 8, B and D).

In contrast, pretreatment with NSF (ASLL) peptide did not affect the activated mβ_2AR-P417L-induced initial increase (Fig. 8, E and G). However, the increase was sustained and did not display a late G_i-induced decrease of contraction rate (Fig. 8, E and H). When myocytes expressing mβ_2AR-P417L were treated with PDZ (DSAL) peptide, the activated receptor induced a significantly greater initial increase in contraction rate than the control (Fig. 8, F and G), and the increase was sustained and did not show a late G_i-induced decrease (Fig. 8, F and H). Together, these data showed that although disrupting the binding to PDZ protein (such as NHERF/EBP50) affects the receptor coupling to both G_s and G_i, dis-

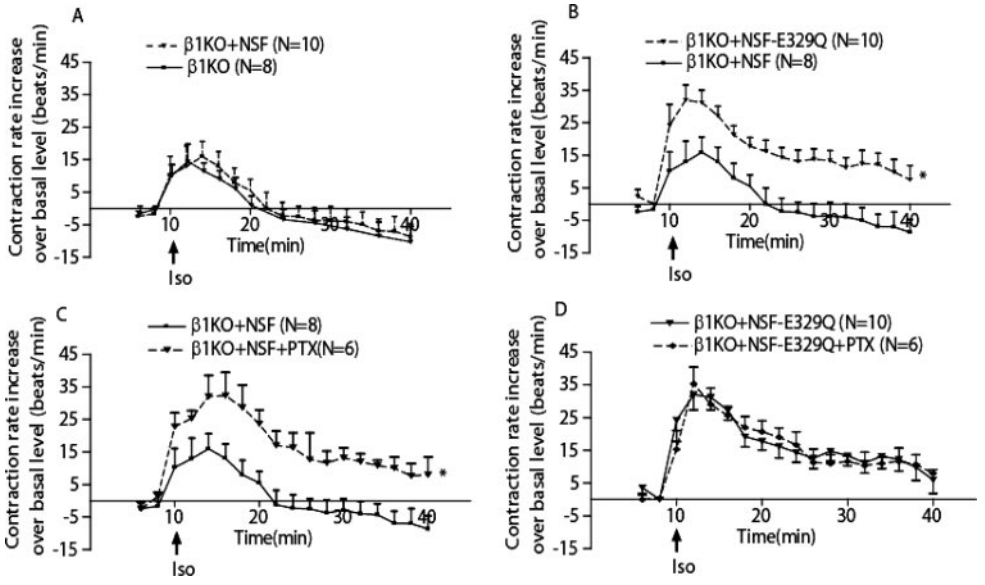

Fig. 5. Dominant-negative NSF-E329Q mutant inhibits the mβ_2AR coupling to G_i protein. Spontaneously beating cardiac myocytes from β_1AR KO mice were transfected with a wild-type NSF (A and C) or NSF-E329Q (B and D) mutant adenovirus as indicated. The cells were administered 10 μM isoproterenol with inhibition of G_i by PTX. Overexpressing the NSF E329Q mutant enhanced the contraction-rate increase induced by isoproterenol stimulation. Additional PTX treatment did not further enhance the contraction-rate increase induced by the mβ_2AR. The data represent the mean ± S.E. of experiments from at least three different myocyte preparations. *, $p < 0.05$, time course significantly different by two-way ANOVA.

rupting the binding to NSF selectively affects the receptor coupling to G_i in cardiomyocytes.

Discussion

In the present study, several approaches were used to test whether NSF regulates β_2AR trafficking and physiological signaling. This idea was extended from the studies of β_2AR-selective interactions with NSF and PDZ proteins. A distal portion of the cytoplasmic C terminus of the hβ_2AR selectively binds to several PDZ domain-containing proteins, such as the cytoskeleton-associated protein NHERF/EBP50, which is implicated in receptor recycling (Cao et al., 1999). However, a subsequent study confirmed the importance of the PDZ ligand for receptor recycling to the cell surface but identified a distinct non-PDZ interaction of this sequence with NSF that was required for proper endocytic recycling (Cong et al., 2001). Although the reported difference could result from the difference between the derived HEK293 cell lines, we have tried to address the functional roles of these receptor-protein interactions in primary cultured cardiomyocytes—a native environment that may have a more precise regulation of receptor function. We have shown previously that the carboxyl-terminal sequence of mβ_2AR was also required for efficient plasma membrane recycling and for receptor coupling to G_i in cardiomyocytes (Xiang and Kobilka, 2003). In this study, we showed that the binding to NSF enhanced both internalization and recycling rates of β_2AR and increased the receptor coupling to G_i signaling in cardiomyocytes (Figs. 2, 3, and 8). We further distinguished the effects of NSF and PDZ binding on β_2AR signaling in myocytes. Although the binding to NSF increases receptor/G_i coupling, the binding to PDZ proteins affects receptor coupling to both G_s and G_i proteins (Fig. 8).

It is interesting that, at the receptor's distal carboxyl terminus, the mβ_2AR (DSPL) differs from the hβ_2AR (DSLL) at the −1 position, at which the hβ_2AR has a leucine critical for binding to NSF. Because the mβ_2AR has a proline residue at the same relative position of the receptor cytoplasmic tail, we predicted that the receptor could not bind to NSF. Our experiments confirmed a very low affinity binding of NSF to the mβ_2AR cytoplasmic tail (Fig. 1). We used a gain-of-function approach by replacing the proline with a leucine to generate a mutant mβ_2AR-P417L. This mutant has a distal terminus identical with that of the hβ_2AR (hβ_2AR mimic) and displayed recovered binding to NSF (Fig. 1). The direct NSF-binding seemed to increase the rates of both agonist-induced endocytosis and recycling of the mβ_2AR-P417L in cardiomyocytes (Fig. 2 and 3). We cannot exclude the possible contribution by the small increase in binding affinities of the mutant mβ_2AR-P417L for PDZ proteins or new binding partners. However, our beating assay data supported that the increased trafficking rates are probably caused by the fact that the mutant mβ_2AR-P417L gained binding to NSF (Fig. 8).

Consistent with the trafficking data, the mβ_2AR-P417L and the hβ_2AR also displayed a more profound coupling to G_i than the mβ_2AR in cardiomyocytes (Fig. 7). We have established previously that activated mβ_2AR undergo sequential coupling to G_s and G_i to modulate cardiomyocyte contraction rate, and the recycling of mβ_2AR is necessary for coupling to G_i (Xiang and Kobilka, 2003). By using membrane-permeable peptides to selectively inhibit the receptor binding to NSF or PDZ proteins, we will be able to distinguish the subtle effects of a specific binding on receptor signaling. Although disruption of PDZ binding affects receptor coupling to both G_s and G_i, disruption of NSF binding selectively inhibits receptor coupling to G_i (Fig. 8). It is interesting that despite that the mβ_2AR does not bind to NSF well, the NSF peptide ASLL affected the receptor signaling (Fig. 8A). This may result from a low basal interaction between the mβ_2AR and NSF. On the other hand, NSF peptide ASLL is capable of binding to PDZ proteins (Cong et al., 2001); thus, it may compete against DSPL on the mβ_2AR, which is not a perfect PDZ ligand because of the structure of proline. In contrast, the

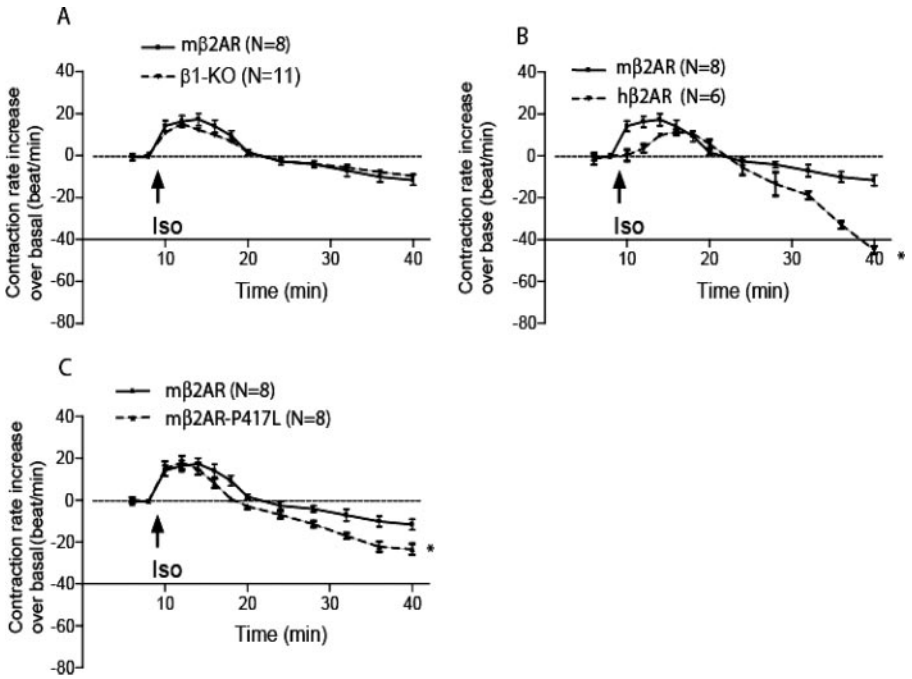

Fig. 6. Differences in β_2AR contraction-rate responses to isoproterenol in neonatal cardiac myocytes from β_1/β_2AR KO mice. The hβ_2AR and mβ_2AR-P417L exhibit different contraction rate profiles than the mβ_2AR at comparable expression levels. Spontaneously beating, cardiac myocytes from β_1/β_2AR KO mice were infected with a FLAG-tagged mβ_2AR (A), hβ_2AR (B), or mβ_2AR-P417L (C) recombinant adenovirus as indicated and infused with 10 μM isoproterenol. Contraction rates were measured and normalized as the change over baseline. The data represent the mean ± S.E. of experiments from at least three different myocyte preparations. *, $p < 0.05$, time course significantly different by two-way ANOVA.

binding between DSLL of the mβ_2AR-P417L and PDZ proteins is less likely to be affected by the NSF peptide (Fig. 8E). Thus, the effect on the mβ_2AR-P417L signaling by the NSF peptides suggested that the binding to NSF affects the receptor/G_i coupling, which is consistent with its role in the modulation of receptor recycling.

NSF was identified as an ATPase, binding to SNARE complexes required for membrane fusion, thus playing critical roles in protein trafficking of many membrane receptors (Whiteheart and Matveeva, 2004). In agreement, we showed that NSF ATPase activity was essential for mβ_2AR trafficking and signaling in cardiomyocytes (Fig. 4 and 5). It is interesting that NSF can bind to β-arrestin, an adaptor-like protein linking most GPCRs to clathrin-coated vesicles for endocytosis (McDonald et al., 1999). NSF binding to β-arrestin, like binding to classic SNARE substrates, is an ATP-dependent event (McDonald et al., 1999). Thus, NSF could play a role together with β-arrestin in recruiting the cargo receptors into clathrin-coated vesicles for budding. This process can be fine-tuned if NSF directly binds membrane cargo receptors, including hβ_2AR (Heydorn et al., 2004). In addition, the binding of NSF to the hβ_2AR is enhanced in the ATP-bound form (Gage et al., 2005), and the NSF ATPase activity dissociates the AMPA receptor from PDZ proteins allowing endocytosis (Osten et al., 1998; Hanley et al., 2002). Therefore, NSF can facilitate receptor recruitment into clathrin-coated vesicle by both direct binding to the cargo receptor and its ATPase activity. In the case of mβ_2AR, the activated receptor recruits β-arrestin; this brings NSF to the receptor. NSF ATPase activity helps to dissociate the receptor from PDZ proteins to enter clathrin-coated vesicles, and later NSF regulates the vesicle fusion to endosome. In comparison, hβ_2AR can directly bind to the NSF. When NSF is recruited to the receptor/arrestin complexes, it can compete against the receptor binding to PDZ proteins. This competition can lead to an increase in internalization rates (Fig. 3). During the receptor recycling, NSF binding can bridge the cargo receptor to SNARE complexes, which facilitate the docking of recy-

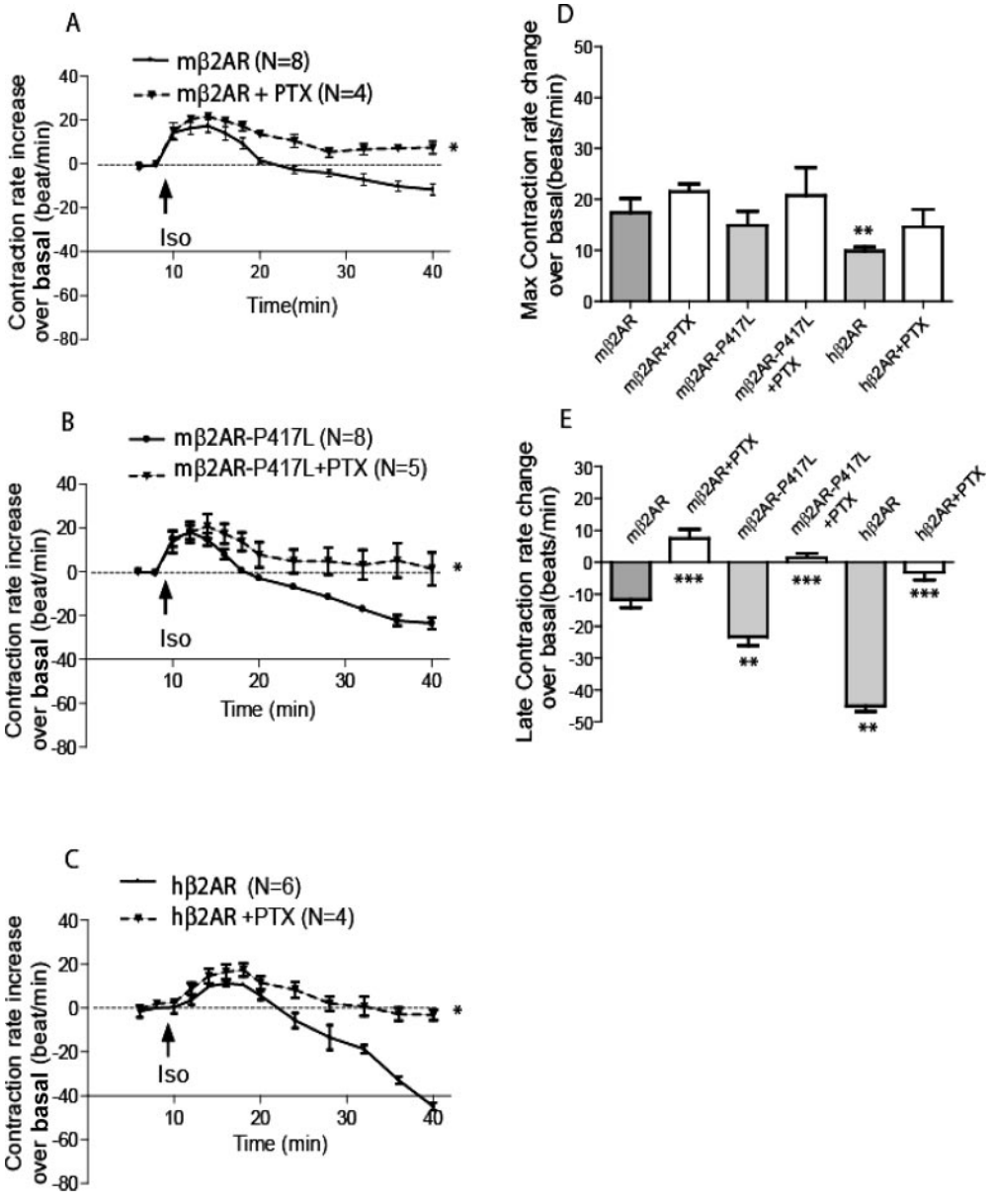

Fig. 7. NSF binding enhances the G_i signaling components of β_2ARs. Spontaneously beating cardiac myocytes from β_1/β_2AR KO mice were transfected with a FLAG-tagged mβ_2AR (A), mβ_2AR-P417L (B), or hβ_2AR (C) adenovirus as indicated. The cells were administered 10 μM isoproterenol with inhibition of G_i with PTX. PTX treatment did not affect initial response usually mediated by receptor/G_s coupling (D) but significantly enhanced the contraction rate during the late stimulation induced by the G_i coupling to the activated hβ_2AR, mβ_2AR, or mβ_2AR-P417L (E). The data represent the mean ± S.E. of experiments from at least three different myocyte preparations. *, $p < 0.05$, time course significantly different by two-way ANOVA. **, $p < 0.05$, unpaired t test significantly different on initial maximum contraction rate increases or late contraction-rate decreases mediated by different β_2ARs. ***, $p < 0.05$, unpaired t test significantly different on late contraction rate decreases after PTX treatment.

cling vesicles to plasma membrane, hence enhancing the recycling rate of hβ_2AR and mβ_2AR-P417L but not mβ_2AR. In this study, we only measured the cell surface receptor level during endocytosis and recycling. Any additional role of NSF in receptor trafficking among endosomal compartments remains to be addressed.

It is noteworthy that the subtle effects of NSF-hβ_2AR binding on trafficking and signaling is not conserved throughout mammals; such an effect can be overlooked easily in an experimental procedure. Although NSF is a common factor involved in membrane receptor trafficking, the context of the NSF-receptor complex can further complicate the type and degree of receptor regulation. These regulations will probably include the binding of the receptor to PDZ-domain containing proteins and cytoskeleton-associated proteins and additional binding of NSF to other trafficking proteins such as SNARE complexes and arrestin. Further studies using NSF mutants with selective ablation of binding to the β_2AR or other proteins such as arrestin will help to dissect any roles of individual protein-protein interactions on the β_2AR trafficking and signaling in cardiomyocytes or physiological settings.

Indeed, an effect of NSF binding to the hβ_2AR is likely to be complicated by competitive binding of PDZ proteins on the same sequence at the carboxyl-terminal end (Cong et al., 2001). A PDZ binding can have multiple effects on membrane receptor distribution and trafficking. One effect of PDZ binding is to stabilize and restrict the receptors at distinct subcellular domains. This is supported by the recent evidence that overexpressing NHERF-1/EBP50 reduced the agonist-induced internalization of two GPCRs, the parathyroid hormone receptor type-1 and thromboxane A(2)β receptor in HEK293 cells (Rochdi and Parent, 2003; Sneddon et al., 2003). By binding to cytoskeleton and/or scaffold proteins, the receptors can associate with signaling components and form complexes to either facilitate or restrict signal trans-

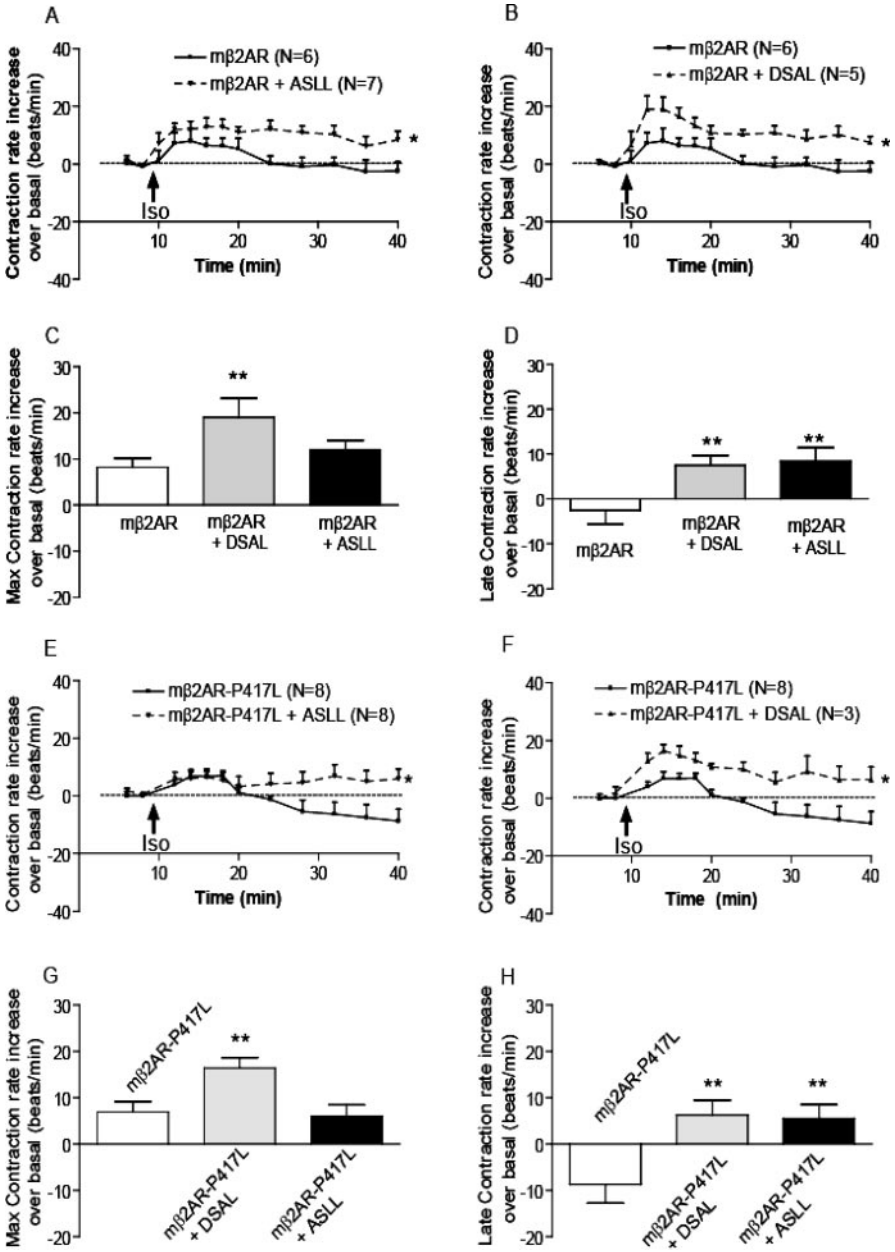

Fig. 8. Selective disruptions of NSF and NHERF-1/EBP50 binding have distinct effects on β_2AR signaling. Spontaneously beating cardiac myocytes from β_1/β_2AR KO mice were transfected with a FLAG-tagged mβ_2AR (A–D) or mβ_2AR-P417L (E–H) adenovirus as indicated. The cells were administered 10 μM isoproterenol with pretreatment of membrane-permeable NSF peptide ASLL and PDZ peptide DSAL to disrupt the receptor binding to NSF and PDZ protein, respectively. NSF peptide ASLL significantly affected the receptor-mediated contraction response during the late stimulation, which are usually mediated by receptor/G_i coupling (D and H). In contrast, PDZ peptide affected both initial contraction rate increase mediated by receptor/G_s coupling (C and G) and the late contraction-rate response mediated by receptor/G_i coupling (D and H). The data represent the mean ± S.E. of experiments from at least three different myocyte preparations. *, $p < 0.05$, time course significantly different by two-way ANOVA. **, $p < 0.05$, unpaired t test significantly different on initial maximum contraction-rate increases or late contraction-rate decreases after treatment with peptides.

duction. Consistent with the notion, our previous and current studies support that disrupting the PDZ binding to the β_2ARs enhances the receptor coupling to G_s in cardiomyocytes (Xiang and Kobilka, 2003). In contrast, the PDZ protein GRIP/ABP binding has been shown to play a role in the stabilization of an intracellular pool of AMPA receptors that have been internalized with stimulation, thus inhibiting their recycling to the synaptic membrane (Braithwaite et al., 2002). Therefore, depending on the receptors and their binding partners, the PDZ domain-containing proteins can stabilize the receptor complexes at either the cell surface or intracellular compartments to fine-tune the receptor function in a given cell type. The third effect of PDZ binding seems to promote receptor trafficking to another subcellular location. Both PICK1 and NHERF-1/EBP50 have been shown to be critical for AMPA receptor and β_2AR recycling back to the cell surface (Cao et al., 1999; Xiang and Kobilka, 2003; Lu and Ziff, 2005). In cardiomyocytes, selective disruption of PDZ binding with point mutations or with membrane-permeable peptide blocks receptor recycling and also inhibits receptor coupling to G_i ((Xiang and Kobilka, 2003) and Fig. 8). This PDZ-promoted trafficking may simply be a result of PDZ sequestration of receptors away from a competing trafficking fate, which could generalize PDZ interactions as hindrances to trafficking. The function of PDZ binding on receptor endocytosis and recycling could be further complicated by agonist-dependent phosphorylation of the receptor C-terminal end by G-protein receptor kinases and subsequent receptor dephosphorylation by pH-sensitive phosphatases (Sibley et al., 1986; Pitcher et al., 1995, 1998; Cao et al., 1999). The significance of this interplay in cardiomyocytes remains to be seen.

When the hβ_2AR was expressed in murine cardiomyocytes, the receptor displayed sequential coupling to G_s and G_i to regulate the myocyte contraction rate (Fig. 6). This result reinforced the notion from our previous studies that the recycling of the β_2AR is part of a mechanism necessary for the receptor to switch from G_s to G_i (Xiang et al., 2002, 2005; Xiang and Kobilka, 2003). Both the human and murine β_2ARs displayed a dual coupling to both G_s and G_i proteins in cardiac myocytes. Our studies revealed a species-dependent difference between human and murine β_2ARs. The hβ_2AR seemed to have a lower efficiency in coupling to the G_s pathway and a significantly higher efficiency in coupling to G_i than the mβ_2AR when regulating the myocyte contraction rate (Figs. 6 and 7). Our results suggest that the profound G_i coupling is in part due to the increased binding to NSF. The mechanism of the low G_s coupling efficiency is not clear, although the higher receptor endocytosis rate could be an indication of enhanced desensitization. Another clue lies in the differences between receptor species. Despite the fact that the mβ_2AR-P417L had recovered the ability to bind NSF, much of the signaling properties of this mutant mβ_2AR still resembled those of the mβ_2AR rather than the hβ_2AR (Fig. 6). The differences of other structural domains on the hβ_2AR and mβ_2AR must thus account for the differences observed between the mβ_2AR-P417L and the hβ_2AR in cardiomyocytes. The notable regions include both the third loop and the proximal region of the carboxyl tail, which can directly influence G protein coupling. Another species-dependent difference is that a unique sugar-modification site located on the second extracellular domain of the hβ_2AR, but not rodent β_2ARs, promotes receptor degradation upon long-term agonist stimulation (Mialet-Perez et al., 2004). When overexpressed in mice, the hβ_2AR seems to enhance the cardiac contraction in animal hearts without developing heart failure (Milano et al., 1995). The β_2AR/G_s signaling is proapoptotic (Zhu et al., 2001), whereas the β_2AR/G_i signaling plays an antiapoptotic role in both mouse hearts and cultured mouse cardiac myocytes (Zhu et al., 2001; Patterson et al., 2004). Thus, the more preferential coupling of the hβ_2AR to G_i over G_s observed in our experiments could explain the lack of pathologic changes observed with overexpression of the hβ_2AR in the hearts of mice. Further studies characterizing the differences between the hβ_2AR and mβ_2AR are needed to advance our understanding of adrenergic physiology in vivo.

In conclusion, the present results indicate that NSF ATPase activity is necessary for agonist-dependent β_2AR trafficking in cardiomyocytes, whereas NSF binding enhances the receptor transportation rates. Both the direct binding to NSF and its ATPase activity are important for the receptor coupling to G_i. Our data also showed different affinities of NSF binding to β_2ARs from different species, and the direct binding to NSF contributes to the differences of receptor signaling in cardiomyocytes. Our data further revealed distinct effects of NSF and PDZ binding on β_2AR signaling. In contrast to the selective effect on G_i coupling by the receptor binding to NSF, the receptor binding to PDZ proteins affects the receptor coupling to both G_s and G_i proteins. The present results add to the growing appreciation of diversified cellular factors as part of comprehensive mechanisms to fine-tune GPCR signaling and membrane trafficking in native mammalian cardiomyocytes.

Acknowledgments

We acknowledge Anthony Bretscher (Cornell) for anti-EBP50 and Sidney Whiteheart (University of Kentucky) for valuable discussion and for anti-NSF.

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