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## NSF Regulates $\beta_2$ Adrenoceptors Trafficking and Signaling in Cardiomyocytes

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**Abbreviations:** GPCR, G protein-coupled receptor;  $\beta_2$ AR,  $\beta_2$ -adrenergic receptor;  $\beta_1$ AR,  $\beta_1$ -adrenergic receptor; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; NSF, N-ethylmaleimide-sensitive factor; PDZ, PSD-95/Discs-large/ZO-1; NHERF/EBP50, Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor/ezrin/radixin/moesin-binding phosphoprotein of 50 kDa; GST, glutathione S-transferase; PTX, pertussis toxin; NEM, N-ethylmaleimide.

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## Abstract

Recycling of G protein-coupled receptors (GPCRs) determines the functional resensitization of receptors and is implicated in switching  $\beta_2$  adrenoceptor ( $\beta_2$ AR) G protein specificity in cardiomyocytes. The human  $\beta_2$ AR carboxyl end binds to the N-ethylmaleimide-sensitive factor (NSF), an ATPase integral to membrane trafficking machinery. Interestingly, the human  $\beta_2$ AR (h $\beta_2$ AR) carboxyl end pulled down NSF from mouse heart lysates whereas the murine one did not. Despite this difference, both  $\beta_2$ ARs exhibited substantial agonist-induced internalization, recycling, and Gi coupling in cardiomyocytes. The h $\beta_2$ AR, however, displayed faster *rates* of agonist-induced internalization and recycling when compared to the m $\beta_2$ AR; and a more profound Gi component in its contraction response. Replacing the m $\beta_2$ AR proline (-1) with a leucine generated a gain-of-function mutation, m $\beta_2$ AR-P417L, with a rescued ability to bind NSF, faster internalization and recycling than the m $\beta_2$ AR, and a significant enhancement in Gi signaling, which mimics the h $\beta_2$ AR. Selective disruption of the m $\beta_2$ AR-P417L binding to NSF inhibited the receptor coupling to Gi. Meanwhile, inhibiting NSF with N-ethylmaleimide blocked the m $\beta_2$ AR recycling following agonist-induced endocytosis. Expressing the NSF E329Q mutant lacking ATPase activity inhibited the m $\beta_2$ AR coupling to Gi in cardiomyocytes. Our results revealed a dual regulation on h $\beta_2$ AR 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  $\beta_2$ ARs.

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## Introduction

Beta adrenoceptors play a pivotal role in regulating cardiomyocyte contraction through distinct signaling pathways. The  $\beta_1$ AR couples to Gs protein(s) which increases cAMP/PKA activity and the contraction rate whereas the activated  $\beta_2$ AR sequentially couples to both Gs and Gi in neonatal cardiomyocytes, creating a biphasic change in contraction.  $\beta_2$ AR Gi coupling appears to be dependent on receptor trafficking, which includes both endocytosis and recycling. Inhibiting either process blocks receptor coupling to Gi in cardiomyocytes (Xiang and Kobilka, 2003; Xiang et al., 2002).

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 downregulate 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  $\beta_2$ AR has a type I PDZ ligand at its carboxyl-terminal end that is necessary for recycling, and sufficient to reroute the  $\delta$ -opioid receptor from a degradative to a recycling pathway (Cao et al., 1999; Gage et al., 2001). In cultured, neonatal, mouse cardiomyocytes, this sequence is also required for the temporal switch from Gs to Gi-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

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trafficking of this receptor dictates not only cellular resensitization, but also signal transduction specificity. Despite progress in understanding the  $\beta_2$ AR recycling process, numerous questions concerning the core mechanism and physiological variations remain.

While the recycling sequence at the  $\beta_2$ AR C-terminus has been shown to bind PDZ domains in NHERF family proteins (NHERF-1/EBP50 and NHERF-2/E3KARP) (Cao et al., 1999; Hall et al., 1998), 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 in order 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  $\beta$ -arrestin, an adaptor protein involved in GPCR desensitization and endocytosis upon agonist stimulation.  $\beta$ -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  $\beta_2$ AR was inferred to inhibit recycling of receptors, while imparting NSF binding on the  $\delta$ -opioid receptor slightly enhanced its ability to recycle (Cong et al., 2001; Gage et al., 2005). While there is also evidence to show that PDZ interactions promote receptor recycling (9), and are functionally important for Gi coupling in cardiomyocytes (Xiang and Kobilka, 2003), it is not clear how NSF may affect  $\beta_2$ AR trafficking and signaling in these cells.

Here, we used neonatal mouse cardiomyocytes as a model system to address these questions.

Interestingly, the NSF binding sites on the  $\beta_2$ AR were not conserved among mammalian species,

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providing a naturally occurring divergence in NSF binding to exploit. The -1 position of the  $\beta_2$ AR carboxyl terminus is proline in m $\beta_2$ AR and leucine in h $\beta_2$ AR. Due to this single amino acid difference m $\beta_2$ AR binding to NSF was not detectable. Nevertheless, in spite of the lack of detectable binding of the m $\beta_2$ AR carboxyl terminus to NSF in biochemical assays, we found that inhibition of NSF activity with N-ethylmaleimide (NEM) inhibited murine  $\beta_2$ AR (m $\beta_2$ AR) recycling in spite of this poor affinity. In addition, both human and murine  $\beta_2$ ARs sufficiently recycled after endocytosis, and coupled to Gi pathways in cardiomyocytes. The different affinities for NSF appeared 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  $\beta_2$ AR recycling and coupling to Gi in cardiomyocytes, indicating that NSF is required for proper trafficking and signaling of  $\beta_2$ ARs in cardiomyocytes independent of a high-affinity interaction with the receptor. This study strengthens the relationship between  $\beta_2$ AR 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  $\beta_2$ AR in pcDNA3 (Invitrogen) 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- $\beta_2$ AR and GST- $\beta_2$ AR-Ala proteins (encompassing amino acids 328-413 of the human  $\beta_2$ AR, and the latter with an additional Ala added to the C-terminus) have also been reported (Cao et al., 1999). A comparable murine GST- $\beta_2$ AR construct was created by insertion of a PCR product of the region encoding amino acids 328-418 of the FLAG-m $\beta_2$ AR construct, using primers containing EcoRI

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and HindIII appendages and performing the appropriate digestion and ligation into pGEX-KG (Pharmacia). The human NSF coding sequence was similarly ligated into pEGFP-N1 (Clontech) following SacI digestion of the vector and a PCR product containing a 3' SacI appendage and including the 5' SacI restriction site from the source vector, NSF in pBluescriptR (ATCC). The P417L mutation was introduced into the FLAG-m $\beta_2$ AR and GST-m $\beta_2$ AR constructs via the QuikChange® Site-Directed Mutagenesis Kit (Stratagene), as was the E329Q NSF mutation into the pEGFP-N1 construct. Plasmid amplification was done in DH5 $\alpha$  *Escherichia coli* and all sequences were verified by dideoxynucleotide sequencing (UCSF Biomolecular Resource Center).

#### *Cell Culture and Transfection*

Spontaneously beating neonatal cardiomyocytes were prepared from hearts of 1-day-old  $\beta_1/\beta_2$ AR-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-m $\beta_2$ AR has been described (Xiang et al., 2002), and the FLAG-m $\beta_2$ AR/P417L, FLAG-h $\beta_2$ AR, GFP-NSF, and GFP-NSF-E329Q adenoviral vectors were generated with the same pAdEasy system (Q.biogen, Carlsbad, CA). Neonatal myocytes were infected with viruses at a multiplicity of infection of 100, after being cultured for 24 hours. The receptor expression levels were determined by ligand binding assays as described (Xiang et al., 2002). They were expressed at equivalent levels in cardiac myocytes (FLAG-m $\beta_2$ AR 147.3  $\pm$  22 fmole/mg; FLAG-m $\beta_2$ AR/P417L 171.6  $\pm$  9.1 fmole/mg; and FLAG-h $\beta_2$ AR 160.3  $\pm$  21.8 fmole/mg membrane).

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### *GST Pulldowns*

The various GST- $\beta_2$ AR fusion proteins were produced in BL21 *Escherichia coli* and bound to glutathione-Sepharose agarose beads (Amersham Biosciences). Beads containing 10  $\mu$ g of the full-length fusion protein (assessed by densitometry of Coomassie-stained protein resolved by SDS-PAGE) were incubated for 4 hours at 4°C in 0.5 mL of clarified extracts from frozen mouse hearts with atria removed (Pel-Freez Biologicals), prepared to ~10mg/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, 10 mM Tris pH 7.4, complete Roche protease inhibitor cocktail), and protein was eluted in LDS Sample Buffer (Invitrogen) with DTT added to 20 mM. Samples were divided in two for SDS-PAGE, transfer to nitrocellulose, and Western blots using rabbit anti-EBP50 antibodies (courtesy of Dr. Anthony Bretscher, Cornell University) or the mouse 2E5 anti-NSF antibody (courtesy of Dr. Sidney W. Whiteheart, University of Kentucky).

### *Immunofluorescence Microscopy*

Myocyte images were obtained using a similar setup on a Zeiss Axioplan 2 microscope. Fluorescent measurements of the myocyte receptor trafficking were made by a ratiometric normalization of fluorescent intensities measured using Metamorph software (Universal Imaging). Epitope-tagged receptors were detected using M1 anti-FLAG antibody (Sigma). Selective detection of surface relative to total pools of receptor, and its use to estimate receptor recycling, have been described (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-488 (Molecular Probes) using standard procedures as described previously (Tanowitz and von Zastrow, 2003). Secondary staining was performed using a commercial goat

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anti-mouse IgG Alexa-594 conjugate (Molecular Probes). 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- $\beta_2$ AR. Media was refreshed 1 hour before 10  $\mu$ M isoproterenol (Sigma) stimulation for 10 or 30 minutes. Periods of agonist washout following 30 minute isoproterenol stimulations were also performed for an additional 30 or 60 minutes as indicated.

### *Myocyte Contraction Rate Assay*

Measurement of spontaneous contraction rates from myocytes expressing either the endogenous or the indicated FLAG- $\beta_2$ AR were carried out with and without the use of PTX as described previously (Devic et al., 2001). In some assays, NEM was applied 30 minutes before addition of isoproterenol. Tat peptide, Tat- $\beta_2$ -DSAL consisting of Tat linked to GRQGFSSDSAL of  $\beta_2$ AR, Tat- $\beta_2$ -ASLL consisting of Tat linked to GRQGFSSASLL of  $\beta_2$ AR 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  $\mu$ M peptide for 25 min, before isoproterenol (10  $\mu$ M; Sigma) exposure.

### *Statistical Analysis*

Curve-fitting and statistical analyses were performed using Prism (GraphPad Software, Inc.).

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## Results

### *NSF has higher binding affinity to human $\beta_2$ AR than murine $\beta_2$ AR*

To understand the molecular mechanism of the NSF effect on  $\beta_2$ AR signaling in cardiomyocytes, the interaction between  $\beta_2$ AR and NSF from heart lysate was examined. NSF, a hexameric ATPase involved in membrane fusion, can bind to the carboxyl terminus of the h $\beta_2$ AR. The protein-binding region on this receptor involve 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 h $\beta_2$ AR. Interestingly, several rodent  $\beta_2$ ARs, including the m $\beta_2$ AR, are identical to the h $\beta_2$ AR in the carboxyl-binding region except at one residue (leucine -1 of the h $\beta_2$ AR, DSLL) that is required for binding to NSF, but not to NHERF-1/EBP50 (Cong et al., 2001). Rather, the m $\beta_2$ AR has a proline at the -1 position (DSPL). To determine whether both the human and murine  $\beta_2$ ARs have a similar capacity to bind NSF, GST-fusion proteins, including various  $\beta_2$ AR 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 h $\beta_2$ AR, but not the m $\beta_2$ AR (Fig.1A). In contrast, NHERF-1/EBP50 bound to the cytoplasmic tail of both the h $\beta_2$ AR and m $\beta_2$ AR under these conditions (Fig.1B). As a negative control for non-specific binding, an addition of a single alanine residue to the h $\beta_2$ AR carboxyl terminus (GST-h $\beta_2$ AR-Ala) was tested as well. As previously reported, this mutant failed to exhibit the PDZ domain-mediated and NSF protein binding (Fig. 1A and 1B, and (Cao et al., 1999)). In addition, we attempted to 'rescue' an NSF interaction with the m $\beta_2$ AR tail by substitution of the m $\beta_2$ AR proline 417 with a leucine residue (m $\beta_2$ AR-P417L).

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The m $\beta_2$ AR-P417L pulled down similar amounts of NSF from lysates when compared to the h $\beta_2$ AR, indicating that the m $\beta_2$ AR-P417L cytoplasmic tail fully rescued binding to NSF (Fig.1A). Similarly this mutant m $\beta_2$ AR-P417L pulled down NHERF-1/EBP50 from mouse heart lysates (Fig.1B).

*The binding of NSF and the NSF ATPase activity have distinct effects on  $\beta_2$ AR trafficking in cardiomyocytes*

In order to examine whether NSF plays any role in  $\beta_2$ AR trafficking in cardiomyocytes, we analyzed the localization of flag-tagged  $\beta_2$ ARs in cardiac myocytes. The m $\beta_2$ AR, m $\beta_2$ AR-P417L, and h $\beta_2$ AR were transiently expressed in cardiac myocytes using recombinant adenovirus. Immunofluorescence studies showed that all three receptors had a cell-surface staining during a non-stimulated 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 and data not shown). Interestingly, when we measured the short-term decrease in cell-surface receptors after agonist stimulation in cardiac myocytes, we found that the m $\beta_2$ AR-P417L had a faster rate ( $t_{1/2}$  2.63  $\pm$  0.05 minutes) of cell-surface-receptor decrease than the m $\beta_2$ AR ( $t_{1/2}$  10.93  $\pm$  0.01 minutes, Fig.3). Since the receptor level change in the short time points after agonist stimulation is primarily determined by agonist-induced endocytosis, this data suggested a faster rate of endocytosis for the m $\beta_2$ AR-P417L than for the m $\beta_2$ AR in cardiac myocytes. The h $\beta_2$ AR had a similar rate ( $t_{1/2}$  3.4  $\pm$  0.03 minutes) of cell-surface-receptor decrease when compared to the

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m $\beta_2$ AR-P417L (Fig.3). However, after 30 minutes of agonist stimulation, we observed a similar amount of surface receptor decreases with the m $\beta_2$ AR (30.54 + 2.90 %), the m $\beta_2$ AR-P417L (28.43 ± 1.69 %), and the h $\beta_2$ AR (24.94 ± 2.74 %). The observed decrease of receptor density at 30 minutes 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 h $\beta_2$ AR and m $\beta_2$ AR-P417L recovered cell-surface staining almost completely after a 60-minute incubation (Fig.2). In contrast, the m $\beta_2$ AR 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 appeared to return to the surface within 60 minutes. When we examined the cell-surface-receptor density at different time points with the fluorescent ELISA assay, the m $\beta_2$ AR exhibited a lower recovery of cell-surface-receptors after recycling for 60 minutes than the m $\beta_2$ AR-P417L mutant and the h $\beta_2$ AR (Fig.2B, \* p < 0.05). A significant difference in surface recovery was also observed using ratiometric image measurements at 60 minutes after agonist washout (data not shown). These data indicate that the m $\beta_2$ AR, although capable of undergoing agonist-induced internalization and recycling in cardiomyocytes, differs in rates of recycling when compared to the h $\beta_2$ AR and m $\beta_2$ AR-P417L.

It has been well established that NSF ATPase activity play 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

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following removal of agonist for 60 minutes 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 inhibits endogenous m $\beta$ <sub>2</sub>AR coupling to Gi pathway in cardiomyocytes*

Our finding that NEM inhibits  $\beta$ <sub>2</sub>AR 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 m $\beta$ <sub>2</sub>AR 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 and Matveeva, 2004; Whiteheart et al., 1994). When the endogenous m $\beta$ <sub>2</sub>AR in the  $\beta$ <sub>1</sub>AR-KO myocyte was stimulated by isoproterenol, the activated receptor induced a biphasic contraction-rate response with an initial increase mediated by G<sub>s</sub> coupling followed by a sustained G<sub>i</sub>-dependent decrease to drop the contraction rate below basal level (Fig.5A and (Xiang et al., 2002)). When wild type NSF was expressed in  $\beta$ <sub>1</sub>AR-KO cardiac myocytes, we did not observe any significant change in the endogenous m $\beta$ <sub>2</sub>AR-mediated contraction rate response (Fig. 5A). In contrast, when the NSF E329Q mutant was overexpressed in cardiomyocytes, the contraction rate mediated by the m $\beta$ <sub>2</sub>AR was significantly higher than the control, and did not display a decrease below the basal level (Fig. 5B). This response profile was similar to that observed with an inhibition of G<sub>i</sub> by PTX (Fig. 5C). Indeed, additional treatment of PTX did not generate any further increases in contraction

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rates (Fig. 5D). Therefore, the NSF E329Q behaved as a dominant negative to block the receptor coupling to Gi in cardiomyocytes. In addition, when myocytes are pretreated with NEM to inhibit the NSF ATPase activity, we also observed similar effects to those by NSF E329Q mutant on m $\beta_2$ AR signaling mediated contraction rate response (data not shown).

*The divergent C-termini of the human and murine  $\beta_2$ AR have different effects on contraction rate responses in neonatal cardiomyocytes*

Our previous studies have shown that the localization and trafficking of the m $\beta_2$ AR 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  $\beta_2$ AR affected the receptor trafficking *rates* after agonist stimulation 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 m $\beta_2$ AR was expressed in  $\beta_1\beta_2$ AR-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 drop the contraction rate below basal level (Fig.6A and (Xiang et al., 2002)). This contraction-rate change is equivalent to that induced by the endogenous m $\beta_2$ AR in  $\beta_1$ AR-KO myocytes (Fig. 6A). The m $\beta_2$ AR-P417L induced a similar contraction rate response profile and initial increase when compared to the m $\beta_2$ AR in  $\beta_1\beta_2$ AR-KO myocytes (Fig. 6C and 7D). However, the contraction rate decreased faster, and the contraction rate was lower than that induced by the m $\beta_2$ AR during late stimulation in cardiac myocytes (Fig. 6C and 7E). In addition, when stimulating the h $\beta_2$ AR expressed in  $\beta_1\beta_2$ AR-KO myocyte with isoproterenol, the activated receptor also induced a biphasic, contraction-rate change with an initial increase followed by a sustained decrease (Fig. 6B). Though interestingly, the initial contraction-rate increase was

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smaller than that induced by the activated  $m\beta_2AR$  and  $m\beta_2AR$ -P417L (Fig. 6B and 7D); more strikingly, the late decrease in contraction rate induced by the exogenous  $h\beta_2AR$  was greater than that induced by the  $m\beta_2AR$  and  $m\beta_2AR$ -P417L (Fig. 6B and 7E).

The profound contraction-rate decrease induced by the  $m\beta_2AR$ -P417L and the  $h\beta_2AR$  suggests that these receptors may have enhanced coupling to  $G_i$  and/or reduced coupling to  $G_s$  when compared to the  $m\beta_2AR$ . 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  $\beta_2AR$ s before isoproterenol stimulation. Upon inhibiting  $G_i$  with PTX, the isoproterenol-stimulated  $m\beta_2AR$  induced a slightly greater, but not significant contraction rate increase in myocytes in comparison to the control, and prevented the late  $G_i$  dependent contraction rate decrease (Fig. 7A and (Xiang et al., 2002)). PTX treatment also inhibited the contraction rate decrease mediated by the  $m\beta_2AR$ -P417L or the  $h\beta_2AR$  during the late phase of stimulation (Fig. 7B and 7C). These data suggest that compared to the activated  $m\beta_2AR$ , the  $m\beta_2AR$ -P417L had an enhanced  $G_i$  coupling upon isoproterenol stimulation; and the activated  $h\beta_2AR$  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  $\beta_2AR$  activation-induced contraction rates in cardiomyocyte.*

To further probe the effect of the  $\beta_2AR$  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  $m\beta_2AR$  or

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m $\beta_2$ AR-P417L (the h $\beta_2$ AR mimic) in  $\beta_1\beta_2$ AR-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 m $\beta_2$ AR 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. 8A and 8C). The increase was sustained during stimulation, and lacked a late decrease mediated by receptor/Gi coupling in control cells (Fig. 8A and 8D). When m $\beta_2$ AR expressing myocytes were treated with PDZ (DSAL) peptide, the activated receptor induced a significantly greater initial increase than the control (Fig 8B and 8C), and the increase was sustained and lacked a late Gi-dependent decrease (Fig. 8B and 8D).

In contrast, pretreatment with NSF (ASLL) peptide did not affect the activated m $\beta_2$ AR-P417L induced initial increase (Fig. 8E and 8G). However, the increase was sustained, and did not display a late Gi-induced decrease of contraction rate (Fig. 8E and 8H). When myocytes expressing m $\beta_2$ AR-P417L were treated with PDZ (DSAL) peptide, the activated receptor induced a significant greater initial increase in contraction rate than the control (Fig 8F and 8G), and the increase was sustained, and did not show a late Gi-induced decrease (Fig. 8F and 8H). Together, these data showed that while disrupting the binding to PDZ protein (such as NHERF/EBP50) affects the receptor coupling to both Gs and Gi, disrupting the binding to NSF selectively affects the receptor coupling to Gi in cardiomyocytes.

## Discussion

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In the present study, several approaches were used to test whether NSF regulates  $\beta_2$ AR trafficking and physiologic signaling. This idea was extended from the studies of  $\beta_2$ AR-selective interactions with NSF and PDZ proteins. A distal portion of the cytoplasmic C-terminus of the h $\beta_2$ AR 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). While 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 previously shown that the carboxyl-terminal sequence of m $\beta_2$ AR was also required for efficient plasma membrane recycling, and for receptor coupling to Gi in cardiomyocytes (Xiang and Kobilka, 2003). In this study, we showed that the binding to NSF enhanced both internalization and recycling rates of  $\beta_2$ AR, and increased the receptor coupling to Gi signaling in cardiomyocytes (Fig. 2, 3, and 8). We further distinguished the effects of NSF and PDZ binding on  $\beta_2$ AR signaling in myocytes. While the binding to NSF increases receptor/Gi coupling, the binding to PDZ proteins affects receptor coupling to both Gs and Gi proteins (Fig. 8).

Interestingly, at the receptor's distal carboxyl terminus, the m $\beta_2$ AR (DSPL) differs from the h $\beta_2$ AR (DSLL) at the -1 position, at which the h $\beta_2$ AR has a leucine critical for binding to NSF. Since the m $\beta_2$ AR 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 m $\beta_2$ AR cytoplasmic tail (Fig. 1). We utilized a gain-of-

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function approach by replacing the proline with a leucine to generate a mutant  $m\beta_2AR$ -P417L. This mutant has a distal terminus identical to that of the  $h\beta_2AR$  ( $h\beta_2AR$  mimic), and displayed recovered binding to NSF (Fig.1). The direct NSF-binding appeared to increase the rates of both agonist-induced endocytosis and recycling of the  $m\beta_2AR$ -P417L in cardiomyocytes (Fig. 2 and 3). We can not exclude possible contribution by the small increase in binding affinities of the mutant  $m\beta_2AR$ -P417L for PDZ proteins, or new binding partners for the mutant receptor. However, our beating assay data supported that the increased trafficking rates are likely due to the fact that the mutant  $m\beta_2AR$ -P417L gained binding to NSF (Fig. 8).

Consistent with the trafficking data, the  $m\beta_2AR$ -P417L and the  $h\beta_2AR$  also displayed a more profound coupling to  $G_i$  than the  $m\beta_2AR$  in cardiomyocytes (Fig. 7). We have previously established that activated  $m\beta_2AR$  undergo sequential coupling to  $G_s$  and  $G_i$  to modulate cardiomyocyte contraction rate and the recycling of  $m\beta_2AR$  is necessary for coupling to  $G_i$  in cardiomyocytes (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. While 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). Interestingly, despite that the  $m\beta_2AR$  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  $m\beta_2AR$  and NSF. Alternatively, NSF peptide ASLL is capable of binding to PDZ proteins (Cong et al., 2001), thus it may compete against DSPL on the  $m\beta_2AR$  which is not a perfect PDZ ligand due to the structure of proline. In contrast, the binding between DSLL of the  $m\beta_2AR$ -P417L and PDZ proteins is less likely affected by the NSF peptide (Fig. 8E). Thus, the effect on the  $m\beta_2AR$ -P417L signaling by the NSF peptides suggested that the

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binding to NSF affects the receptor/Gi coupling, which is consistent with its role in modulation of receptor recycling.

NSF was identified as an ATPase, binding to SNAP receptor (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 m $\beta$ <sub>2</sub>AR trafficking and signaling in cardiomyocytes (Fig 4 and 5). Interestingly, NSF can bind to  $\beta$ -arrestin, an adaptor-like protein linking most GPCRs to clathrin-coated vesicles for endocytosis (McDonald et al., 1999). NSF binding to  $\beta$ -arrestin, like binding to classical SNARE substrates, is an ATP-dependent event (McDonald et al., 1999). Thus, NSF could play a role together with  $\beta$ -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 h $\beta$ <sub>2</sub>AR (Heydorn et al., 2004). In addition, the binding of NSF to the h $\beta$ <sub>2</sub>AR 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 (Hanley et al., 2002; Osten et al., 1998). 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 m $\beta$ <sub>2</sub>AR, the activated receptor recruits  $\beta$ -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, h $\beta$ <sub>2</sub>AR 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 docking of recycling vesicles to plasma membrane; hence enhance the recycling

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rate of h $\beta_2$ AR and m $\beta_2$ AR-P417L, but not m $\beta_2$ AR. 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 remain to be addressed.

It is noteworthy that the subtle effects of NSF-h $\beta_2$ AR binding on trafficking and signaling is not conserved throughout mammals; such an effect can be overlooked easily in an experimental procedure. While 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 likely include the binding of the receptor to PDZ-domain containing proteins and cytoskeleton associated proteins, as well as 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  $\beta_2$ AR or other proteins such as arrestin will help dissect any roles of individual protein-protein interactions on the  $\beta_2$ AR trafficking and signaling in cardiomyocytes or physiological settings.

Indeed, an effect of NSF binding to the h $\beta_2$ AR 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) beta 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 transduction.

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Consistent with the notion, our previous and current studies support that disrupting the PDZ binding to the  $\beta_2$ ARs enhance the receptor coupling to Gs 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 appears to promote receptor trafficking to another subcellular location. Both PICK1 and NHERF-1/EBP50 have been shown to be critical for AMPA receptor and  $\beta_2$ AR recycling back to the cell surface (Cao et al., 1999; Lu and Ziff, 2005; Xiang and Kobilka, 2003). In cardiomyocytes, selective disruption of PDZ binding with point mutations or with membrane permeable peptide blocks receptor recycling, and also inhibits receptor coupling to Gi ((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 (GRKs) and subsequent receptor dephosphorylation by pH-sensitive phosphatases (Pitcher et al., 1998; Pitcher et al., 1995; Sibley et al., 1986)(Cao et al., 1999). The significance of this interplay in cardiomyocytes remains to be seen.

When the  $\beta_2$ AR was expressed in murine cardiomyocytes, the receptor displayed sequential coupling to Gs and Gi to regulate the myocyte contraction rate (Fig.6). This result reinforced the

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notion from our previous studies that the recycling of the  $\beta_2$ AR is part of a mechanism necessary for the receptor to switch from Gs to Gi (Xiang and Kobilka, 2003; Xiang et al., 2005; Xiang et al., 2002). Both the human and murine  $\beta_2$ ARs displayed a dual coupling to both Gs and Gi proteins in cardiac myocytes. Our studies revealed a species-dependent difference between human and murine  $\beta_2$ ARs. The h $\beta_2$ AR appeared to have a lower efficiency in coupling to the Gs pathway and a significantly higher efficiency in coupling to Gi than the m $\beta_2$ AR when regulating the myocyte contraction rate (Fig.6 and 7). Our results suggest that the profound Gi coupling is in part due to the increased binding to NSF. The mechanism of the low Gs coupling efficiency is not clear, though 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 m $\beta_2$ AR-P417L had recovered the ability to bind NSF, much of the signaling properties of this mutant m $\beta_2$ AR still resembled those of the m $\beta_2$ AR rather than the h $\beta_2$ AR (Fig.6). The differences of other structural domains on the h $\beta_2$ AR and m $\beta_2$ AR must thus account for the differences observed between the m $\beta_2$ AR-P417L and the h $\beta_2$ AR 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 h $\beta_2$ AR, but not rodent  $\beta_2$ ARs, promotes receptor degradation upon long-term agonist stimulation (Mialet-Perez et al., 2004). When overexpressed in mice, the h $\beta_2$ AR appears to enhance the cardiac contraction in animal hearts without developing heart failure (Milano et al., 1995). The  $\beta_2$ AR/Gs signaling is pro-apoptotic (Zhu et al., 2001), whereas the  $\beta_2$ AR/Gi signaling plays an anti-apoptotic role in both mouse hearts and cultured mouse cardiac myocytes (Patterson et al., 2004; Zhu et al., 2001). Thus, the more preferential coupling of the h $\beta_2$ AR to Gi over Gs observed in our experiments could explain the lack of pathologic changes observed with over-expression of the h $\beta_2$ AR in the

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hearts of mice. Further studies characterizing the differences between the h $\beta_2$ AR and m $\beta_2$ AR are needed in order to advance our understanding of adrenergic physiology *in vivo*.

In conclusion, the present results indicate that NSF ATPase activity is necessary for agonist-dependent  $\beta_2$ AR 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 Gi. Our data also showed different affinities of NSF binding to  $\beta_2$ ARs 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  $\beta_2$ AR signaling. In contrast to the selective effect on Gi coupling by the receptor binding to NSF, the receptor binding to PDZ proteins affects the receptor coupling to both Gs and Gi 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.

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### Footnote

Y.W. and B.L. contributed to this study equally.

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## Figure Legends

**Figure 1: The binding of m $\beta_2$ AR and h $\beta_2$ AR to NSF and NHERF-1/EBP50.** The binding of m $\beta_2$ AR and h $\beta_2$ AR to NSF and NHERF-1/EBP50 from mouse heart extracts. GST pull downs were performed as described under Experimental Procedures. Western detection of NHERF-1/EBP50 pulled down by the indicated GST- $\beta_2$ AR 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 3 or more experiments.

**Figure 2: NSF binding enhances recycling of FLAG- $\beta_2$ ARs in neonatal cardiac myocytes from  $\beta_1/\beta_2$ AR-KO mice.** (A) Human and murine  $\beta_2$ ARs internalize and recycle in cardiac myocytes. Cardiac myocytes expressing a FLAG-tagged h $\beta_2$ AR, m $\beta_2$ AR, or m $\beta_2$ AR-P417L were stained with M1 primary antibody conjugated to the Alexa-488 fluorophore to observe a starting ‘total’ receptor population. Following no treatment (0), 30’ 10  $\mu$ M isoproterenol treatment (30), or 30’ isoproterenol treatment followed by a surface antibody strip and 60’ of agonist removal (30+60), cells were stained under non-permeable 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 3 experiments. (B) NSF-binding  $\beta_2$ ARs recycle faster. Surface levels of the three  $\beta_2$ ARs 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 mins of

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isoproterenol stimulation; 3. 30 minutes isoproterenol followed by 30 minutes of drug removal; and 4. 30 minutes isoproterenol followed by 60 minutes of drug removal). Surface levels are normalized as percent of untreated cell surface fluorescence and error bars reflect standard deviations over 3 experiments. \*,  $P < 0.05$ ; significantly different between m $\beta_2$ AR and h $\beta_2$ AR or m $\beta_2$ AR-P417L by *t*-test.

**Figure 3: NSF Binding enhances the internalization kinetics of FLAG- $\beta_2$ ARs expressed in neonatal cardiac myocytes from  $\beta_1/\beta_2$ AR KO mice.** Surface levels of the three  $\beta_2$ ARs were quantified by fluorescent measurement of M1-Alexa 488 associated with the cell surface receptors after the indicated periods of 10  $\mu$ M isoproterenol administration. Data was normalized as percent *decrease* of untreated cell surface fluorescence and error bars reflect standard deviations over 3 experiments. The data represent the mean  $\pm$  SE of experiments from at least three different myocyte preparations.

**Figure 4: Inhibiting NSF with NEM blocks the FLAG- $\beta_2$ AR recycling after agonist induced endocytosis in cardiomyocytes.** (A) Murine  $\beta_2$ ARs internalize and recycle in cardiac myocytes. Cardiac myocytes expressing a FLAG-tagged m $\beta_2$ AR were treated as described under Experimental Procedures and Figure 3. Images are representative of 3 experiments. Inhibiting NSF with NEM blocks the FLAG- $\beta_2$ AR recycling after agonist-induced endocytosis in cardiomyocytes. (B) Surface levels of the m $\beta_2$ ARs 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

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mins of isoproterenol stimulation; 3. 30 minutes isoproterenol followed by 30 minutes of drug removal; and 4. 30 minutes isoproterenol followed by 60 minutes of drug removal). Surface levels were normalized as percent of untreated cell surface fluorescence and error bars reflect standard deviations over 3 experiments. \*,  $P < 0.05$ ; significantly different between cells with and without NEM treatment by *t*-test.

**Figure 5: Dominant negative NSF E329Q mutant inhibits the  $m\beta_2AR$  coupling to Gi protein.** Spontaneously-beating cardiac myocytes from  $\beta_1AR$  KO mice were transfected with a wild-type NSF or NSF E329Q mutant adenovirus as indicated. The cells were administered 10  $\mu M$  isoproterenol with inhibition of Gi 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  $m\beta_2AR$ . The data represent the mean  $\pm$  SE of experiments from at least three different myocyte preparations. \*,  $P < 0.05$ ; time course significantly different by two-way ANOVA.

**Figure 6: Differences in  $\beta_2AR$  contraction rate responses to isoproterenol in neonatal cardiac myocytes from  $\beta_1/\beta_2AR$  KO mice.** The  $h\beta_2AR$  and  $m\beta_2AR$ -P417L exhibit different contraction rate profiles than the  $m\beta_2AR$  at comparable expression levels. Spontaneously-beating, cardiac myocytes from  $\beta_1/\beta_2AR$  KO mice were infected with a FLAG-tagged  $m\beta_2AR$  (A),  $h\beta_2AR$  (B), or  $m\beta_2AR$ -P417L (C) recombinant adenovirus as indicated and infused with 10  $\mu M$  isoproterenol. Contraction rates were measured and normalized as the change over baseline. The data represent the mean  $\pm$  SE

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of experiments from at least three different myocyte preparations. \*,  $P < 0.05$ ; time course significantly different by two-way ANOVA.

**Figure 7: NSF Binding enhances the Gi signaling components of  $\beta_2$ ARs.**

Spontaneously-beating cardiac myocytes from  $\beta_1/\beta_2$ AR KO mice were transfected with a FLAG-tagged m $\beta_2$ AR (A), m $\beta_2$ AR-P417L (B), or h $\beta_2$ AR (C) adenovirus as indicated. The cells were administered 10  $\mu$ M isoproterenol with inhibition of Gi with PTX. PTX treatment did not affect initial response usually mediated by receptor/Gs coupling (D), but significantly enhanced the contraction rate during the late stimulation induced by the Gi coupling to the activated h $\beta_2$ AR, m $\beta_2$ AR, or m $\beta_2$ AR-P417L (E). The data represent the mean  $\pm$  SE 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  $\beta_2$ ARs. \*\*\*,  $P < 0.05$ ; unpaired t-test significantly different on late contraction rate decreases after PTX treatment.

**Figure 8. Selectively disruptions of NSF and NHERF-1/EBP50 binding have distinct effects on  $\beta_2$ AR signaling.**

Spontaneously-beating cardiac myocytes from  $\beta_1/\beta_2$ AR KO mice were transfected with a FLAG-tagged m $\beta_2$ AR (A-D) or m $\beta_2$ AR-P417L (E-H) adenovirus as indicated. The cells were administered 10  $\mu$ M 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

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stimulation, which are usually mediated by receptor/Gi coupling (D and H). In contrast, PDZ peptide affected both initial contraction rate increase mediated by receptor/Gs coupling (C and G) and the late contraction rate response mediated by receptor/Gi coupling (D and H). The data represent the mean  $\pm$  SE 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.

Figure 1

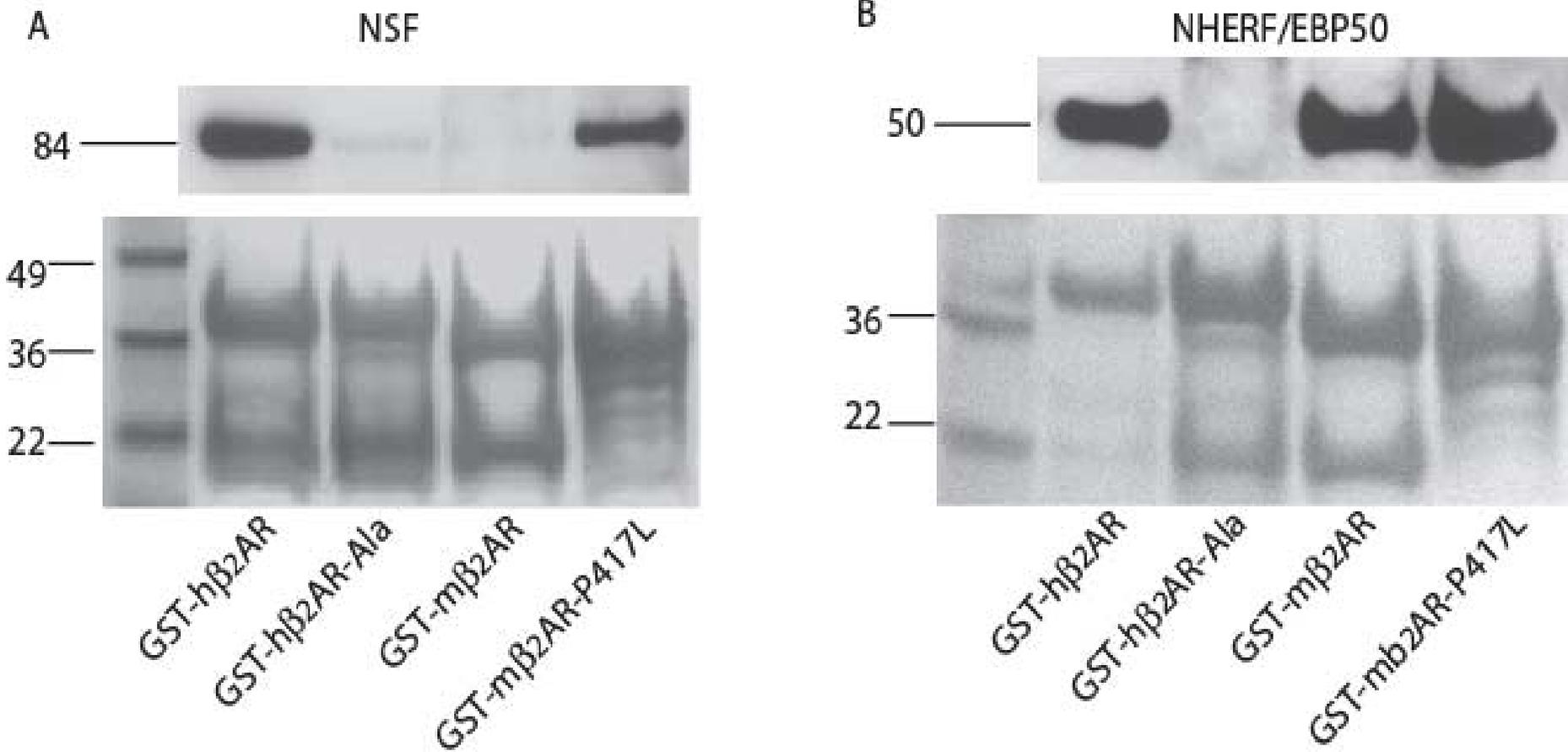
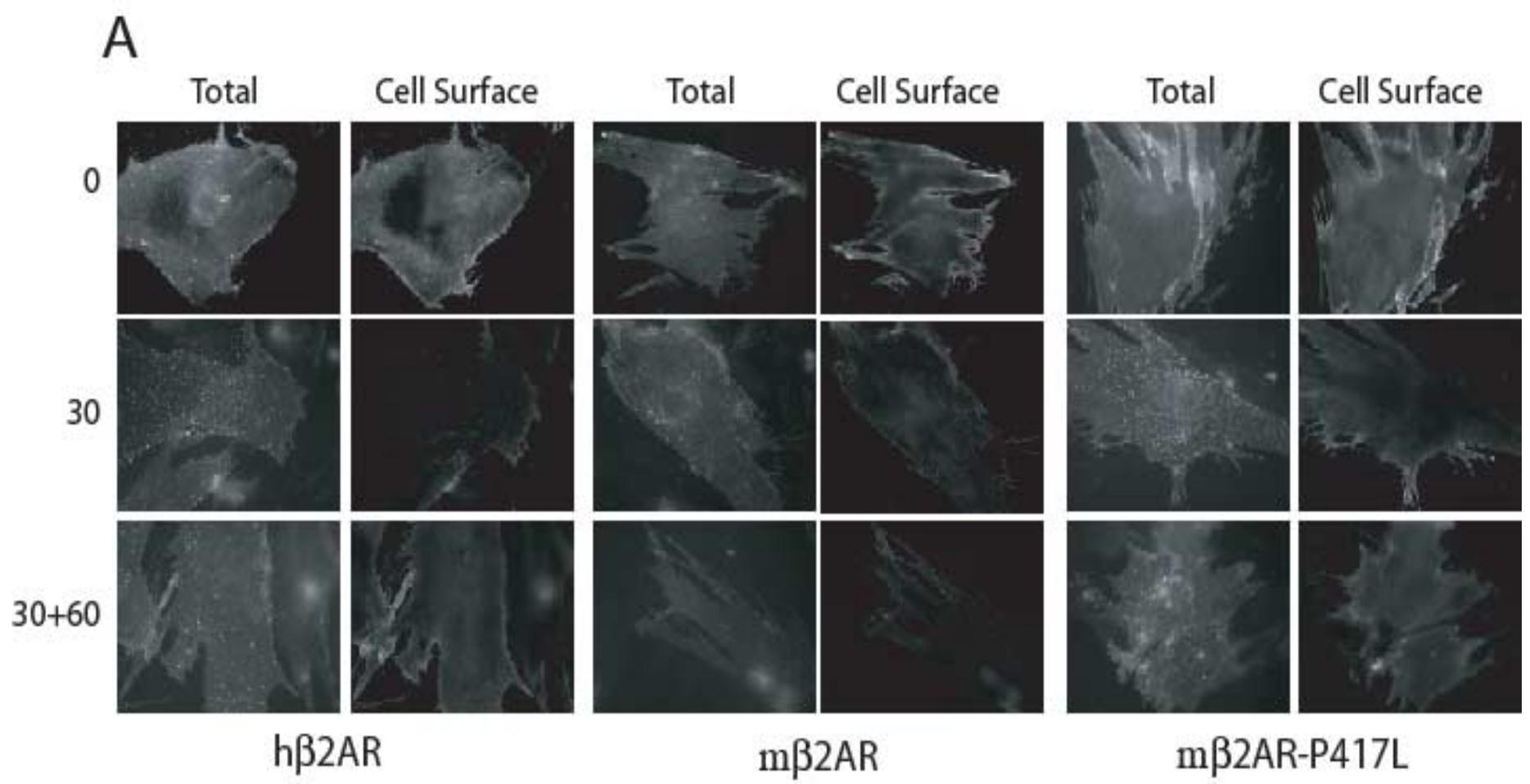


Figure 2



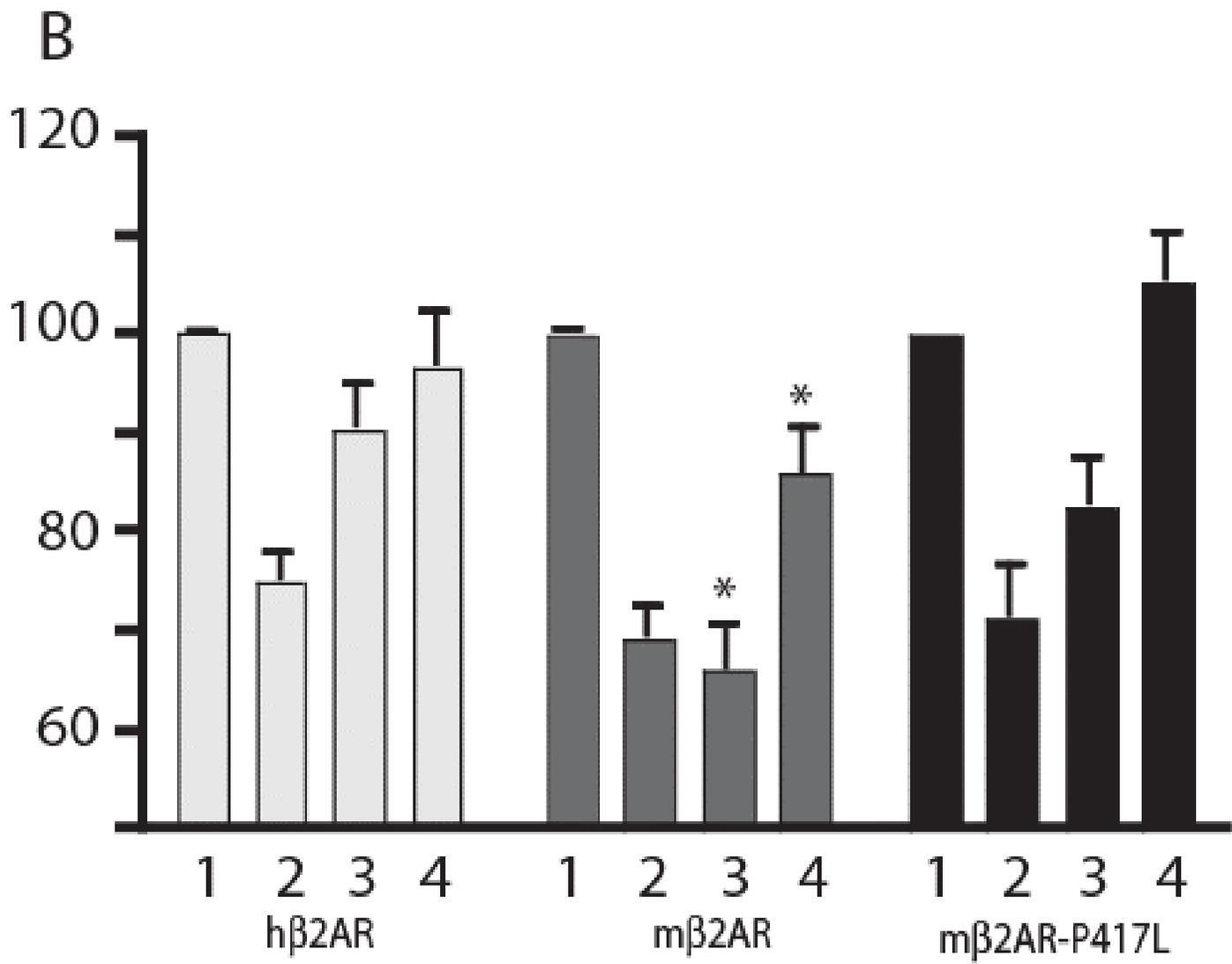


Figure 3

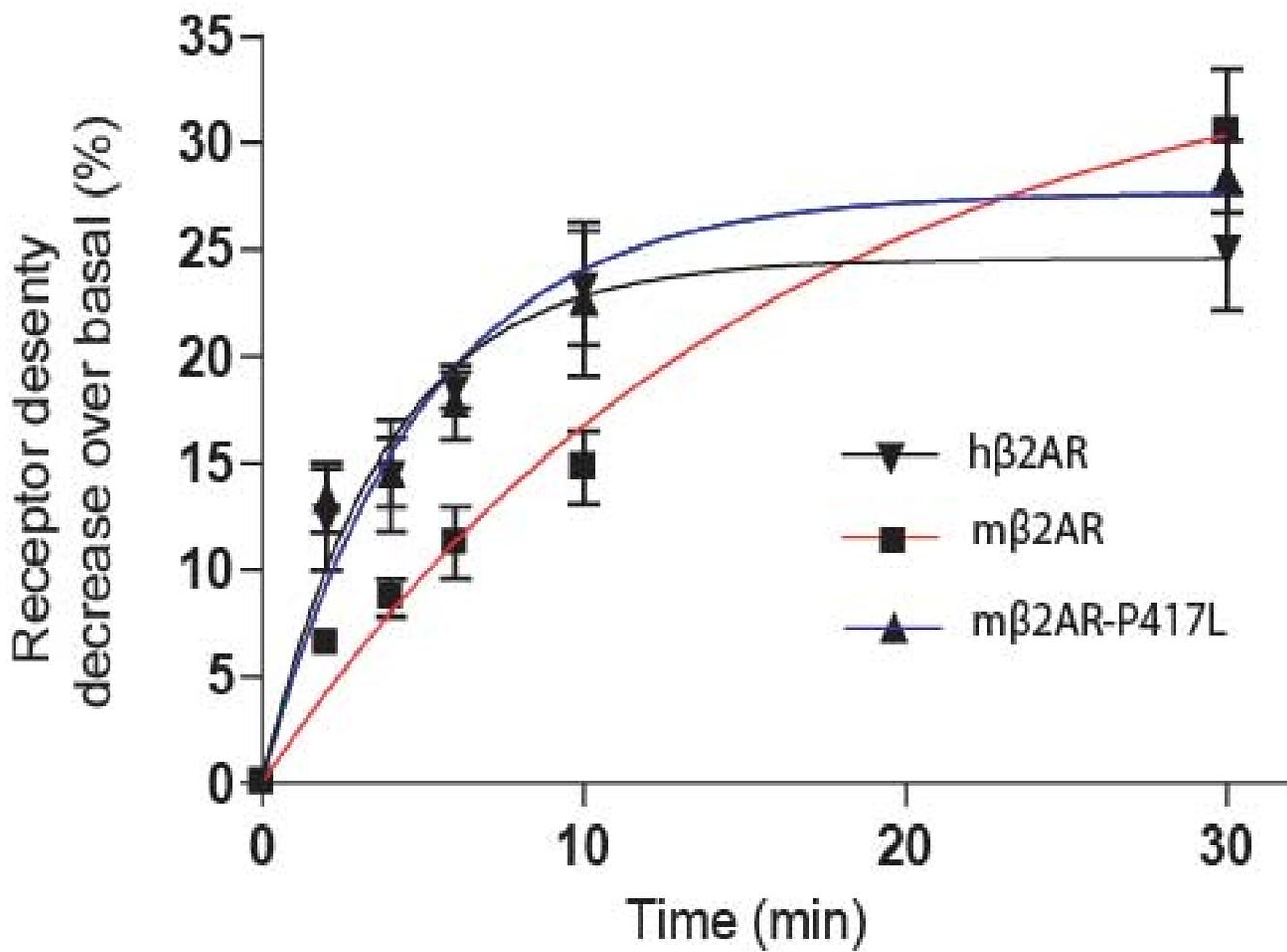
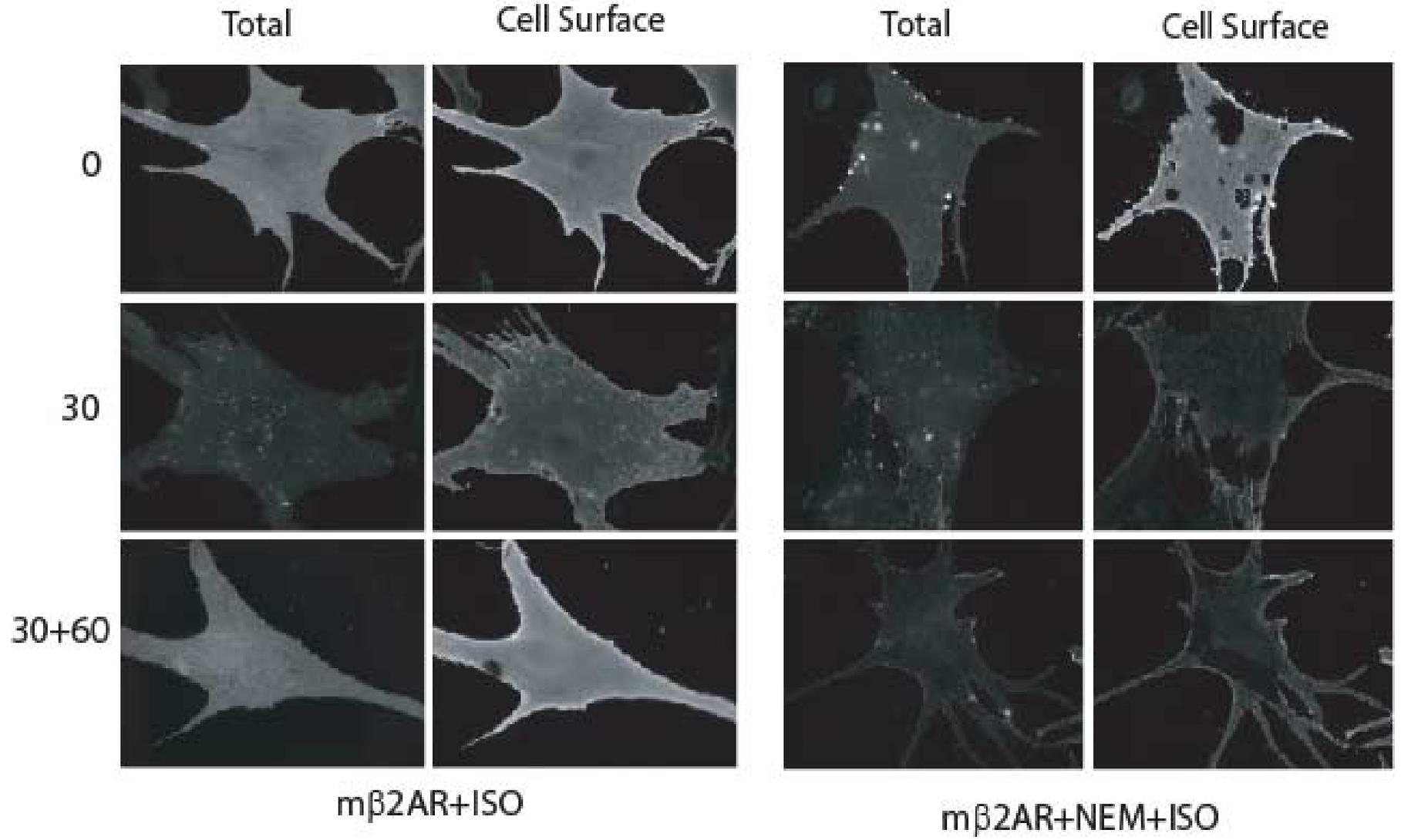


Figure 4

A



B

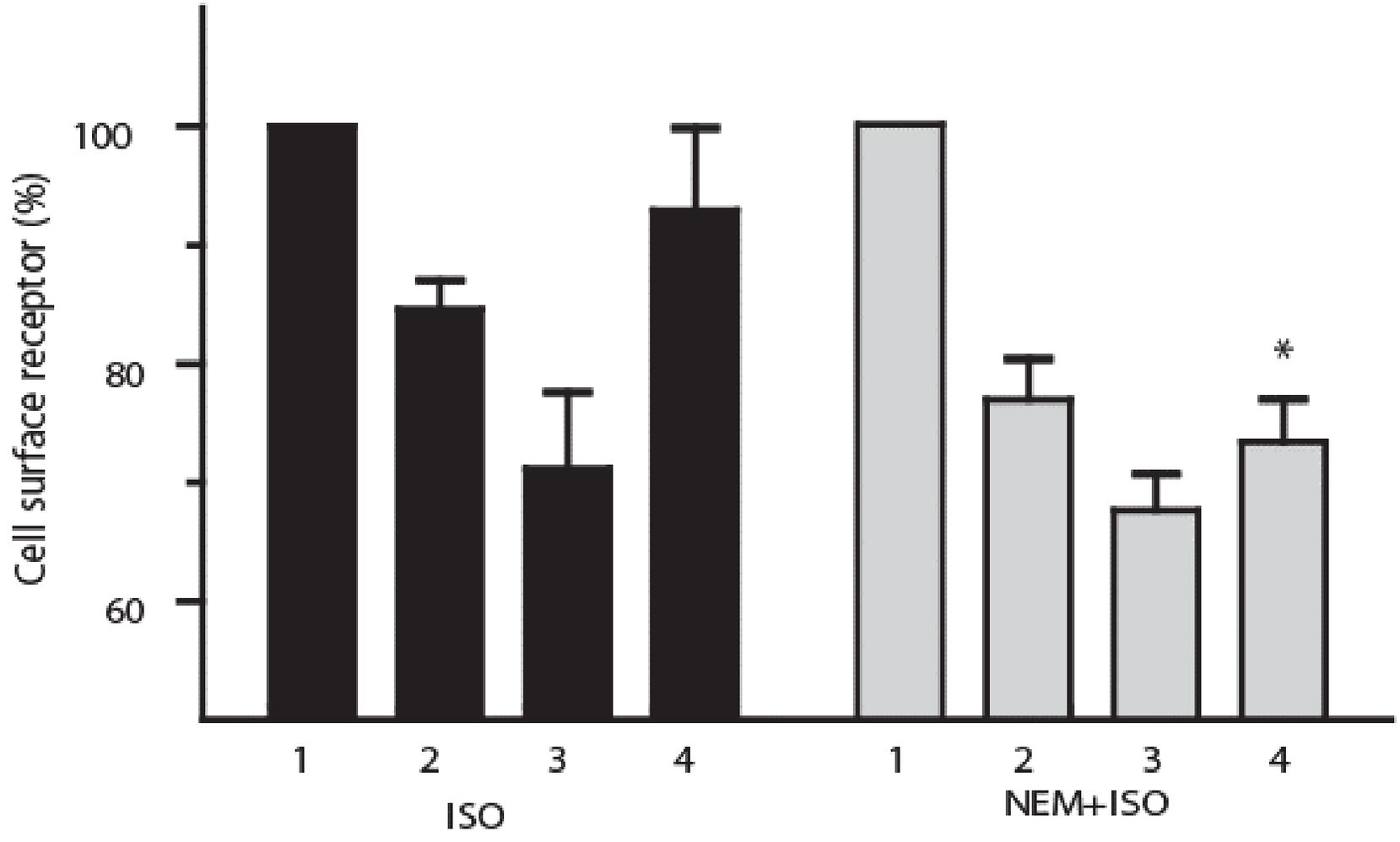


Figure 5

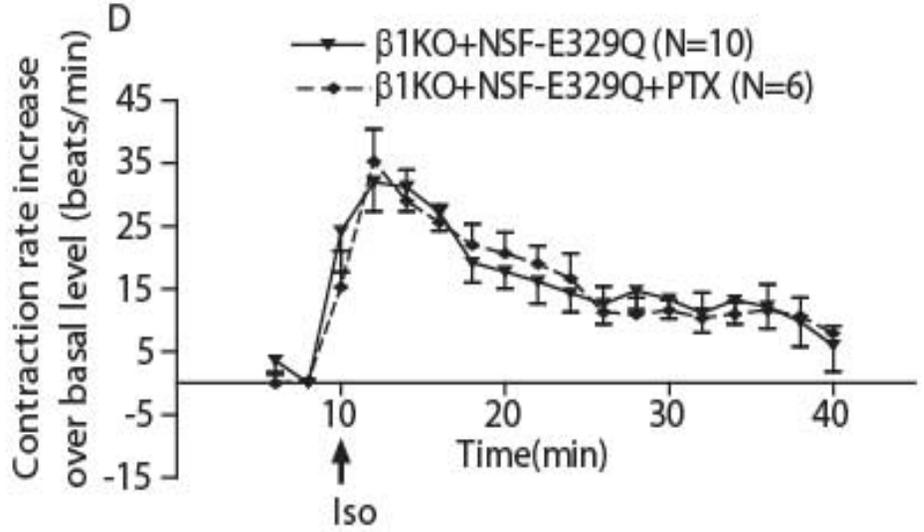
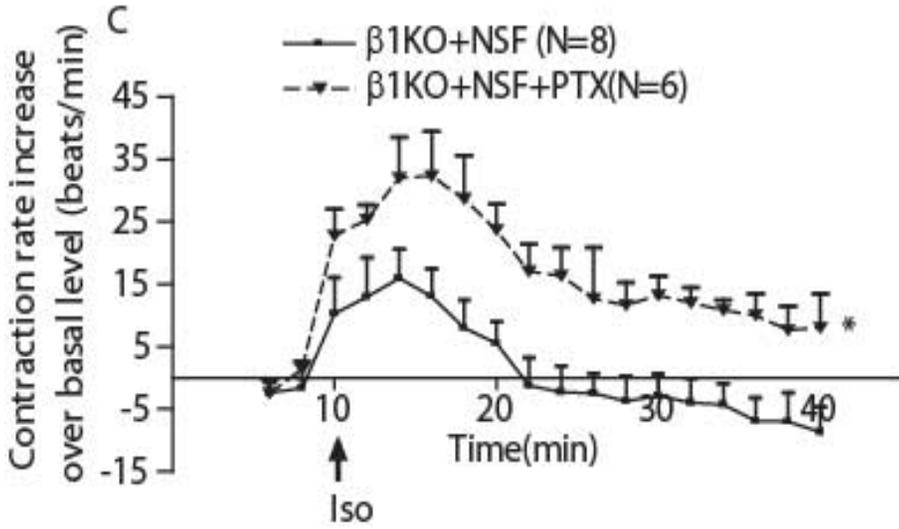
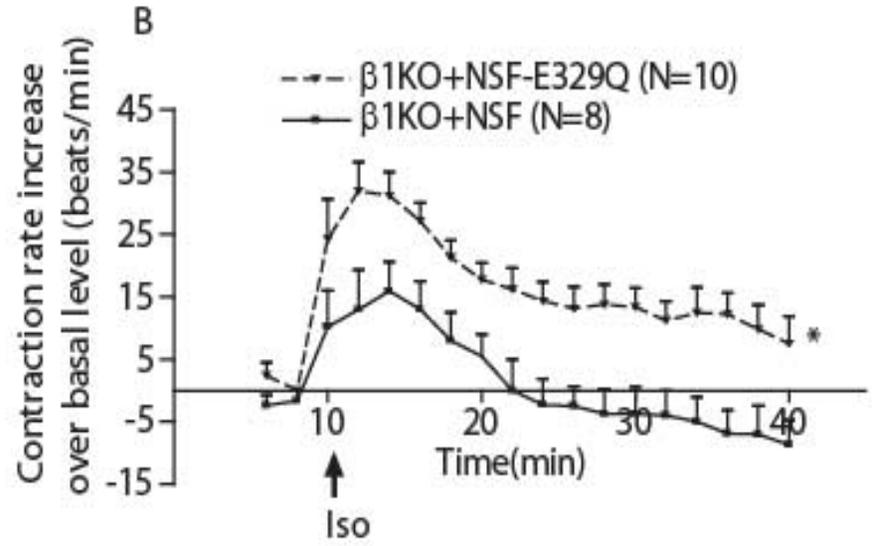
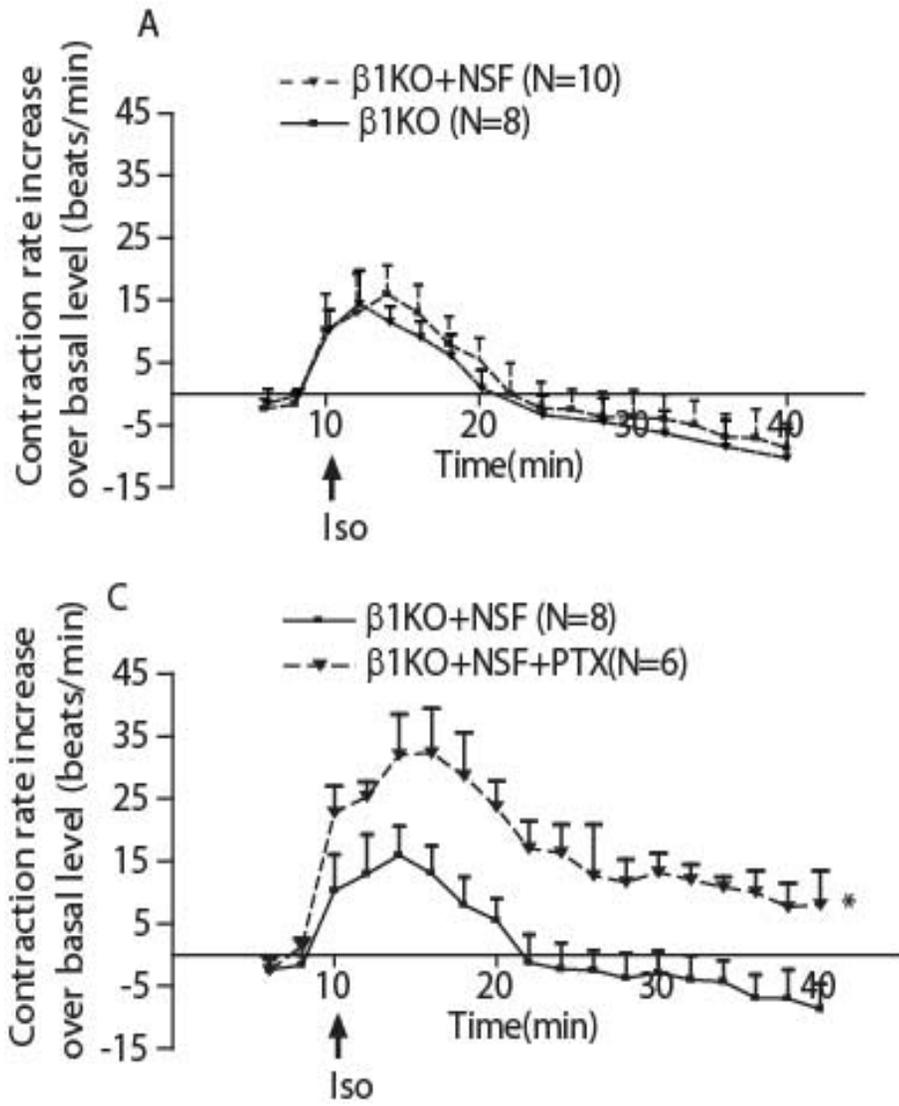
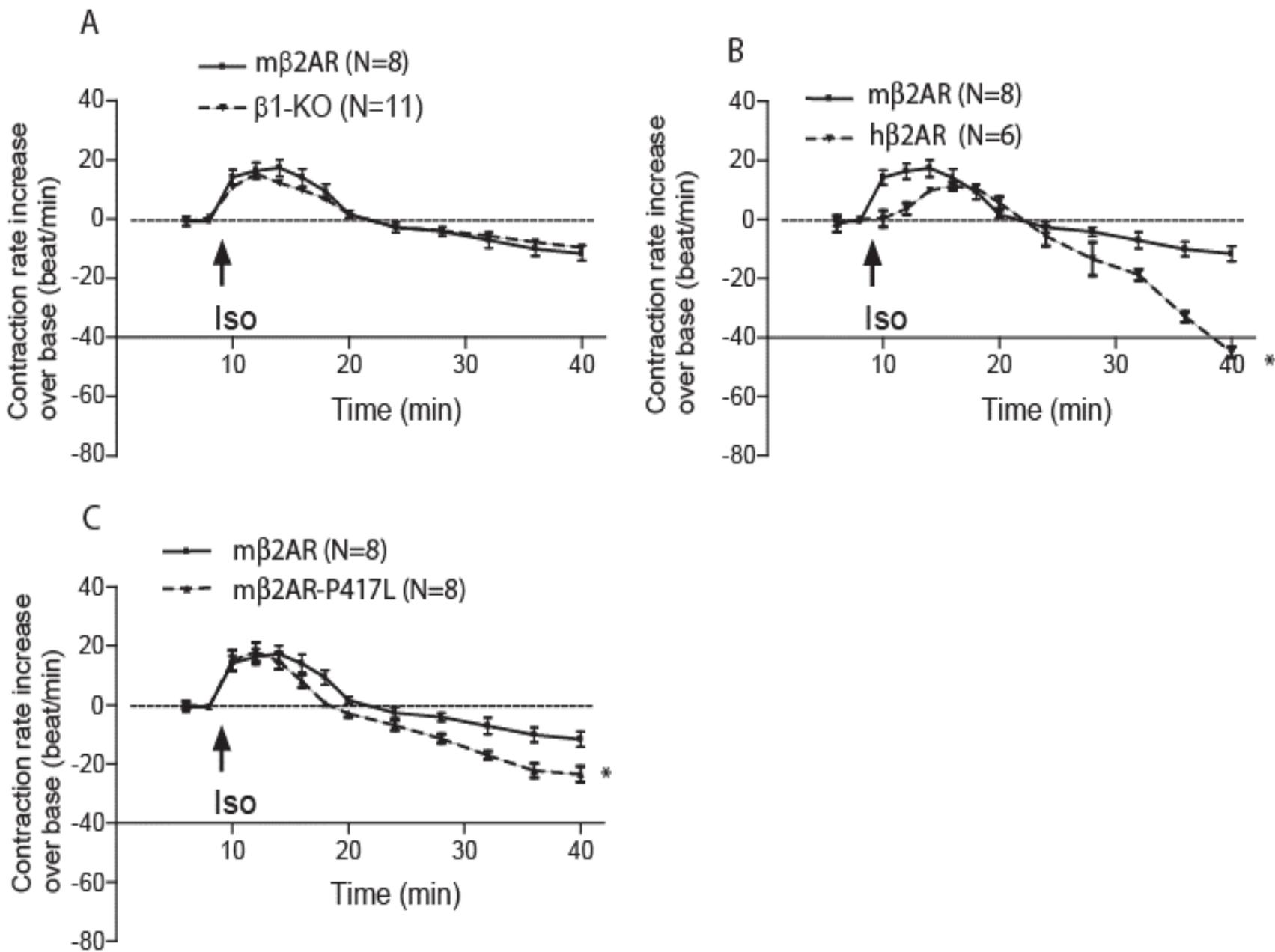


Figure 6



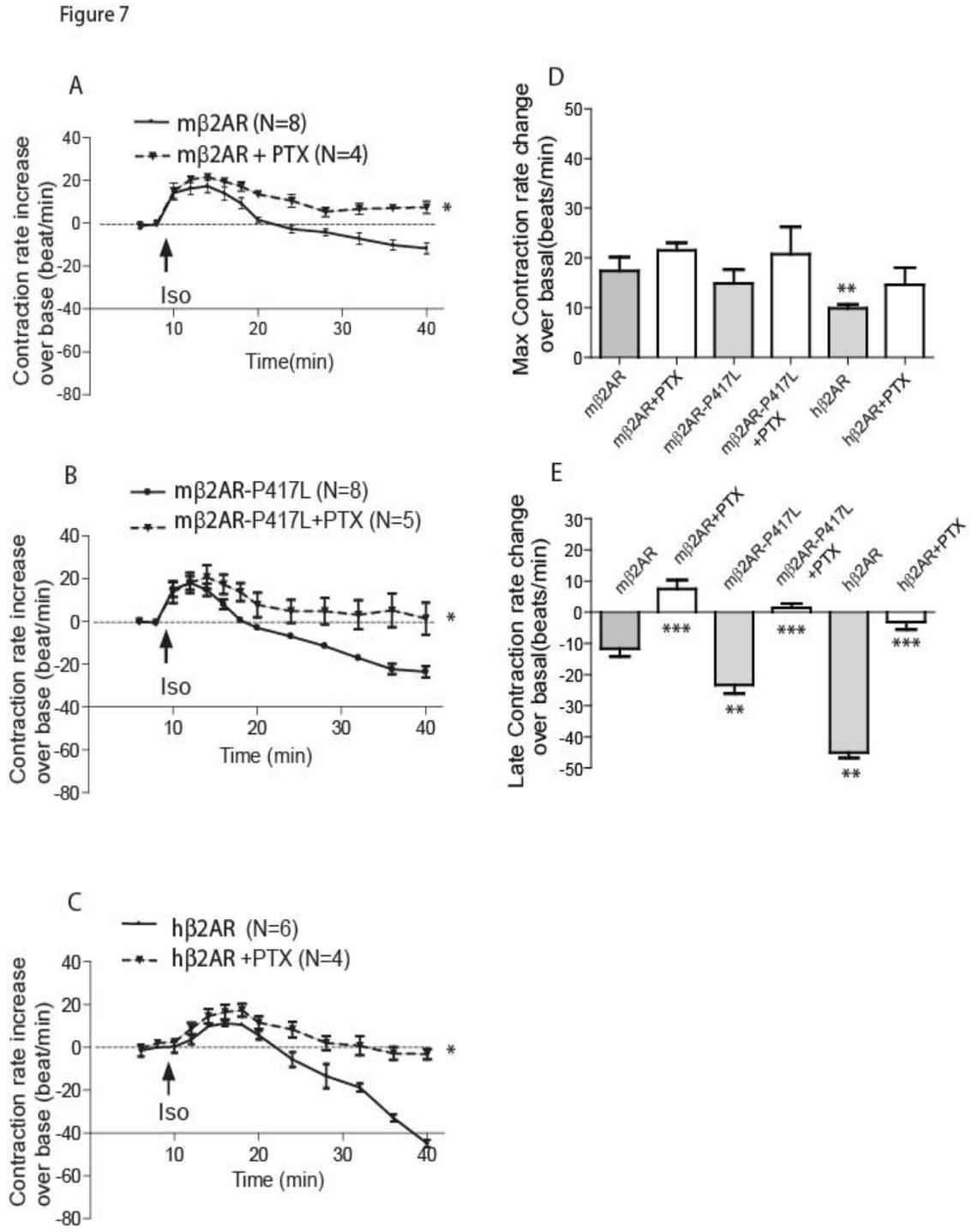


Figure 8

