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The effects of the β -agonist isoproterenol on the down-regulation, functional responsiveness, and trafficking of β_2 -adrenergic receptors with amino-terminal polymorphisms

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Abstract

The β_2 -adrenergic receptor (β_2 AR) is an important target for respiratory and cardiovascular disease medications. Clinical studies suggest that amino-terminal polymorphisms of the β_2 AR may act as disease modifiers. We hypothesized that polymorphisms at amino acids 16 and 27 result in differential trafficking and down-regulation of β_2 AR variants following β -agonist exposure. The functional consequences of the four possible combinations of these polymorphisms in the human β_2 AR (designated β_2 AR-RE, -GE, -RQ and -GQ) were studied using site-directed mutagenesis and recombinant expression in HEK 293 cells. Ligand binding assays demonstrated that after 24 h exposure to 1 μ M isoproterenol, isoforms with Arg16 (β_2 AR-RE and β_2 AR-RQ) underwent increased down-regulation compared to isoforms with Gly16 (β_2 AR-GE and β_2 AR-GQ). Consistent with these differences in down-regulation between isoforms, prolonged isoproterenol treatment resulted in diminished cyclic AMP response to subsequent isoproterenol challenge in β_2 AR-RE relative to β_2 AR-GE. Confocal microscopy revealed that the receptor isoforms had similar co-localization with the early endosomal marker EEA1 following isoproterenol treatment, suggesting that they had similar patterns of internalization. None of the

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Competing interests

Dr. Kurten received a grant from Astra-Zeneca AB (2008–2009). The other authors declare that they have no competing interests.

Authors' contributions

YK designed the studies, performed experiments, wrote and revised the manuscript. KS, LB, and PLP performed functional assay. AK performed sequencing from mammalian clones. SMJ, LEC and RCK conceived the studies, secured funding support, participated in the design and troubleshooting of the experiments and in the revision of the manuscript.

isoforms exhibited significant co-localization with the recycling endosome marker Rab11 in response to isoproterenol treatment. Furthermore, we found that prolonged isoproterenol treatment led to a higher degree of co-localization of β_2 AR-RE with the lysosomal marker Lamp1 compared to that of β_2 AR-GE. Taken together, these results indicate that a mechanism responsible for differential responses of these receptor isoforms to β -agonist involves differences in the efficiency with which agonist-activated receptors are trafficked to lysosomes for degradation, or differences in degradation in the lysosomes.

Keywords

β_2 -adrenergic receptor; receptor down-regulation; N-terminal polymorphisms; loss of binding sites; cyclic AMP response; confocal microscopy

1. Introduction

Asthma is a chronic airway disease that affects approximately 20 million Americans (Cheng and Arnold, 2008) and is characterized by airway inflammation and hyperresponsiveness to contractile agents. The β_2 -adrenergic receptor (β_2 AR) is the principal target for bronchodilator medications used to treat asthma. Agonist stimulation of β_2 AR inhibits contractile processes, resulting in bronchoprotection and bronchodilation. The β_2 AR is a prototypical G-protein coupled receptor (GPCR) (Rosenbaum et al., 2007). When activated, β_2 ARs are coupled to a stimulatory G-protein complex, thereby liberating the G_{sa} subunit that in turn activates adenylyl cyclase, catalyzing the conversion of ATP to the second messenger cyclic AMP. The resulting elevations in intracellular cyclic AMP activate several cytosolic kinases to induce relaxation of airway smooth muscle and bronchodilation (Bai and Sanderson, 2006).

Two general classes of β -agonists are currently in use: long-acting “controller” medications and short-acting “quick-relief” medications. Treatment of asthma by short-acting β -agonists (SABA) provides immediate short-lived reversal (“quick relief”) of airway obstruction whereas long-acting β -agonists (LABA) are used to sustain bronchoprotection. Chronic β -agonist administration is associated with deleterious side effects including hypersensitivity to contractile agents, decreased bronchodilation and poor clinical control (Inman and Adelstein, 1969; Conolly et al., 1971; Tsagaraki et al., 2006). The clinical phenomenon of tolerance thus limits the therapeutic efficacy of β -agonists. Both desensitization (a loss in cellular signalling without a net change in receptor number) and β_2 AR down-regulation (a net loss of receptors) are potential mechanisms for clinical tolerance.

Amino-terminal polymorphisms in the gene encoding the human β_2 AR have been associated with changes in respiratory function and in clinical disease markers (Israel et al., 2001; Palmer et al., 2006; Wechsler et al., 2006). The wild-type allele of the β_2 AR gene was initially characterized as encoding Arg and Gln at amino acid positions 16 and 27, respectively (Kobilka et al., 1987). However, Gly can be substituted for Arg at amino acid position 16, and Glu can be substituted for Gln at position 27. Although the frequency of these polymorphisms is the same in the normal population as in the asthmatic population (Dewar et al., 1998; Reihnsaus et al., 1993), randomized clinical trials revealed that the Arg16

polymorphism is associated with worsened lung function in patients treated with either short- or long-acting β -agonists (Israel et al., 2001; Wechsler et al., 2006). These studies also examined the effect of polymorphisms at positions 16 and 27 on patient responses to regular versus as-needed use of β -agonists. Regular use, as opposed to as-needed use, reduced peak expiratory flow in patients homozygous for Arg at position 16. These findings were corroborated by another study for albuterol (SABA) but not salmeterol (LABA) (Taylor et al., 2000). In keeping with the aforementioned studies, it was demonstrated that the Arg16 polymorphism predisposed young asthmatics treated with salmeterol to exacerbations (Palmer et al., 2006). Arg16 has also been associated with subsensitivity of response to bronchoprotection (Lee et al., 2004). By contrast, the clinical significance of polymorphisms at position 27 is unclear (Israel et al., 2001; Palmer et al., 2006). Some studies did not find an association between β_2 AR N-terminal polymorphisms and response to asthma therapy (Bleecker et al., 2006, 2007). Taken together, the data indicate that clinical response to β -agonist medications, including susceptibility to adverse effects, may be modulated by receptor genotype. In particular, patients homozygous for Arg at position 16 (~17% of Caucasian and 20% of African-Americans) might benefit from alternate asthma treatment strategies. It is therefore important to understand the molecular events underlying differential responses to β -agonist treatment to facilitate the development of more effective therapies.

The functional consequences of amino-terminal polymorphisms of the human β_2 AR have been studied by site-directed mutagenesis and recombinant expression of these receptors in Chinese hamster fibroblasts (CHW cells), which normally do not express β_2 AR (Green et al., 1994). In these studies, all four polymorphic variants of the β_2 AR (Arg16+Glu27, Gly16+Glu27, Arg16+Gln27 and Gly16+Gln27 designated herein as β_2 AR-RE, -GE, -RQ and -GQ, respectively) displayed normal agonist binding and stimulation of adenylyl cyclase activity. However, they differed in agonist-promoted down-regulation of receptor numbers. Prolonged agonist exposure resulted in increased down-regulation of β_2 AR-GQ (Gly16+Gln27) and β_2 AR-GE (Gly16+Glu27) compared to β_2 AR-RQ (Arg16+Gln27). On the other hand, β_2 AR-RE (Arg16+Glu27) was shown to be resistant to down-regulation. No difference in β_2 AR mRNA level was detected in this study, indicating that differences in receptor degradation may account for down-regulation. Polymorphic receptors were also studied in primary cultures of human airway smooth muscle (HASM) cells, with similar results (Green et al., 1995). Cell lines homozygous for Arg16, Gly16, or Glu27 were selected for this study. No cell line was found to be homozygous for Gln27, nor was any cell line homozygous at both alleles. Prolonged agonist exposure resulted in profound functional desensitization (cyclic AMP response) of β_2 AR-Rx (Arg16) and β_2 AR-Gx (Gly16) compared to β_2 AR-xE (Glu27). This study is notable because β_2 AR are endogenously expressed in the native context, but the combinations of alleles (and especially the heterozygous alleles at the other locus) preclude definitive interpretation. Taken together, the results of these studies indicate that amino-terminal polymorphisms of the β_2 AR may impart altered susceptibility to agonist-promoted down-regulation.

The present study used recombinant human β_2 AR with amino-terminal polymorphisms in a human expression system to study the effects of β -agonist on the cellular trafficking of the receptors in response to β -agonist. We used confocal microscopy to examine trafficking of

the polymorphic receptors to elucidate the mechanism(s) for differences in receptor down-regulation underlying differential response to therapy. Yellow fluorescent protein (YFP) was used as a reporter to facilitate detailed temporal and spatial analysis of the receptor responses to β -agonist treatment. We found that the Arg16 isoforms were down-regulated to a greater extent than the Gly16 isoforms. The results suggest that this could be due to enhanced trafficking to lysosomes and/or degradation in lysosome.

2. Methods

2.1 Cell Culture, Plasmids, and Transfection

The human embryonic kidney cell line, HEK 293, was maintained in Dulbecco's modified Eagle's medium/Ham's F12 (50:50) (Cellgro, Herndon, VA) supplemented with 5% calf serum, 1% antibiotic/antimycotic in a 5% CO₂ incubator at 37°C. The human β_2 AR coding sequence was previously amplified from genomic DNA. The human β_2 AR cDNA was cloned in pEYFP-N1 vector (Clontech, Mountain View, CA) (Jones et al., 2003; Schnackenberg et al., 2006). Insertion of the β_2 AR cDNA into vector results in addition of the YFP tag at the C-terminus proximal to the stop codon. The β_2 AR cDNA codes for Arg at amino acid position 16 and Gln at position 27 and is designated as β_2 AR-RQ. β_2 AR-RQ was used as the template to generate β_2 AR-GQ (Gly at amino acid position 16, Gln at amino acid position 27) and β_2 AR-RE (Arg at amino acid position 16, Glu at amino acid position 27) by using Quik Change II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) - 3 step protocol with primers 5'-cttcttgctggcaccacatggaagccatgcg-3' (sense), 5'-cgcgatggcttcattgggtgccagcaagaag-3' (antisense) changing Arg to Gly at position 16 and 5'-acgacgtcacgcaggagaggacgaggtgtg-3' (sense), 5'-cacacctgctcctctcctgctgacgtcgt-3' (antisense) changing Gln to Glu at position 27, respectively. β_2 AR-GQ was used as the template to generate β_2 AR-GE (Gly at amino acid position 16, Glu at amino acid position 27) in the same manner. The identity of newly generated constructs was confirmed by automated sequencing and BLAST analysis. pEYFP-N1 vector (Clontech, Mountain View, CA) contains a G418 resistance cassette for positive selection in mammalian cells. Stable transfections were made by transfecting HEK 293 cells at 60–80% confluency with 1 μ g of cDNA in 35 mm dishes using the calcium phosphate precipitation method (Chen and Okayama, 1988). Forty-eight hours after transfection, cells were subcultured and maintained in complete medium containing 500 μ g/ml G418 (Life Technologies, Inc.). After 4 weeks, antibiotic resistant clones expressing YFP were selected and reseeded in 48-well plates. Isolated colonies were maintained on medium supplemented with 200 μ g/ml G418. Clones were screened to determine the level of receptor expression by using a radioligand binding assay. Cell lines expressing approximately equivalent numbers of receptor for each of the polymorphic clones were selected for further studies. To confirm that the receptor expressed is of expected genotype, clones were sequenced directly from the cell lines chosen for the studies. Briefly, genomic DNA from the selected clones was extracted using DNeasy Blood and Tissue Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer instructions. The target DNA sequence of the ADRB2 containing region with single nucleotide polymorphisms was amplified from genomic DNA using Phusion High Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) and a set of primers to generate an amplicon of 787 bp in length. The amplified products were electrophoresed on an agarose

gel (1%) and visualized with ethidium bromide staining to ascertain whether the amplification had been successful. The amplified product was excised from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN Inc.). The amplified fragments were sequenced using automated sequencing with a set of primers permitting analysis of both polymorphic loci.

2.2 Receptor binding assay using intact cells

For receptor binding assays, polymorphic clones were grown in 6-well plates to near confluence and treated with 1 μ M isoproterenol (Sigma-Aldrich, St. Louis, MO) for 20 min, 2 h and 24 h. Cell monolayers were lifted with cold PBS supplemented with 5 mM EDTA using a rubber policeman and washed twice with PBS by centrifugation. Cells were counted and 1,000,000 of cells for each treatment were taken for the assay. Cells were incubated in triplicate with a single saturating concentration of [3 H]dihydroalprenolol (DHA) (~5 nM) (Perkin Elmer, Boston, MA; specific activity = 117.8 Ci/mmol) for 20 min at 30°C. Incubations were terminated by vacuum filtration through Whatman glass fiber filters (Optics Planet, Inc., Northbrook, IL) pre-soaked in assay buffer (50 mM Tris, 2 mM MgCl_2 , pH 7.4) and repeated washes with ice-cold assay buffer. Bound radioactivity was determined by liquid scintillation analyzer (Packard BioScience Company, Meriden, CT). Nonspecific binding was determined by using 0.1 μ M (–)-propranolol (Sigma, St. Louis, MO).

2.3 Measurement of β_2 AR-mediated stimulation of adenylyl cyclase activity

The conversion of the [3 H]adenine-labeled ATP pools to cyclic AMP was used as a measure of isoproterenol effect on cyclic AMP levels as described previously (Prather, et al., 1994). Briefly, measurements were made with β_2 AR-RE and β_2 AR-GE clones seeded into 6-well plates. Cells were near 100% confluence. Cells were untreated or treated with 1 μ M isoproterenol for 24 h. On the day of the assay, media were removed and replaced with incubation mixture (warmed to 37°C) (DMEM containing 0.09% NaCl, 500 μ M 3-isobutyl-1-methylxanthine, and 2 μ Ci/well [3 H]-adenine) for 1 h. At the time of the assay, plates were placed in an ice-water bath for 5 min. The incubation mixture was then removed and replaced with ice-cold assay mixture [Krebs-Ringer HEPES buffer (110 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , 25 mM glucose, 55 mM sucrose, and 10 mM HEPES, at pH 7.4) containing 500 μ M 3-isobutyl-1-methylxanthine] and 1 μ M isoproterenol. The plates were then incubated at 37°C for 15 min and placed back in the ice-water bath for 5 min. After termination of incubations with 50 μ l of 3.3 N perchloric acid and subsequent addition of [32 P]cyclic AMP as an internal standard, radioactive cyclic AMP was separated from other 3 H-labeled nucleotides by a double-column chromatographic method. Seven milliliters of scintillation fluid was then added and samples were immediately counted in a Beckman LS2800 scintillation counter.

2.4 Fluorescence microscopy

For indirect immunofluorescence microscopy, cells were grown on glass coverslips, treated with 1 μ M isoproterenol for the time points indicated in the experiments. For indirect immunofluorescence using Lamp1 antibody, cells were fixed with freshly prepared 3.6% paraformaldehyde, 0.024% saponin, 2.5 mM EDTA, 1 mM sodium vanadate in PBS, pH 7.2 for 15 min. Free aldehyde groups were reduced with 0.1% sodium borohydride in PBS for

10 min, and cells were rinsed with PBS, blocked and permeabilized in buffer containing 5% BSA, 0.012% saponin, 1 mM sodium vanadate, pH 7.2 for 30 min. For indirect immunofluorescence using EEA1, Rab11 and β_2 AR antibody, cells were fixed with freshly prepared 3.6% paraformaldehyde in PBS, pH 7.32 for 15 min. Blocking/ permeabilization buffer contained 5% BSA, 0.1% Triton X -100, pH 7.32. Organelle markers were visualized using the labelled avidin-biotin method. Samples were incubated with primary antibody followed by separate incubations with biotinylated secondary antibody and with Texas-Red labeled Avidin D (Vector Laboratories Inc., Burlingame, CA). Optimal dilutions of the antibodies were determined in titration experiments. Antibodies were diluted in the blocking/ permeabilization buffer and samples washed with PBS after each incubation. The nuclei were stained with 20 nM 4,6-diamidinophenylindole (DAPI). Antibody dilutions and sources were as follows: mouse monoclonal anti-Lamp1 (H4A3, 8, Developmental Studies Hybridoma Database), 1:100; mouse monoclonal anti-EEA1 (Transduction Laboratories, Lexington, KY), 1:300; rabbit polyclonal anti-Rab11 (Abcam, Inc., Cambridge, MA), 1:200; and chicken polyclonal anti- β_2 AR (Abcam, Inc.), 1:500. Secondary goat anti-rabbit (Vector Laboratories, Inc.), goat anti-mouse (Vector Laboratories, Inc.), and rabbit anti-chicken (Abcam, Inc.) biotinylated antibodies were used at a dilution of 1:200. Samples were mounted in Fluoromount-G mounting medium (Electron Microscopy Sciences, Hatfield, PA) and visualized by dual confocal microscopy using the LSM510 multitasking configuration (YFP, λ_{ex} = 516 nm, λ_{em} = 530–600 nm BP; Texas Red, λ_{ex} = 561 nm, λ_{em} = 575 nm LP). A Zeiss Plan-Apo 63x 1.40NA oil immersion objective was used for image acquisition. The acquisition settings were kept constant between specimens. Images were stored as a tagged image format and were analyzed as previously described (Kurten et al., 2001). Co-localization was quantified by calculating the percentage of overlap of YFP-tagged β_2 AR on the specific intracellular compartment being analyzed. Using ImageJ, the yellow and red channels of the RGB image were thresholded and converted into binary images. The binary images were multiplied to generate a binary overlap image. Only co-localized pixels above threshold give a nonzero value in the product image. The number of pixels in the product image were divided by the number of pixels in the β_2 AR-YFP image and converted to percentage of co-localization of β_2 AR with the compartment marker.

2.5 Flow cytometry

For flow cytometry experiments, cells were seeded in 6-well plates. Cells were about 80% confluence. Cells were untreated or treated with 1 μ M isoproterenol for 24 h, trypsinized, collected and analyzed on BD FACSCalibur flow cytometer (BD Biosciences). YFP fluorescence was detected in FL1 channel with CellQUEST Pro Software (Becton-Dickinson). Data were analyzed using FlowJo 8.6.6 software.

2.6 Data Analysis/ Statistical Methods

In radioligand binding experiments, [3 H]DHA binding to cells at each time point was measured in triplicate. Each “n” represented data from one set of cell culture plates (one condition). Experiments were performed at n = 4. The n value of an experiment represents experiments performed on different days. In trafficking experiments, 10 – 12 images were analysed for each time point in each experiment. Experiments were performed at n = 3 on three separate days. Bartlett’s, Cochran’s, and Hartley’s tests were applied to assess

homogeneity of variances between experiments. Data are presented as the mean $\pm$ S.E.M. based on data averaged from 3 independent experiments. To analyse data from experiments with multiple time points, two-way analysis of variance followed by HSD Tukey's post hoc test was used for all possible pairwise comparisons with $p < 0.05$ accepted as significant.

1. Results

3.1 Characterization of polymorphic clones

To study the effect of β -agonist on trafficking and down-regulation of the β_2 AR with N-terminal polymorphisms *in vitro*, HEK 293 cells were stably transfected with YFP-tagged β_2 AR polymorphic cDNA. The parent HEK 293 cell line was selected because it expresses a negligible amount of endogenous β_2 AR (von Zastrow and Kobilka, 1992), is suitable for trafficking and radioligand binding experiments, and has a human origin. Clones were designated β_2 AR-RQ, β_2 AR-GQ, β_2 AR-RE, and β_2 AR-GE, where the first letter stands for the amino acid at position 16 (R for arginine and G for glycine) and the second letter stands for the amino acid at position 27 (Q for glutamine and E for glutamic acid). Stable transfectants were assessed for β_2 AR expression using the hydrophobic β -antagonist [3 H]DHA. Clones expressing approximately equivalent levels of the YFP-tagged β_2 AR were selected for further experiments. β_2 AR-RQ and β_2 AR-GQ clones expressed 840,000 and 758,000 receptors/ cells, respectively, and β_2 AR-RE and β_2 AR-GE expressed 650,000 and 840,000 receptors/ cells, respectively. Sequencing of DNA directly from the mammalian cell clones chosen for the experiments was performed to verify that the receptor expressed is of the expected genotype. The YFP tag permits a detailed spatial and temporal analysis of the trafficking of the receptor isoforms. Previous studies have shown that the pharmacological properties of GFP- and YFP-tagged β_2 ARs are similar to the wild type receptor (Barak et al., 1997; Kallal et al., 1998; Angers et al., 2000). To verify that the YFP tag is a valid surrogate marker to trace β_2 AR, we performed indirect immunofluorescence using a well-characterized β_2 AR-specific antibody (Koryakina et al., 2008). Confocal microscopy confirmed that the cells expressed the YFP-tagged β_2 AR on their surface in the absence of agonist and that the YFP-tagged β_2 AR was internalized after treatment with the β -agonist isoproterenol. Antibody staining for the β_2 AR confirms that the YFP signal traces the β_2 AR (Figure 1).

3.2 Prolonged isoproterenol treatment resulted in enhanced down-regulation of isoforms with arginine at amino acid position 16

The effect of isoproterenol on density of the β_2 AR with amino-terminal polymorphisms was determined by measuring receptor levels using a cell permeable β -antagonist that detects both cell surface and internalized receptors. Isoproterenol treatment for 20 min resulted in little change in receptor number. Exposure to isoproterenol for 2 h led to a 10 – 20% loss of binding sites, but no differences in receptor density were noted between isoforms. However, 24 h treatment with isoproterenol resulted in significant differences in receptor down-regulation, depending on the isoform (Figure 2). Isoforms with Arg at amino acid position 16 underwent increased down-regulation: β_2 AR-RQ and β_2 AR-RE had 50.6% and 70.2% reduction in receptor binding sites, respectively, whereas those with Gly at position 16 were relatively resistant to down-regulation: β_2 AR-GQ and β_2 AR-GE lost 27.7% and 23.2% of

binding sites, respectively (Figure 2). Isoproterenol treatment did not affect cell viability as assessed by trypan blue staining. β_2 AR-RE exhibited the most striking reduction in receptor number, indicating that glutamic acid at position 27 has a greater tendency to agonist-induced down-regulation in the presence of agrinine at position 16. However, β_2 AR-GQ and β_2 AR-GE isoforms had similar down-regulation profiles, implying that any effect of the amino acid at position 27 to enhance down-regulation is suppressed by the dominant effect of glycine at position 16. Our subsequent experiments were focused on β_2 AR-RE and β_2 AR-GE because these isoforms exhibited the most and the least agonist-induced receptor down-regulation, respectively.

The receptor down-regulation was analyzed in further detail by performing time-course studies for the chosen isoforms. β_2 AR-RE and β_2 AR-GE clones were treated with isoproterenol and radioligand binding measured after 2, 4, 8, 12, 18, and 24 h. Both clones down-regulated over the time-course study (Figure 3). After 4 h of treatment, β_2 AR-RE receptor levels down-regulated more than those of β_2 AR-GE. This difference in receptor down-regulation was maintained throughout the time-course studies and was the greatest after 24 h treatment. As a complimentary approach to assess receptor down-regulation, we monitored change in YFP fluorescence by flow cytometry. Prolonged exposure to isoproterenol resulted in a shift in YFP fluorescence intensity compared to untreated cells in both clones (Supplement Figure 1A and B). β_2 AR-RE isoform had 55% reduction in mean fluorescence intensity, whereas the decrease was only 20.4% in β_2 AR-GE (Supplement Figure 1C). Thus, down-regulation of the receptor detected in binding experiments correlated well with decrease in intensity of YFP fluorescence.

3.3 Chronic isoproterenol treatment results in reduced cyclic AMP response to β -agonist for β_2 AR-RE compared to β_2 AR-GE

We determined the extent to which differences in agonist-induced down-regulation for β_2 AR-RE and β_2 AR-GE corresponded to differences in their functional activation of adenylyl cyclase to elevate intracellular cyclic AMP. Cells were either untreated or chronically (24 h) treated with isoproterenol, and cyclic AMP accumulation was then measured in response to acute isoproterenol challenge. In untreated cells, isoproterenol treatment for 15 minutes stimulated significant increases in cyclic AMP accumulation in both clones, indicating that both β_2 AR-RE and β_2 AR-GE mediate the appropriate physiological response. With long-term (24 h) isoproterenol treatment, cyclic AMP accumulation in response to acute agonist challenge was reduced by 61.7% in β_2 AR-RE expressing cells and by 48.3% in β_2 AR-GE expressing cells (Figure 4). Thus, differences in down-regulation of the receptor isoforms as defined by ligand binding are paralleled by changes in functional responses.

3.4 β_2 AR-RE and β_2 AR-GE exhibit similar degrees of co-localization with the early endosome marker EEA1

After agonist exposure, β_2 ARs are internalized to endosomes (von Zastrow and Kobilka 1992, 1994; Gagnon et al., 1998). Some β_2 ARs are then recycled back to the plasma membrane (Pippig et al., 1995; Hanyaloglu et al., 2005), others utilize an indirect recycling route through the pericentriolar recycling endosome (Moore et al., 2004), and some are

sorted to the lysosome for degradation (Kallal et al. 1998; Moore et al., 1999). We hypothesized that differences in the down-regulation of the isoforms were due to differences in trafficking. To follow trafficking of the YFP-tagged β_2 AR, we used cell compartment specific markers: early endosome antigen (EEA1) to define early endosomes, lysosomal integral membrane protein Lamp1 to define lysosomes, and the small GTPase Rab11 to define recycling endosomes.

To assess the internalization of receptors, cells were treated with isoproterenol, and samples were analyzed every 15 min during the first one and a half hours, and after 2, 6, 12, and 18 h. In unstimulated cells, the receptors were located on the cell surface, and co-localization with EEA1 was marginal (Figure 5K). Addition of agonist results in β_2 AR endocytosis observed as a sharp increase in co-localization with EEA1 after 15 min of isoproterenol treatment (Figure 5A–J, K), reaching a maximum of approximately 55% at that time point. Co-localization then gradually declined during next 2 h, returning to basal levels after 2 h of agonist treatment. Over the time course of the assay, there was no difference in the transit of β_2 AR-RE and β_2 AR-GE through the early endosome compartment (Figure 5). This indicates that altered endocytosis does not account for the enhanced down-regulation of β_2 AR-RE compared to β_2 AR-GE.

3.5 Differential recycling through Rab11-positive compartment does not account for the difference in down-regulation of the polymorphic receptors

Rab GTPases are associated with distinct cellular compartments where they function as regulators of intracellular transport (Trischler et al., 1999). Therefore, Rab proteins can be used as compartment-specific markers. At least two different pathways are proposed for recycling of receptors to the plasma membrane: direct shuttling from the sorting endosome (short cycle), or through Rab11-positive perinuclear recycling endosome (long cycle) (Ullrich et al., 1996). Similarly to the EEA1 studies, we performed comprehensive time-course studies of co-localization with Rab11. Little co-localization of β_2 AR with Rab11 was detected in untreated cells, amounting to 3 – 4% for both isoforms, with no change after isoproterenol treatment at any analyzed time point (Figure 6). These results suggest that the β_2 AR normally does not transit through the recycling endosome compartment defined by Rab11 in response to β -agonist. This is in general agreement with previous studies (Moore et al., 1999; Innamorati et al., 2001). Even if the small fraction (3 – 4%) of the receptor localized to the recycling endosome at steady state is significant, any traffic through this compartment is agonist-independent and identical for both isoforms of the receptor analyzed.

3.6 β_2 AR-RE co-localizes with the lysosomal marker Lamp1 to a greater extent than does β_2 AR-GE

A significant body of evidence suggests that under conditions of prolonged agonist exposure, the β_2 AR may be trafficked to lysosomes for degradation (Kurz and Perkins, 1992; Moore et al., 1999). We therefore evaluated the lysosomal targeting of β_2 AR-RE and β_2 AR-GE to determine if this accounted for the differential down-regulation. Cells were treated with isoproterenol for 18 h and samples were analyzed for receptor co-localization with Lamp1 every hour. The time-course of co-localization of β_2 AR with Lamp1 was

essentially identical for β_2 AR-RE and β_2 AR-GE: co-localization was marginal in untreated cells, but it progressively increased by 2 h of treatment, remained elevated for several hours, and decreased after 14 h of treatment (Figure 7). However, a larger fraction of β_2 AR-RE than β_2 AR-GE co-localized with Lamp1. With exception of the 7 and 11 h time points, the differences in co-localization between β_2 AR-RE and β_2 AR-GE were statistically significant ($p < 0.05$) from 4 – 12 h. The results suggest that enhanced co-localization of β_2 AR-RE compared to β_2 AR-GE with the lysosomal marker is due to enhanced trafficking to lysosome where the receptor is degraded.

DISCUSSION

In this study, we used a YFP-tagged β_2 AR to perform detailed spatial and temporal analyses of trafficking and down-regulation of β_2 AR polymorphic at amino acid positions 16 and 27. Radioligand binding experiments revealed that the four isoforms (with all possible combinations at positions 16 and 27) had different down-regulation profiles. Prolonged agonist treatment resulted in increased down-regulation of isoforms with arginine at position 16 (β_2 AR-RQ and β_2 AR-RE) compared to those with glycine (β_2 AR-GQ and β_2 AR-GE). β_2 AR-RE had the highest degree of reduction in receptor binding sites indicating that glutamic acid at position 27 either enhances down-regulation in the presence of arginine at position 16, or glutamine at position 27 suppresses this effect. The present experiments do not discriminate between these possibilities because the β_2 AR-GQ and β_2 AR-GE isoforms had very similar degrees of down-regulation, indicating that glycine at position 16 suppresses down-regulation and has a dominant effect over the amino acid at position 27.

To define the mechanisms underlying enhanced down-regulation, β_2 AR-GE and β_2 AR-RE, which lack the modulatory effects of residue 27, were selected for more detailed experiments. We performed an extensive time-course study of down-regulation for the chosen polymorphic receptors, β_2 AR-GE and -RE. The time-course study demonstrated increased down-regulation of the β_2 AR-RE compared to β_2 AR-GE. This difference in down-regulation became apparent at 8 h time point of isoproterenol treatment and increased over the time-course. In addition to ligand binding assay, we used flow cytometry to assess down-regulation of YFP-tagged receptors. Similar to the above receptor binding studies, β_2 AR-RE had enhanced decrease in YFP fluorescence intensity compared to β_2 AR-GE as a result of prolonged isoproterenol treatment.

We assessed cyclic AMP accumulation to confirm that differences in down-regulation of β_2 AR-GE and β_2 AR-RE translated into differences in functional response. The results of this functional assay demonstrate that β_2 AR-RE exhibited a significantly greater inhibition of cyclic AMP accumulation in response to acute challenge after chronic agonist treatment compared to β_2 AR-GE. Although it is yet to be determined if the ~13.4% difference between the ability of β_2 AR-RE and β_2 AR-GE clones to modulate cyclic AMP levels results in physiological effects, these experiments show that differences in receptor down-regulation translate into differences in signaling that could, in turn, translate into differences in clinical responses to β -agonist in humans.

We hypothesised that differences in down-regulation of the polymorphic receptors were due to differential trafficking of the receptors. To test this hypothesis, we followed trafficking of the receptors in response to isoproterenol and co-localized receptors with cell compartment specific markers EEA1, Rab11 and Lamp1 to identify early endosomes, recycling endosomes, and lysosomes, respectively. Isoproterenol-stimulated transit through the early endosome was identical for β_2 AR-GE and β_2 AR-RE. The decline in receptor co-localization with EEA1 was paralleled by an increase in co-localization with the lysosomal marker Lamp1 and consistent with targeting of the receptors to lysosomes. As receptors leave early endosomes, they can either recycle back to the plasma membrane via rapid or slow recycling pathways (Ullrich et al., 1996; Innamorati et al., 2001; Volpicelli et al. 2002), or they can be targeted to the lysosome for degradation. In the rapid recycling, receptor is recycled to the plasma membrane directly from the early endosome, whereas in the slow recycling receptor traverses perinuclear recycling endosome compartment in order to reach plasma membrane (Trischler et al., 1999).

Under conditions of chronic exposure to β -agonist, the rate of receptor recycling is decreased (Moore et al., 1999a; Moore et al., 1999b). It was shown previously in HEK 293 cells that after 18 h of isoproterenol exposure, 40–50% of the β_2 ARs do not return to the cell surface and are thought to be degraded (Moore, et al. 1999a). Under conditions of prolonged presence of β -agonist, a significant fraction of the β_2 ARs are specifically sorted from the endosome to the lysosome, where the receptors are degraded by cysteine proteases (Moore et al., 1999b). This provides a possible mechanism for the loss of receptor binding sites observed during prolonged (at least several hours) treatment with agonist. The use of the cysteine protease inhibitor leupeptin blocked receptor down-regulation, providing strong evidence for a mechanistic link between down-regulation (defined as a loss of receptor binding sites) and trafficking of the receptors to the lysosome. In our experiments, we detected extensive co-localization of receptors with the lysosomal marker Lamp1 after only 1–2 h of isoproterenol treatment. Importantly, a significant proportion of β_2 ARs were localized to the lysosomal compartment for over 12 hours, supporting the notion that the β_2 AR is not an immediate target for degradation. Eventual degradation by cysteine proteases (Moore et al., 1999b) may explain the decline in receptor co-localization with Lamp1 that we observed after 14 h treatment. The β_2 AR-RE isoform had a higher degree of co-localization with the lysosomal marker compared to β_2 AR-GE. Importantly, in the ligand binding assay the β_2 AR-RE was down-regulated more compared to β_2 AR-GE starting at about 8 h after isoproterenol treatment. Because the decrease in binding sites parallels increase in co-localization with the lysosomal marker, these results indicate that the differences in the down-regulation of the polymorphic receptors detected in the radioligand binding assay could be due to differences in the rate of lysosomal targeting and/or degradation.

Earlier studies have shown that small GTPase Rab11 is associated with the recycling endosome, an organelle that contains recycling molecules targeted to the plasma membrane. Rab11 is implicated in controlling the “recycling” of the transferrin receptor, the M_4 muscarinic acetylcholine receptor and the V_2 vasopressin receptor (Ullrich et al., 1996; Innamorati et al., 2001; Volpicelli et al., 2002) through this compartment. Previous study failed to demonstrate an appreciable co-localization of β_2 AR with Rab11 following agonist

treatment (Innamorati et al., 2001; Moore et al., 2004). However, in cells over-expressing Rab11, β_2 AR was diverted from the lysosomal degradation pathway and extensively co-localized with Rab11 (Moore et al., 2004) following prolonged (6 h) exposure to a β -agonist. This finding implicates a possible role of Rab11 in the recycling of β_2 AR. However, an alternative interpretation is that over-expression of Rab11 may shift trafficking pathways from the lysosome to the recycling endosome compartment, and the extensive co-localization of the β_2 AR with Rab11 detected at these conditions could be due to expansion of the recycling endosome compartment. In an 18 h time-course of continuous agonist exposure we did not detect an appreciable co-localization of the polymorphic receptors with Rab11 (within the limits of our methodology), suggesting that the receptors probably did not transit through the perinuclear recycling endosomal compartment. As discussed earlier, the receptors could also be recycled directly from the sorting endosome to the plasma membrane (Innamorati et al., 2001). In the current study we have not attempted to investigate changes in the receptor localization between sorting endosomes and the plasma membrane. It is also possible that multiple recycling pathways exist that recycle the receptor back to the plasma membrane by interacting with a variety of trafficking factors. A recent study demonstrated that sorting between the recycling and degradative pathways occurs at the level of receptor entry into retromer tubules, and this process is assisted by adaptor proteins (Temkin et al., 2011). As discussed above, interpretation of our trafficking experiments is restricted because of the lack of thorough data on the recycling of the polymorphic variants. Thus, increased co-localization of β_2 AR-RE with the lysosomal marker under prolonged agonist treatment could be due to increased targeting to lysosomes or slower recycling. In the latter scenario, receptors could be diverted from the recycling pathway and thus more likely to be targeted to lysosome.

Structural features involved in desensitization and down-regulation of the β_2 AR are primarily located on the intracellular carboxyl terminal tail and the third intracellular loop. The mechanisms by which residues in the ectodomain of the receptor may influence its down-regulation are unknown. The crystal structure of an engineered β_2 AR was reported (Cherezov et al., 2007), but the amino terminal domain (1–28 amino acids) appeared to be disordered. The β_2 AR amino-terminus contains two sites of N-glycosylation at amino acid positions 6 and 15. It has been shown that glycosylation is not required for either ligand binding or functional coupling, but it has been suggested that it may play a role in receptor trafficking from the endoplasmic reticulum to the plasma membrane (Collins et al., 1991). In the hamster β_2 AR, the asparagines at positions 6 and 15 were shown to be important for membrane insertion, and their mutation resulted in reduced expression of the receptors on the cell surface (Rands et al., 1990). Furthermore, in the β_2 AR of humans and higher order primates, a third glycosylation site is present in the second extracellular loop at position 187 (Mialet-Perez et al., 2004). Mutation of asparagine to glycine at amino acid position 187 abrogated lysosomal targeting despite prolonged agonist treatment. Thus, this site was identified as a motif important for lysosomal targeting of internalized β_2 AR. Although the mechanisms are undefined, ectodomain modifications can have significant effects on processes that are usually thought to involve interactions between the cytoplasmic domains of transmembrane receptors.

The present findings are not in agreement with a previous study that also used radioligand binding as an index of receptor down-regulation (Green et al., 1994). In that study, after 24 h exposure to 10 μ M isoproterenol, glycine isoforms down-regulated more than arginine isoforms, and the isoform with glutamic acid at position 27 (Arg16Glu27) was resistant to down-regulation. The reasons for the discrepancies between this and our study are not immediately apparent but could be related to the differences in expression systems. The CHW cell line is of hamster origin, whereas the HEK 293 cells used in the present study has human origin. We have also used a lower dose (1 μ M) of isoproterenol in our study. The same group performed studies on human airway smooth muscle cells using cell lines that were homozygous at either locus 16 or 27. Cells homozygous for Gly16 (and also heterozygous at amino acid position 27) underwent enhanced down-regulation of the receptor number following exposure to 10 μ M isoproterenol, whereas Glu27 was relatively resistant to down-regulation (Green et al., 1995). Both Gly16 and Arg16 underwent similar desensitization as assessed by cyclic AMP response, but the desensitization of Glu27 was abrogated. This study is important because it was performed in primary culture, however, heterozygosity at the other locus and the limited number of combinations of alleles may impede interpretation of the results.

Our data are in line with a study on the effect of β_2 AR polymorphism in mast cells (Kay et al., 2007). Additional effects of β -agonists in the airway include suppression of airway inflammation by inhibiting the release of pro-inflammatory mediators from mast cells (O'Connor et al., 1994). Thus, in the context of asthma, influence of the β_2 AR polymorphism on the response of mast cells might be important. The potential effects of polymorphisms on functional response to the β -agonist isoproterenol in mast cells were investigated both at the individual SNPs found on the 5'UTR and coding region of the β_2 AR gene and at the haplotype levels. The efficacy, potency, as well as functional desensitization of inhibition IgE-mediated histamine release were assessed. From all individual SNPs analyzed in the study, only the polymorphism at amino acid position 16 significantly influenced the desensitization of the response in mast cells. Mast cells expressing the Gly16/Gly16 receptor were found less prone to functional desensitization of agonist-mediated response in mast cells than were those expressing the Arg16/Arg16 receptor, and heterozygotes had an intermediate phenotype. Interestingly, none of the above parameters were influenced by haplotype. The results of our study are also in agreement with a number of clinical studies showing that Arg16 homozygotes had a less favourable response as a result of regular use of a β -agonist (Israel et al., 2001; Lee et al., 2004; Palmer et al., 2006; Taylor et al., 2000; Wechsler et al., 2006).

There are certain limitations in the present study. Our ligand binding experiments did not distinguish the contribution of *de novo* receptor synthesis in the total number of receptors, but in our studies transcription was driven by a viral promoter and was not likely to be influenced by treatment with a β -agonist. Also it was previously shown in CHW cells that the rates of receptor synthesis after irreversible alkylation were not different between polymorphic receptors (Green et al., 1994). Further, in this study, we used nonselective β -agonist isoproterenol. It is not known whether our findings with isoproterenol are reflective of bronchodilators commonly used in clinic.

The β_2 AR gene contains 17 SNPs in the 5' upstream region, 7 SNPs in the coding region, and one SNP and a variable poly-C tract in the 3'UTR (Panebra et al., 2010). It has been suggested that studying the effect of the β_2 AR polymorphisms in haplotypes rather than separate SNPs might be more relevant (Liggett, 2006). Indeed, a recent study elegantly demonstrated that the context in which an individual SNP exists in the gene is important (Panebra et al., 2010). In this study, eight common haplotypes were established to assess expression and agonist-promoted down-regulation in COS7 cells, and two haplotypes exhibited slightly greater agonist-induced down-regulation relative to the other six. Since the polymorphic variants in our study were generated by amplification of the coding region from genomic DNA and site-directed mutagenesis, it is not possible to directly compare the results of these studies. One of the limitations of our study is that the approach to generate the polymorphic variants that we utilized may have resulted in haplotypes that are not common in the general population. However, it is interesting to note that both haplotypes that showed subtle increase in agonist-promoted down-regulation had an Arg16 polymorphism (Panebra et al., 2010).

This work is a step forward in elucidating possible mechanism(s) responsible for differences in the function of the β_2 AR containing N-terminal polymorphisms. Our trafficking experiments provide a mechanistic explanation for the difference in down-regulation of β_2 AR in response to β -agonists and hence the function of the receptors. This comprehensive analysis of the trafficking of the polymorphic receptors, paralleled with the results of the ligand binding assay, leads us to propose that the difference in receptor down-regulation after prolonged exposure to isoproterenol is due to increased lysosomal degradation of isoforms with arginine at position 16. Whether this increased lysosomal degradation is a consequence of enhanced targeting to lysosomes or of a reduction in the recycling step remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Antibody staining for the β_2 AR confirms the specificity of the YFP signal
HEK 293 cells stably expressing YFP-tagged human β_2 AR were either untreated (A, B, C) or treated (D, E, F) with isoproterenol for 4 h, fixed, stained with Ab-13989 antibody and imaged by confocal microscopy. A, D - β_2 AR-YFP; B, E – staining with β_2 AR-specific antibody (ab13989, Abcam); C, F – merged images.

Figure 2. β_2 ARs with arginine at position 16 demonstrated increased down-regulation in response to prolonged isoproterenol treatment

Cells were treated with 1 μ M isoproterenol for 20 min, 2 h and 24 h and radioligand binding assays were performed using [3 H]DHA. Values are expressed as percent of the untreated population and are means $\pm$ S.E. of 4 independent experiments, each experiment was performed in triplicate. P values were calculated using HSD Tukey's test. The values are statistically significant at 24 h treatment for pairs: RQ and GQ (* $p=0.001$), RE and GE (** $p=0.0001$), and RQ and RE (* $p=0.001$).

Figure 3. β_2 AR-RE down-regulated more over time with prolonged agonist treatment compared to β_2 AR-GE

Cells expressing β_2 AR-RE and β_2 AR-GE were treated with 1 μ M isoproterenol for 24 h and receptor numbers were analysed using a radioligand assay after 2, 4, 8, 12, 18, and 24 h of isoproterenol treatment. The experiment was repeated twice with similar results. Results of the representative experiment are shown. Values are expressed as mean $\pm$ S.D.

Figure 4. β_2 AR-RE had a reduced cyclic AMP response compared to β_2 AR-GE after chronic β -agonist treatment

β_2 AR-RE and β_2 AR-GE clones were treated with 1 μ M isoproterenol for 24 h, and cyclic AMP accumulation was measured in response to acute isoproterenol challenge. The experiment was performed three times. Values are expressed as percent of the untreated population and are means $\pm$ S.E.M.

Figure 5. Isoforms demonstrated similar co-localization with early endosome marker EEA1 in the time-course studies

β_2 AR-RE and β_2 AR-GE clones were treated with 1 μ M isoproterenol and processed for confocal microscopy using an anti-EEA1 antibody every 15 min during the first one and a half hours, and after 2, 6, 12 and 18 h. A–J - Representative images are shown for selected time points. K - Quantification of time-course of co-localization with EEA1. Images were analyzed as described in Materials and Methods and results plotted on the graph. Multiple images were acquired and analysed for each time point in each experiment. Data are presented as means $\pm$ S.E.M. based on data averaged from 3 independent experiments performed on separate days.

Figure 6. Polymorphic receptors show little co-localization with the Rab11-positive endosomal compartment

Cells, β_2 AR-RE and β_2 AR-GE clones, were treated with 1 μ M isoproterenol and samples were stained with an anti-Rab11 antibody and confocal microscopy every 15 min during first one and a half hour and then every hour between 2 and 18 h. Data were acquired similar as described in Figure 5. A–J - Representative images are shown for selected time points.

Figure 7. β_2 AR-RE co-localized more strongly with the lysosomal marker Lamp1 than did β_2 AR-GE

Cells, β_2 AR-RE and β_2 AR-GE clones, were treated with 1 μ M isoproterenol and samples were processed for IIF with anti-Lamp1 antibody and confocal microscopy every hour for a total of 18 hours. A–J - Representative images are shown for selected time points. K- Quantification of time-course of co-localization with Lamp1. In general, the difference in co-localization was statistically significant between 4 – 12 h of treatment ($p < 0.05$); time points 7 h ($p = 0.195$) and 11 h ($p = 0.062$) were exceptions.