Salmeterol Stimulation Dissociates β_2 -Adrenergic Receptor Phosphorylation and Internalization

Robert H. Moore, Ellen E. Millman, Veronica Godines, Nicola A. Hanania, Tuan M. Tran, Hui Peng, Burton F. Dickey, Brian J. Knoll, and Richard B. Clark

Department of Pediatrics and Department of Pulmonary and Critical Care Medicine, Baylor College of Medicine; Department of Integrative Biology and Pharmacology, University of Texas Health Sciences Center-Houston; Department of Biology and Biochemistry and Department of Pharmacology and Pharmaceutical Sciences, University of Houston; and Division of Pulmonary Medicine, M.D. Anderson Cancer Center, Houston, Texas

Salmeterol is a long-acting β_2 -adrenergic receptor (β_2 AR) agonist commonly used in the treatment of asthma and chronic obstructive pulmonary disease. It differs from other β -agonists in that it has a very low intrinsic efficacy, especially when compared with the other available long-acting β -agonist, formoterol. Receptor desensitization and down-regulation has been described with the chronic use of β -agonists. This effect may not be the same with all β -agonists and may be related to their stabilization of altered receptor states. The extreme hydrophobicity and high-affinity quasi-irreversible binding of salmeterol have rendered studies examining the mechanisms by which it mediates receptor desensitization, down-regulation, and internalization difficult. We determined the capacity of salmeterol to induce β_2 AR endocytosis, G protein-coupled receptor kinase (GRK)-site phosphorylation, degradation, and β -arrestin2 translocation in HEK293 cells as compared with other agonists of varying intrinsic efficacies. Despite stimulating GRK-mediated phosphorylation of Ser355,356 after 30 min and 18 h to an extent similar to that observed with agonists of high intrinsic efficacy, such as epinephrine and formoterol, salmeterol did not induce significant β_2 AR internalization or degradation and was incapable of stimulating the translocation of enhanced green fluorescent protein- β -arrestin2 chimera (EGFP- β -arrestin2) to the cell surface. Salmeterol-induced receptor endocytosis was rescued, at least in part, by the overexpression of EGFP- β -arrestin2. Our data indicate that salmeterol binding induces an active receptor state that is unable to recruit β -arrestin or undergo significant endocytosis or degradation despite stimulating considerable GRK-site phosphorylation. Defects in these components of salmeterol-induced receptor desensitization may be important determinants of its sustained bronchodilation with chronic use.

Keywords: β_2 -adrenergic receptor; salmeterol; phosphorylation; internalization; desensitization

β_2 -adrenergic agonists are important therapeutic agents for the relief of bronchoconstriction in asthma and chronic obstructive pulmonary disease (COPD). Short-acting agents are usually used for rescue of symptoms, whereas long-acting agents are reserved for maintenance therapy. Salmeterol is a β_2 -selective agonist that provides sustained bronchodilation and protection against provocative stimuli for at least 12 h after a single dose (1), resists washing, and recovers activity after a temporary blockade of

CLINICAL RELEVANCE

Salmeterol is commonly used in the treatment of asthma and COPD, yet its molecular pharmacologic properties are poorly understood. This work provides information regarding the pharmacologic properties of salmeterol at the cellular and molecular levels.

β_2 -adrenergic receptors (β_2 ARs) by reversible antagonist binding (2). Salmeterol has the same saligenin active group as the β_2 -agonist albuterol but contains a long hydrophobic aralyloxyalkyl side chain terminating in a phenyl ring (3). The persistent membrane association of salmeterol, which seems to be due to a high-affinity interaction of this hydrophobic tail with a receptor "exosite" in the vicinity of transmembrane segments 6 and 7 that is distinct from the site that interacts with the saligenin group (4, 5), have made the study of its pharmacologic properties at the cellular and molecular levels problematic. These properties of salmeterol may lead to a unique receptor activation profile, as has been reported for a number of other agonist-G protein-coupled receptor interactions (6, 7).

We previously have shown that salmeterol-induced β_2 AR desensitization in HEK293 and BEAS-2B human bronchial epithelial cell lines is reduced relative to the full agonist epinephrine or the partial agonist albuterol (8). β_2 AR desensitization is a multi-step process that initiates with the phosphorylation of multiple serine residues in the receptor's third intracellular loop by protein kinase A and in the cytoplasmic tail by G protein-coupled receptor kinases (GRKs). Protein kinase A-mediated phosphorylation of receptor and receptor desensitization is termed "heterologous" because it occurs in response to increases in cAMP induced by any means. In contrast, homologous desensitization caused by phosphorylation of the receptor by members of the GRK family seems to require agonist occupancy of the receptor and to be limited to a serine/threonine-rich region between amino acids 355 and 364 in the receptor's cytoplasmic tail (9–12). Agonist occupancy and GRK-site phosphorylation synergistically act to create a receptor conformation that allows high-affinity binding by an arrestin protein at the activation and phosphorylation domains (13, 14). Arrestin-bound β_2 ARs are fully uncoupled from G_s protein and are internalized into early endosomes by a process termed endocytosis, a step that contributes to receptor desensitization and likely triggers the dissociation of β -arrestin, a prerequisite for receptor resensitization with continued agonist (15). With prolonged exposure to agonists, the number of cellular β_2 ARs is reduced by a process termed downregulation, which is mediated by receptor degradation in lysosomes that depends on receptor internalization and by reduced receptor expression (16–18).

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Correspondence and requests for reprints should be addressed to Robert H. Moore, M.D., Department of Pediatrics, Baylor College of Medicine, 6621 Fannin, CCC1040, Houston, TX 77030. E-mail: rmoore@bcm.tmc.edu

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By directly measuring the K_d and EC_{50} of adenylyl cyclase (AC) activation, we previously have estimated the intrinsic efficacy of salmeterol activation of AC to be $\sim 12\%$ that of the full agonists epinephrine and isoproterenol, $\sim 20\%$ that of the highly lipophilic long-acting agonist formoterol, $\sim 50\%$ that of the structurally similar agonist albuterol, and ~ 4 -fold greater than that of the very weak partial agonist ephedrine (10). Because with most β -agonists studied to date the initial rate of GRK-site phosphorylation correlates well with agonist intrinsic efficacy for AC activation and because receptor desensitization is likely a critical determinant of the ability of a β_2 -agonist to provide sustained bronchodilation over time, we sought to compare the ability of salmeterol with that of a variety of agonists of differing intrinsic efficacies to induce endocytosis, β -arrestin translocation, and receptor degradation.

MATERIALS AND METHODS

Cell Culture and Reagents

The 12B6 line was derived from HEK293 cells and expresses a β_2 AR with an N-terminal hemagglutinin (HA) epitope tag and with Arg at position 16 and Gln at position 27. The receptors are expressed at an approximate density of 300,000 receptors per cell (gift of B. Kobilka, Palo Alto, CA). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS and 400 μ g/ml of G418. Mouse monoclonal IgG against the HA epitope (mAb HA.11) was purchased from Covance (Berkeley, CA). Rabbit polyclonal antibodies against the β_2 AR C-tail and phosphoserines 355,356 (α -pS355,356) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat FITC- and tetramethyl rhodamine isothiocyanate (TRITC)-anti-mouse IgG were obtained from Molecular Probes (Eugene, OR) and goat-anti-mouse IgG conjugated to alkaline phosphatase from Jackson Immunological Research (West Grove, PA). Paraformaldehyde and glutaraldehyde were obtained from Electron Microscopy Sciences (Ft. Washington, PA). FuGENE6 transfection reagent was obtained from Roche Molecular Biochemicals (Mannheim, Germany). β_2 AR agonists were purchased from Sigma Chemical Co. (St. Louis, MO), except for RR-formoterol, which was obtained from Sepracor (Marlborough, MA); albuterol hemisulfate, which was obtained from ICN Biomedical (Aurora, OH); and salmeterol, which was obtained from GlaxoSmithKline (Uxbridge, Middlesex, UK). All other reagents were from Sigma. The enhanced green fluorescent protein- β -arrestin2 chimera (EGFP- β -arrestin2) construct was a gift of Dr. M. Caron (Durham, NC).

Immunofluorescence Microscopy

12B6 cells growing on glass cover slips were fixed, labeled with primary and secondary antibodies, and mounted as previously described (19). β_2 ARs were detected using mHA.11 (5 μ g/ml) followed by fluorescently tagged goat α -mouse IgG secondary antibody (5 μ g/ml). For studies of EGFP- β -arrestin2 translocation, cells were treated with the indicated agonist for 2 min and immediately fixed and mounted for visualization. Images were acquired at optical section depths of 150 nm using a Zeiss Axiophot inverted microscope (Zeiss, Göttingen, Germany) with a 100 $\times$ lens (1.4 numerical aperture), optimized by deconvolution using DeltaVision software (Applied Precision, Inc., Issaquah, WA), and transferred to Adobe Photoshop for digital processing.

Measurement of Surface β_2 ARs by ELISA

12B6 cells growing in fibronectin-coated, 96-well clusters were exposed to agonists for times up to 60 min at 37°C and fixed with 0.5% glutaraldehyde for 15 min. Agonist concentrations were ~ 10 -fold greater than the range of measured K_{δ} for the β_2 AR: ~ 400 –700 nM for (-)-epinephrine, ~ 150 –300 nM for (-)-isoproterenol, 500–600 nM for albuterol, 5–7 nM for RR-formoterol, 1–2 nM for salmeterol, and $\sim 2,800$ nM for ephedrine (8, 14, 20). The following steps were done at room temperature, with washes using PBS. The fixed cells were washed twice and blocked with 5% normal goat serum (NGS) for 1 h. Antibody mHA.11 was added at 0.5 μ g/ml in PBS with 5% NGS for 1 h, and the cells were washed and incubated with goat- α -mouse IgG-alkaline phosphatase (diluted 1:2,000 in PBS, 5% NGS) for 1 h. The wells were washed in

a buffer containing 580 mM NaCl, 50 mM ethanolamine, and 5 mM MgCl₂ (pH 9.5). The reaction was developed for 20 min in the same buffer with 1 mg/ml *p*-nitrophenol phosphate and stopped with 3 N NaOH before measurement of the optical density (OD₄₁₀). The background optical density in 12B6 cells, determined without primary antibody, was $< 1\%$ of that obtained when the primary antibody was present. The background OD in untransfected HEK293 cells, determined using primary and secondary antibodies, was $< 1\%$ that of 12B6 cells plated at a similar density. Mean ODs in 12B6 cells before agonist treatment or treated with 0.1 mM ascorbic acid and 1 mM thiourea (AT) alone generally ranged from 0.35 to 0.40. The linearity of the assay was confirmed by measuring the signal after the plating of 12B6 cells at 5-fold dilutions. The resulting ODs as a fraction of a $1 \times$ dilution (3×10^4 cells, set to 1.0) are as follows: $0.2 \times$ cells = 0.28; $0.04 \times$ cells = 0.05; and $0.008 \times$ cells = -0.001 .

Effect of EGFP- β -Arrestin2 Overexpression on Receptor Internalization

12B6 cells growing on glass cover slips were placed in complete medium with 3% FBS and transfected with 2 μ g of pEGFP- β -arrestin2 or pEGFP using 3 μ l of FuGENE 6 transfection reagent according to the manufacturer's instructions. Forty-eight hours later, cells were treated with the designated agonist for 15 min or left untreated as a control. Cells were fixed, labeled with primary and secondary antibodies, and mounted as described previously, except that β_2 ARs were detected using the monoclonal antibody mHA.11 (5 μ g/ml) followed by goat α -mouse IgG-TRITC secondary antibody (5 μ g/ml). Images were acquired as described previously. For experiments involving EGFP- β -arrestin2 overexpression, cells of similar brightness were selected for imaging.

Determination of β_2 AR Degradation

β_2 AR degradation in 12B6 cells was determined as previously described (21). In brief, cells growing on 6-well clusters were treated with EZ-link sulfo-NHS-biotin (0.5 mg per well) for 30 min at room temperature to biotinylate surface receptors. The biotinylated cells were treated with the indicated agonist for 22 h, washed, and harvested in solution containing leupeptin (10 μ g/ml). Cells were pelleted and solubilized at 4°C in solubilization buffer (20 mM Hepes [pH 7.4], 300 mM NaCl, 5 mM EDTA, 0.8% n-dodecyl- β -D-maltoside, and Complete EDTA-free protease inhibitor [Sigma] at standard concentration). Lysates were centrifuged at $16,000 \times g$ to remove cellular debris. From each sample, 50 μ g of protein was added to 50 μ l streptavidin agarose beads (Sigma) at 4°C for 1 h to bind biotinylated receptors, and the beads were pelleted by centrifugation. The final pellets were resuspended in Laemmli buffer, heated to 65°C for 15 min, and centrifuged at $11,000 \times g$ to free biotinylated receptors from the streptavidin-agarose. A 10- μ g aliquot of eluted protein from each sample was treated with 2 μ l peptide N-glycosidase F (New England Biolabs, Beverly, MA) for 90 min at 37°C, and the samples were electrophoresed and transferred to Immobilon-P membranes. The membranes were probed with the anti- β_2 AR C-terminus polyclonal antibody at a dilution of 1:1,000 and detected using an Alpha Innotech Fluorochem 8800 CCD camera system (1,000-fold dynamic range; Alpha Innotech, San Leandro, CA). The CCD camera in the Fluorochem 8800 has been calibrated by performing immunoblots with serial dilutions of lysates from cells expressing β_2 ARs. We found the range to be linear up to and possibly beyond 3.3×10^6 integrated density values. In our experiments, the integrated density values usually ranged from 5×10^4 to 5×10^5 .

Agonist-induced cell loss was assessed by direct visualization of cells using light microscopy and by measuring total protein concentrations after treatments with the various ligands. In separate experiments using similar conditions, cells were harvested and counted using a Beckman-Coulter Z2 particle counter. Cell morphology, total protein levels, and cell counts were similar after treatment with AT alone or with each agonist.

Measurement of GRK-Site Phosphorylation

GRK-site receptor phosphorylation was determined as previously described (10). 12B6 cells growing in 6-well clusters were treated with 10 μ M epinephrine in AT, 100 nM RR-formoterol, or 50 nM salmeterol or with AT carrier alone for 30 min or 18 h. Cells were harvested, pelleted

by centrifugation at 2,000 rpm, and solubilized in buffer containing 20 mM HEPES (pH 7.4), 300 mM NaCl, 0.8% *n*-dodecyl- β -D-maltoside, 5 mM EDTA, 3 mM EGTA, 20 mM sodium pyrophosphate, 10 mM sodium fluoride, 25 mM imidazole, 10 μ g/ml benzamidine, 10 μ g/ml trypsin inhibitor, 100 nM okadaic acid, 10 μ g/ml leupeptin, and 14 mM β -mercaptoethanol. The eluate was digested with 1,500 units of *N*-glycosidase F for 2 h at 37°C and heated at 60°C for 15 min in SDS-sample buffer. Samples were resolved on SDS-PAGE (12% gel) and transferred to nitrocellulose for immunoblotting. The membranes were probed with a rabbit β_2 AR phosphoserine 355, 356-specific antibody at a dilution of 1:500 and detected by chemiluminescence. After quantification by densitometry, the immunoblots were stripped and reprobed with a β_2 AR-specific rabbit antibody at a dilution of 1:1,000 to quantify total receptors. The results reflect the increase in receptor phosphorylation at serines 355, 356 normalized to total receptor load with epinephrine-stimulated, GRK-site phosphorylation being set as 100%.

RESULTS

After exposure to a full agonist, β_2 ARs rapidly move into punctate intracellular vesicles, consistent with early endosomes (19). To examine the receptor response to other ligands, 12 β 6 cells were treated for 15 min with full and partial agonists of varying coupling efficiencies. The fixed and permeabilized cells were labeled with an anti-HA antibody to detect HA-tagged β_2 ARs and examined by immunofluorescence microscopy. In untreated cells, β_2 ARs were located predominantly on the cell surface, as indicated by the linear appearance of the staining (Figure 1A). Treatment with epinephrine caused the appearance of intracellular, vesicular β_2 ARs (Figure 1B), consistent with earlier results using isoproterenol (19). RR-formoterol (Figure 1C) and albuterol (Figure 1D) caused the appearance of intravesicular β_2 ARs. In contrast, no intracellular vesicles were detected after treatment with salmeterol at 20 nM (Figure 1E) (a concentration at least 10-fold higher than its K_d) (8), or after treatment with the weak partial agonist ephedrine at 100 μ M (Figure 1F).

To determine whether salmeterol and ephedrine could evoke the endocytosis of β_2 ARs at too low a rate to be detectable by immunofluorescence microscopy, we used a quantitative ELISA technique. Agonist-treated 12 β 6 cells were fixed but not permeabilized before binding the anti-HA antibody so that only surface β_2 ARs (which are tagged at the extracellular N-terminus) were detected. Treatment with epinephrine for 60 min caused a 59% loss of the surface β_2 ARs, whereas treatment with albuterol caused a 24% loss (Figure 2). These percentages reflect the steady-state level of surface β_2 ARs predicted by the rate con-

stants for agonist-induced endocytosis and recycling and are in close agreement with our previous measurements using radioligand binding assays (22). The partial agonist RR-formoterol evoked the endocytosis of 55% of receptors, similar to epinephrine and consistent with its high intrinsic efficacy as compared with albuterol. In contrast, there was a < 5% loss of surface receptors over this time course using salmeterol, which was not statistically different from control, and there was no detectable loss using ephedrine (Figure 2).

That salmeterol did not trigger significant receptor endocytosis was surprising in view of the considerable phosphorylation known to be induced by this agonist at GRK sites serines 355 and 356 (10). Recent data indicate that the time courses for GRK-site phosphorylation induced by 50 nM salmeterol or 50 nM epinephrine are almost identical (10). To further assess the relative efficiency of salmeterol and epinephrine in stimulating the endocytosis of β_2 ARs, 12 β 6 cells were treated with 5 μ M or 50 nM epinephrine or 50 nM salmeterol for varying times up to 30 min and receptor endocytosis analyzed by ELISA (Figure 3A). In cells exposed to 5 μ M epinephrine, a saturating concentration, almost 60% of the receptors rapidly were removed from the cell surface. In cells treated with 50 nM epinephrine, a nonsaturating concentration (estimated fractional occupancy 10–15%), $\sim$ 20% of the β_2 ARs underwent endocytosis, with steady-state internalization being reached within 10 min, whereas < 5% of the β_2 ARs were internalized after 30 min in cells treated with 50 nM salmeterol, a saturating concentration.

Because β_2 AR endocytosis requires the binding of GRK-phosphorylated receptors by β -arrestin (13, 23), we sought to determine if salmeterol was capable of promoting β -arrestin recruitment to the membranes. 12 β 6 cells transiently expressing an EGFP- β -arrestin2 chimera were exposed to vehicle, 5 μ M epinephrine, 50 nM epinephrine, or 50 nM salmeterol for 2 min and were fixed and visualized for EGFP- β -arrestin2 translocation from the cytosol to the plasma membrane. Epinephrine at 5 μ M and 50 nM triggered the translocation of an EGFP- β -arrestin2 to small clusters at the plasma membrane, consistent with clathrin-coated pits (Figure 3B) (12). In cells stimulated with 50 nM salmeterol, little or no EGFP- β -arrestin2 translocation to the plasma membrane was observed. Albuterol (6 μ M) and formoterol (40 nM) stimulated EGFP- β -arrestin2 translocation to the cell surface (data not shown).

Figure 1. Immunofluorescence microscopy of 12 β 6 cells treated with various agonists. Cells were treated for 15 min with agonists and were permeabilized and labeled with anti-HA primary and FITC-secondary antibodies to visualize β_2 ARs. (A) AT alone (CON). (B) 5 μ M epinephrine (EPI). (C) 40 nM RR-formoterol (FOR). (D) 6 μ M albuterol (ALB). (E) 20 nM salmeterol (SAL). (F) 100 μ M ephedrine (EPH). The images shown are representative microscopic fields of four or five independent experiments. Bar = 10 μ m.

Figure 2. Quantification of surface β_2 ARs using ELISA. 12 β 6 cells growing in 96-well plates were treated for varying times with ligands and fixed and assayed for surface β_2 ARs. Closed squares, 5 μ M epinephrine (EPI). Open squares, 40 nM RR-formoterol (FOR). Closed diamonds, 20 nM salmeterol (SAL). Open circles, 100 μ M ephedrine (EPH). Closed triangles, 6 μ M albuterol (ALB). The figure depicts the means $\pm$ SEM from three separate experiments.

The high-affinity binding of β -arrestin to β_2 ARs is believed to require the synergistic interaction between at least two sites, an activation recognition domain and a phosphorylation recognition domain (13). In the absence of agonist activation or phosphorylation of GRK sites, the β -arrestin–receptor interaction is of low affinity and unlikely to promote receptor endocytosis

(13). Because 50 nM salmeterol and 50 nM epinephrine induced similar levels of GRK-site β_2 AR phosphorylation (10) but were very different in their capacity to trigger receptor endocytosis and β -arrestin translocation (Figure 3), we surmised that salmeterol may be less capable of promoting β -arrestin binding via the agonist activation site. We predicted that if this were true, overexpressing β -arrestin2 might increase the β -arrestin–receptor interaction by mass action and promote some receptor endocytosis, as previously has been shown for β_2 AR mutants defective in endocytosis (24). In contrast, the very weak partial agonist ephedrine stimulates little GRK-site phosphorylation (10, 20) and therefore would not be expected to promote receptor endocytosis after the overexpression of β -arrestin2. To test these predictions, 12 β 6 cells transiently expressing EGFP- β -arrestin2 (Figures 4B and 4C) or EGFP alone (Figure 4A) were treated with 50 nM salmeterol (Figures 4A, 4B, 4D, and 4E) or 100 μ M ephedrine (Figures 4C and 4F) for 15 min, fixed, and labeled with antibody to identify β_2 ARs. As shown in Figures 4B and 4E, in cells overexpressing EGFP- β -arrestin2, a marked redistribution of β_2 ARs from the plasma membrane into punctate vesicles can be seen after a 15-min exposure to salmeterol as compared with EGFP-transfected cells (Figures 4A and 4D). In contrast, the overexpression of β -arrestin2 had no effect on the surface distribution of β_2 ARs in cells treated with ephedrine (Figures 4C and 4F), consistent with the observed lack of rapid receptor phosphorylation by GRK induced by this agonist (10, 20). The lack of apparent β -arrestin2 redistribution to the cell surface in salmeterol-treated cells despite significant receptor internalization likely reflects the relatively low amount of arrestin associated with receptors relative to free cytoplasmic arrestin and some loss of arrestin–receptor interaction after detergent permeabilization of cells during the immunolabeling procedure. That β -arrestin2 is not

Figure 3. Effect of salmeterol and low-dose epinephrine on β_2 AR internalization and EGFP- β -arrestin2 translocation. (A) 12 β 6 cells growing in 96-well plates were treated for varying times with ligands and fixed and assayed for surface β_2 ARs by ELISA. Closed triangles, 5 μ M epinephrine (EPI). Closed squares, 50 nM epinephrine. Open circles, 50 nM salmeterol (SAL). * P < 0.05 as compared with 50 nM epinephrine. (B) 12 β 6 cells transiently expressing EGFP- β -arrestin2 were treated with vehicle, 5 μ M epinephrine, 50 nM epinephrine, or 50 nM salmeterol for 2 min. Cells were immediately fixed and imaged using deconvolution microscopy with sections of 150 nm in the z-plane. Images show sections from the bottom of the cell (i.e., at the coverslip) or through the center of the cell ($\sim$ 3–4 μ m above the coverslip). Bar = 10 μ m.

Figure 4. Overexpression of EGFP- β -arrestin2 promotes receptor endocytosis in salmeterol-treated cells. 12 β 6 cells transiently overexpressing EGFP- β -arrestin2 or untransfected controls were treated with 50 nM salmeterol or 100 μ M ephedrine for 15 min and were fixed and labeled with anti-HA and TRITC-labeled secondary antibodies to visualize β_2 ARs. (A–C) EGFP. (D–F) β_2 ARs. A and D show β_2 ARs in EGFP vector-transfected cells exposed to salmeterol for 15 min. B and E show paired images of EGFP- β -arrestin2 and β_2 ARs channels obtained from salmeterol-treated cells. C and F show paired images of EGFP- β -arrestin2 and β_2 ARs channels obtained from ephedrine-treated cell. The fields shown are representative of two independent experiments. Bar = 10 μ m.

observed in internal vesicles is expected given that arrestin is known not to traffic with β_2 ARs after receptor endocytosis (25).

Although salmeterol stimulates considerable rapid GRK-site phosphorylation of β_2 ARs (10), its clinical effects last for 12 h or more (26). We sought to determine if the sustained effects of salmeterol were due to diminished GRK-site phosphorylation as compared with other agonists after prolonged treatment. Receptors were treated with saturating concentrations of salmeterol, epinephrine, or formoterol for 30 min or 18 h, and the extent of GRK-mediated phosphorylation was measured using a phosphoserine-specific antibody (anti-pSer355,356). After 30 min, the extent of GRK-site phosphorylation stimulated by salmeterol was similar to that triggered by epinephrine and formoterol, a relationship that was maintained even after 18 h of agonist treatment (Figure 5A). This finding indicates that the ability of salmeterol to induce prolonged bronchodilation is not related to defects in GRK-site phosphorylation.

During prolonged exposures to agonist, the number of cellular β_2 ARs is significantly reduced by a process termed “down-regulation,” which contributes to chronic receptor desensitization (17). In 12 β 6 cells, β_2 AR down-regulation is largely mediated by receptor degradation in lysosomes rather than reduced receptor synthesis and likely is dependent on receptor internalization (17, 27). Given that the extent of GRK-site phosphorylation induced by salmeterol was similar to that stimulated by the stronger agonists epinephrine and formoterol, we determined if β_2 AR internalization and downregulation after prolonged exposures to agonist is dependent on agonist intrinsic efficacy. Because over time receptor synthesis may affect the measurement of β_2 AR internalization by the ELISA, we assessed whether salmeterol stimulated some receptor internalization after a prolonged exposure to agonist using immunofluorescence microscopy. 12 β 6 cells were treated with AT alone or with saturating concentrations of the indicated agonists for 18 h and fixed and labeled to identify β_2 ARs as described previously (Figure 5B). In cells treated with epinephrine or formoterol, receptors are largely localized in punctuate vesicles, likely reflecting early endosomes and lysosomes. However, in cells treated with salmeterol, little or no intracellular receptors are seen, similar to cells treated with vehicle alone.

Although salmeterol does not induce perceivable β_2 AR internalization after 18 h, we questioned whether there was a low level of internalization that might be adequate to induce some receptor degradation in lysosomes. To address this question, we

directly measured β_2 AR degradation in cells after an extended exposure of 22 h to various agonists (Figure 5C). The protocol measures the degradation only of receptors that were present on the cell surface at the time of agonist addition and is not affected by synthesis of new receptors. Although the weak partial agonists salmeterol and ephedrine only slightly stimulated receptor degradation to an extent that was not significantly different from control cells, receptor degradation was significantly enhanced in cells treated with the much stronger partial agonist formoterol and the full agonist isoproterenol.

DISCUSSION

The role of salmeterol in the treatment of chronic asthma and COPD is well established (1, 28, 29). Its clinical usefulness comes in part from its ability to provide sustained bronchodilation that is maintained even during chronic use (26). One factor explaining the long action of salmeterol is its prolonged association with the β_2 AR due to its lipophilicity and the interaction of its tail region with the β_2 AR (5). However, this property alone does not entirely explain the lack of desensitization to its bronchodilating effect that has been observed in clinical trials compared with what has been observed with the use of agonists of high intrinsic efficacy, such as formoterol (1, 26, 30, 31). Our data provide additional insights into the unique pharmacologic properties of salmeterol and suggest that its inability to induce the membrane translocation of β -arrestin, and subsequent receptor endocytosis and degradation, may be related to its low intrinsic efficacy and may contribute to its prolonged bronchodilating effects.

Experimental evidence supports the view that the rate of GPCR endocytosis is governed by the initial rate of receptor phosphorylation by GRK after agonist binding (32). In our previous study (10), we found that the initial rate of GRK-site β_2 AR phosphorylation correlated with agonist intrinsic efficacy for AC activation, and after a 2-min treatment salmeterol induced GRK-site phosphorylation to an extent that was less than that observed with saturating concentrations of the full agonist epinephrine and the partial agonists formoterol and albuterol. Consistent with this previous finding, in this study salmeterol induced less β_2 AR endocytosis than any of these other β -agonists. However, in our previous study, we found that the extent of salmeterol-induced GRK-site phosphorylation was similar to that observed after a concentration of epinephrine (50 nM) that produces a fractional receptor occupancy of 10–15%. Based on these data,

Figure 5. (A) GRK-site phosphorylation after chronic exposure to agonists. 12 β 6 cells were treated with AT alone or AT + 10 μ M epinephrine (EPI), 100 nM RR-formoterol (FOR), or 50 nM salmeterol (SAL) for 0.5 or 18 h. β_2 ARs were resolved by SDS-PAGE and immunoblotted with anti-pS(355,356) Ab and anti-C-tail Ab. Phosphorylation data were normalized to receptor levels and expressed as the percent of epinephrine-induced response, which was set to 100%. The resulting figure depicts the means $\pm$ SEM from three separate experiments. (B) Internalization of β_2 ARs after prolonged exposures to agonist. 12 β 6 cells were treated with AT or AT + 5 μ M epinephrine (EPI), 100 μ M RR-formoterol (FOR), or 50 nM salmeterol (SAL) for 18 h, fixed, and labeled to identify receptors as described. Bar = 10 μ m. (C) Degradation of β_2 ARs after treatment with various agonists. 12 β 6 cells were cultured in 6-well clusters and surface biotinylated as described in MATERIALS AND METHODS. The cells were treated for 22 h with AT (CON) or with one of the following agonists: 10 μ M isoproterenol (ISO), 50 nM salmeterol (SAL), 100 μ M ephedrine (EPH), or 100 nM RR-formoterol (FOR). After agonist treatment, the cells were lysed, and receptors were recovered with streptavidin-agarose. Equal protein loads were electrophoresed and immunoblotted with antibody to the receptor C-terminus. The receptors typically migrated as two bands, one of which results from proteolysis of the epitope tag. In each lane, the density of both bands was measured and summated using a Fluorochem 8800 CCD camera system. $n = 4$, except for formoterol, where $n = 3$. * $P < 0.05$ as compared with control.

by two observations: (1) Salmeterol is unable to trigger the rapid translocation of EGFP- β -arrestin2 to the cell surface (see Figure 3B), and (2) salmeterol induces substantial β_2 AR internalization in cells overexpressing EGFP- β -arrestin2 after a 15-min treatment period (see Figure 4), indicating that the overexpression of β -arrestin, at least in part, overcomes active receptor conformations that are poorly coupled with β -arrestin, as suggested by recently reported data (33). Our findings are consistent with those in a recently published study in which β_2 AR and β -arrestin2 binding was assessed by β -galactosidase complementation, and salmeterol was shown to inhibit β -arrestin-receptor interaction (34). Further, studies of the binding properties of several β_2 -agonists indicate that the aromatic rings of catechols, such as epinephrine and isoproterenol, bind to a different site within the β_2 AR as compared with the aromatic rings of noncatechols, such as albuterol (35). As a result, albuterol induces an active receptor conformation that is distinct from that induced by epinephrine and isoproterenol. Such results provide a mechanism by which saligenins, such as salmeterol and albuterol, stimulate less receptor endocytosis as compared with catechols, such as isoproterenol and epinephrine. However, the level of β_2 AR endocytosis triggered by salmeterol is much lower than that induced by albuterol despite their structurally identical aromatic rings. Although a component of this reduction could be ascribed to salmeterol being somewhat less efficient in activation of AC, it is not consistent with the near complete lack of endocytosis or with the comparison to 50 nM epinephrine-induced endocytosis.

Our data infer that salmeterol binding to the β_2 AR must stabilize a unique receptor activation domain that differs considerably from that of epinephrine, formoterol, or albuterol in its interactions with AC and GRK versus its effects on β -arrestin translocation and receptor endocytosis and down-regulation. Such discrepancies would not be predicted based simply on the two-state receptor model or on this agent's saligenin aromatic ring or lipophilicity (10, 20). It is possible that the binding of the hydrophobic arylalkoxyalkyl side chain of salmeterol to a second site of the β_2 AR is responsible for this proposed unique conformation. Also, we cannot exclude the possibility that

we predicted that the receptor endocytosis after these two treatments would be similar if GRK site phosphorylation was the sole determinant of endocytosis. To the contrary, although 50 nM epinephrine triggered significant β_2 AR endocytosis and β -arrestin translocation, the saturating concentration of salmeterol did not induce appreciable receptor endocytosis or β -arrestin translocation.

We therefore concluded that salmeterol-bound β_2 ARs, though phosphorylated by GRK, must be deficient in their interaction with endogenous β -arrestin. This conclusion is supported

salmeterol stimulates the phosphorylation of distinct sites as compared with other agonists. However, our data indicate that the extents of phosphorylation of two key GRK-sites, ser355 and ser356, after 30-min or 18-h treatments with salmeterol are similar to those measured after treatment with epinephrine or formoterol. Further, the repertoire of potential GRK-sites within the receptor's C-terminus seems to be limited, as demonstrated by recent mass spectrometry studies (11). Thus, salmeterol may represent another example of a growing list of agonists that induce unique patterns of intracellular trafficking of GPCRs by stabilizing altered receptor states, as recently reviewed (6). There is mounting evidence that several β_2 AR agonists cause unique conformations based on fluorescence studies (36) and GTP shifts in agonist affinities (37).

With prolonged exposures to agonist, some β_2 ARs are lost from the cell, further reducing the number of receptors available for signal transduction. This downregulation process has been noted in cells from human subjects after 24 h of regular treatment with the partial agonist metaproterenol (38). Given that β -agonists, including salmeterol, are used regularly in the treatment of chronic asthma, β_2 AR downregulation likely has a key role in the development of chronic drug desensitization. Although there are likely several mechanisms that contribute to β_2 AR downregulation, receptor degradation in lysosomes after receptor internalization has a central role in this process (17). In this study, we show that salmeterol induces much less receptor degradation than other agonists, including isoproterenol and formoterol. This finding is likely due to the inability of salmeterol to stimulate significant β_2 AR endocytosis after acute or prolonged exposures, which has been shown to be required for the major component of efficient receptor downregulation (16, 27). It is possible that this inability to induce β_2 AR degradation contributes to the prolonged activity of salmeterol and the maintenance of bronchodilation even after months of therapy. The failure of salmeterol to trigger receptor degradation is not a result of diminished steady-state, GRK-mediated receptor phosphorylation, suggesting that these two receptor events are not tightly coupled.

Our findings that salmeterol is unable to stimulate significant β_2 AR endocytosis differs from our previously published results (8). In the previous study, we showed that the extent of salmeterol-induced receptor internalization was $\sim 50\%$ after 30 min and was comparable to that seen with albuterol. However, in that study, receptor internalization was determined by measuring the loss of binding of the β_2 AR antagonist [3 H]CGP12177, whose binding is dependent on the displacement of agonist. Given the high affinity of salmeterol for the β_2 AR and its ability to inhibit the binding of other agonists, it is likely that the apparent receptor internalization observed in that study was spurious and reflected the inefficient displacement of salmeterol by CGP12177. In the current study, we measured receptor endocytosis using immunologic methods (ELISA and immunofluorescence microscopy), which are not dependent on agonist binding and which clearly demonstrate a marked defect in salmeterol-induced β_2 AR endocytosis.

The low intrinsic efficacy of salmeterol and the lack of endocytosis induced by this agonist may have important clinical implications. Of interest is the observation that both of the asthma mortality epidemics in New Zealand and the United Kingdom were associated with the use of high-dose formulations of agonists of high intrinsic efficacy (39, 40). Toxicity from these drugs may result from their stimulation of nontarget tissues containing low levels of β_2 ARs that are not effectively stimulated by agonists of low intrinsic efficacy. Alternatively, the high degree of β_2 AR desensitization induced by a full agonist might result in clinically significant tachyphylaxis. For instance, formoterol, an agonist of

high intrinsic efficacy, has been shown in clinical trials to provide prolonged bronchodilation in subjects with asthma, but some tachyphylaxis to its bronchodilating properties has been observed (41). Consistent with this observed receptor desensitization in clinical settings, the ability of formoterol to stimulate GRK-site phosphorylation exceeds that of salmeterol (10), and formoterol induces β -arrestin translocation and β_2 AR endocytosis and degradation similarly to the full agonists epinephrine and isoproterenol. Thus, agonists of low intrinsic efficacy may have advantages in the maintenance therapy of asthma and COPD (42). Such agonists cause less desensitization by virtue of their low efficacies, and salmeterol seems to have a further reduction in the component of receptor desensitization attributable to rapid endocytosis, interaction with β -arrestin, and in chronic receptor degradation. The clinical importance of these factors and the optimal level of intrinsic efficacy that achieves adequate response while minimally activating nontarget tissues and inducing desensitization remains to be determined. Furthermore, factors other than intrinsic efficacy, such as genetic variations in the β_2 AR and race, may contribute to the potential toxicity observed with the prolonged use of β -agonists, including salmeterol, and these need to be evaluated further in future studies (43, 44).

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