Salmeterol Stimulation Dissociates β2-Adrenergic Receptor Phosphorylation and Internalization


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 β2-adrenergic receptor (β2AR) 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 β2AR 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 β2AR 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 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: β2-adrenergic receptor; salmeterol; phosphorylation; internalization; desensitization

β2-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 β2-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 β2-adrenergic receptors (β2-ARs) by reversible antagonist binding (2). Salmeterol has the same saligenin active group as the β2-agonist albuterol but contains a long hydrophobic araloyloxyalkyl 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 β2AR 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). β2AR 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 β2ARs are fully uncoupled from G 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 β2-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).

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.
By directly measuring the Kᵦ and EC₅₀ of adenylyl cyclase (AC) activation, we previously have estimated the intrinsic efficacy of salmeterol activation of AC to be ~12% that of the full agonists epinephrine and isoproterenol, ~20% that of the highly lipophilic long-acting agonist formoterol, ~50% that of the structurally similar agonist albuterol, and ~4-fold greater than that of the very weak partial agonist epididymis (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-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 β₂AR with an N-terminal hemagglutinin (HA) epitope tag and with Arg at position 16 and Glu at position 27. The receptors are expressed at an approximate density of 300,000 receptors per cell (gift of B. Koblika, Palo Alto, CA). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS and 400 μg/ml of G418. Mouse monoclonal IgG against the HA epitope (mAb HA.11) was purchased from Roche Molecu-ular Biology (Uxbridge, Middlesex, UK). All other reagents were from Sigma. The structurally similar agonist albuterol, and 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-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.

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 μg of pEGFP-β-arrestin2 or pEGFP using 3 μl of FuGENE6 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 β₂ARs were detected using the monoclonal antibody mHA.11 (5 μg/ml) followed by goat α-mouse IgG-TRITC secondary antibody (5 μg/ml). Images were acquired as described previously. For experiments involving EGFP-β-arrestin2 overexpression, cells of similar brightness were selected for imaging.

Determination of β₂AR Degradation

β₂AR 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 μg/ml). Cells were pelleted and solubilized at 4°C in solubilization buffer (20 mM Heps [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 × g for 20 min at 4°C to remove cellular debris. From each sample, 5 μg of lysate was added and the streptavidin (Sigma) was bound 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 × g to remove cellular debris. From each sample, 5 μg of lysate was added and the streptavidin (Sigma) was bound 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 × g to free biotinylated receptors from the streptavidin-agarose. A 10-μg aliquot of eluted protein from each sample was treated with 2 μl 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 antibody for C-terminus polycyclonal 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 β₂ARs. We found the range to be linear up to and possibly beyond 3.3 × 10⁵ integrated density values. In our experiments, the integrated density values usually ranged from 5 × 10⁴ to 3.3 × 10⁵.

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 μM 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
Results

After exposure to a full agonist, β2ARs rapidly move into punctate intracellular vesicles, consistent with early endosomes (19). To examine the receptor response to other ligands, 12β6 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 β2ARs and examined by immunofluorescence microscopy. In untreated cells, β2ARs 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 β2ARs (Figure 1B), consistent with earlier results using isoproterenol (19). RR-formoterol (Figure 1C) and albuterol (Figure 1D) caused the appearance of intravesicular β2ARs. 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 Kd) (8), or after treatment with the weak partial agonist ephedrine at 100 nM (Figure 2).

To determine whether salmeterol and ephedrine could evoke the endocytosis of β2ARs at too low a rate to be detectable by immunofluorescence microscopy, we used a quantitative ELISA technique. Agonist-treated 12β6 cells were fixed but not permeabilized before binding the anti-HA antibody so that only surface β2ARs (which are tagged at the extracellular N-terminus) were detected. Treatment with epinephrine for 60 min caused a 49% loss of the surface β2ARs, whereas treatment with albuterol caused a 24% loss (Figure 2). These percentages reflect the steady-state level of surface β2ARs predicted by the rate constants 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 β2ARs, 12β6 cells were treated with 5 μM 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 μM 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%), ~ 20% of the β2ARs underwent endocytosis, with steady-state internalization being reached within 10 min, whereas < 5% of the β2ARs were internalized after 30 min in cells treated with 50 nM salmeterol, a saturating concentration.

Because β2AR 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. 12β6 cells transiently expressing an EGFP-β-arrestin2 chimera were exposed to vehicle, 5 μM 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 μM 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 μM) and formoterol (40 nM) stimulated EGFP-β-arrestin2 translocation to the cell surface (data not shown).
Figure 2. Quantification of surface β2ARs using ELISA. 1.2β6 cells growing in 96-well plates were treated for varying times with ligands and fixed and assayed for surface β2ARs. Closed squares, 5 μM epinephrine (EPI). Open squares, 40 nM RR-formoterol (FOR). Closed diamonds, 20 nM salmeterol (SAL). Open circles, 100 μM ephedrine (EPH). Closed triangles, 6 μM albuterol (ALB). The figure depicts the means ± SEM from three separate experiments.

The high-affinity binding of β-arrestin to β2ARs 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 β2AR 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 β2AR 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, 12β6 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 μM ephedrine (Figures 4C and 4F) for 15 min, fixed, and labeled with antibody to identify β2ARs. As shown in Figures 4B and 4E, in cells overexpressing EGFP-β-arrestin2, a marked redistribution of β2ARs 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 β2ARs 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
observed in internal vesicles is expected given that arrestin is known not to traffic with β2ARs after receptor endocytosis (25).

Although salmeterol stimulates considerable rapid GRK-site phosphorylation of β2ARs (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 β2ARs is significantly reduced by a process termed “down-regulation,” which contributes to chronic receptor desensitization (17). In 12β6 cells, β2AR 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 β2AR internalization and downregulation after prolonged exposures to agonist is dependent on agonist intrinsic efficacy. Because over time receptor synthesis may affect the measurement of β2AR internalization by the ELISA, we assessed whether salmeterol stimulated some receptor internalization after a prolonged exposure to agonist using immunofluorescence microscopy. 12β6 cells were treated with AT alone or with saturating concentrations of the indicated agonists for 18 h and fixed and labeled to identify β2ARs 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 β2AR 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 β2AR 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 β2AR due to its lipophilicity and the interaction of its tail region with the β2AR (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 β2AR 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 β2AR 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,
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 β2-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 β2-ARs, though phosphorylated by GRK, must be deficient in their interaction with endogenous β-arrestin. This conclusion is supported 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 β2-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 β2-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 β2-agonists indicate that the aromatic rings of catechols, such as epinephrine and isoproterenol, bind to a different site within the β2-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 β2-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 β2-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 aralyloxyalkyl side chain of salmeterol to a second site of the β2-AR is responsible for this proposed unique conformation. Also, we cannot exclude the possibility that

Figure 5. (A) GRK-site phosphorylation after chronic exposure to agonists. 12β6 cells were treated with AT alone or AT + 10 μM epinephrine (EPI), 100 nM RR-formoterol (FOR), or 50 nM salmeterol (SAL) for 0.5 or 18 h. β2-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 ± SEM from three separate experiments. (B) Internalization of β2-AR after prolonged exposures to agonist. 12β6 cells were treated with AT or AT + 5 μM epinephrine (EPI), 100 μM RR-formoterol (FOR), or 50 nM salmeterol (SAL) for 18 h, fixed, and labeled to identify receptors as described. Bar = 10 μm. (C) Degradation of β2-ARs after treatment with various agonists. 12β6 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 μM isoproterenol (ISO), 50 nM salmeterol (SAL), 100 μM epinephrine (EPI), 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 summed using a Fluorochem 8800 CCD camera system. n = 4, except for formoterol, where n = 3. *P < 0.05 as compared with control.
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 β2AR agonists cause unique conformations based on fluorescence studies (36) and GTP shifts in agonist affinities (37).

With prolonged exposures to agonist, some β2ARs 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 β2-agonists, including salmeterol, are used regularly in the treatment of chronic asthma, β2AR downregulation likely has a key role in the development of chronic drug desensitization. Although there are likely several mechanisms that contribute to β2AR 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 β2AR 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 β2AR 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 β2AR endocytosis differs from our previously published results (8). In the previous study, we showed that the extent of salmeterol-induced receptor internalization was ~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 β2AR antagonist [3H]CGP12177, whose binding is dependent on the displacement of agonist. Given the high affinity of salmeterol for the β2AR 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 β2AR 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 β2ARs that are not effectively stimulated by agonists of low intrinsic efficacy. Alternatively, the high degree of β2AR 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 patients 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 β2AR 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 β2AR and race, may contribute to the potential toxicity observed with the prolonged use of β2-agonists, including salmeterol, and these need to be evaluated further in future studies (43, 44).

Conflict of Interest Statement: R.H.M. received lecture fees yearly from 2003–2006 for speaking at events sponsored by GlaxoSmithKline. E.E.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.A.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.M.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.J.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.F.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.B.C. received two grants from Glaxo in 1995 ($25,000 and $75,000) and received $1,500 for a talk for Novartis Pulmonary Group, October 18, 2005.

References

10. Tran TM, Friedman J, Qunaibi E, Baumeur F, Moore RH, Clark RB. Characterization of agonist stimulation of cAMP-dependent protein kinase and G protein-coupled receptor kinase phosphorylation of the


