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Salmeterol-induced desensitization, internalization and phosphorylation of the human β_2-adrenoceptor

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1 Partial agonists of the β_2-adrenoceptor which activate adenylyl cyclase are widely used as bronchodilators for the relief of bronchoconstriction accompanying many disease conditions, including bronchial asthma. The bronchodilator salmeterol has both a prolonged duration of action in bronchial tissue and the ability to reassert this activity following the temporary blockade of human β_2-adrenoceptors with antagonist.

2 We have compared the activation and desensitization of human β_2-adrenoceptor stimulation of adenylyl cyclase induced by salmeterol, adrenaline and salbutamol in a human lung epithelial line, BEAS-2B, expressing β_2-adrenoceptor levels of 40–70 fmol mg^{-1}, and in human embryonic kidney (HEK) 293 cell lines expressing 2–10 pmol mg^{-1}. The efficacy observed for the stimulation of adenylyl cyclase by salmeterol was only $\cong$10% of that observed for adrenaline in BEAS-2B cells expressing low levels of β_2-adrenoceptor, but similar to adrenaline in HEK 293 cells expressing very high levels of receptors. Salmeterol pretreatment of these cells induced a rapid and stable activation of adenylyl cyclase activity which resisted extensive washing and β_2-adrenoceptor antagonist blockade, consistent with binding to a receptor exosite and/or to partitioning into membrane lipid.

3 The desensitization and internalization of β_2-adrenoceptors induced by the partial agonists salmeterol and salbutamol were considerably reduced relative to the action of adrenaline. Consistent with these observations, the initial rate of phosphorylation of the receptor induced by salmeterol and salbutamol was much reduced in comparison to adrenaline.

4 Our data suggest that the reduction in the rapid phase of desensitization of β_2-adrenoceptors after treatment with salmeterol or salbutamol is caused by a decrease in the rate of β_2-adrenoceptor kinase (βARK) phosphorylation and internalization. In contrast, the rate of cyclic AMP-dependent protein kinase (PKA)-mediated phosphorylation by these partial agonists appears to be similar to adrenaline.

Keywords: Salmeterol; β_2-adrenoceptor; desensitization; internalization; phosphorylation

Introduction

Agonist activation of β_2-adrenoceptors increases adenylyl cyclase activity through the stimulatant protein G_s. Partial β_2-adrenoceptor agonists are traditionally defined as those agents exhibiting reduced efficacy for the activation of adenylyl cyclase relative to full β_2-adrenoceptor agonists such as adrenaline and isoprenaline. Partial β_2-adrenoceptor agonists are among the most commonly prescribed drugs in the treatment of asthma. Numerous clinical studies indicate that the partial agonist salmeterol provides sustained bronchodilatation and protection against exercise-induced asthma and nighttime exacerbation of asthma for at least 12 h following a single dose (Ball *et al*., 1991; Cheung *et al*., 1992; Johnson *et al*., 1993; Nials *et al*., 1993; Sears, 1993). The ability of this drug to produce prolonged bronchodilatation of tracheal smooth muscle has been the subject of extensive studies (reviewed in Coleman *et al*., 1996). The bronchodilatation caused by salmeterol is unusual compared with short-acting β_2-adrenoceptor agonists, such as salbutamol and adrenaline, in that it is resistant to washing and even shows the ability to recover activity following temporary blockade of β_2-adrenoceptors by antagonists. Salmeterol is extremely lipophilic: it is taken up into liposomes in less than 1 min, and displays a partition coefficient of $\approx$1/23,000 (water/lipid) with a correspondingly slow release ($t_{1/2}$ = 30 min) (Rhodes *et al*., 1992). Although membrane partitioning and reassertion have been noted for other lipophilic agonists such as formoterol (for review, see Anderson, 1993), salmeterol has a longer duration of action and its effects are sustained even at submaximally effective concentrations (Naline *et al*., 1994).

In an effort to explain its persistent activation of the β_2-adrenoceptor, two hypotheses have been proposed: that salmeterol's membrane association may involve high-affinity binding to a specific site on or near the β_2-adrenoceptor which is distinct from the activating site (the exosite hypothesis) (Ball *et al*., 1991; Johnson *et al*., 1993; Nials *et al*., 1993); or, salmeterol may partition into the lipid bilayer in a dose-dependent manner (the membrane diffusion microkinetic hypothesis) (Anderson, 1993).

Supporting the exosite hypothesis is the finding that mutation of a small portion of the fourth transmembrane domain of the β_2-adrenoceptor substantially reduces, but does not abolish, persistent salmeterol association and receptor activation (Green *et al*., 1996). On the other hand, aliphatic side chain analogues of salmeterol, with no agonist efficacy, do not prevent the reassertion of salmeterol-induced relaxation

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after a temporary block with a reversible antagonist (Bergendahl *et al*., 1995a).

To be long-acting, an agonist must persist at the site of action and not cause extensive receptor desensitization. There is no evidence from studies of bronchodilatation that chronic salmeterol treatment induces tolerance (desensitization) to its bronchodilating effects, although there is evidence that tolerance develops to its protective effects against a bronchoconstrictor stimulus (Cheung *et al*., 1992; Ramage *et al*., 1994; Bhagat *et al*., 1995). One of the primary actions of β_2-adrenoceptor agonists in the relaxation of smooth muscle is the activation of adenylyl cyclase (Johnson *et al*., 1993), and the relaxant effects of salmeterol on airway smooth muscle are directly correlated with the activation of adenylyl cyclase and subsequent intracellular accumulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Ellis *et al*., 1995). However, there have been few insights into the cellular mechanisms of salmeterol activation and desensitization of β_2-adrenoceptor stimulation of adenylyl cyclase. Our group previously found that the hamster β_2-adrenoceptor stably transfected into mouse L cells showed persistent activation of adenylyl cyclase after pretreatment of cells with salmeterol. Salmeterol also appeared to cause a reduced desensitization relative to adrenaline (Clark *et al*., 1996). However, the molecular basis for the reduction was not understood.

To probe the mechanisms involved in the reduced desensitization we initiated a comparative study of salmeterol-, salbutamol-, and adrenaline-induced desensitization in two human cell lines, the BEAS-2B human lung epithelial cell line, and sublines of the human embryonic kidney cell line, HEK 293, which has been stably transfected with either the 12CA5 epitope-modified human β_2-adrenoceptor (12β6) (Von Zastrow & Kobilka, 1992), or with the 12β6 β_2-adrenoceptor additionally modified by the insertion of 6 histidine residues on the C-terminus (HAβAR6HIS). The main reasons for selecting these cell lines were as follows: firstly, they express the human β_2-adrenoceptor in human-derived cell lines. This provides a more straightforward comparison of our cellular studies to those of the action of salmeterol in human lung tissue than our previous study of the hamster receptor expressed in mouse L cells that have no endogenous β_2-adrenoceptor. Further, it is well known that even minor changes in β_2-adrenoceptor structure, as is found in a comparison of the hamster and human receptors, could significantly alter the characteristics of agonist binding, activation and desensitization. Secondly, the effect of receptor number on the action of salmeterol could be explored because these cell lines express over a 100 fold difference in human β_2-adrenoceptor levels, since the BEAS-2B cell line expresses the β_2-adrenoceptor at 40–70 fmol mg^{-1} membrane protein (similar to airway smooth muscle, Green *et al*., 1996), and the HEK cell lines express 2–10 pmol mg^{-1}. Thirdly, the HEK 293 lines with overexpressed β_2-adrenoceptors provide excellent model cell lines for the study of internalization and phosphorylation of the receptor.

Our investigation of the desensitization of β_2-adrenoceptors in these cell lines in response to salmeterol, salbutamol and adrenaline revealed that the rapid desensitization provoked by adrenaline treatment of cells was greater than that observed for salmeterol and salbutamol, and that for salmeterol this difference persisted for hours. We found that the initial rate of phosphorylation of the β_2-adrenoceptor induced by either salmeterol or salbutamol was reduced relative to adrenaline. The extent of internalization of the β_2-adrenoceptor by the two partial agonists was also reduced relative to adrenaline. These data, coupled with recent evidence that the βARK/β-arrestin pathway is necessary for internalization (Tsuga *et al*., 1994; Ferguson *et al*., 1996; Goodman *et al*., 1996), suggest that the reduced desensitization observed with salmeterol is primarily a function of reduced homologous (βARK/β-arrestin and internalization), but not heterologous (PKA-mediated) desensitization. We conclude that the longevity of salmeterol action in these cell lines is a complex function not only of the tenacity of its association with the receptor following intact cell treatment, but also of a reduced extent of receptor desensitization.

Methods

Preparation of the 6-histidine-modified β_2-adrenoceptor

The 12β6 clone of HEK 293 cells overexpressing the haemagglutinin (HA) epitope-tagged β_2-adrenoceptors were obtained from Brian Kobilka (Stanford University, Palo Alto, CA). The HA epitope, an 11 amino acid sequence containing the antigen to the 12CA5 antibody, was introduced into the N-terminus following the first two amino acids (met-gly). The β_2-adrenoceptor in this cell line is overexpressed to 7–10 pmol mg^{-1} membrane protein. The HA epitope-modified receptor was further modified by insertion of 6 histidines at the C-terminus following the last two amino acids (leu-leu), and these residues were followed by a stop codon. This modification was introduced with the polymerase chain reaction (PCR). The PCR products were gel purified, digested with Sal I (which also cuts Acc I sites), and recloned into the pBC12B1 expression vector and digested with Acc I and Sal I. The modified receptor, cloned into pBC12B1, was sequenced to check for errors in the PCR and to verify the predicted structure.

Transfection of the 6-histidine-modified β_2-adrenoceptor into HEK 293 cells

The HEK 293 cell line was chosen in part because it expresses an endogenous β_2-adrenoceptor at a very low level (6–10 fmol mg^{-1}). This low expression does not interfere with characterization of the overexpressed receptor at levels over 100 fold higher. HEK cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% foetal bovine serum (FBS) at 37°C in 5% CO_2. The expression vector containing the 6-histidine-modified receptor was transfected into HEK 293 cells, along with the neomycin-expressing plasmid pSV2neo, by calcium phosphate co-precipitation in a mass ratio of 100:1. Cells were shocked the next day with 25% glycerol for 1 min, and one day later were split into 96 well dishes into selection medium (DMEM containing 10% FBS and geneticin at 400 μg ml^{-1}). Stable transformants expressing the receptor were selected by use of an intact cell [^{3}H]-CGP-12177 binding assay as described below. One cell line expressing 2–3 pmol of the β_2-adrenoceptor mg^{-1} membrane protein was selected for use in the internalization and phosphorylation experiments. This clone is referred to as HAβAR6HIS. The extent of agonist-induced desensitization of the β_2-adrenoceptor in this cell line did not differ significantly from that observed either in the BEAS-2B cell line, or in the12β6 cell line (data not shown). BEAS-2B cells were purchased from the American Type Culture Collection, and were cultured in Ham's F-12 with 10% FBS.

Cell treatments and membrane preparation

Cells were pretreated with the various hormones, drugs and appropriate control buffers in 150 mm tissue culture dishes in

growth medium for the times indicated in the individual experiments. All incubations of cells with agonists were performed with a final concentration of 1.0 mM thiourea and 0.1 mM sodium ascorbate (AT) to prevent oxidation of the catecholamine. After pretreatment the medium was removed, and the cells were washed 6 times with 10 ml of ice-cold HME buffer (20 mM HEPES pH 8, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 2 mM tetrasodium pyrophosphate, 10 μg ml^{-1} trypsin inhibitor, 0.1 mg ml^{-1} bovine serum albumin). The cells were then scraped into 8 ml of HME + 10 μg ml^{-1} leupeptin, and homogenized with 7 strokes of a type B Dounce homogenizer on an ice bath. The homogenates were layered over step gradients of 23% and 43% sucrose in HE buffer (20 mM HEPES pH 8, 1 mM EDTA) in a cold room, and centrifuged for 40 min at 25,000 r.p.m. in a Beckman SW28.1 rotor at 4°C. The membranes at the 23/43% interface were removed, frozen in liquid nitrogen and stored at −80°C. The membranes were diluted in HE buffer before the assays.

Adenylyl cyclase assays

Adenylyl cyclase activity in the membrane preparations was measured as previously described (Whaley *et al*., 1994; Yuan *et al*., 1994) with the free Mg^{2+} concentration set at 0.3 mM, and a final concentration of ATP at 0.1 mM. All adenylyl cyclase incubations were for 10 min at 30°C.

Agonist binding assays and measurement of receptor levels

Receptor numbers (B_{max}) and the K_d for [^{125}I]-CYP were determined by Scatchard analysis of [^{125}I]-CYP binding to membranes as previously described (Whaley *et al*., 1994; Yuan *et al*., 1994) with the following modifications. The concentration of [^{125}I]-CYP was varied from 1 to 200 pM, and the radioligand was diluted in HE buffer plus 50 μM phentolamine, 0.1 mM sodium ascorbate and 1 mM thiourea. Nonspecific binding was assessed by the inclusion of alprenolol at 1.0 μM.

The K_d values for agonist binding to the β_2-adrenoceptor were determined by displacement of 20–40 pM [^{125}I]-CYP in the presence of 10 μM GTP by use of the Cheng-Prussoff correction. Data analysis was performed with the computer program GraphPad. The K_d values for salmeterol, adrenaline and salbutamol binding to the endogenous β_2-adrenoceptors in the HEK 293 cells were 1.5 ± 0.5, 711 ± 104 and 528 ± 99 nM, respectively, and were similar, within experimental variations, to the K_ds measured for the epitope-modified receptors.

Receptor internalization

HAβAR6HIS or 12β6 cells growing in six-well culture dishes were incubated with AT or agonists for 0–30 min. The attached cells were washed 3 times (30 s total time) in 37°C DMEM and then 4 times with ice-cold DMEM. The warm washes considerably reduced the level of salmeterol carried over into the binding assay. Cell surface receptor number was determined by incubation at 0–3°C with 10–20 nM [^{3}H]-CGP-12177 for 1 h in the absence of presence of 1.0 μM alprenolol (Hertel *et al*., 1983). After three washes to remove excess ligand the cells were removed by treatment with trypsin/EDTA and the bound radioactivity was measured after removal of the cells by scintillation counting. Nonspecific binding was determined in the presence of 1.0 μM alprenolol, and competition by quasi-irreversibly associated salmeterol was assessed by the inclusion of 100 μM metoprolol or 500 μM atenolol. Total receptor levels were assessed by [^{3}H]-CGP-12177 binding in the presence of 0.2% digitonin (Slowiejko *et al*., 1994), which permeabilizes the cells and allows access of the ligand to internalized receptors.

Receptor phosphorylation

HAβAR6HIS cells were grown to confluency and labelled with [^{32}P]-H_3PO_4. Cells were then treated with various agonists, solubilized and subjected to a two step purification procedure which utilized Ni-NTA-agarose and wheat germ agglutinin-agarose as previously described (January *et al*., 1997). SDS polyacrylamide gel electrophoresis was performed on the purified receptor by use of 7.5% polyacrylamide gels with the addition of pre-stained low molecular weight standards (BioRad). Gels were dried and ^{32}P was quantified by a Molecular Dynamics Phosphorimager Model 860 and Imagequant software. The gels were exposed to the phosphorscreen from 3–24 h. The fold stimulation of phosphorylation was determined by calculation of the ratio of the agonist-treated samples to the vehicle-treated controls. ^{32}P area/volume quantitation by Imagequant could be converted to counts per minute (c.p.m.) by spotting a known amount of ^{32}P to the dried gels. Within an individual experiment, where independent samples were processed in triplicate, the standard error was minimal. For example, in one experiment, ^{32}P c.p.m. for 5 min with 10 μM adrenaline ($n = 3$) were 265 ± 8.5. The identity of the β_2-adrenoceptor band was confirmed by (i) its absence in the parental HEK 293 lines carried through an identical purification, and (ii) Western blotting of the purified receptor following electrotransfer using anti-HA polyclonal antibody as the primary antibody(BABCO) and horseradish peroxidase-conjugated goat anti-rabbit (BioRad) as the secondary antibody.

Calculation of % desensitization

Recently we verified experimentally that the following equation describes the change in the parameters of the adenylyl cyclase assay which accompany a change in receptor number (Whaley *et al*., 1994; 1995):

$$\frac{V_{max}}{EC_{50}} = \frac{V_{100}\,\frac{k_1}{k_{-1}}\,r}{K_d} \qquad \textit{Equation } 1$$

where V_{max} is the maximal adenylyl cyclase activity observed for saturating concentrations of agonist; EC_{50} is the concentration of agonist which yields half the maximal adenylyl cyclase activity; k_1, the coupling efficiency, is the rate constant for activation of adenylyl cyclase by β_2-adrenoceptor; k_{-1} is the rate constant for adenylyl cyclase inactivation; r is the total receptor number; V_{100} is the theoretical value representing adenylyl cyclase activity when k_1 is infinite; and K_d is the binding constant for agonist and β_2-adrenoceptor. k_{-1} is a first order rate constant which is independent of receptor and agonist levels and therefore can be assumed to be invariant. Any changes observed in the term k_1/k_{-1} can be attributed to variations in k_1.

Since the ability of an agonist to induce adenylyl cyclase activation is dependent on both the coupling efficiency (k_1) and the receptor number (r), we expressed the total capacity of β_2-adrenoceptors to activate adenylyl cyclase as k_1r. We defined k_1r as the coupling capacity for agonist-bound β_2-

adrenoceptors at a given receptor density and calculate this as follows:

$$\frac{k_1}{k_{-1}}\mathrm{r} = \frac{V_{max}\ K_d}{V_{100}\ EC_{50}} \qquad \textit{Equation 2}$$

Since k_{-1} is a constant, (k_1/k_{-1})r is a quantitative measure of the coupling capacity of β_2-adrenoceptors in the membrane preparation.

β_2-Adrenoceptor desensitization in response to high occupancy by strong agonists is characterized by the phosphorylation of β_2-adrenoceptors by PKA and βARK (Clark, 1986; Perkins *et al*., 1991; Inglese *et al*., 1993; Yuan *et al*., 1994), which have been shown to uncouple receptors from their ability to activate adenylyl cyclase. Quantitatively this represents a decrease in the coupling efficiency (k_1). In addition, during desensitization surface receptors are lost due to internalization and downregulation (Clark, 1986; Perkins *et al*., 1991; Moore *et al*., 1995), causing a decrease in r. Since k_1 and r are both expected to decrease, we used the change in the receptor's coupling capacity to determine the extent of desensitization which has occurred following agonist exposure. This can be expressed quantitatively as a ratio of (k_1/k_{-1})r for desensitized receptor to (k_1/k_{-1})r for naive receptor. By definition, V_{100} is the same for the desensitized and naive receptor. In addition, Strasser *et al*. (1984, 1986) have shown that the β_2-adrenoceptor low affinity K_d for agonist does not change when the receptor is uncoupled, due to the phosphorylation by a kinase which occurs during desensitization. Therefore, the equation to calculate β_2-adrenoceptor desensitization as the ratio of $\{(k_1/k_{-1})\mathrm{r}\}_D$ and $\{(k_1/k_{-1})\mathrm{r}_N$ can be simplified to the following form:

$$\%\ \text{desensitization} = \left(1 - \frac{(\frac{k_1}{k_{-1}}\mathrm{r})_D}{(\frac{k_1}{k_{-1}}\mathrm{r})_N}\right) \times 100\% \qquad \textit{Equation 3}$$

$$= \left(1 - \frac{V_{max_D}\ EC_{50_N}}{EC_{50_D}\ V_{max_N}}\right) \times 100\%$$

where 'D' indicates measurements for the desensitized receptor and 'N' indicates measurements made for the naive receptor. This allows the calculation of the % desensitization for the receptor following agonist pretreatments by use of the V_{max} and EC_{50} values which are measured for β_2-adrenoceptor before and after desensitization. In our previous study of salmeterol-induced desensitization of the hamster β_2-adrenoceptor (Clark *et al*., 1996), the extent of desensitization was estimated by the changes in the EC_{50} only. The formulation given above used in the present work gives a more quantitative measure of desensitization, especially in cells expressing low levels of receptor, such as the BEAS-2B, where changes in V_{max} are more pronounced (Whaley *et al*., 1994).

Materials

Salmeterol and metoprolol were provided by Glaxo Research and Development (U.K.). Salmeterol was dissolved in a minimal volume of glacial acetic acid and then diluted and stored at a concentration of 10 mM in phosphate-buffered saline (PBS) (2.68 mM KCl, 1.47 mM KH_2PO_4, 152 mM NaCl and 8.06 mM Na_2HPO_4). Adenosine 5′-triphosphate (ATP), salbutamol, alprenolol and adrenaline were obtained from Sigma Chemical Co. [α^{32}P]-ATP and [^{3}H]-CGP-12177 ((±)-4-(3-*t*-butylamino-2-hydroxypropoxy)denzimidazol-2-one) were obtained from Dupont/NEN. GTP and monoclonal antibody 12CA5 were obtained from Boehringer Mannheim. [^{125}I]-iodocyanopindolol ([^{125}I]-CYP) was prepared as described previously (Whaley *et al*., 1994). Ni-NTA agarose was obtained from Qiagen. Wheat germ agglutinin agarose was obtained from Vector Laboratories, and n-dodecyl-β-D-maltoside was obtained from Calbiochem.

Results

Salmeterol as a partial agonist in the activation of adenylyl cyclase

The effect of various concentrations of salmeterol and adrenaline on the stimulation of adenylyl cyclase in membranes prepared from the high (12β6) and low (BEAS-2B) β_2-adrenoceptor-expressing cell lines is shown in Figure 1. Salmeterol activation of adenylyl cyclase in membranes from the cultured cell lines occurred without a lag, in contrast to slow onset of relaxation of smooth muscle in clinical studies and in tissue preparations (Johnson *et al*., 1993; Naline *et al*., 1994; Anderson *et al*., 1996). In the BEAS-2B membranes, the efficacy for salmeterol was only approximately 10% of that of adrenaline (activity above basal), as would be expected for a partial agonist. However, in membranes from the 12β6 line (expressing 100 fold higher β_2-adrenoceptor levels) the V_{max} values for salmeterol and adrenaline activation of adenylyl cyclase were identical within experimental error. The data in Figure 1 also demonstrate that there was a profound (>200 fold) increase in the EC_{50} for adrenaline activation of adenylyl cyclase in the BEAS2B cells relative to the 12β6 cells (2–3 nM). These results agree well with the predictions of our recent work that the EC_{50} for agonist activation of adenylyl cyclase increases as β_2-adrenoceptor levels decrease, and that agonist efficacy (V_{max}) decreases when receptor levels become very low (Whaley *et al*., 1994; 1995). The EC_{50} for salmeterol from Figure 1 was in the range of 1–2 nM. However, we do not feel that this is an accurate measure in these cell lines because low concentrations were skewed by absorption of the agonist to the walls of the tubes and diffusion into membranes. Further, salmeterol stimulation of adenylyl cyclase in the BEAS-2B cells was too weak to obtain reliable EC_{50} values. For these reasons

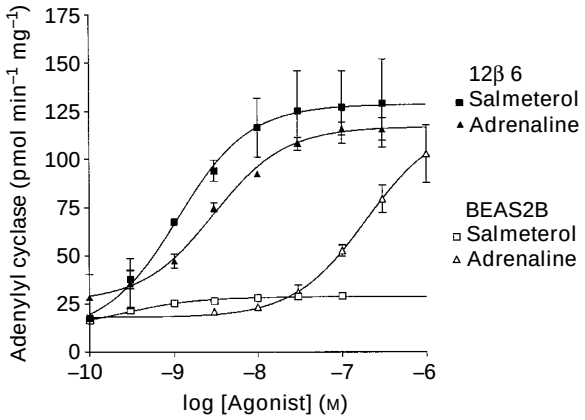

Figure 1 Comparison of adrenaline and salmeterol activation of adenylyl cyclase in membranes from cells expressing low and very high β_2-adrenoceptor levels. Membranes prepared as described in Methods from BEAS-2B cells expressing 40–70 fmol β_2-adrenoceptor mg^{-1} (open symbols) or 12β6 cells expressing 7–10 pmol β_2-adrenoceptor mg^{-1} (solid symbols). Adenylyl cyclase assays were performed in triplicate at the various concentrations of either salmeterol or adrenaline. The graph shows the averages and range of the data from two experiments.

we have been unable to calculate the coupling efficiency of salmeterol activation of the β_2-adrenoceptor using the rigorous formations we previously introduced (Whaley *et al*., 1994; Yuan *et al*., 1994; January *et al*., 1997). However, based on the dramatic drop in the V_{max} for salmeterol in the BEAS-2B cells, the coupling efficiency of salmeterol was estimated to be approximately 10% that of adrenaline.

Salmeterol pretreatment of cells results in a stable, concentration-dependent activation of adenylyl cyclase

Previous studies demonstrated that salmeterol causes a prolonged relaxation of airway smooth muscle even after washout of free drug ($t_{1/2} > 10$ h), suggesting, but not demonstrating, a sustained activation of adenylyl cyclase (Ball *et al*., 1991; Johnson *et al*., 1993; Nials *et al*., 1993). To investigate this directly, we pretreated both the 12β6 and BEAS-2B cell lines with 1, 10 or 100 nM salmeterol for 5 min, washed the cells extensively, and then assayed basal and adrenaline-stimulated adenylyl cyclase activity in purified membrane preparations. As shown in Figure 2, salmeterol pretreatment caused a concentration-dependent, stable activation of 'basal' membrane adenylyl cyclase activity (i.e., the activity without adrenaline stimulation) which survived the extensive washing and membrane purification, similar to our previous observations with the hamster β_2-adrenoceptor expressed in L cells (Clark *et al*., 1996). This effect was far more pronounced in the high expression 12β6 cells (solid lines in Figure 2), but was also observable to a lesser extent for the BEAS-2B cells (dashed lines in Figure 2). In contrast, neither 10 μM adrenaline nor 5 μM salbutamol pretreatment caused an increase in basal activity with either cell line (data not shown).

Desensitization of β_2-adrenoceptors induced by salmeterol, salbutamol and adrenaline in the BEAS-2B cell line

It has been demonstrated that the extent of β_2-adrenoceptor desensitization can be determined by the measurement of changes in the EC_{50} and V_{max} for adrenaline stimulation of adenylyl cyclase following agonist pretreatment (Clark, 1986; Perkins *et al*., 1991; Inglese *et al*., 1993; Yuan *et al*., 1994). With typical agonists such as adrenaline or salbutamol that are completely washed out after pretreatment of cells, the resulting β_2-adrenoceptor desensitization causes an increase in the EC_{50} which may also be accompanied by a decrease in the observed V_{max}. The adrenaline dose-response curves in Figure 2 indicate that salmeterol pretreatment caused concentration-dependent increases in the EC_{50} for adrenaline activation of adenylyl cyclase derived from both the BEAS-2B and 12β6 cell lines. In addition, decreases in the V_{max} were observed as the concentration of salmeterol pretreatment increased.

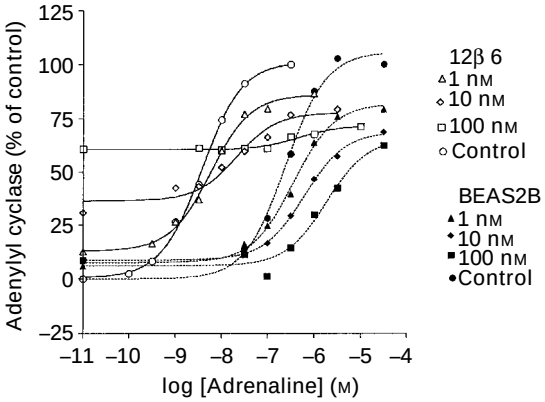

Figure 2 Effect of 5 min salmeterol pretreatment of BEAS-2B (solid symbols) and 12β6 cells (open symbols) on basal and adrenaline-stimulated adenylyl cyclase activity. BEAS-2B and 12β6 cells at confluence were pretreated with either vehicle, 1 nM, 10 nM, or 100 nM salmeterol for 5 min and membranes prepared as described in Methods. Adenylyl cyclase activity was assayed in the presence of various concentrations of adrenaline. The values shown are the mean of triplicate determinations and data are expressed as % of maximum control activity (100 and 125 pmol min^{-1} mg^{-1} for BEAS-2B and 12β6 cells, respectively). The s.e.mean of triplicate determinations did not exceed 4.5%.

Assessment of salmeterol-induced desensitization was complicated by the persistence of salmeterol in the membranes following pretreatment of cells with this agonist as shown in Figure 2. Under these conditions, an increase in the EC_{50} and a decrease in the V_{max} for adrenaline results in part from competition between the very poor agonist salmeterol and the strong agonist adrenaline for the β_2-adrenoceptor ligand binding pocket. We propose that the changes in EC_{50} and V_{max} observed following salmeterol pretreatment are caused by a combination of salmeterol-induced desensitization and salmeterol competition for adrenaline stimulation of adenylyl cyclase during the assay. To determine the changes in the activation parameters which may be due to desensitization, following pretreatment by salmeterol, requires separating ligand competition effects from those due to desensitization.

Previous studies of airway smooth muscle (Ball *et al*., 1991; Johnson *et al*., 1993; Nials *et al*., 1993) and our studies of L cells (Clark *et al*., 1996) showed that salmeterol's relaxant activity is blocked by the presence of a reversible antagonist, but that reassertion of this activity occurs following washout of the antagonist. This suggested that the binding of salmeterol to the β_2-adrenoceptor activation site during salmeterol pretreatment of cells could be blocked by inclusion of a reversible β_2-adrenoceptor antagonist, without altering the ability of salmeterol to bind to the membrane and/or to a putative receptor exosite. The presence of the antagonist prevents the initiation of β_2-adrenoceptor desensitization during salmeterol pretreatment while still leaving salmeterol present in the membranes to compete with adrenaline during the adenylyl cyclase assay. By comparing the effects of pretreatment with salmeterol in the presence and absence of the reversible antagonist metoprolol, we could determine the extent of salmeterol-induced desensitization and differentiate this effect from the effects of salmeterol competition.

BEAS-2B cells were pretreated for various times with either 5 μM salbutamol, 10 μM adrenaline or 10 nM salmeterol in the presence or absence of 100 μM metoprolol. Membranes were then prepared and stimulation of adenylyl cyclase by adrenaline measured. These concentrations of agonists (8–10 fold over K_d) were chosen because they result in similar levels of receptor occupancy (>85%). Therefore, even though adrenaline was used at a 1000 fold higher concentration than salmeterol, occupancy is similar since the K_ds for the two agonists are 1.5 nM and 711 nM, respectively. Typical data are shown in Figure 3 for a 5 min pretreatment with either salmeterol (in the absence or presence of metoprolol), salbutamol or adrenaline. Pretreatment of the BEAS-2B cells with any of the agonists caused an increase in the EC_{50} and a decrease in the V_{max} for subsequent adrenaline stimulation of adenylyl cyclase. The adrenaline treatment caused a comparable increase in the EC_{50} to that induced by salmeterol or salbutamol, but a much larger decrease in the V_{max}, indicating that adrenaline induced much greater desensitization than

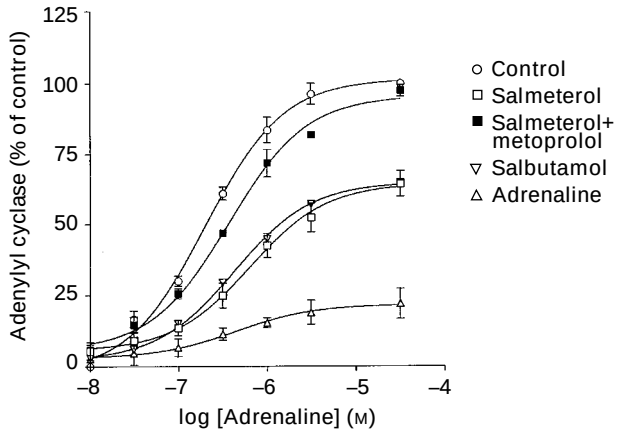

Figure 3 Determination of the ability of salmeterol, salbutamol and adrenaline to induce desensitization of β_2-adrenoceptor stimulation of adenylyl cyclase. BEAS-2B cells were pretreated for 5 min with 10 nM salmeterol, 10 nM salmeterol plus 100 μM metoprolol, 5 μM salbutamol, 10 μM adrenaline or AT (control). Membranes prepared as described in Methods were assayed for adenylyl cyclase activity in triplicate for each experiment in the presence of various concentrations of adrenaline as indicated. The graph shows the averages and range of the data from two experiments. The data are expressed as % of maximum control activity (108 pmol min^{-1} mg^{-1}).

either salbutamol or salmeterol. The changes with salmeterol or salbutamol alone were similar, with the notable exception that salmeterol but not salbutamol caused a significant increase in basal activity.

Inclusion of metoprolol with the sameterol pretreatment blocked most of the decrease in V_{max} and reduced the salmeterol-induced increase in the EC_{50} for adrenaline stimulation. In contrast, metoprolol did not prevent the small but consistent salmeterol-induced increase in 'basal' activity. Thus, metoprolol appears to block the salmeterol-induced desensitization of the β_2-adrenoceptor by blocking binding at the active site during pretreatment, as is made evident by the much higher V_{max} observed when metoprolol was present during pretreatment. However, the persistent increase in basal activity indicates that metoprolol does not interfere with salmeterol binding to the membrane or to a putative receptor exosite. Pretreatment with metoprolol alone had no significant effect on adrenaline activation of adenylyl cyclase (data not shown). Therefore, the decrease in V_{max} and the increase in EC_{50} seen for the salmeterol plus metoprolol curve can be assumed to be due to the carryover of salmeterol from the pretreatment to the adenylyl cyclase assay. The additional decrease in V_{max} and slight increase in EC_{50} when metoprolol was absent indicates that significant β_2-adrenoceptor desensitization occurs with salmeterol pretreatment.

Quantitative comparison of the desensitization induced by salmeterol, salbutamol and adrenaline in the BEAS-2B cell line

To compare more rigorously the desensitization induced by the partial agonists salbutamol and salmeterol with that induced by the full agonist adrenaline, we quantified the desensitization induced by the three agonists for pretreatment times of 2 min to 6 h. Figure 4a, a summary of these experiments, shows the % desensitization of adrenaline stimulation as a function of the time of agonist pretreatment. The data for salmeterol in Figure 4a were corrected for the contribution to apparent desensitization by competition from

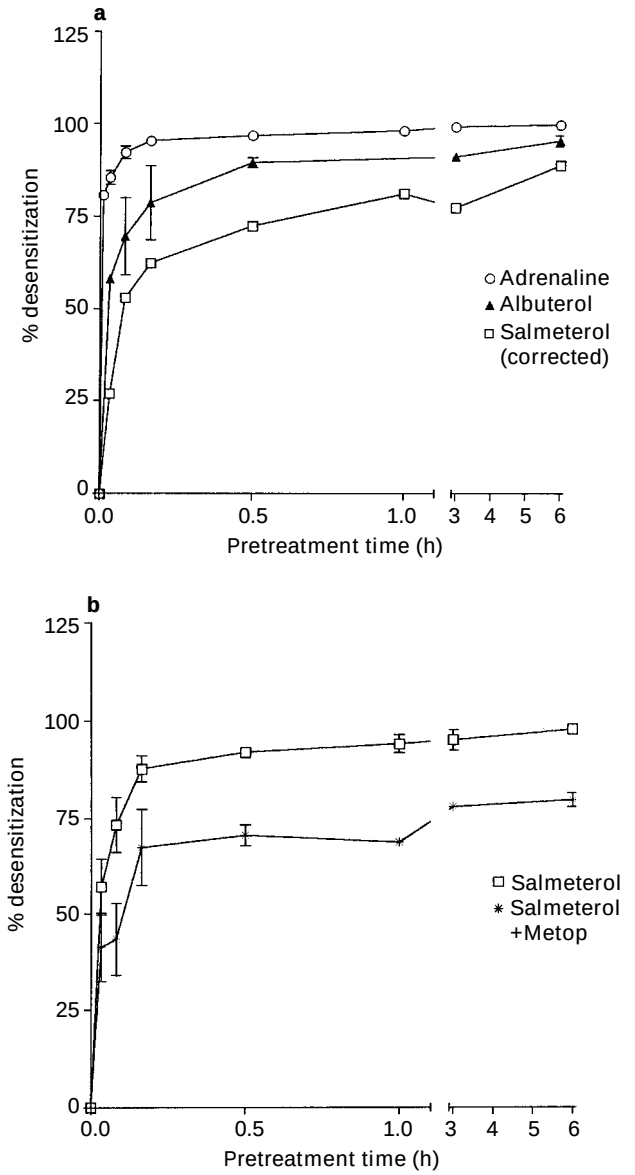

Figure 4 BEAS-2B cells were treated for various times (2, 5, 10, 30 min, or 1, 3 and 6 h) with either 10 nM salmeterol, 10 nM salmeterol plus 100 μM metoprolol (Metop), 5 μM salbutamol or 10 μM adrenaline. Membranes were prepared as described in Methods. For each membrane preparation at each time point we performed complete dose-response for adrenaline (seven concentrations) activation of adenylyl cyclase. Each concentration of adrenaline was assayed in triplicate (s.e. for triplicates were routinely less than 5%). The EC_{50} and V_{max} were determined by Graph Pad analysis of the dose-response data. % desensitization of each time point was calculated with equation 3 as described in the Methods section. The 'corrected salmeterol' curve (a) shows the desensitization induced by salmeterol after correction of the apparent salmeterol-induced desensitization (b) by that observed in the presence of metoprolol (b). The corrected salmeterol % desensitization was calculated with equation 3 after first dividing the ratio, $(k_1r/k_{-1})_D/(k_1r/k_{-1})_N$, determined from the mean of the data of salmeterol-treated cells by this same ratio determined for cells treated with salmeterol + metoprolol. The % desensitization for each time point is the average value for 2–6 experiments with the exception of those where $n=1$. The parentheses indicate the s.e.mean for $n \geqslant 3$, and the range of the values where $n=2$. Error bars are not shown for the salmeterol data in (a), since these values are corrected for the apparent desensitization that occurs after treatment with salmeterol plus metoprolol as given above. The number of experiments for each treatment time shown in (b) was as follows for salmeterol and salmeterol plus metoprolol, respectively (time in min or h is followed by the n value in parentheses): 2 min (3,2); 5 min (6,4); 10 min (3,2); 30 min (3,2); 1 h (2,1); 3 h (3,2); 6 h (2,3). For the adrenaline and albuterol data shown in (a), n values were respectively: 2 min (2,2); 5 min (2,4); 10 min (2,2); 30 min (2,2); 1 h (1,0); 3 h (1,2); 6 h (1,3).

salmeterol released from its membrane binding sites during the assays. This competition was assessed from the data in Figure 4b that shows the time course of the salmeterol-induced desensitization in the presence and absence of metoprolol. The apparent desensitization observed with salmeterol plus metoprolol shows the extent of the competition of salmeterol carried over into the assays. The salmeterol data in Figure 4b were corrected for this competition (see legend) giving the data shown in Figure 4a.

The extent of adrenaline-induced desensitization over the 0–10 min time period was considerably greater than that seen for salmeterol and salbutamol (Figure 4a). Following a 10 min pretreatment with agonist, the desensitization was 95, 79 and 62% with adrenaline, salbutamol and salmeterol, respectively. The corresponding coupling capacity ratios of desensitized β_2-adrenoceptors to naive β_2-adrenoceptors were 0.05, 0.21 and 0.38 for adrenaline, salbutamol and salmeterol, demonstrating an approximate 4 fold reduction in desensitization due to salbutamol and a 7–8 fold reduction in desensitization due to salmeterol relative to adrenaline following a 10 min pretreatment with agonist.

Adrenaline-induced desensitization was near complete after just 20 min, while salbutamol required 6 h to attain a comparable loss of activity (see Figure 4a). Even after 6 h the desensitization after salmeterol treatment remained less than that achieved with adrenaline. An additional observation of interest is that the desensitization in response to salbutamol, salmeterol and adrenaline had a distinctly biphasic character; that is, a relatively rapid phase was followed by a slower phase.

Similar experiments were performed with the 12β6 cell line with similar results; i.e., the adrenaline-induced short-term desensitization was far greater than that caused by salmeterol or salbutamol (data not shown). However, because the high receptor number in the 12β6 cells resulted in very high basal activity of adenylyl cyclase after salmeterol pretreatment (Figure 2), it was difficult to determine accurately an EC_{50} for adrenaline stimulation.

Internalization of the β_2-adrenoceptor

To measure the internalization of the β_2-adrenoceptor, monolayer HAβAR6HIS cells were treated with either 10 μM adrenaline, 6 μM salbutamol or 15 nM salmeterol in the presence or absence of either 100 μM metoprolol or 500 μM atenolol for 2–30 min, and subsequently surface receptor quantified by [^{3}H]-CGP-12177 binding. The antagonists (metoprolol or atenolol) were included with salmeterol to provide an estimate of the extent of competition by salmeterol that was not washed out with [^{3}H]-CGP-12177. On the basis of this measurement the competition by residual salmeterol was found to be in the range of 5–15%. In the absence of agonist, more than 95% of β_2-adrenoceptors were on the cell surface as determined by [^{3}H]-CGP-12177 binding in the presence and absence of digitonin (data not shown). The data in Figure 5 demonstrate that adrenaline caused a greater internalization of the β_2-adrenoceptor than either salbutamol or salmeterol. The average extent of internalization after 30 min treatment with adrenaline, salmeterol and salbutamol was $79 \pm 4\%$ ($n=3$), $53 \pm 6\%$ ($n=3$), and $46 \pm 8\%$ ($n=2$), respectively. Through the use of parallel assays with and without permeabilization with digitonin (to reveal the total receptor population), we found no downregulation (loss of total receptor) after the 30 min incubation with agonists. Similar results were obtained with the 12β6 cell line (data not shown). It should be noted that we attempted to measure the internalization of the β_2-adrenoceptor in suspended cells and found that the extent of internalization induced by the various agonists was greatly reduced relative to the attached cells.

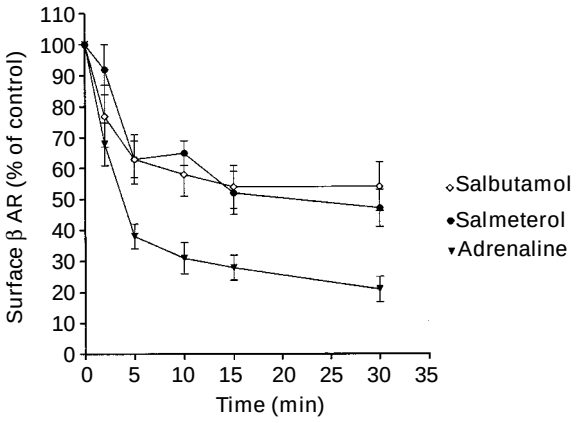

Figure 5 Loss of cell surface β_2-adrenoceptor after treatment of HAβAR6HIS cells with either 10 μM adrenaline, 6 μM salbutamol or 15 nM salmeterol in the presence or absence of antagonists (100 μM metoprolol or 500 μM atenolol) from 2 to 30 min. Surface receptors were measured by [^{3}H]-GCP-12177 binding as described in Methods. The loss of receptor after salmeterol treatment was corrected for the loss in cells treated with salmeterol plus either metoprolol or atenonol. The surface receptor level is expressed as % surface receptor remaining relative to vehicle-treated controls. Data represent the mean and s.e.mean of 3 experiments for adrenaline and salmeterol and the average and range of 2 experiments for salbutamol.

Phosphorylation of the β_2-adrenoceptor after treatment with adrenaline, salbutamol and salmeterol

To determine the extent of the phosphorylation of the β_2-adrenoceptor induced by these agonists, HAβAR6HIS cells were prelabelled with ^{32}P for 3 h and subsequently stimulated with either vehicle (AT), 10 μM adrenaline, 6 μM salbutamol or 15 μM salmeterol for 20 s, 1, 5 and 30 min. Figure 6 shows the results of three experiments and the Imagequant analysis from a typical experiment. Adrenaline caused a rapid 13.5 ± 3.9 fold increase in the extent of phosphorylation of the β_2-adrenoceptor relative to vehicle-treated controls after just 1 min, whereas both salbutamol and salmeterol induced significantly less phosphorylation (4.6 ± 1.0 and 4.2 ± 0.46 fold, respectively) at this time. The level of phosphorylation caused by salbutamol and salmeterol at 1 min was slightly greater than that caused by forskolin (3.5 ± 1.5 fold, data not shown) which activates PKA by directly stimulating adenylyl cyclase. After 5 min the level of phosphorylation caused by all three agonists was identical (≈ 10 fold). After 30 min of treatment the levels achieved by salbutamol (10.5 ± 1.7 fold) and salmeterol (13.4 ± 2.9 fold) actually exceeded that caused by adrenaline (6 ± 0.81 fold). The rapid rise and fall in the phosphorylation caused by adrenaline relative to the partial agonists probably reflects the greatly increased rate of both phosphorylation and dephosphorylation by adrenaline. The greater rate of dephosphorylation in the presence of adrenaline is not understood at present, but may in part be caused by both the greater initial rate of phosphorylation and the greater rate of internalization after treatment with this agonist. The profile of phosphorylation induced by adrenaline that we observed is nearly identical to that obtained previously in S49 lymphoma cells in response to pretreatment with isoprenaline (Strasser *et al.*, 1986).

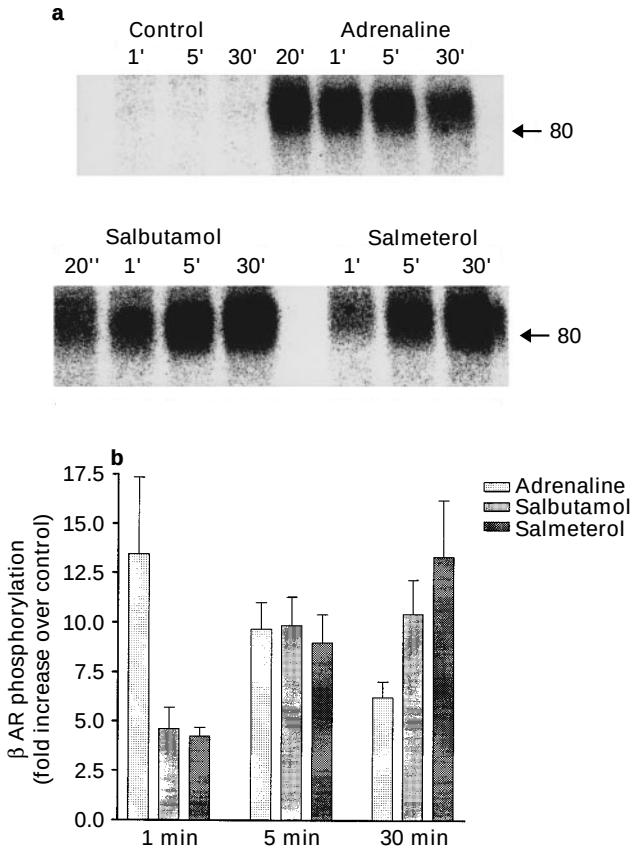

Figure 6 Time course of phosphorylation of the β_2-adrenoceptor (β AR) in HAβAR6HIS after treatment with either vehicle, 10 μM adrenaline, 6 μM salbutamol or 15 nM salmeterol. Cells were labelled with ^{32}P, treated with agonist for 1, 5 or 30 min and purified as described in Methods. Radiolabelled β_2-adrenoceptor was visualized by Imagequant analysis after 24 h phosphorscreen exposure. (a) Representative data from one experiment showing the Imagequant analysis of the time course of phosphorylation after adrenaline, salmeterol and albuterol treatment. (b) Data shown are the mean $\pm$ s.e.mean of 3 separate experiments expressed as the fold increase in β_2-adrenoceptor phosphorylation over control.

Discussion

In the present study the ability of salmeterol to activate and desensitize human β_2-adrenoceptors was determined in human cell lines expressing either a low or high level of receptor. Salmeterol stimulated adenylyl cyclase activity in both of the human cell lines, BEAS-2B and 12β6, and did not exhibit a lag time for onset of this activity under the experimental conditions employed here. Similar results were obtained previously for cyclic AMP accumulation in intact rat B50 neuroblastoma cells (McCrea & Hill, 1993) and bovine tracheal smooth muscle (Ellis *et al*., 1995), and for activation of adenylyl cyclase in mouse L-cells transfected with the hamster β_2-adrenoceptor (Clark *et al*., 1996). The lag observed for salmeterol relaxation of airway smooth muscle *in vivo* and in physiological airway smooth muscle preparations (Naline *et al*., 1994; Anderson *et al*., 1996) is not understood, but may in part be caused by its slow diffusion to its site of action. The persistence of salmeterol's concentration-dependent action following multiple washings appears to be caused in part by its very favourable partitioning into membrane lipid (Rhodes *et al*., 1992; Bergendal *et al*., 1995a,b), perhaps together with some function of a specific β_2-adrenoceptor domain, the exosite (Ball *et al*., 1991; Nials *et al*., 1993; Green *et al*., 1996). Our results do not distinguish between these possibilities. However, we have found a similar persistence of zinterol stimulation following intact cell treatment and subsequent assay of adenylyl cyclase (data not shown). This observation suggests that the persistence of these drugs, under our conditions, after washout is at least in part a factor of their high affinity for the β_2-adrenoceptor (K_d values for these agonists are very similar) combined with their partitioning into the lipid.

The importance of receptor number on β_2-adrenoceptor agonist action

The dramatic difference in the efficacy (V_{max}) of salmeterol activation of adenylyl cyclase in cells with high versus low β_2-adrenoceptor numbers and the 100 fold difference in the potency of adrenaline activation in the two cell lines, demonstrate that receptor levels play a profound and predictable role in the responsiveness of cells to partial and full agonists (Whaley *et al*., 1994; 1995; Yuan *et al*., 1994). Although salmeterol binds to β_2-adrenoceptors with high affinity ($K_d = 1.5$ nM), it is a partial agonist with a low coupling efficiency compared to adrenaline. This results in a greatly reduced efficacy (V_{max}) of salmeterol activation of adenylyl cyclase in the BEAS-2B cells expressing low numbers of receptors. However, in cells such as the 12β6 overexpressing β_2-adrenoceptors, the V_{max} of salmeterol is equivalent to that of adrenaline. These results demonstrate that the efficacy of salmeterol is profoundly affected by the level of β_2-adrenoceptors, consistent with the predictions drawn from our previous studies (Whaley *et al*., 1994). Several previous studies of salmeterol relaxation of human airway smooth muscle (Ball *et al*., 1991; Naline *et al*., 1994) demonstrated that the efficacy of salmeterol is about 60–70% of the full agonists isoprenaline and adrenaline. Therefore, it is possible that β_2-adrenoceptor levels in human airway smooth muscle could be 3–4 fold higher ($\approx$120–280 fmol mg^{-1}) than those found in the BEAS-2B human epithelial cell line, with the caveat that other factors altering coupling efficiency in lung could influence this estimate.

The difference in β_2-adrenoceptor levels provided two other results of interest; first, that the basal activity was much higher following salmeterol pretreatment in the 12β6 cell line, and second that the desensitization was similar in these cell lines. The first observation is consistent with the predictions of Whaley *et al*. (1994); that is, that the EC_{50} for salmeterol (or any agonist) is reduced as receptor levels increase. Therefore any salmeterol carried over in the membrane preparation (either from partition into lipid or binding to the putative exosite) that dissociates from these sites and binds to the active site during assays, will have a lower EC_{50} and a greater efficacy for activation of adenylyl cyclase in the high expression cells relative to low expression. The second observation, that the desensitization was similar over this enormous range of β_2-adrenoceptor levels with these three agonists, demonstrates that the cellular components causing the desensitization are not rate limiting, as we have previously observed in L-cells over a more limited range of β_2-adrenoceptor levels (Yuan *et al*., 1994), and over a 200 fold range of receptor levels in HEK 293 cells (January *et al*., 1997).

Partial agonists cause reduced β_2-adrenoceptor desensitization relative to full agonists

By use of a variety of genetic, biochemical, and pharmacological approaches, it has been shown that β_2-adrenoceptor

desensitization occurs by at least three mechanisms: receptor phosphorylation by protein kinases, internalization of receptors, and reduction of total cellular receptor numbers (downregulation) (Clark, 1986; Palczewski & Benovic, 1991; Perkins *et al*., 1991; Inglese *et al*., 1993). Exposure to low concentrations of β_2-adrenoceptor agonists (or other drugs such as forskolin that activate PKA) induces a rapid ($t_{1/2} \approx 1$ min) partial desensitization mediated by PKA phosphorylation of the receptor on the third intracellular loop (Clark *et al*., 1988; 1989; Yuan *et al*., 1994), and, with prolonged treatment, a much slower downregulation of β_2-adrenoceptors (Perkins *et al*., 1991; Proll *et al*., 1993) that exhibits a $t_{1/2}$ of 1–4 h. Higher concentrations of agonist resulting in significant receptor occupancy induce (in addition to the PKA effect) a rapid ($t_{1/2}$ of 0.2–0.4 min) homologous desensitization that has been proposed to result from the phosphorylation of receptors, presumably on the carboxyl-terminal domain by G protein-coupled receptor kinase (GRK), followed by the binding of a receptor capping protein called β-arrestin (Palczewski & Benovic, 1991; Perkins *et al*., 1991). High occupancy of the receptor also drives internalization of β_2-adrenoceptors ($t_{1/2}$ of 2–4 min) that is appreciably slower than protein kinase-mediated phosphorylations.

Our studies show that over the time period of 1 to 5 min salbutamol and salmeterol induced less β_2-adrenoceptor desensitization than that seen following pretreatment with adrenaline. It is likely that the initial rapid salmeterol- and salbutamol-induced desensitization is caused primarily by PKA phosphorylation, since the activation of PKA requires only small increases in cyclic AMP. Also, the extent of the desensitization after completion of the rapid phase of salmeterol-induced desensitization is similar to that observed in other systems undergoing PKA-mediated desensitization (Kunkel *et al*., 1987; Clark *et al*., 1988; 1989; Yuan *et al*., 1994). This conclusion was supported by the measurement of phosphorylation of the β_2-adrenoceptor. The extent of phosphorylation caused by salmeterol and salbutamol was only 1/3 the level achieved by adrenaline after 1 min of treatment and did not significantly exceed that caused by forskolin that activates PKA via direct activation of adenylyl cyclase (January *et al*., 1997).

After the rapid phase of desensitization of β_2-adrenoceptor stimulation of adenylyl cyclase we found that the desensitization caused by salbutamol and salmeterol appeared to be distinctly biphasic, such that the difference between their desensitization and that caused by adrenaline by 30 min was somewhat diminished. Again this observation was consistent with the phosphorylation data which demonstrated that, while there was a significantly reduced initial rate of phosphorylation by salbutamol and salmeterol relative to adrenaline, these agonists eventually achieved a level after 30 min which approached that achieved by adrenaline after just 1 min, and which was significantly greater than the level in the presence of adrenaline after 30 min. To summarize, these results are most consistent with the interpretation that the partial agonists salmeterol and salbutamol do in fact provoke GRK-mediated phosphorylation, but at a much slower rate than adrenaline, whereas all three agonists appear to activated PKA-mediated phosphorylation consistent with their full activation of adenylyl cyclase at these saturating concentrations.

For salmeterol and salbutamol the reduction in desensitization relative to adrenaline is probably also caused in part by the decreased receptor internalization, as measured by [^{3}H]-CGP-12177 binding. From these data it appears that the extent of receptor internalization, desensitization and phosphorylation is a function of agonist coupling efficiency. This conclusion is supported by recent studies from our group (January *et al*., 1997) showing that the extent of β_2-adrenoceptor internalization and desensitization in the HAβAR6HIS cell line correlates with the coupling efficiency of a range of partial agonists that included ephedrine and dobutamine, agonists that display coupling efficiencies only 2 and 4% of that of adrenaline respectively. We found no significant internalization by ephedrine and only 15–20% with dobutamine. In addition, Su *et al*. (1980) showed that the partial agonists soterenol and zinterol caused less desensitization than the full agonist isoprenaline. Toews & Perkins (1984) showed that the partial agonists terbutaline and soterenol caused less internalization relative to isoprenaline, as measured by their ability to reduce the apparent affinity of intact cell agonist binding. It has also been demonstrated that albuterol causes less agonist-induced internalization relative to isoprenaline (Morrison *et al*., 1996). Our conclusion is supported by the work of Benovic *et al*. (1988) which demonstrated that *in vitro* phosphorylation of β_2-adrenoceptors by βARK was reduced with partial compared to full agonists.

It has been appreciated for some time that β_2-adrenoceptor desensitization by the βARK/β-arrestin pathway and internalization in response to full agonists, such as adrenaline and isoprenaline, require high occupancy of the receptor (Benovic *et al*., 1988; Palczewski & Benovic, 1991; Inglese *et al*., 1993). Since agonist-receptor interaction is required for β_2-adrenoceptor internalization, this desensitization mechanism is believed to target only the receptor (R) in its activated conformation (R^*) (Strasser *et al*., 1985). The increased coupling efficiency of adrenaline over salbutamol and salmeterol is believed to be due to the ability of full agonists to shift a higher percentage of R into R^* compared to a weak agonist. Therefore, even though receptor occupancy is >85% with the three agonists under our experimental conditions, adrenaline would make a proportionately greater percentage of the receptor available as substrate for the βARK pathway and internalization, while salbutamol and salmeterol would provide smaller percentages. Since evidence from several recent studies suggests that βARK-mediated phosphorylation and β-arrestin binding are required for internalization (Tsuga *et al*., 1994; Ferguson *et al*., 1996; Goodman *et al*., 1996), in addition to the high occupancy requirement, it follows that both are lowered by partial agonist stimulation.

Implications of the persistent activation of adenylyl cyclase and the reduced extent of desensitization by salmeterol

Our results suggest that the long duration of action of salmeterol is a function of both its persistence and a reduced extent of desensitization, properties that might be expected to confer the ability to induce persistent bronchodilatation to the extent that cell studies can be extrapolated to tissue. However, since salmeterol binding triggers PKA-mediated desensitization and, at a reduced rate, βARK-mediated phosphorylation, coupled with internalization and receptor downregulation, significant β_2-adrenoceptor desensitization does occur at least in cell cultures. The biphasic nature of the salmeterol-induced desensitization that is revealed after the initial rapid phase (0–5 min) probably primarily reflects the delayed βARK-mediated event, and the contribution from internalization. At present it is difficult to extrapolate our observations to clinical pharmacology. It appears that functional desensitization of human lung tissue may not be significant (see review by Barnes, 1995). We would add the caveat that the type of

sensitive measurements possible in isolated cell studies are not possible as yet in studies of human tissue *in situ*.

Our observations concerning the increase in salmeterol efficacy at increased receptor levels indicate that this may lead to variation among individuals in the efficacy and potency of partial agonists to produce bronchodilatation when β_2-adrenoceptor levels vary. The response of an individual may also vary depending on prior exposure to β_2-adrenoceptor agonist and bronchoconstrictor stimuli (Cheung *et al*., 1992; Ramage *et al*., 1994; Bhagat *et al*., 1995), or to the existence of polymorphisms of the β_2-adrenoceptor as described by Green *et al*. (1996). Further, the effects of desensitization will vary considerably; that is, individuals with higher initial levels of β_2-adrenoceptor would predictably show an apparent resistance to desensitization if receptor levels are sufficiently high and the desensitization does not appreciably alter the efficacy of weak partial agonists. Strategies designed to augment β_2-adrenoceptor levels, such as with corticosteroid treatment, may have important effects in attenuating desensitization at least superficially.

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