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Stable Activation and Desensitization of β_2-Adrenergic Receptor Stimulation of Adenylyl Cyclase by Salmeterol: Evidence for Quasi-irreversible Binding to an Exosite

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SUMMARY

The relaxation of tracheal smooth muscle by the β_2-adrenergic receptor (βAR) agonist salmeterol displays several unusual properties: (i) slow onset of action ($t_{1/2}$ = 5–15 min), (ii) prolonged activation ($t_{1/2}$ = 8–14 hr), and (iii) the ability to recover from βAR blockade. These properties led to the hypothesis that salmeterol binds with very high affinity to an exosite in addition to the βAR activating site. Despite extensive characterization of salmeterol-induced bronchodilation, little is known about the molecular actions of salmeterol. We report the unique properties of salmeterol binding to the βAR, activation of adenylyl cyclase, and desensitization of the hamster βAR expressed in L cells. First, we found that salmeterol activation of adenylyl cyclase, although rapid and potent (low EC_{50} relative to epinephrine), was nevertheless remarkably inefficient relative to the full agonist epinephrine. Reduced coupling efficiency of salmeterol was demonstrated using formulations recently introduced by our group. Second, we found that pretreatment of L cells with salmeterol led to a stable activation of adenylyl cyclase that survives extensive wash procedures and sucrose step gradient purification of plasma membrane fractions. This activation of basal adenylyl cyclase did not require salmeterol binding to the classic active site during pretreatment, as it occurred in the presence of an excess of a βAR antagonist. Third, we found that the rapid phase of salmeterol-induced desensitization was much reduced relative to epinephrine, consistent with its poor coupling efficiency and with its prolonged activation of adenylyl cyclase. These unique properties of salmeterol support the proposal that it binds reversibly to the activating or active site and as well to an extremely high affinity exosite from which it has access to the active site.

The unusual characteristics of the β_2-adrenergic agonist salmeterol led us to examine for the first time its binding to the βAR, its activation of adenylyl cyclase, and the desensitization that results from its treatment of cells. Like other β_2-adrenergic agonists, it induces bronchodilation of tracheal smooth muscle. However, the salmeterol-induced bronchodilation is atypical in that it is slow in onset of action, very prolonged, and resistant to washout (1–5). The tenacity of salmeterol's action is revealed by experiments in which salmeterol relaxation of smooth muscle reappears after blockade by βAR antagonist and washout of the antagonist without readdition of salmeterol (2–4). These unusual characteristics led to the hypothesis (1, 2) that salmeterol binds to two sites, the saligenin moiety binding to the classic βAR active site that leads to activation of adenylyl cyclase and the hydrophobic tail binding to another site, called the exosite, as illustrated in Fig. 1. In this model, the extremely high affinity binding of the tail to an exosite keeps salmeterol tethered in the vicinity of the receptor.

The exosite, so far ill defined, is proposed to be either a domain of the βAR or a site in close proximity to the βAR such as plasma membrane lipid (1, 2). The hydrophobicity of salmeterol has been well documented (2–5), and Rhodes *et al.* (6) demonstrated that it has a partition coefficient (water/lipid) of 1:23,000. These observations suggest that the stable very high affinity binding revealed by prior studies could be caused entirely by the partition of the drug into membranes. However, the possibility remains that the exosite could also involve a domain of the βAR because compounds nearly identical to salmeterol, with the exception of the number of CH_2—residues in the alkyloxalkyl tail, do not display the prolonged relaxation characteristic of salmeterol (2). Whatever the nature of the exosite, salmeterol action after blockade by antagonists would be restored by flapping in and out of the active site from its tethered position in the vicinity.

The prolonged bronchodilation observed with salmeterol has led to the speculation that it does not cause desensitization of the βAR; however, the limitations of tissue studies precluded the resolution of this. In the present study, this issue has been addressed using a cellular model consisting of the hamster βAR expressed in L cells, a system we previously characterized with regard to desensitization (7–12). The de-

ABBREVIATIONS: βAR, β_2-adrenergic receptor; ICYP, [^{125}I]iodocyanopindolol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

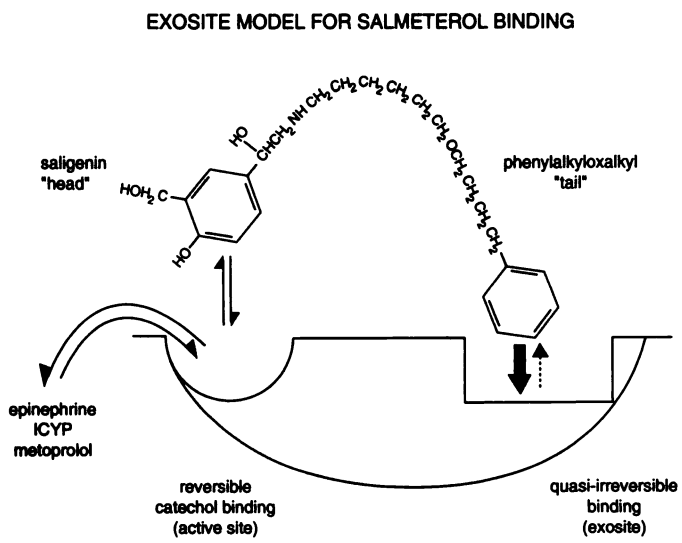

Fig. 1. Exosite model for salmeterol binding.

sensitization of βAR stimulation of adenylyl cyclase in L cells is characterized by an increase in the EC_{50} and, with sufficient desensitization, decreases in V_{max}. A problem we anticipated in the study of salmeterol-induced desensitization was that exosite-bound salmeterol would complicate analysis. That is, because salmeterol is a partial agonist it would be expected to compete with epinephrine, thereby increasing the EC_{50} for epinephrine activation of adenylyl cyclase (13). Because the EC_{50} is also increased by desensitization, a method was needed to determine the extent to which the EC_{50} shift was caused by exosite-bound salmeterol competing with epinephrine versus that caused by salmeterol-induced desensitization.

This problem has been addressed in this study by our demonstration that salmeterol activation of the βAR, but not exosite binding, is blocked by βAR antagonists. This allowed a means of distinguishing the effects of the competition of exosite-bound salmeterol with the full agonist epinephrine from those caused by desensitization. Another problem addressed was the determination of the coupling efficiency of salmeterol. To accomplish this, we drew on our recent studies in which we demonstrated that βAR activation of adenylyl cyclase can be quantitatively described by a modification of the Cassel-Selinger model over a 2000-fold range of βAR levels (7, 8). In addition, we derived formulations to more precisely define coupling efficiency for either mutant receptors or partial agonists that factors out confounding problems of changes in K_d and receptor number. Using this formulation, we found that salmeterol has a very poor coupling efficiency relative to epinephrine. This in turn provided a rationale for the reduced desensitization we document with salmeterol treatment of L cells.

Experimental Procedures

Materials. Salmeterol and metoprolol were provided by Glaxo Research and Development. Salmeterol solutions were prepared as follows: Salmeterol was first dissolved in a minimal volume of glacial acetic acid and then diluted and stored at a concentration of 10 mM in phosphate-buffered saline composed of 2.68 mM KCl, 1.47 mM KH_2PO_4, 152 mM NaCl, and 8.06 mM Na_2HPO_4. [α-^{32}P]ATP was obtained from DuPont/NEN. ATP was obtained from Sigma Chemical Co., and GTP was obtained from Boehringer Mannheim. Epinephrine (Sigma) was dissolved in a solution of 100 mM thiourea and 10 mM ascorbate, pH 7, and used in both pretreatments and assays at a 1:100 dilution. ICYP was prepared as described previously (7).

Cell incubations and membrane preparations. L cells expressing the wild-type hamster βAR (11) obtained from Drs. Richard Dixon and Catherine Strader (Merck) were cultured in T150 flasks at 37° with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 200 μg/ml geneticin. Cells were pretreated with the various hormones, drugs, and appropriate control buffers in 150-mm dishes in growth medium for the times indicated in the individual experiments. All incubations of cells with epinephrine were performed with a final concentration of 1.0 mM thiourea and 0.1 mM ascorbate to prevent oxidation of the catecholamine. Controls were incubated with the ascorbate/thiourea. Controls for salmeterol pretreatment included PBS at a 1000-fold dilution. For incubations with salmeterol in combination with metoprolol, the antagonist was added 10 sec before salmeterol. After pretreatment the medium was removed, and the cells were washed four 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 trypsin inhibitor, 0.1 mg/ml bovine serum albumin). The cells were then scraped into 8 ml of HME plus 10 μg/ml leupeptin and homogenized with seven 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 rpm in a Beckman SW28.1 rotor at 4°. The membranes at the 23/43% interface were removed, frozen in liquid nitrogen, and stored at −80°. The membranes were diluted in HE buffer before assays.

Adenylyl cyclase and receptor binding assays. Adenylyl cyclase activity in the membrane preparations was measured as previously described (7, 8) with the free Mg^{2+} concentration set at 0.3 mM. The final concentration of ATP in the assay was 0.1 mM. All adenylyl cyclase incubations were for 10 min at 30°. Under these conditions, cAMP synthesis was essentially linear for the 10-min incubation regardless of whether membrane preparations were derived from salmeterol-pretreated cells.

The B_{max} for the βAR and the K_d for ICYP in the membranes were determined by Scatchard analysis of ICYP binding as previously described (7, 8) with the following modifications. The concentration of ICYP varied from 1 to 200 pM. ICYP was diluted in HE buffer plus 50 μM phentolamine, 0.1 mM ascorbate, and 1 mM thiourea. The high and low affinity K_d values for salmeterol were determined by salmeterol displacement of 20–30 pM ICYP in the absence and presence of 10 μM GTP, respectively, using the Cheng-Prusoff correction and GraphPad to analyze the data. Nonspecific binding was assessed by inclusion of 1.0 μM alprenolol. Incubations were for 45 min. The extent of the GTP shift was quantified by comparing the ratios of the low affinity to the high affinity dissociation constants for agonist binding in the absence of GTP.

Definition of coupling efficiency and calculation of the coupling efficiency ratio. The term "coupling efficiency" has been very loosely used in the past without consideration of the consequences of either receptor levels or changes in the K_d for agonists or mutant receptors. In previous studies of salmeterol, coupling efficiency was defined solely on the basis of its V_{max} compared with that of the full agonist isoproterenol. We have shown in our recent work (7, 8) that this is inadequate. We defined coupling efficiency as k_{on}, the rate constant for activation of adenylyl cyclase in the Cassel-Selinger model, and presented several means of calculating coupling efficiency. In the present work, we used the following equation:

$$\frac{k_{on}r}{k_{off}} = \frac{K_d}{EC_{50}} - 1$$

where k_{on} and k_{off} are, respectively, the rate constants for activation and inactivation in the Cassel-Selinger scheme for adenylyl cyclase activation, r is the receptor density, K_d is the dissociation constant for the agonist, and EC_{50} is the concentration for half-maximal response. It should be noted that in this model, the K_d used is that measured in the presence of GTP since GTP is present during adenylyl cyclase assays at saturating levels (10 μM). For a specific membrane preparation, $k_{off}r$ and r are independent of the identity of the agonist so that a comparison of the ($k_{on}r/k_{off}$) terms is equivalent to a direct comparison of the k_{on} terms.

Results

Determination of the EC_{50} for salmeterol activation of adenylyl cyclase and the K_d for salmeterol binding to the βAR active site. While previous studies of salmeterol relaxation of smooth muscle suggested that salmeterol was a partial agonist based on its reduced efficacy, unambiguous determination of partial agonism requires measurement of the EC_{50} for salmeterol stimulation of adenylyl cyclase in combination with the K_d for salmeterol binding. These data allow the calculation of the coupling efficiency ratio as described in Experimental Procedures. We have previously shown that the ratio is a rational quantitative measure of β_2-adrenergic agonism (7, 8). The data in Fig. 2A show that salmeterol activation of adenylyl cyclase displayed an EC_{50} of 2.0 nM (the mean of three experiments was 1.8 ± 0.25 nM), ~10-fold lower (more potent) than that of epinephrine (the mean EC_{50} of three experiments was 22.7 ± 1.6 nM), but that it did not achieve the same V_{max} as the hormone. In contrast to the very slow onset of salmeterol-induced tracheal smooth muscle relaxation, we found that salmeterol activation of adenylyl cyclase did not display a significant lag (data not shown).

The K_d for salmeterol binding to the βAR was determined by competition with ICYP (Fig. 2B). The K_d describing the equilibrium between salmeterol free in solution and that bound to the active site (and/or salmeterol bound to the exosite with the saligenin moiety binding reversibly to the active site) was determined to be 3.48 ± 1.5 nM in the presence of GTP (average of four experiments, similar to that shown in Fig. 2B). The affinity of salmeterol for the active site was much greater than that of epinephrine, which for this experiment was 365 nM, in agreement with the value of 343 ± 61 nM we previously reported (7). Using these K_d and EC_{50} values, the ratio of the coupling efficiency for salmeterol relative to epinephrine was found to be 0.07, demonstrating that salmeterol was only 7% as efficient an agonist as epinephrine.

In the experiment shown in Fig. 2B, we also evaluated the GTP shift for salmeterol versus epinephrine because it is well established that partial agonists display a smaller GTP shift relative to full agonists (14, 15). Consistent with the partial agonist behavior of salmeterol activation of adenylyl cyclase, we found that salmeterol exhibited a smaller GTP shift than epinephrine. Fig. 2B shows a representative example of GTP shifts for salmeterol and epinephrine. In this case, the ratio of the low to the high affinity dissociation constants was 69 for epinephrine and 4.2 for salmeterol. Similar results were obtained in four other experiments in which the range of the rightward shift was 40–50 for epinephrine and 2.0–5.2 for salmeterol.

Characteristics of salmeterol pretreatment of intact cells on basal and epinephrine-stimulated adenylyl cyclase. Intact L cells were treated for 5–300 sec with 10 or 100 nM salmeterol. After cell lysis and purification of membranes, basal activity and the dose response of epinephrine stimulation of adenylyl cyclase were measured. The data in Fig. 3 (A and B) show typical experiments. Fig. 3 (C and D) show the changes in basal activity and the EC_{50} for epinephrine averaged from two or more experiments. Basal activity was significantly increased, reaching a maximum value within 1 min of pretreatment (4.5-fold and 3-fold for the 100 and 10 nM pretreatments, respectively), after which there was a decline to lower levels.

The EC_{50} values for epinephrine stimulation over the 5-min treatment, determined from Eadie-Hofstee plots of the data, progressively increased, reaching values 5- and 15-fold over the controls for the 10 and 100 nM treatments, respectively (Fig. 3D). With the 1–5-min salmeterol pretreatment, some decline in the V_{max} for epinephrine stimulation was observed, usually in the range of 10–30% (Fig. 3, A and B). In

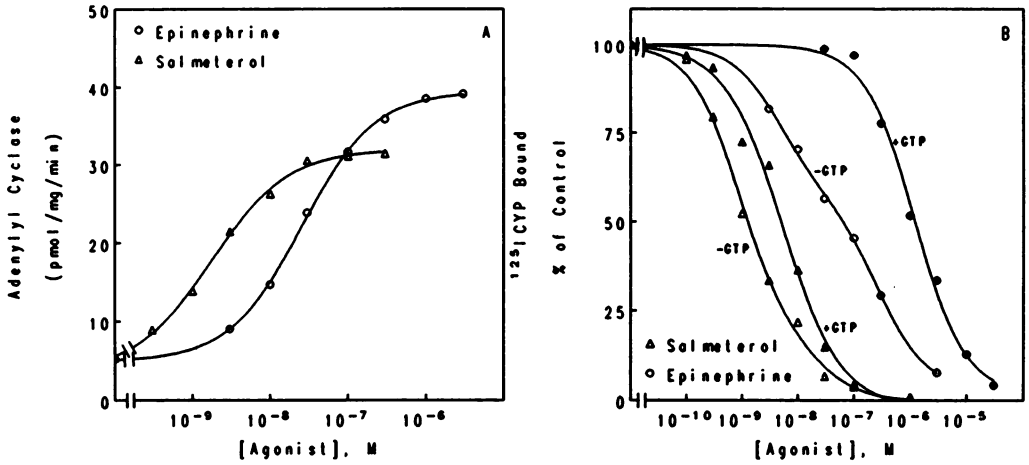

Fig. 2. Comparison of salmeterol and epinephrine stimulation of adenylyl cyclase, and displacement of ICYP binding. A, Adenylyl cyclase activity was measured as a function of increasing concentrations of salmeterol (0.3–300 nM) or epinephrine (3 nM to 3 μM). Values shown are the mean of triplicate determinations from one experiment. B, Comparison of salmeterol (*triangles*) and epinephrine (*circles*) displacement of ICYP binding in the absence (*open symbols*) or presence (*closed symbols*) of 10 μM GTP. The ICYP concentration was 23 pM. Values are shown as the percent of ICYP binding in the absence of displacing agonist (percent of control).

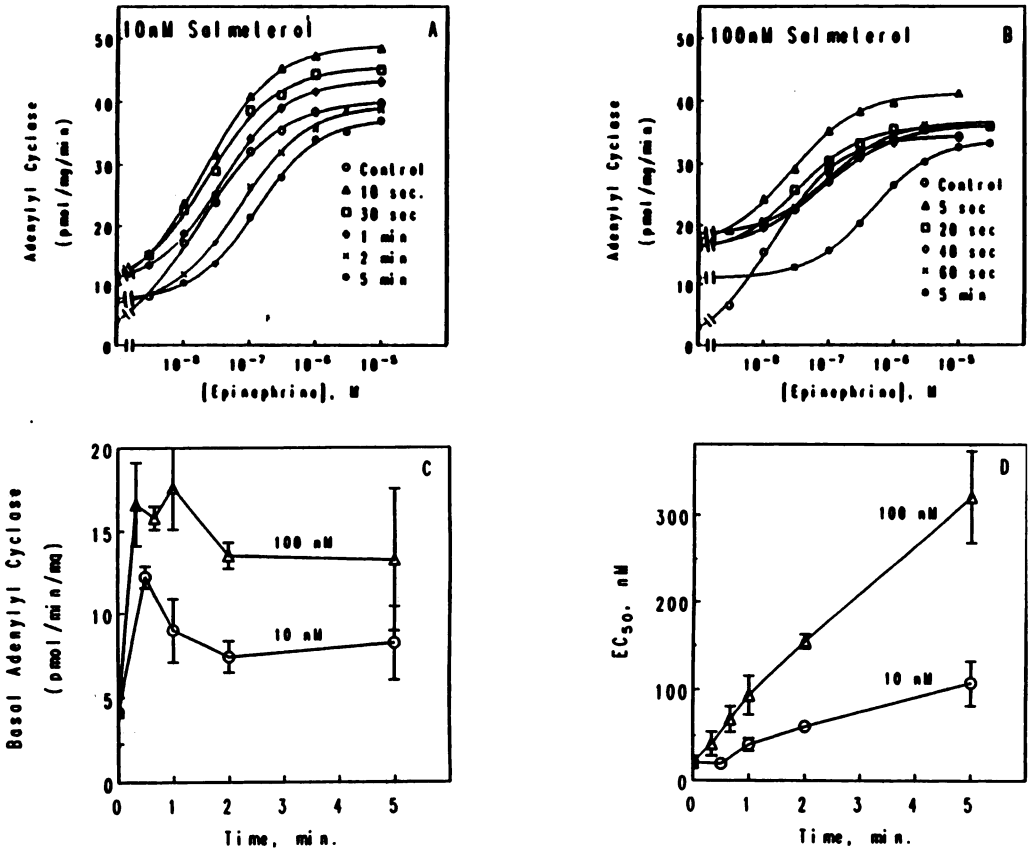

Fig. 3. Effect of the time of pretreatment of L cells with salmeterol on basal and epinephrine-stimulated adenylyl cyclase. A and B, L cells were incubated either with no additions (control) or with 10 nM (A) or 100 nM (B) salmeterol for the times indicated. Cells were washed and lysed, and plasma membranes were isolated on sucrose step gradients. Basal and epinephrine-stimulated adenylyl cyclase activities were measured as described in Experimental Procedures. Values shown are the mean of triplicate determinations of a single experiment. C and D, Data are the mean of three or more experiments except for the 30-sec point at 10 nM salmeterol and the 40-sec and 2-min points at 100 nM salmeterol, which are the mean of two experiments. *Bars*, standard deviation of the mean of three or more experiments and the range where $n = 2$. EC_{50} values for epinephrine stimulation were derived from Eadie-Hofstee plots of the data.

other experiments, we have shown that the effects of 10 nM salmeterol pretreatment beyond 5 min eventually approach those achieved with just 5 min with 100 nM salmeterol; i.e., the EC_{50} for epinephrine stimulation of adenylyl cyclase increased 7-fold after 20 min. Pretreatment of cells with 1 nM salmeterol caused a 1.6 ± 0.7-fold increase in basal activity after 30 min and a 2.6 ± 0.017-fold increase in the EC_{50} for epinephrine stimulation.

To provide further evidence for salmeterol-induced desensitization of adenylyl cyclase, we examined the effect of 100 nM salmeterol pretreatment on the GTP shift of salmeterol displacement of ICYP binding to the βAR. We found that the GTP shift was almost completely eliminated with a 5- or 30-min treatment with 100 nM salmeterol; i.e., the shift was 1.04 ± 0.11 (five experiments, data not shown). To compare the rate and extent of salmeterol-induced desensitization with those of the full agonist epinephrine, L cells were pretreated with 10 μM epinephrine for 1–5 min, and basal adenylyl cyclase and epinephrine dose-response data were obtained. As shown in Fig. 4, epinephrine caused a rapid and progressive desensitization; i.e., the EC_{50} increased 15-fold after 5 min, and the V_{max} was decreased by 50%, effects that were very similar to those of 100 nM salmeterol. However, in contrast to salmeterol pretreatment, basal activity progressively declined to 20% of control basal values after 5 min since epinephrine (unlike salmeterol) is rapidly washed out.

Salmeterol-induced desensitization and down-regulation with prolonged salmeterol pretreatment. Salmeterol not only caused the rapid desensitization of βAR stimulation of adenylyl cyclase but also induced a much more slowly developing but profound further desensitization and down-regulation after prolonged treatment with either 10 or

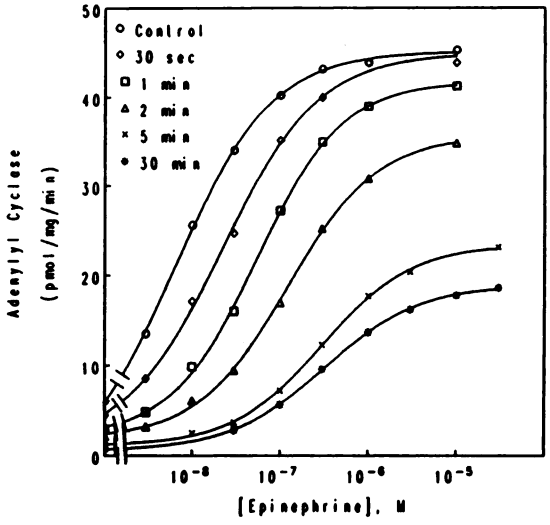

Fig. 4. Effect of the time of pretreatment of L cells with 10 μM epinephrine on epinephrine-stimulated adenylyl cyclase. L cells were incubated with 10 μM epinephrine in 1 mM thiourea and 0.1 mM ascorbate or with the carrier only (*control*) for the times indicated and then washed and homogenized. The membranes were purified as described in Experimental Procedures. Basal and epinephrine-stimulated adenylyl cyclase activities were measured. The values shown are the mean of triplicate determinations of a single experiment.

100 nM salmeterol for 24 hr (Fig. 5A). As a result, epinephrine stimulation was reduced to barely detectable levels, the EC_{50} was increased >20-fold, and the V_{max} was decreased ~85%. The B_{max} for ICYP binding was reduced 74% from 194 to 50 fmol/mg with the 10 nM treatment. In three similar experiments, we found an average down-regulation of the βAR of

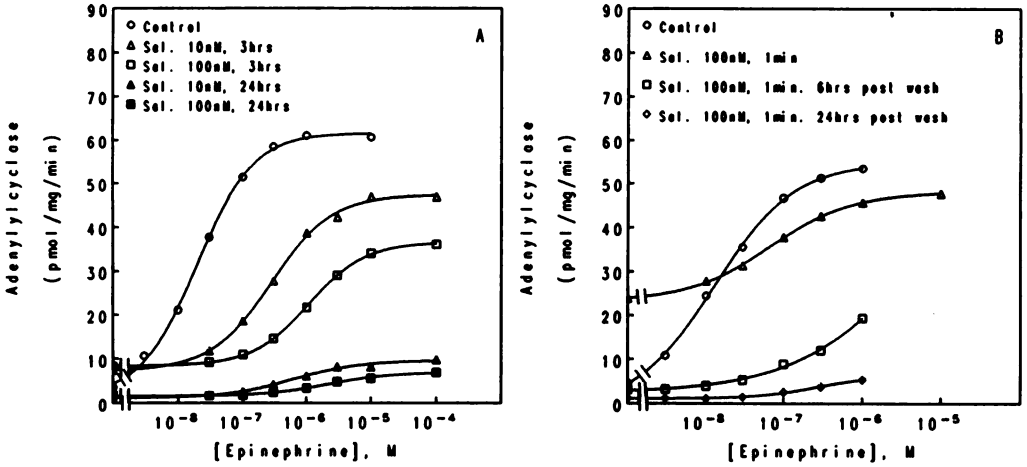

Fig. 5. Desensitization and down-regulation of the βAR after continuous treatment with salmeterol or 1-min treatment followed by washout and reincubation. A, L cells were treated for 3 or 24 hr with 10 or 100 nM salmeterol. The control sample received no treatment. The cells were then washed, and membranes were prepared and adenylyl cyclase measured as described. B, L cells were treated for 1 min with 100 nM salmeterol and then washed six times with 10 ml of medium (37°) and reincubated in medium for either 6 hr or 24 hr as indicated. Cells were then washed, membranes were prepared, and basal and epinephrine-stimulated adenylyl cyclase was measured as described. The values shown are the mean of triplicate determinations from one experiment.

85.3 ± 5.5% (± standard error). Although we have not performed extensive time courses, we have found that approximately half of the receptors are lost after a 3-hr treatment (44.6% and 48.4% down-regulation was observed in two experiments).

Basal adenylyl cyclase activity was also decreased ~2-fold relative to controls and >90% relative to the level reached after 1 min of salmeterol treatment (see Fig. 3C). Because basal activity is presumably in large part attributable to salmeterol tightly bound to the membrane or an exosite from which it has access to the activating site, it was to be expected that the desensitization and down-regulation of the βAR would result in the observed decline in the basal activities. In this experiment cells were also pretreated for 3 hr with the two concentrations of salmeterol. The shift in the EC_{50} and the changes in basal activity were not much greater with the 3-hr pretreatment than the effects of a 20-min pretreatment with 100 nM salmeterol (data not shown).

Desensitization and down-regulation do not require continuous incubation with free salmeterol. An additional observation of considerable interest was that treatment with 100 nM salmeterol for just 1 min, followed by six washes with growth medium at 37° and continued incubation in the absence of salmeterol for 24 hr, resulted in a desensitization that was barely distinguishable from that in cells incubated with salmeterol for the entire 24-hr period (Fig. 5B). In contrast, a 1-min treatment with 10 μM epinephrine followed by the same wash procedure and subsequent 24-hr incubation caused no significant desensitization (data not shown). These data demonstrate that salmeterol (but not epinephrine) remains tightly bound to the cells even after many hours at 37°, consistent with the 12-hr $t_{1/2}$ for the off time of its relaxation of smooth muscle (2–4).

Tethered salmeterol competes with ICYP binding. A comparison was made for the K_d and B_{max} values for ICYP binding to membranes prepared from untreated L cells and cells pretreated for 30 min with 100 nM salmeterol. The salmeterol treatment caused a 2.1-fold increase in the K_d for ICYP from 8.4 ± 1.4 to 17.8 ± 1.8 pM. The B_{max} was decreased 13.7% from 413 ± 65 to 357 ± 48 fmol/mg. To control for the possibility that desensitization of the βAR might alter the K_d for ICYP, we also compared the K_d for ICYP after 5.0 μM epinephrine-induced desensitization. This treatment did not alter the K_d for ICYP.

Incubation of L cells with a reversible βAR antagonist prevents the salmeterol-induced desensitization. The effect of metoprolol on salmeterol-induced changes in adenylyl cyclase is shown in Fig. 6, which is the average of three experiments with 8-min salmeterol pretreatment; Fig. 7, which shows the time course of salmeterol action with and

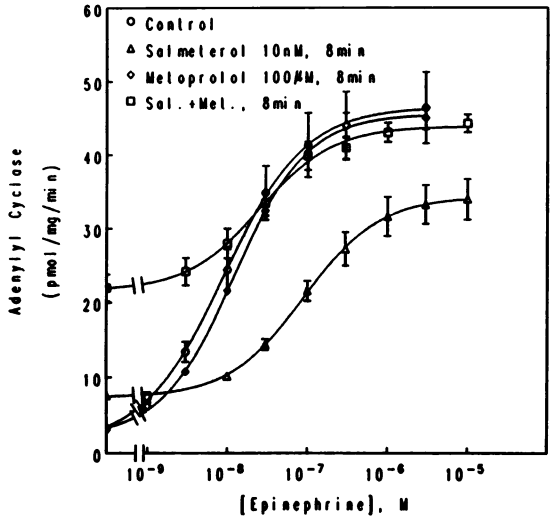

Fig. 6. Effect of the antagonist metoprolol on the salmeterol-induced increase in basal adenylyl cyclase and the increase in the EC_{50} for epinephrine stimulation. L cells were pretreated for 8 min with either carrier (*control*), 100 μM metoprolol, 10 nM salmeterol, or the combination of metoprolol and salmeterol (for the combination, metoprolol was added 10 sec before the addition of salmeterol). After the pretreatments, cells were washed and homogenized, and membranes were prepared as described in Experimental Procedures. Values shown are the mean ± standard error of three experiments, each assayed in triplicate.

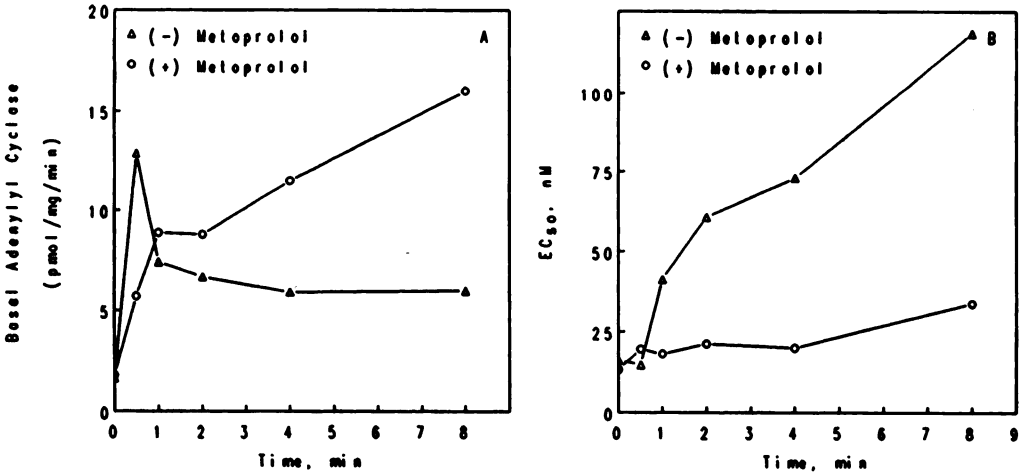

Fig. 7. Effect of metoprolol on the time course of the 10 nM salmeterol-induced increases in basal adenylyl cyclase and the EC_{50} for epinephrine-stimulated activity. L cells were pretreated with 10 nM salmeterol in the presence or absence of 100 μM metoprolol for the times indicated, membranes were prepared, and adenylyl cyclase activities were measured as discussed. EC_{50} values for epinephrine stimulation were determined from Eadie-Hofstee plots of epinephrine concentration data similar to that shown in Fig. 6.

without metoprolol on basal adenylyl cyclase and EC_{50} values for epinephrine stimulation; and Table 1, which shows a summary of the data from three or more experiments. Pretreatment of L cells with 10 nM salmeterol in the presence of 100 μM metoprolol attenuated the increase in the EC_{50} for epinephrine stimulation of adenylyl cyclase determined from dose response data. The typical 4–6-fold increase in the EC_{50} after 8 min of 10 nM salmeterol was reduced to ~1.5-fold by metoprolol (Fig. 6, Table 1). In contrast, basal adenylyl cyclase activity was steadily increased by the combination of the drugs relative to salmeterol alone, at least for times of >1 min of treatment (Fig. 7, Table 1). After 8 min of treatment, metoprolol (100 μM) and salmeterol (10 nM) elevated basal 3-fold over the effect of salmeterol alone and 5-fold relative to controls. Incubation of cells with metoprolol alone caused no significant effect on either the EC_{50} or basal activity, demonstrating that it was removed with the wash and membrane preparation.

Similar results were obtained with 100 nM salmeterol in combination with metoprolol; that is, the 15–17-fold increase in the EC_{50} in the absence of metoprolol was reduced to 6–8-fold in its presence. The level of activation of basal adenylyl cyclase reached the maximal level that could be expected for salmeterol activation of naive adenylyl cyclase as shown in Fig. 2 (~30 pmol/min/mg). In control experiments, we demonstrated that metoprolol caused a full inhibition of salmeterol stimulation with a K_i of 260 nM. Furthermore, the increased basal adenylyl cyclase activity after salmeterol pretreatment of cells in the presence of metoprolol was similarly inhibited by the addition of metoprolol to the assay solutions.

TABLE 1

Dissociation by metoprolol of the salmeterol-induced increase in basal AC activity and the EC_{50} for epinephrine activation of AC

Pretreatment		Basal AC	EC_{50}
Salmeterol	Metoprolol		
		pmol/min/mg	*nM*
0	0	4.2 ± 1.9 (26)	20.0 ± 7 (26)
10 nM	0	7.2 ± 0.7 (6)	106.0 ± 42 (6)
0	100 μM	3.3 ± 1.0 (3)	12.9 ± 5.4 (3)
10 nM	100 μM	20.5 ± 3.9 (4)	28.2 ± 5.4 (4)
100 nM	0	13.2 ± 4.3 (8)	320.0 ± 52 (6)
100 nM	100 μM	30.6 ± 5.5 (3)	149.0 ± 46 (3)

L cells were pretreated for 5 or 8 min with 10 nM or 100 nM salmeterol in the presence or absence of 100 μM metoprolol. All values are mean ± standard deviation. The number of experiments is given in parentheses.

Discussion

Previous studies of salmeterol relaxation of airway smooth muscle led to the conclusions that its action on βAR was slow in onset, that it had an extremely prolonged duration of action and did not desensitize, that it was a partial agonist (V_{max} between 40–60% of a full agonist), and that its properties were consistent with two binding sites, as shown in Fig. 1 (2–4). Although our data are consistent with the two-site model, the direct examination of salmeterol binding to the βAR and its activation and desensitization of adenylyl cyclase reported here have provided new molecular insights and rather different conclusions.

Coupling efficiency of salmeterol. With regard to partial agonism, we found that the coupling efficiency for salmeterol activation of adenylyl cyclase was only ~7% that of epinephrine, whereas previous estimates based solely on efficacy (V_{max}) of salmeterol relaxation versus the full agonist isoproterenol had led to the conclusion that salmeterol was 40–60% as efficacious as isoproterenol. We have found that the V_{max} for response to an agonist depends on the receptor concentration present in the cell or preparation (7, 8). Moreover, when the receptor number is high, even weak agonists cause essentially full stimulation of the adenylyl cyclase system. To have an index that assesses relative agonist efficiency and is independent of receptor number, we used the efficiency with which the agonist catalyzes the activation of the cyclase, i.e., the rate constant for activation in the Cassel-Selinger scheme. The actual practical index is the k_{on} to k_{off} ratio that is given by the measurable expression: $(K_d/EC_{50} - 1)/r$, where r is the receptor density and K_d and EC_{50} have their customary definitions. Because k_{off} is independent of the nature of the agonist, the k_{on}/k_{off} ratio is directly proportional to the effectiveness of the agonist in promoting βAR activation of adenylyl cyclase. The considerable difference in these two means of estimating agonist effectiveness underlies the importance of using coupling efficiency rather than efficacy for comparisons of agonists (7, 8). This may have profound implications on desensitization as well.

Exosite and active site binding of salmeterol. The model shown in Fig. 1 proposing that salmeterol binds to two

sites was based on prior characterization of the bronchodilation induced by salmeterol. Our data support this model, in particular, the demonstration that active site binding but not exosite binding (revealed by the increased basal adenylyl cyclase activity) is blocked by the antagonist metoprolol. Nevertheless, the nature of the exosite remains unknown. Although it is clear that salmeterol partitions into lipid and that this contributes to the exosite, it is unresolved whether there is a site in the immediate vicinity of the βAR that allows active site binding by the tethered drug. This ambiguity raises interesting theoretical problems concerning the measurement of the K_d for salmeterol by displacement of ICYP. For example, it is possible that the βAR can bind two molecules of salmeterol simultaneously. This would alter slightly the free concentration in binding assays and shift leftward our measurement of the true K_d for the active site. It is also possible that the K_d for active site binding of the putative tethered salmeterol differs from that of salmeterol in solution. Furthermore, it is certain that the partition of salmeterol into the lipid will alter the concentration of the solution salmeterol and also cause an apparent rightward shift in the EC_{50} for displacement of ICYP.

In the present study, we measured a K_d for salmeterol binding to the active site by ICYP displacement at equilibrium. The assumption of equilibrium is valid since it is clear from the rapidity of metoprolol inhibition of salmeterol activation of adenylyl cyclase or of bronchodilation that the salmeterol half-time for dissociation from the active site is in the order of minutes. If salmeterol bound the active site with a half-time for dissociation of hours, as appears to be the case with exosite binding, estimates of its binding affinity by competition with ICYP would not be meaningful. To the extent that the concentration of salmeterol is altered by exosite binding or by other complications discussed above, the measured K_d will deviate from the true K_d. However, the deviation should not be more than what we have observed in binding assays after salmeterol pretreatment, where an approximate doubling of the K_d was observed.

Salmeterol activation of adenylyl cyclase. A property of salmeterol action quite different for adenylyl cyclase activation versus relaxation of airway smooth muscle was the absence of any lag in salmeterol activation of adenylyl cyclase either in cell-free assays with membranes or with intact cell pretreatments followed by adenylyl cyclase assay. In terms of the absence of a detectable lag in activation of adenylyl cyclase, salmeterol behaves as a typical βAR agonist. In light of these data, we suggest that the relatively slow onset of salmeterol's relaxation of tracheal smooth muscle ($t_{1/2}$ = 3–5 min) was caused by slow salmeterol penetrance to the active site. This is obviously not a problem in our studies of isolated cells or adenylyl cyclase. The persistence of salmeterol activation of basal adenylyl cyclase after salmeterol pretreatment of cells, extensive washing, and sucrose step gradients is another unusual action of salmeterol relative to the typical more hydrophilic agonists. Although this basal adenylyl cyclase activity partially declines (desensitizes) after 1 min of pretreatment, it remains significantly elevated over basal levels, even after 3 hr.

Salmeterol-induced desensitization. Given the absence of evidence for any decline in salmeterol bronchodilation in clinical studies (2–4), one of the most surprising results of this study was our discovery that salmeterol caused some desensitization of βAR stimulation of adenylyl cyclase. There are four observations in support of this conclusion: the decrease in basal adenylyl cyclase with time, the increase in EC_{50} for epinephrine activation, the diminution of the GTP shift in agonist affinity, and a profound down-regulation of receptors with prolonged pretreatment times. The significance of the loss of basal adenylyl cyclase, the loss of the GTP shift, and the down-regulation is quite straightforward. However, the interpretation of the EC_{50} shifts is more complex. This is because salmeterol carried over into the assay in the membranes from the very tight binding to the exosite acts as an antagonist to epinephrine activation of adenylyl cyclase (13) since it has access to the active site but is a very weak agonist. Through the use of metoprolol to block active site binding and desensitization, we were able to estimate that there is a 1.5–2-fold and 4–5-fold shift in the EC_{50} due to competition by exosite-bound salmeterol after the 10 and 100 nM salmeterol pretreatments, respectively. Correcting the 4–6-fold and 16–18-fold increases in the EC_{50} we observed for epinephrine stimulation of adenylyl cyclase for the competition caused by the 10 and 100 nM salmeterol pretreatments (5 min) leaves a 3–4-fold shift attributable to the rapid phase of desensitization. This is significantly less than the desensitization we observe with 10 μM epinephrine over the same time of pretreatment (Fig. 4) with comparable occupancy of receptors. It is important to emphasize that occupancy with 10 μM epinephrine is similar to that with 100 nM salmeterol (~30-fold over the K_d). Partial agonists likely act by shifting the receptor to the activated state to a lesser degree than do full agonists (18). Therefore, it is to be expected that homologous desensitization, which is dependent on high occupancy and the activated state of the receptor (19–21), would be reduced.

How do we reconcile the salmeterol-induced desensitization of the βAR in L cells with the clinical observations of no desensitization? In our model cell system, our observation that salmeterol desensitization is reduced relative to epinephrine is certainly consistent with the prolonged action of salmeterol in the clinic and is also consistent with our observation that the effect of salmeterol on basal adenylyl cyclase persists through extensive washing and sucrose step gradient purification of membranes. As the experiment in Fig. 5A demonstrates, basal activity remains elevated even after 3 hr of continuous treatment with 100 nM salmeterol, and this is certainly consistent with the persistence of the action of salmeterol in clinical studies (2–4). Second, in airways there may be just enough salmeterol at the active site so that adenylyl cyclase, although only partially activated, is sufficiently activated to maintain elevated cAMP levels and continuous activation of cAMP-dependent protein kinase. In contrast, our test system, with the continuous presence of salmeterol at high levels, likely overestimates the desensitization that occurs in clinical studies. Third, our recent studies of the human βAR in BEAS-2B cells and overexpressed in HEK293 cells indicate that the human receptor is also desensitized far less by salmeterol than by epinephrine.[1]

[1] R. B. Clark, C. Allal, B. S. Whaley, B. J. Knoll, R. H. Moore, B. F. Dickey, and R. Barber. Prolonged activation of the human β_2-adrenergic receptor by salmeterol: A function of persistent membrane association and greatly reduced desensitization. Submitted for publication.

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