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Comparison of Duration of Agonist Action at β_1- and β_2-Adrenoceptors in C6 Glioma Cells: Evidence that the Long Duration of Action of Salmeterol Is Specific to the β_2-Adrenoceptor

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SUMMARY

The C6 glioma cell line, which expresses β_1- and β_2-adrenoceptors at a ratio of 80:20, was used to investigate the durations of action of formoterol at β_1-adrenoceptors and of salmeterol at both β_1- and β_2-adrenoceptors in an attempt to determine whether the sustained duration of action of salmeterol was unique to β_2-adrenoceptors or, as with formoterol, resulted from its lipophilic nature and partitioning into the bulk lipid of the plasma membrane. In this cell line, formoterol, like the nonselective β-adrenoceptor agonist isoprenaline, behaved as a potent, full agonist at β_1-adrenoceptors and did not seem to exhibit a high degree of selectivity for β_2-adrenoceptors. Salmeterol seemed to stimulate cAMP accumulation in C6 cells predominantly via activation of the subpopulation of β_2-adrenoceptors. However, at high (micromolar) agonist concentrations, salmeterol also activated β_1-adrenoceptors, albeit with low potency and efficacy. At high concentrations (30 μM), salmeterol attenuated cAMP responses mediated by activation of β_1-adrenoceptors by isoprenaline (K_P = 1.6 μM), indicating that salmeterol exhibited a low affinity for β_1-adrenoceptors in C6 cells. In multiple washout experiments, cAMP responses to isoprenaline and formoterol waned with increasing numbers of washing processes. Therefore, it seemed that formoterol relied on its moderately lipophilic nature to partition into bulk lipid of the plasma membrane to produce sustained activity, particularly at high agonist concentrations. Salmeterol was found to persist at β_2-adrenoceptors in C6 cells despite washing cell monolayers up to four times. To determine the duration of action of salmeterol at β_1-adrenoceptors expressed on the same cells, use was made of full/partial agonist interactions. In cells exposed to a single washout of agonist-containing medium, salmeterol (30 μM) lost its ability to attenuate responses to the more efficacious agonist, isoprenaline. This observation provided convincing evidence to support the hypothesis that salmeterol exhibits sustained agonist activity at β_2-adrenoceptors, but not β_1-adrenoceptors, expressed on the same cells. Therefore, the sustained activity of salmeterol at β_2-adrenoceptors seems to be unique and does not result solely from its partitioning into the bulk lipid of the plasma membrane.

Salmeterol behaves as a potent and selective β_2-adrenoceptor agonist in isolated preparations of guinea pig trachea and human bronchus, which contain predominantly β_2-adrenoceptors (1–4). It exhibits extremely persistent effects at β_2-adrenoceptors in these tissues and mediates smooth muscle relaxation for periods of >12 hr (1, 4). This behavior has been attributed to the binding of salmeterol at both the active site of the β_2-adrenoceptor and to an exosite within the β_2-adrenoceptor protein itself or at a perireceptor site in the local lipid surrounding the β_2-adrenoceptor (2, 5, 6). An alternative hypothesis, suggested by Anderson *et al.* (7), is that the long duration of action of salmeterol is due to its highly lipophilic nature and that this might account for the persistence of salmeterol action observed *in vitro*. However, an important question regarding the pharmacological profile of salmeterol is whether it can bind to and produce sustained agonist activity at other receptors, e.g., the β_1-adrenoceptor subtype. Unfortunately it has been difficult to examine the pharmacology of salmeterol in β_1-adrenoceptor-containing isolated tissues, such as rat atria, because it is a very weak agonist in this system (>10,000-fold weaker than isoprenaline; Refs. 1 and 8), and it exhibits very low efficacy (4% of the maximal response elicited by isoprenaline; Ref. 8).

β-Adrenoceptor subtypes often coexist in the same tissue, such as β_2- and β_1-adrenoceptors in guinea pig trachea (9), and can be coexpressed on cultured cells (10, 11), although one subtype often predominates. For example, the SK-N-MC

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ABBREVIATIONS: HBH, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered Hanks' solution; ICI 118551, erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropyl-aminobutane-2-ol.

human neuroepithelioma cell line contains adrenoceptors of both the β_1-subtype (12) and the β_3-subtype (13, 14). The C6 rat glioma cell line (15) has been shown to express both β_1- and β_2-adrenoceptors (16–20). Radioligand binding studies have shown that β_1- and β_2-adrenoceptors are present at a ratio of 80:20 in C6 cells (16, 20). In addition, previous pharmacological investigations with C6 cell monolayers have shown that cAMP accumulation, in response to stimulation with isoprenaline, is mediated predominantly via β_1-adrenoceptors (20, 21). However, β_2-selective adrenoceptor agonists, such as zinterol, are also capable of mediating cAMP accumulation in C6 cells, albeit to a smaller extent than when isoprenaline is used as agonist, presumably in response to stimulation of the subpopulation of β_2-adrenoceptors (20).

In the current study, we compared in the C6 cell line the pharmacological profiles of the adrenoceptor agonists formoterol and salmeterol with that of the nonselective β-adrenoceptor agonist isoprenaline. The aim was to determine whether the long duration of action of salmeterol is unique to β_2-adrenoceptors, due to specific binding of the phenylalkyloxyalkyl tail of the molecule to an exosite or a perireceptor environment (2, 5), or is a result of its highly lipophilic nature and its retention in the bulk lipid of the plasma membrane (7). If the latter hypothesis is correct, salmeterol should exhibit a long duration of action at both β_1- and β_2-adrenoceptors despite washout of agonist-containing medium from cell monolayers. However, if the former hypothesis is accurate, salmeterol will not persist for long periods at β_1-adrenoceptors after agonist removal.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium, fetal calf serum, and Hanks' solution were obtained from Northumbria Biologicals (UK). 8-[^{3}H]Adenine (specific activity, 1147 GBq mmol^{-1}) and 8-[^{14}C]cAMP (specific activity, 11.3 GBq mmol^{-1}) were obtained from Amersham International (Buckinghamshire, UK). Dowex 50W (H^+ form, 200–400 mesh) and glutamine were obtained from Bio-Rad (Hemel Hempstead, UK) and Flow Laboratories (Thame, UK), respectively. Neutral alumina (type WN-3), imidazole, (±)-isoprenaline hydrochloride, and atenolol were purchased from Sigma (UK). The gifts of salmeterol free base and formoterol (Glaxo Research and Development Ltd., Stevenage, UK), ICI 118551 hydrochloride (ICI Pharmaceuticals, Macclesfield, UK), and rolipram (Schering A.G., Berlin, Germany) are gratefully acknowledged.

Cell culture. C6 cells and B50 cells (European Collection of Animal Cell Cultures, Salisbury, UK) were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine and 10% (v/v) fetal calf serum at 37° under an atmosphere of 10% CO_2 in humidified air in 75-cm^3 flasks (Costar). Experiments were performed in 24-well cluster dishes when cells had reached confluence (3 days after seeding).

[^{3}H]cAMP accumulation. Measurement of agonist-stimulated [^{3}H]cAMP accumulation was performed with the protocol described previously (22). Briefly, C6 cell monolayers were incubated for 2 hr at 37° in 1 ml/well HBH (20 mM) containing [^{3}H]adenine (74 kBq/well). Before the addition of agonists, cells were incubated for 30 min in 1 ml/well HBH containing the phosphodiesterase type IV inhibitor rolipram (100 μM; Ref. 23). Unless otherwise stated, rolipram (100 μM) was included in all incubations. Where appropriate, antagonists (in 10 μl of HBH) were added to cell monolayers during this 30-min equilibration period. Agonists (in 10 μl of HBH) were then added to the assay system, and the incubation was continued for the required length of time. Incubations were terminated by the addition of 50 μl of concentrated HCl to each well, which lysed the cells. [^{3}H]cAMP content of the supernatant buffer from each well was isolated through sequential ion exclusion Dowex-alumina chromatography as described previously (22).

For the studies of the washout of different agonists, cell monolayers were exposed to agonists for 10–30 min. Subsequently, monolayers were subjected to one to four washing procedures at set time intervals. Each wash procedure involved discarding the existing medium from each well, washing the cells twice with 1 ml HBH, and replacing the original medium with prewarmed HBH (1 ml) that contained rolipram (100 μM). After the final incubation period, assays were terminated through the removal of the incubation medium from each well and replacement of the contents with an additional 1-ml aliquot of HBH, which contained 50 μl concentrated HCl to facilitate cell lysis. Intracellular levels of [^{3}H]cAMP were then determined as described above. Time-matched control experiments were run conjointly, in which cell monolayers were exposed continuously to identical concentrations of agonists, to control for any ongoing desensitization.

Data analysis. cAMP data was corrected for interwell variability in cell number and recovery of the tracer, [^{14}C]cAMP, from Dowex-alumina chromatography. Agonist and antagonist concentration-response curves were fitted to a logistic equation through computer-assisted curve fitting with the use of the computer program Inplot 4 (GraphPad) as described previously (22). This analysis was used to calculate the dose-response parameters E_{max}, EC_{50}, IC_{50}, and Hill coefficients. The relative potency of each agonist was measured with reference to isoprenaline (relative potency = 1) with the equation EC_{50} (isoprenaline)/EC_{50} (test agonist). Agonist efficacy was determined in terms of the maximal cAMP response compared with the maximal response to isoprenaline (100%). Apparent antagonist dissociation constants (K_B) were determined, assuming competitive antagonism, with the use of one of two methods: (i) from parallel shifts of agonist concentration-response curves using the relationship $K_B = D/(K_2/K_1 - 1)$ (27), where D is the concentration of antagonist, K_1 is the concentration of agonist producing half-maximal response, and K_2 is the concentration of agonist producing the same response in the presence of antagonist; or (ii) with a modification of the null method described by Lazereno and Roberts (26). Briefly, a concentration-response curve to an agonist was generated, and a concentration (C) of agonist was chosen that gave a response of >50% of the maximum agonist response. The concentration was then determined of antagonist (IC_{50}) that was required to reduce the response to this concentration (C) of agonist by 50%. The agonist concentration-response curve was fitted to a logistic equation as described above, and a concentration of agonist was identified (C') that yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_B was then determined from the relationship $C/C' = IC_{50}/K_B + 1$. Partial agonist dissociation constants (K_P) were estimated according to the method of Stephenson (28). Dose ratios were calculated to compare the rate of offset of action of agonist action from β-adrenoceptors in C6 cells with the following equation:

$$\text{Dose ratio} = \frac{EC_{50} \text{ of CRC to agonist after number of washes}}{EC_{50} \text{ of CRC to agonist in time-matched control cells}}$$

Data represent mean ± standard error of triplicate or quadruplicate determinations obtained in varying numbers of experiments. In some experiments, data are represented as a percentage of the maximal time-matched control response, and results are expressed as mean ± standard error from the combined data of experiments. Significance testing was performed with the use of Student's t tests and two-way analysis of variance. The significance of differences in the fitted parameters (E_{max} or EC_{50}) of concentration-response curves was determined through analysis with the use of the program ALLFIT (29) of the effect on the residual sums of squares (for nonlinear regression analysis) of forcing them to be equal.

Results

Comparison of agonist-stimulated [^{3}H]cAMP accumulation in C6 cells. Isoprenaline, formoterol, and salmeterol stimulated the formation of [^{3}H]cAMP in a concentration-dependent manner (10-min incubation period) with a rank order of potency of isoprenaline > formoterol > salmeterol. Stimulation of C6 cells with isoprenaline (10 μM) led to a large increase in [^{3}H]cAMP levels (42.9 ± 6.1-fold over basal; EC_{50} = 14.6 ± 1.7 nM; 14 experiments). Formoterol (EC_{50} = 111.7 ± 37.0 nM) also behaved as a highly efficacious agonist in this system. Formoterol (10 μM) produced 92.0 ± 0.4% (three experiments) of the response mediated by isoprenaline (10 μM) when cAMP accumulation mediated by each agonist was directly compared within the same experiment. Salmeterol (10 μM) also produced substantial increases in [^{3}H]cAMP levels in C6 cells. The actual accumulation of [^{3}H]cAMP (in dpm) stimulated by salmeterol varied among experiments but was always significantly lower ($p < 0.01$; unpaired t test) than that obtained with isoprenaline in the same experiment. Thus, salmeterol (EC_{50} = 264.0 ± 63.5 nM) generated only 13.9 ± 0.9% (six experiments) of the cAMP responses to isoprenaline (10 μM) measured in the same experiment.

Antagonism of [^{3}H]cAMP responses to agonists in C6 cells. In an attempt to determine the contribution of each β-adrenoceptor subtype to the cAMP response mediated by each agonist in C6 cells, the response to a near-maximally effective concentration of each agonist was antagonized by preincubation of C6 monolayers with various concentrations of either the β_1-selective antagonist atenolol (24) or the β_2-selective antagonist ICI 118551 (25) for a 30-min period. Both atenolol and ICI 118551 attenuated the [^{3}H]cAMP response to isoprenaline (1 μM) and formoterol (1 μM) in a concentration-dependent manner. The mean apparent dissociation constants (K_B) values obtained with the null method described by Lazereno and Roberts (26) are shown in Table 1 and were indicative of a primarily β_1-adrenoceptor-mediated response. Attenuation of the cAMP responses to a submaximally effective concentration of salmeterol (1 μM) seemed to be more complicated (Fig. 1). The cAMP response to salmeterol was potently antagonized by increasing concentrations of ICI 118551 (Fig. 1b). The apparent K_B value calculated from this analysis was 7.7 ± 1.8 nM (seven experiments; Table 1). This result suggested that a large proportion of the cAMP response generated by salmeterol occurred via activation of β_2-adrenoceptors. The inhibition by atenolol of responses to salmeterol (1 μM) was unexpected. With increasing concentration, atenolol attenuated the agonist-mediated response, but complete inhibition of cAMP accumulation was never achieved (Fig. 1d). However, the use of atenolol (10 μM) was able to significantly attenuate ($p < 0.05$) cAMP responses to salmeterol (1 μM), by 30.3 ± 7.5% (three experiments). These observations provided evidence that the cAMP response mediated by salmeterol, at the concentration used in this study, occurred predominantly via β_2-adrenoceptors, although a variable proportion of the response was mediated through activation of β_1-adrenoceptors. The fact that high concentrations of ICI 118551 reduced the response of salmeterol (1 μM) to basal levels (Fig. 1b), however, suggests that these high concentrations of ICI 118551 also antagonize the β_1-adrenoceptor component of the response. This finding is consistent with the pK_B of 7.15 obtained for ICI 118551 (Table 1) for antagonism of the β_1-adrenoceptor response to isoprenaline in these cells. This suggests that high concentrations of ICI 118551 cannot be used to study the β_1-adrenoceptor component of the response to salmeterol in isolation from its larger β_2 component.

An alternative approach was used to further examine the abilities of ICI 118551 and atenolol to attenuate isoprenaline-and salmeterol-stimulated cAMP accumulation in C6 cells. Cell monolayers were preincubated with either ICI 118551 (0.3 μM) or atenolol (10 μM) for 30 min before the addition of increasing concentrations of salmeterol or isoprenaline. In the presence of ICI 188551 (0.3 μM), the concentration-response curves to both salmeterol and isoprenaline shifted to the right (Fig. 2). However, the degree of the shift was greater in salmeterol-stimulated cells as the major proportion of the cAMP response mediated by each concentration of this agonist was mediated via β_2-adrenoceptors (Fig. 2b). The pK_B values for ICI 118551, calculated from the parallel shifts of the concentration-response curves to salmeterol and isoprenaline, were 8.01 ± 0.08 (three experiments) and 6.97 ± 0.08 (three experiments), respectively, which is consistent with the estimated K_B values obtained according to the method of Lazereno and Roberts (26). Pretreatment of cells with atenolol (10 μM) led to contrasting attenuation of the cAMP responses to salmeterol and isoprenaline in this cell line (Fig. 3). The inclusion of atenolol (10 μM) in the assay system abolished the cAMP response to isoprenaline at concentrations of <10 nM and attenuated cAMP responses at concentrations of <10 μM (Fig. 3a). The pK_B value for atenolol calculated from this analysis was 6.92 ± 0.16, which is also consistent with the estimated K_B value obtained with the null method of Lazereno and Roberts (26) in isoprenaline-stimulated cells. In contrast, atenolol (10 μM) was ineffective in inhibiting responses to salmeterol at <1 μM (Fig. 3b). However, at high agonist concentrations (1–30 μM), atenolol was observed to inhibit cAMP accumulation (Fig. 3b). As such, atenolol (10 μM) significantly attenuated the maximum cAMP response to salmeterol (30 μM), by 34.3 ± 3.6% (three experiments; $p < 0.05$).

Full/partial agonist interactions. Previous studies have demonstrated that an agonist of low efficacy (e.g., sal-

TABLE 1

Effects of selective β-adrenoceptor antagonists on the [^{3}H]cAMP responses to a range of β agonists in C6 cell monolayers

Cells were preincubated with increasing concentrations of either the β_1- or β_2-selective antagonists atenolol and ICI 118551, respectively, for 30 min before the addition of a fixed concentration of agonist (as shown in parentheses). Apparent K_B values were calculated with the null method described by Lazereno and Roberts (26). pK_B values ($-\log_{10} K_B$) have been quoted for each antagonist in this table. In the presence of atenolol, responses to selective β_2-adrenoceptor agonists were not completely inhibited. The maximum percentage inhibition of the response to agonist by 10 mM atenolol was calculated. Data represent mean ± standard error from n experiments.

Agonist	pK_B Apparent		Loss of maximum response in presence of atenolol (10 μM)	n
	ICI 118551	Atenolol		
			%	
Isoprenaline (1 μM)	7.15 ± 0.14	7.31 ± 0.17		3
Formoterol (1 μM)	7.30 ± 0.25	7.15 ± 0.24		3
Salmeterol (1 μM)	8.27 ± 0.20		30.3 ± 7.5 (3)	7

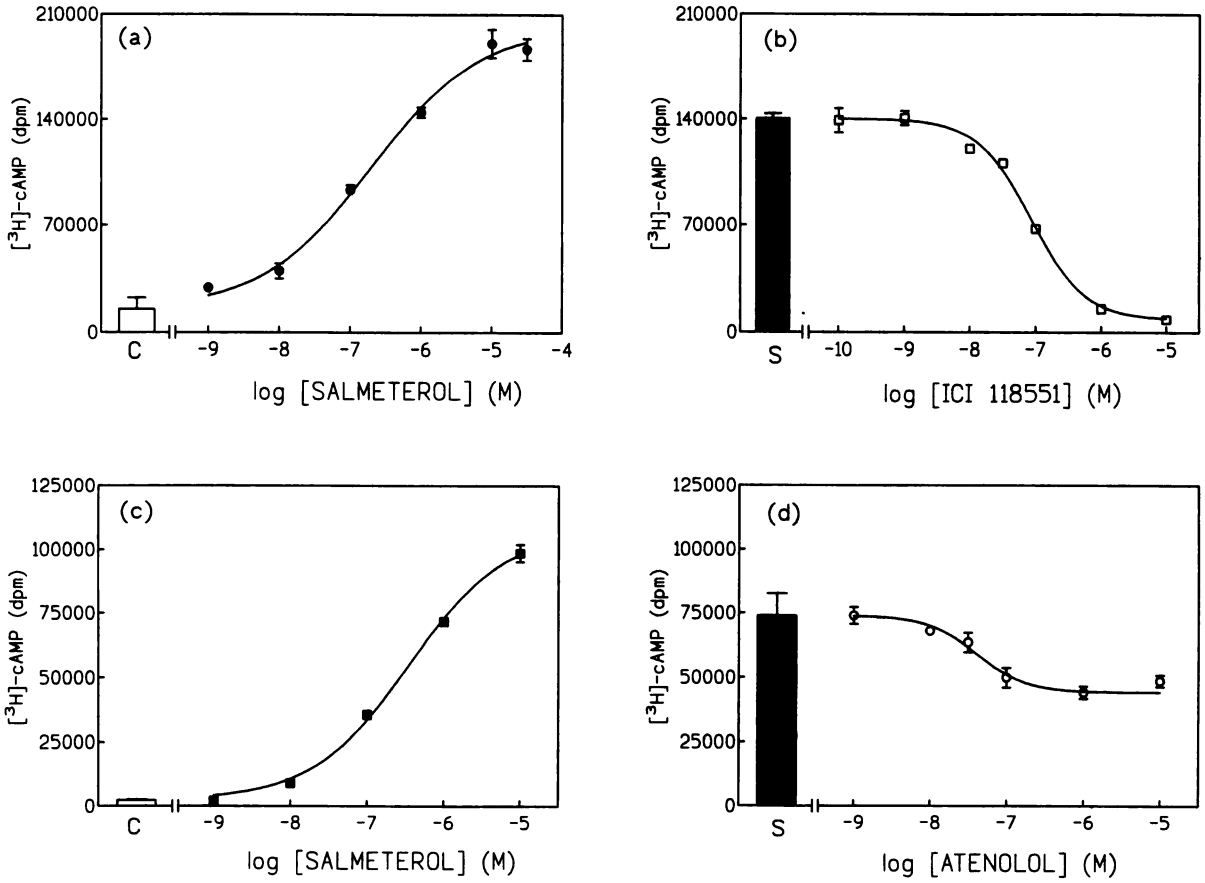

Fig. 1. a and c, Concentration-response curve to salmeterol (●, ■) in stimulating total [^{3}H]cAMP accumulation in C6 cell monolayers. *C*, Basal accumulation of cAMP. b, Inhibition curve to the β_2-selective antagonist ICI 118551 (□) in cells stimulated with salmeterol (1 μM). *S*, Response to salmeterol (1 μM). Cell monolayers were preincubated with a range of concentrations of ICI 118551 for 30 min before the addition of salmeterol (1 μM) for an additional 10 min. Data represent mean ± standard error from four determinations in a single experiment. d, Attenuation of the cAMP response to salmeterol (1 μM) by the β_1-selective antagonist atenolol (○). S, Response to salmeterol (1 μM) in the absence of antagonist. Cell monolayers were preincubated with increasing concentrations of atenolol for 30 min before the addition of salmeterol (1 μM) for an additional 10 min period. Data for a and b or c and d were obtained in the same experiment.

meterol) attenuates the responses of a more efficacious agonist (isoprenaline) in B50 cells (22). Salmeterol exhibited a low efficacy in the C6 cell line. Although a large proportion of the cAMP response to salmeterol was mediated via β_2-adrenoceptors, at high agonist concentrations (>0.1 μM) salmeterol also activated β_1-adrenoceptors to some degree (as shown by antagonist studies). Because responses to isoprenaline seemed to be mediated almost exclusively via β_1-adrenoceptors in the C6 cells, experiments were designed to estimate the dissociation constant (K_P) for salmeterol at β_1-adrenoceptors within this cell line.

When C6 cell monolayers were simultaneously stimulated with various concentrations of isoprenaline in the presence of a supramaximally effective concentration of salmeterol (30 μM; 10-min incubation period), cAMP responses to concentrations of isoprenaline of >10 nM were attenuated by salmeterol, and the concentration-response curve to isoprenaline shifted to the right (Fig. 4a). Based on concentration-response curves, salmeterol (30 μM) was found to stimulate only a slightly higher accumulation of cAMP in C6 cells than salmeterol (1 μM). This lower concentration of salmeterol had no effect on isoprenaline responses. Therefore, it seemed that salmeterol exhibited very low efficacy at β_1-adrenoceptors in this cell line. Analysis of these data enabled a value of 1.60 ± 0.26 μM (four experiments) to be calculated for the K_P of salmeterol at β_1-adrenoceptors in C6 cells.

Effect of incubation medium washout on salmeterol-stimulated [^{3}H]cAMP accumulation at β_2-adrenoceptors. To examine the duration of action of salmeterol solely at β_2-adrenoceptors in this cell line, each concentration-response curve to salmeterol was performed in the presence of atenolol (10 μM). This β_1-selective antagonist was included in experiments to remove any β_1-adrenoceptor component in the cAMP response mediated by high concentrations of salmeterol. The results of a single experiment are shown in Fig. 5. Salmeterol exhibited a long duration of action at β_2-adrenoceptors in C6 cell monolayers at each agonist concentration that was used, despite the cell monolayers being washed for up to four times. The combined data from three experiments also demonstrate the persistence of salmeterol at β_2-adrenoceptors in these cell monolayers (Table 2). After one to four washing processes, no significant differences (based on two-way analysis of variance) were observed between washed and time-matched control curves (Fig. 5). After four washes, the apparent concentration-response curve to salmeterol had shifted only 2.8-fold to the right, and the response to each agonist concentration (even at low concentrations) was similar to control levels of intracellular [^{3}H]cAMP (Table 2). It

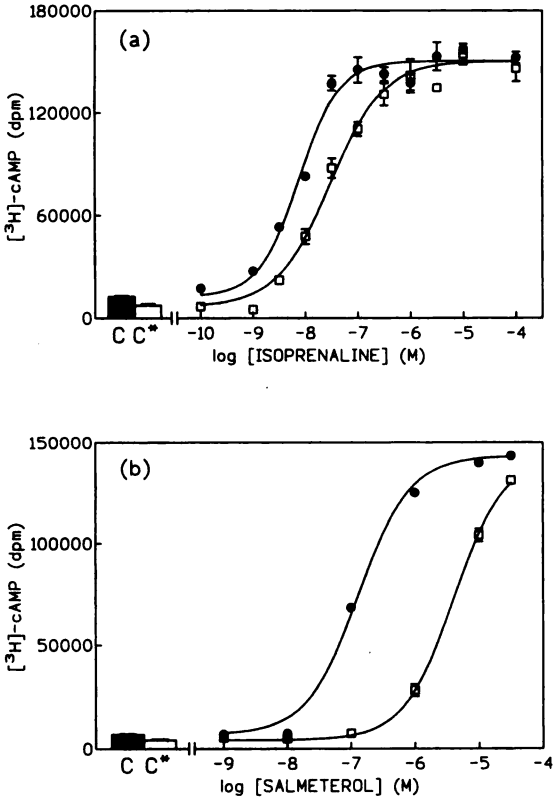

Fig. 2. Effect of the β_2-selective antagonist ICI 118551 on the concentration-response curves for agonist-stimulated total [^{3}H]cAMP accumulation in C6 cells stimulated with (a) isoprenaline and (b) salmeterol. Cell monolayers were preincubated in the absence (●) and presence (□) of ICI 118551 (0.3 μM) before the the addition of increasing concentrations of agonist for an additional 10 min. *C*, Basal accumulation of [^{3}H]cAMP in the absence of ICI 118551; *C**, basal accumulation of [^{3}H]cAMP in the presence of ICI 118551. Data for a and b were obtained in different experiments and represent the mean ± standard error of quadruplicate determinations in a single experiment. These experiments were repeated on two additional occasions, yielding similar results. Apparent K_B values for ICI 118551 were calculated with the use of the Gaddum equation (27). A summary of the mean data from the three experiments is given in the text.

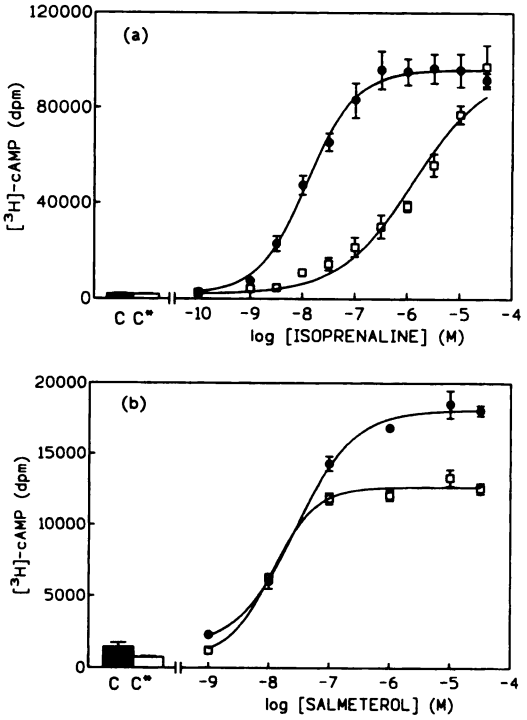

Fig. 3. Effect of the β_1-selective antagonist atenolol on the concentration-response curves for total [^{3}H]cAMP accumulation in response to stimulation with (a) isoprenaline and (b) salmeterol. C6 cell monolayers were preincubated for 30 min in the absence (●) or presence (□) of atenolol (10 μM) before the addition of increasing concentrations of agonist for an additional 10 min. *C*, Basal accumulation of [^{3}H]cAMP in the absence of atenolol; *C**, basal accumulation of [^{3}H]cAMP, in the presence of atenolol. Data for a and b were obtained in different experiments and represent mean ± standard error of quadruplicate determinations in a single experiment. Similar results were obtained in two additional experiments for each agonist. a, The apparent K_B value for atenolol was calculated with the use of the Gaddum equation (27). A summary of the mean data from the three experiments is given in the text.

was notable, however, that there was some degree of desensitization of the β_2-adrenoceptor response in these experiments (Fig. 5d), which limited the time over which these washout experiments could be conducted. After a 60-min incubation with salmeterol, the mean response to salmeterol (30 μM) from the three individual experiments was only 56.8 ± 6.5% of the control response measured at 30 min.

Effect of incubation medium washout on isoprenaline- and formoterol-stimulated [^{3}H]cAMP accumulation at β_1-adrenoceptors. As in B50 cells (22), the hydrophillic agonist isoprenaline exhibited a short duration of action in C6 cells. After a single washout of incubation medium, the apparent concentration-response curve to isoprenaline shifted 2.46 ± 0.27 (three experiments) orders of magnitude to the right (Table 2). After a series of washout procedures, responses to a range of formoterol concentrations (0.1 nM–100 μM) were attenuated in C6 cells, although the rate of loss of formoterol from the β_1-adrenoceptor environment was slower than when isoprenaline was used as agonist (Fig. 6 and Table 2). The apparent concentration-response curve to formoterol shifted only 19.0-fold to the right after a single washout process (Fig. 6a). With increasing numbers of washes (two to four), the cAMP response in terms of each original agonist concentration decreased further (Fig. 6, b–d). After the fourth and final wash, responses equivalent to concentrations of formoterol of <10 μM had been eliminated. Intracellular cAMP was still present in cells initially stimulated with >1 μM formoterol, although the levels of cAMP present were significantly lower than in control cells (Fig. 7d). Significant shifts in EC_{50} values ($p < 0.001$) were observed between control and washed concentration-response curves. The control β_1-adrenoceptor responses to formoterol were better maintained than the β_2 effects of salmeterol, and the responses to 100 μM formoterol were 75.6 ± 5.5% (90 min; Fig. 6b), 59.1 ± 7.5% (120 min; Fig. 6c), and 52.4 ± 4.3% (150 min; Fig. 6d) of the control response measured at 60 min (Fig. 6a) (three experiments).

Use of full/partial agonist interactions to determine duration of action of salmeterol at β_1-adrenoceptors. For control purposes, isoprenaline (0.1 nM–10 μM) was incubated in the presence or absence of salmeterol (30 μM) for 10 min. After assay termination, intracellular levels of [^{3}H]cAMP were determined. In the presence of salmeterol, cAMP responses to concentrations of isoprenaline (>1 nM) were attenuated, and the concentration-response curve was

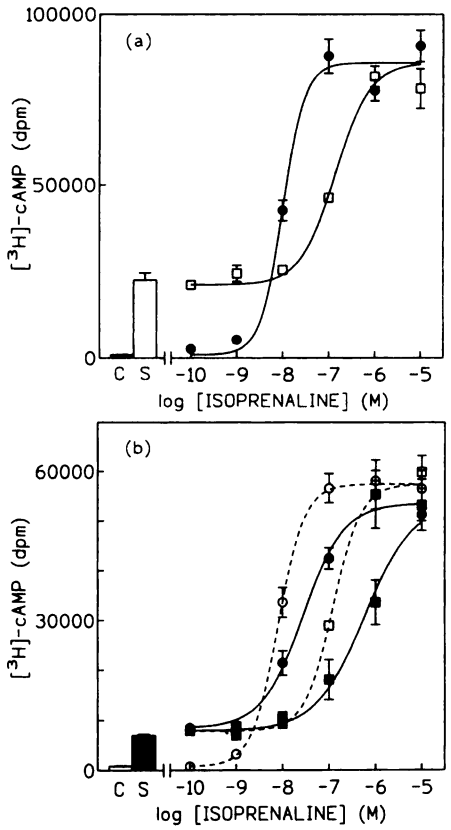

Fig. 4. Attenuation by salmeterol of the total [^{3}H]cAMP response to various concentrations of isoprenaline (a) in the continued presence of salmeterol (30 μM) and (b) after salmeterol washout. a, Agonists were added to cell monolayers simultaneously, and incubations were continued for an additional 10 min. Concentration-response curves are represented in the absence (●) and presence (□) of salmeterol. *C*, Basal accumulation of cAMP; *S*, response to salmeterol. Data represents the mean ± standard error of quadruplicate determinations in a single experiment. Experiments were repeated three times with similar results. b, C6 cell monolayers were incubated with salmeterol (30 μM) for a 10-min period. The incubation medium was then discarded, the cells were washed, and an additional volume (1 ml) of fresh, prewarmed HBH (containing 100 μM rolipram) was applied to each well. After an additional 50-min incubation period, increasing concentrations of isoprenaline (1 nM–10 μM) were applied to cells. After a 10-min incubation period with isoprenaline, assays were terminated, and intracellular levels of [^{3}H]cAMP were measured (●). In a control experiment, salmeterol (30 μM) was incubated with C6 cell monolayers for 60 min before the addition of increasing concentrations of isoprenaline (■) for an additional 10 min. To illustrate the control ability of salmeterol to attenuate responses to isoprenaline, a concentration-response curve to isoprenaline was performed in the presence (□) and absence (○) of salmeterol (30 μM) when both agonists were added to cell simultaneously for 10 min. After assay termination, intracellular levels of [^{3}H]cAMP were measured. The basal accumulation of [^{3}H]cAMP (*open bars*) and the cAMP response mediated by salmeterol alone at t = 10 min (*filled bars*) were measured in each experiment. Data represent mean ± standard error of triplicate determinations in a single experiment. Similar results were obtained in two additional experiments.

shifted to the right (14.1 ± 1.7-fold, three experiments; Fig. 4b). Based on these data, a K_P value of 1.84 ± 0.13 μM (three experiments) was obtained for salmeterol. In test experiments, before the addition of increasing concentrations of isoprenaline for 10 min, cell monolayers were incubated with salmeterol (30 μM) for 10 min, and cells were washed twice with HBH and incubated in an additional volume (1 ml) of prewarmed HBH containing rolipram (100 μM) for a further 50 min to enable new steady state responses to equilibrate. After assay termination, intracellular levels of [^{3}H]cAMP were determined as before. As an additional control, other cell monolayers were exposed to salmeterol (30 μM) continuously for a 60-min period before the addition of isoprenaline to account for ongoing receptor desensitization. In cells that had undergone a washing process after a 10-min incubation period with salmeterol, one of two possible outcomes was anticipated. If salmeterol was long acting at β_1-adrenoceptors in this cell line, the agonist would remain within the vicinity of these receptors after the washout process. Salmeterol would then still be capable of attenuating responses to isoprenaline. The concentration-response curve to isoprenaline would be similar to the control curve obtained when cell monolayers were continually stimulated with salmeterol for a 60-min period. However, if salmeterol was short acting at β_1-adrenoceptors, on washout, salmeterol would no longer be present within the vicinity of the β_1-adrenoceptors and would not be able to attenuate responses to isoprenaline. The concentration-response curve obtained after stimulation of the cells with a range of concentrations of isoprenaline would be similar to that obtained when cells had not been exposed to salmeterol. The results achieved in cells that had undergone this washing process showed that the concentration-response curve to isoprenaline was similar to that observed when the same isoprenaline concentrations were added to cell monolayers for a 10-min period in the absence of salmeterol (Fig. 4b). It did seem, however, that a small degree of desensitization had occurred during the 10-min incubation period with salmeterol (Fig. 4b). Nevertheless, the shift between the concentration-response curves to isoprenaline in washed and time-matched control experiments (19.9 ± 6.7-fold; three experiments) (Fig. 4, *filled symbols*) was not significantly different from the shift observed in the concentration-response curve to isoprenaline in the presence of salmeterol when the agonists were added together for a 10-min period (Fig. 4, *open symbols*). Despite the washout of salmeterol from C6 cells, the direct β_2-adrenoceptor response (observed by the elevated basal cAMP levels) mediated by salmeterol was well maintained throughout the experiment (Fig. 4b).

Use of full/partial agonist interactions to determine the duration of actions of salmeterol at β_2-adrenoceptors in B50 cells. The protocol described above was used to determine the ability of salmeterol to attenuate β_2-adrenoceptor-mediated cAMP responses to isoprenaline after washout of agonist-containing medium in B50 cells, which do not possess β_1-adrenoceptors (21, 22). Salmeterol (1 μM) was capable of shifting the concentration-response curve to isoprenaline (1 nM–100 μM) to the right (123.9 ± 28.4-fold; three experiments; Fig. 7). The pK_P value for salmeterol was calculated to be 17.4 ± 2.6 nM. Salmeterol was incubated with B50 cell monolayers for a 10-min period. After removal of agonist-containing medium, the cells were washed twice with agonist-free buffer for a shorter, 20-min period (due to significant desensitization effects on subsequent isoprenaline responses caused by longer preincubation periods with salmeterol) before the addition of increasing concentrations of isoprenaline. Levels of intracellular [^{3}H]cAMP in washed and control cells were 85.1 ± 8.3% (three experiments) and 83.8 ± 9.3% (three experiments), respectively, at t = 30 min, of those measured at t = 10 min. Under these conditions, in cells that had not undergone a washout process, the concen-

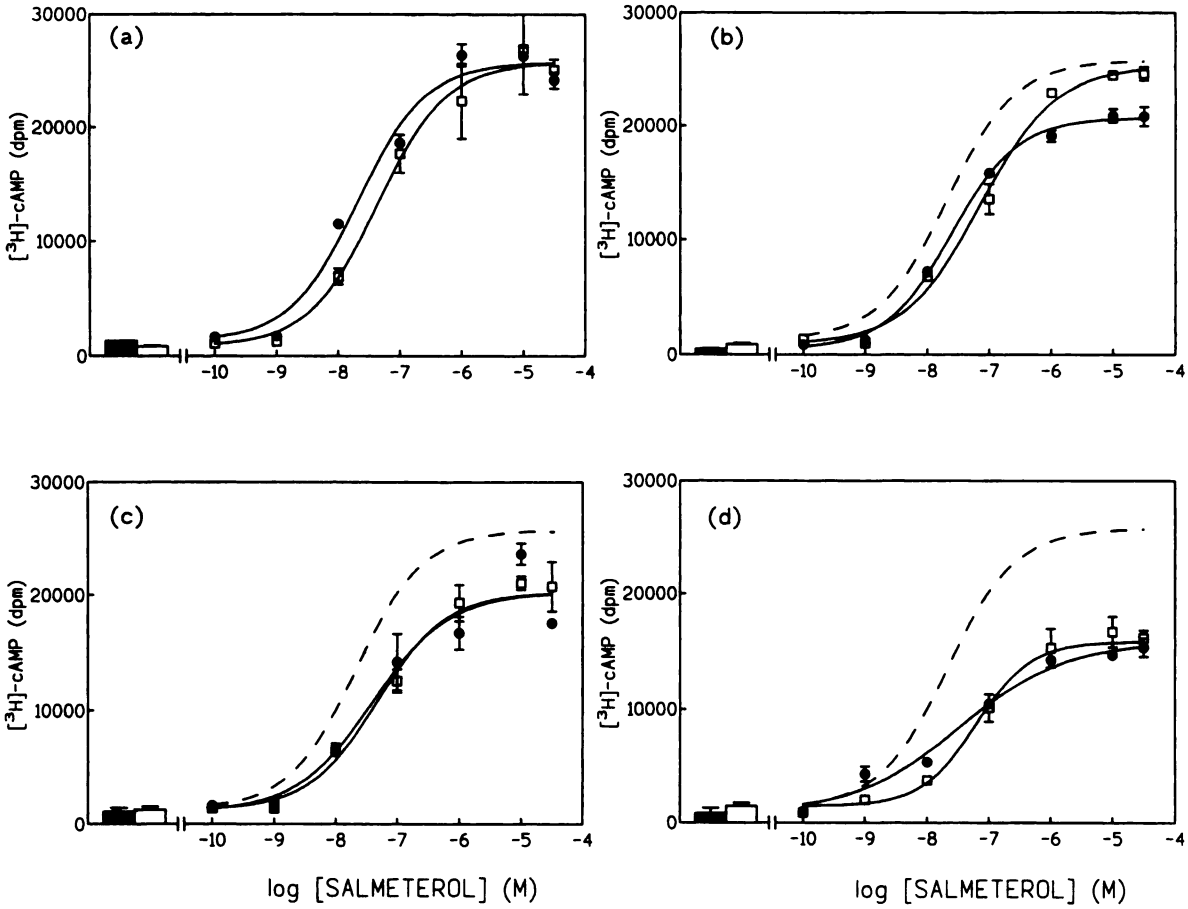

Fig. 5. Effect on the concentration-response curve to salmeterol of washing C6 cell monolayers with agonist-free buffer. Cells were incubated with a range of concentrations of salmeterol in the presence of the β_1-selective antagonist atenolol (10 μM) for 10 min. a, The drug-containing medium was removed from cell monolayers, the cells were washed, and an identical volume (1 ml) of HBH [containing rolipram (100 μM) and atenolol (10 μM)] was added to each well. Intracellular levels of [^{3}H]cAMP were plotted over the initial salmeterol concentration range (□). b–d, Cell monolayers were also washed at t = 10 min. However, these cells were subjected to additional washing processes at (b) t = 20 min, (c) t = 20 and 30 min, and (d) t = 20, 30, and 40 min. Intracellular levels of [^{3}H]cAMP were plotted as in a. Control, time-matched levels of [^{3}H]cAMP in response to continuous stimulation of cells with the same range of concentrations of salmeterol were also determined (●). Incubations were terminated at (a) t = 30 min, (b) t = 40 min, (c) t = 50 min, and (d) t = 60 min. *Dashed curve*, control concentration-response curve from a (30-min incubation period) to allow comparison of the rate of desensitization of cAMP responses to each concentration of salmeterol after each additional increase in incubation period. At the end of each assay period, basal levels of [^{3}H]cAMP in control (*filled bars*) and washed (*open bars*) cell monolayers were measured. Data represent mean ± standard error of triplicate determinations in a single experiment. Data for a–d were obtained in the same experiment. This experiment was repeated on two additional occasions, yielding similar results. Summarized data from all of the repeat experiments are given in Table 2.

tration-response curve to isoprenaline was similar to that observed when salmeterol and isoprenaline had been added simultaneously for a 10-min period (Fig. 7). Based on analysis of the results from three similar experiments, it was clear that the concentration-response curve to isoprenaline in cells that were washed free of salmeterol-containing medium was not significantly different ($p > 0.05$, two-way analysis of variance) than that observed in the control experiment (as illustrated by the results obtained in a single experiment in Fig. 7). In fact, the control concentration-response curve was shifted only 2.9 ± 0.3-fold (three experiments) to the right. The cAMP response to salmeterol was still present after the removal of agonist-containing medium from cell monolayers.

Discussion

The β_1- and β_2-adrenoceptors have been shown to coexist in rat C6 glioma cells (17–20) at a ratio of 80:20 (16, 20). In this study, we determined whether the long-acting β_2-selective adrenoceptor agonist salmeterol was able to produce a sustained cAMP response via both β_1- and β_2-adrenoceptor subtypes in this cell line. Two hypotheses have been put forward to account for the long duration of action of salmeterol in isolated tissues (1–4). The first proposes that salmeterol can bind to both the active site of the β_2-adrenoceptor and, via its phenylalkyloxyalkyl side chain, to an exosite within the β_2-adrenoceptor itself or to a perireceptor site within the local lipid surrounding the β_2-adrenoceptor (2, 5, 6). The alternative hypothesis is that the highly lipophilic nature of salmeterol enables a reservoir of the compound to accumulate within the bulk lipid of the cell membranes and allows salmeterol to access the β_2-adrenoceptor active site from within the membrane environment (7). Clearly, however, if this latter hypothesis is correct, salmeterol should be able to exert a sustained action on any other receptor system with which it can interact. The aim of this study was therefore to determine whether salmeterol is capable of interact-

TABLE 2

Effect of washing cell monolayers with agonist-free medium on the dose ratios calculated from concentration-response curves

Two plates of cell monolayers were incubated with various concentrations of agonist for 30 min (isoprenaline and formoterol) or 10 min (salmeterol) in each of three experiments. One plate was then washed with agonist-free HBH. Assays were terminated after an additional 30- or 20-min period, respectively. In other plates, cells were subjected to one to four washes at 30- or 10-min intervals, respectively. Graphs were plotted representing the cAMP response to each original agonist concentration. Concentration-response curves obtained from washed cell monolayers shifted to the right by variable degrees. The absolute EC_{50} value was determined for each control curve. and the apparent EC_{50} was determined for each washed curve. With these values, a dose ratio was obtained, which was used as a marker with which to compare the rate of offset of agonist action from β_1- (isoprenaline and formoterol) or β_2-adrenoceptors (salmeterol) in C6 cells. Dose ratios (± standard error) were obtained from *n* experiments after each washing process.

Agonist	Dose ratio			
	Wash 1	Wash 2	Wash 3	Wash 4
	log_{10} units (antilog)			
Isoprenaline (3)	2.46 ± 0.27 (288.4)	>3 (>1000)		
Formoterol (3)	1.12 ± 0.24 (13.2)	2.17 ± 0.36 (147.9)	3.29 ± 0.44 (1,949.8)	4.16 ± 0.40 (14,454.4)
Salmeterol (3)	0.29 ± 0.11 (1.9)	0.36 ± 0.14 (2.3)	0.21 ± 0.09 (1.6)	0.52 ± 0.24 (3.3)

ing with the β_1-adrenoceptor present in C6 cells and to compare its duration of action relative to that determined at the β_2-adrenoceptors present in the same cell.

In the presence of the phosphodiesterase type IV inhibitor rolipram (100 μM; Ref. 23), isoprenaline, formoterol, and salmeterol stimulated a large increase in cAMP accumulation in C6 cells over basal levels in a concentration-dependent manner. Isoprenaline was the most potent (EC_{50} = 14.6 nM) of the agonists that we used. From comparisons with other studies that also used C6 cell monolayers, it seemed that the cAMP responses mediated by isoprenaline occurred predominantly via β_1-adrenoceptors (20, 21). Salmeterol and formoterol were also capable of generating [^{3}H]cAMP accumulation in C6 cells. The order of agonist potency was isoprenaline > formoterol > salmeterol. The use of selective β-antagonists demonstrated the extent to which β_1- and β_2-adrenoceptor subtypes contributed to the overall cAMP response mediated by each agonist in this study. Investigations in which the β_1- and β_2-selective antagonists atenolol (24) and ICI 118551 (25), respectively, were used to attenuate response to isoprenaline (1 μM) indicated that the functional response mediated by this agonist occurred via β_1-adrenoceptors. Apparent K_B values of 83 nM (ICI 118551) and 79 nM (atenolol) were within the range expected for interaction of this agonist with β_1-adrenoceptors (16, 21, 24, 25). Formoterol exhibited a similar efficacy to isoprenaline in this cell line, and in inhibition studies with formoterol (1 μM) as agonist, the apparent K_B values for ICI 118551 (50.5 nM) and atenolol (91.6 nM) reflected the fact that formoterol was acting predominantly via β_1-adrenoceptors in this cell line. As in B50 cells (22), salmeterol produced a much smaller maximal response than that elicited by isoprenaline. This small maximal response indicated that most of the cAMP response elicited by salmeterol occurred via the activation of the subpopulation of β_2-adrenoceptors in C6 cells. In antagonist studies, the cAMP responses to a fixed concentration of salmeterol were complicated by the activation of both β_2- and β_1-adrenoceptors. The [^{3}H]cAMP responses to salmeterol (1 μM) were potently antagonized by the β_2-selective antagonist ICI 118551. However, in the presence of atenolol (10 μM), the cAMP response mediated by salmeterol (1 μM) was attenuated by 31%.

To examine more closely the effects of ICI 118551 and atenolol on isoprenaline- and salmeterol-stimulated cAMP accumulation, we constructed concentration-response curves in the presence and absence of a single concentration of antagonist. The concentration of antagonist used was chosen to be capable of inhibiting β_2-adrenoceptor-mediated effects (0.3 μM ICI 118551; Ref. 25) or β_1-adrenoceptor-mediated responses (10 μM atenolol; Ref. 24). As expected, the concentration-response curve to salmeterol was shifted farther to the right in the presence of ICI 118551 (0.3 μM) than when isoprenaline was used as agonist. The apparent pK_B values calculated for ICI 118551 with use of the the Gaddum equation (27) under these conditions were 8.01 (for salmeterol, indicative of interaction with β_2-adrenoceptors) and 6.97 (for isoprenaline, indicative of interaction with β_1-adrenoceptors; 21, 25). These values agreed closely with the K_B values estimated according to the method of Lazereno and Roberts (26). In contrast, in the presence of atenolol (10 μM), the concentration-response curve to isoprenaline was greatly attenuated, yielding a pK_B value of 6.92 for the antagonist, which again was within the range predicted for interaction with β_1-adrenoceptors (24, 25). Atenolol was ineffective in inhibiting cAMP responses to low (nanomolar) concentrations of salmeterol but did inhibit the response to this agonist at high (>0.1 μM) concentrations. Therefore, it seemed that at low concentrations of salmeterol in the C6 cell line, the cAMP responses mediated by this agonist occurred purely through activation of β_2-adrenoceptors. However, at high (micromolar) concentrations, the pharmacology of salmeterol was complex and involved an interaction with both β_1- and β_2-adrenoceptor subtypes.

It is clear, based on a comparison of the size of the cAMP response to salmeterol on β_1-adrenoceptors in C6 cells (30–35% of the maximal response to salmeterol; Fig. 1d and Fig 3b) with the relatively much larger maximal cAMP response to isoprenaline (the maximal response to salmeterol accounting for only 14% of that produced by isoprenaline in the same cell preparation), that salmeterol exhibits low efficacy on β_1-adrenoceptors in C6 cells. Consequently, an attempt was made to determine its affinity for β_1-adrenoceptors in this system with the use of full/partial agonist interactions. In the presence of a supramaximally effective concentration of salmeterol (30 μM), which produced almost no detectable increase in cAMP levels above that elicited by salmeterol (1 μM), the concentration-response curve to isoprenaline was shifted to the right. This provided further evidence that salmeterol occupied β_1-adrenoceptors at high (micromolar) concentrations and that the agonist displayed a low efficacy at this receptor subtype. This is consistent with findings in studies of rat left atria (1). The apparent K_P value obtained for salmeterol (1.6 μM) with these experimental conditions indicated that salmeterol exhibits a much lower affinity for β_1-adrenoceptors than for β_2-adrenoceptors (K_P = 17 nM) (22).

The contributions of β_1- and β_2-adrenoceptors to the sustained duration of action of salmeterol were investigated independently through the use of two different experimental protocols. To determine the duration of action of salmeterol at β_2-adrenoceptors in C6 cells, multiple washout experi-

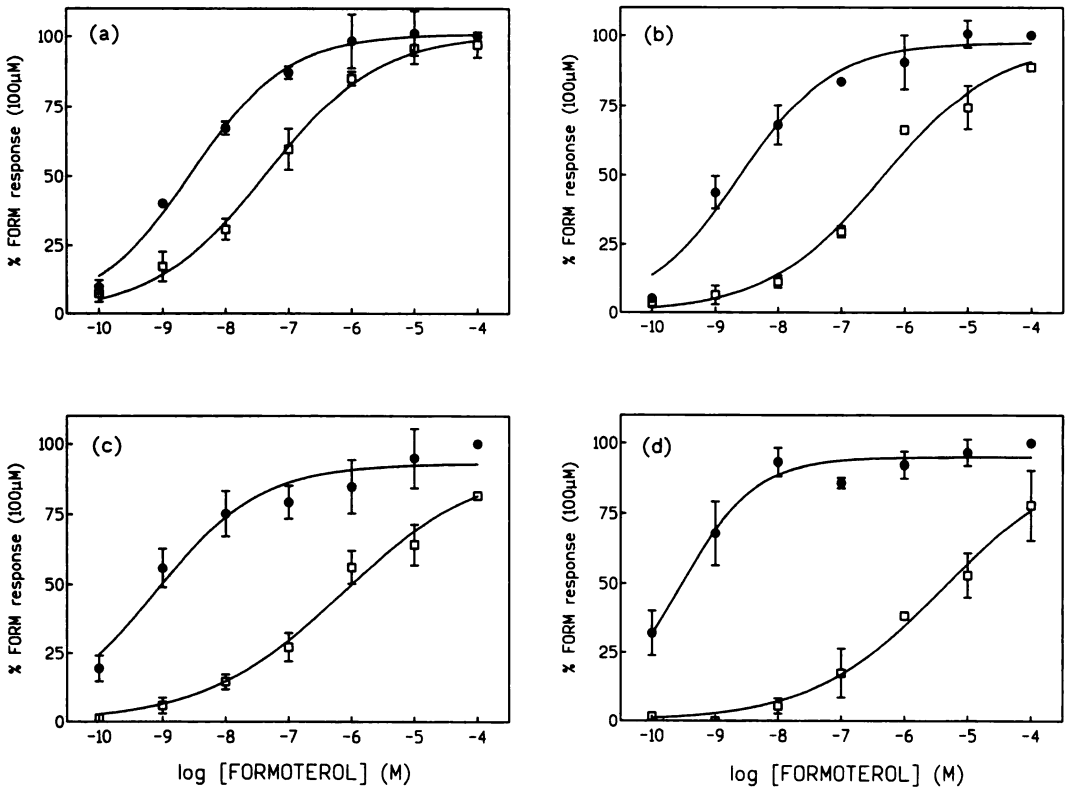

Fig. 6. Effect of washing cell monolayers with agonist-free medium on the concentration-response curve to formoterol in C6 cell monolayers. Cells were incubated with a range of concentrations of formoterol for 30 min. At (a) t = 30 min, (b) t = 30 and 60 min, (c) t = 30, 60, and 90 min, and (d) t = 30, 60, 90, and 120 min, the incubation medium was removed, the cells were washed, and monolayers were incubated in fresh, prewarmed HBH (1 ml) that contained rolipram (100 μM). Time-matched control experiments were also performed, in which the same range of agonist concentrations was used. However, cell monolayers were not washed during each appropriate assay period. Incubations were terminated at (a) t = 60 min, (b) t = 90 min, (c) t = 120 min, and (d) t = 150 min. Intracellular levels of [^{3}H]cAMP were plotted over the original concentrations of formoterol in control (●) and washed (□) cells. Responses are expressed as a percentage (after subtraction of basal values) of the maximum time-matched control response to formoterol (100 μM) in each experiment. Data represent the combined mean ± standard error of triplicate determinations obtained in each of three experiments. In a–d, the concentration-response curves in washed cell monolayers were significantly different ($p < 0.001$, two-way analysis of variance) from control curves. With the use of analysis of residual variances, the difference between curves was found to be due to significant differences between EC_{50} values ($p < 0.001$, a and b). In c and d, the maximum response of the washed curve was insufficiently defined for analysis of residual variances. However, the difference in EC_{50} values between washed and control curves was highly significant ($p < 0.001$) if 100% maximal response was assumed in washed curves.

ments were performed. A range of concentrations of salmeterol were incubated with C6 cell monolayers in the presence of the β_1-selective antagonist atenolol (10 μM) to remove any β_1 component from the cAMP response elicited by high concentrations of salmeterol. As in B50 cells (22), salmeterol exhibited a long duration of action at β_2-adrenoceptors in C6 cells over the entire concentration range used. Unfortunately, inclusion of the β_2-selective antagonist ICI 118115 in the incubation medium could not be used to investigate the duration of action of salmeterol at β_1-receptors with a similar approach because of the β_1-antagonist properties of this compound at the concentrations (0.3 μM) required to completely inhibit the β_2-adrenoceptor component. For example, a pK_B of 7.15 was obtained for ICI 118551 (Table 1) for antagonism of the β_1-adrenoceptor response to isoprenaline in these cells, and it was clear (Fig. 2a) that 0.3 μM ICI 118551 significantly antagonized the cAMP responses to isoprenaline.

Consequently, the examination of the duration of action of salmeterol at β_1-adrenoceptors in C6 cells required the use of a more complex experimental protocol involving the antagonism of the β_1-adrenoceptor cAMP response to isoprenaline by the partial β_1-agonist properties of salmeterol. From the results obtained, it was clear that salmeterol exhibits a short duration of action at β_1-adrenoceptors in this cell line. After washout of agonist-containing medium from salmeterol-stimulated cells, the concentration-response curve to isoprenaline was similar to that obtained in the absence of salmeterol. There was a 20-fold difference in the EC_{50} values for the concentration-response curve to isoprenaline between cells in which the salmeterol-containing incubation medium had been replaced with agonist-free buffer and cells in which salmeterol was continually present. This dose ratio was similar to that calculated from concentration-response curves to isoprenaline in the presence and absence of salmeterol (30 μM).

To check whether the apparently short duration of action of salmeterol on β_1-adrenoceptors was not an artifact of the partial agonist/full agonist experimental paradigm used to evaluate the duration of action of the partial agonist salmeterol, similar experiments were conducted with B50 cells, in which salmeterol is also a partial agonist at the β_2-adrenoceptor (22). In contrast to the actions of salmeterol at β_1-adrenoceptors in C6 cells, this partial agonist continued to attenuate responses to isoprenaline mediated via activation of β_2-adrenoceptors in B50 cells despite washout of agonist-

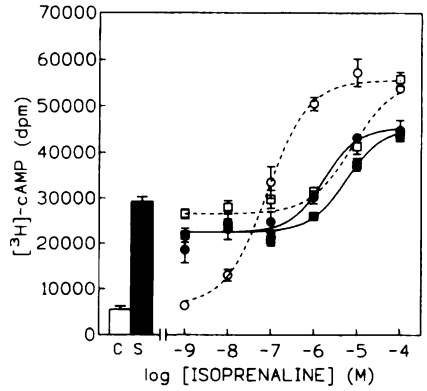

Fig. 7. Effect of washout of agonist-containing medium on the ability of salmeterol to attenuate β_2-adrenoceptor-mediated responses to isoprenaline in B50 cells. B50 cell monolayers were incubated with salmeterol (1 μM) for 10 min. The incubation medium was then discarded, cells were washed, and an additional volume (1 ml) of fresh, prewarmed HBH (containing 100 μM rolipram) was applied to each well. After an additional 20-min incubation period, increasing concentrations of isoprenaline (1 nM–100 μM) were applied to cells for an additional 10 min (●). In a control experiment, salmeterol (1 μM) was incubated with B50 cell monolayers for a 30-min period before the addition of increasing concentrations of isoprenaline (■) for an additional 10 min. To illustrate the ability of salmeterol to attenuate responses to isoprenaline, a concentration-response curve to isoprenaline was performed in the presence (□) and absence (○) of salmeterol (1 μM) when both agonists were added to cell simultaneously for a 10-min period. After assay termination, the existing incubation medium was discarded, cells were lysed in an identical volume (1 ml) of HBH containing 50 mM concentrated HCl, and intracellular levels of [^{3}H]cAMP were measured. The basal accumulation of [^{3}H]cAMP (open bar) and the cAMP response mediated by salmeterol alone at t = 10 min (filled bar) were measured in each experiment. Data represent mean ± standard error of triplicate determinations in a single experiment. Similar results were obtained in two additional experiments.

containing medium, which is indicative of its sustained activity at this receptor subtype.

From these results, it seems that the long duration of action of salmeterol at β_2-adrenoceptors *in vitro*, unlike that for formoterol (7, 30), cannot result solely from its partitioning into, and slow release from, the bulk lipid of the plasma membrane. If this mechanism had been correct, salmeterol, due to its highly lipophilic nature, would also have persisted at β_1-adrenoceptors despite washout of agonist-containing medium from C6 cell monolayers. Therefore, high lipophilicity does not exclusively lead to persistent agonist activity *in vitro*. It is notable from the estimated dissociation constants for salmeterol obtained at the β_2-adrenoceptors (17.4 nM) and β_1-adrenoceptors (1.8 μM) in the C6 cells that salmeterol has a much higher affinity (100-fold) for the active site of the β_2-adrenoceptor than that of the β_1-adrenoceptor. If the exosite binding is in some way linked to active site binding, then it is possible that a similar exosite might exist for the β_1-adrenoceptor. However, it is clear from these studies that at the highest concentrations of salmeterol used at the β_1-adrenoceptor (30 μM), which are 15-fold above its dissociation constant for the β_1-adrenoceptor, there is no sustained action. This is in contrast to the long duration of action observed with 0.1 and 1 μM salmeterol at the β_2-adrenoceptor (Fig. 5), which cover a similar 15-fold increase over the appropriate β_2-binding constant. Consequently, the results obtained in this study favor the existence of an exosite or a perireceptor environment exclusive to β_2-adrenoceptors (5).

Acknowledgments

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