Salmeterol, a long-acting β_2 -adrenoceptor agonist mediating cyclic AMP accumulation in a neuronal cell line

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1 The accumulation of cyclic AMP stimulated by salmeterol, a long-acting β_2 -adrenoceptor agonist and by isoprenaline, a non-selective β -adrenoceptor agonist have been compared in the B50 neuroblastoma cell line.

2 Salmeterol produced a concentration-dependent increase in the accumulation of total [³H]-cyclic AMP in B50 cells yielding an EC₅₀ value of 37 nM which was lower than that obtained with isoprenaline (294 nM). The maximum response to salmeterol was only 46% of that obtained with isoprenaline.

3 The β_2 -adrenoceptor antagonist, ICI 118551, inhibited the responses to both salmeterol (apparent K_D 2.2 nM) and isoprenaline (apparent K_D 1.6 nM). However, the β_1 -adrenoceptor antagonist, atenolol, produced no significant effect at concentrations up to 100 μ M.

4 Salmeterol (1 μ M) changed the concentration-response curve of isoprenaline in the manner of a partial agonist interacting with a full agonist. The K_D of salmeterol obtained from the interaction was 55.6 nM.

5 Whereas salmeterol has a slow onset of action in airway smooth muscle compared to other β_2 -adrenoceptor agonists, in B50 monolayers both salmeterol and isoprenaline produced a rapid increase in cyclic AMP accumulation (*t*_{1/2} 1.1 min and 0.4 min respectively).

6 Despite the existence of cyclic AMP efflux mechanisms that exist in this cell line it was possible to investigate the duration of agonist action by measuring intracellular levels of the second messenger. Replacement of drug-containing medium with fresh buffer led to a rapid reduction in intracellular levels of cyclic AMP in isoprenaline-stimulated cells whereas cyclic AMP accumulation was sustained for much longer periods in salmeterol-stimulated cells. However, the persistent action of salmeterol could be reversed by the addition of a β_2 -selective antagonist.

7 These results confirm that salmeterol has a high affinity, but low efficacy (relative to isoprenaline) for β_2 -adrenoceptors coupled to cyclic AMP accumulation and that the drug persists at its site of action for long periods in the B50 neuronal cell line.

Keywords: Salmeterol; isoprenaline; β_2 -adrenoceptor; cyclic AMP accumulation; B50 neuroblastoma cell line

Introduction

β_2 -Adrenoceptor agonists are currently the most widely used group of bronchodilator drugs employed to relieve airway obstruction in asthma (Barnes *et al.*, 1984). These β_2 -agonists induce the elevation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) content in tissues in response to adenylate cyclase activation via a stimulatory G protein (G_s) (Gilman, 1987; Birnbaumer, 1990). In airway smooth muscle, increased cellular cyclic AMP accumulation leads to the stimulation of cyclic AMP-dependent protein kinase A (PKA) which in turn phosphorylates a range of other proteins, including myosin light chain kinase, associated with the contractile apparatus of the cell (Barnes, 1986) and ion channels (Kume *et al.*, 1989), leading ultimately to smooth muscle relaxation.

Recently, a new bronchodilator drug, salmeterol, has been developed (Bradshaw *et al.*, 1987) which has been shown to be a potent and highly selective β_2 -adrenoceptor agonist and which displays a long duration of action in airway smooth muscle (Brittain, 1990; Ball *et al.*, 1991). The mechanism underlying the long duration of action of salmeterol at β_2 -adrenoceptors remains to be established but it has been postulated that it involves two processes: (a) an interaction between the saligenin head of the molecule and the active site and (b) the binding of the long non-polar N-substituent sidechain within a distinct hydrophobic core region of the receptor protein, which has been termed the 'exo-site' (Jack, 1991). Such an arrangement enables the head group to associate freely with and dissociate from the active site of the

β_2 -adrenoceptor whilst the sidechain remains firmly attached to the receptor protein (Ball *et al.*, 1991). A notable feature of studies performed in strips of airway smooth muscle, however, is the slow onset of action of salmeterol (Ball *et al.*, 1991). This suggests that the duration of action of salmeterol might be partly determined by slow diffusion of this lipophilic molecule to its active site within the smooth muscle segment.

The B50 neuroblastoma cell line (Schubert *et al.*, 1974), derived from the rat central nervous system, has recently been shown to express β_2 -, but not β_1 -adrenoceptors, coupled to cyclic AMP accumulation (Ruck *et al.*, 1990). The aim of this study was to evaluate the pharmacological characteristics of the cyclic AMP response elicited by salmeterol in monolayer cultures of a neuronal cell line, where the onset and offset of agonist action should be less sensitive to diffusional influences than in smooth muscle segments. A preliminary account of some of this work has been communicated to the British Pharmacological Society (McCrea *et al.*, 1992).

Methods

Cell culture

B50 cells (European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wilts.), passages 3–17, were cultured at 37°C under an atmosphere of 10% CO₂ in humidified air in 75 cm³ flasks (Costar). The growth medium was Dulbecco's modified Eagles medium (DMEM) supplemented

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with 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS). Cells were passaged every 4 days using a split ratio of 1:6. Experiments were performed in 24 well cluster dishes, normally 3 days after seeding, on confluent monolayers.

Measurement of [³H]-cyclic AMP accumulation

Cyclic AMP production was assayed by a modification of the method described previously for brain slices (Donaldson *et al.*, 1988). Monolayer cultures were incubated with [³H]-adenine (2 µCi/well) for 2 h at 37°C in 1 ml/well Hanks/HEPES (20 mM) buffer, pH 7.4. Prelabelled cell monolayers were then washed 3 times with Hanks/HEPES buffer (1 ml/well) containing the type IV phosphodiesterase (PDE) inhibitor, rolipram (0.1 mM) (Ruck *et al.*, 1990). Where appropriate, antagonist drugs were equilibrated with the cells for 30 min prior to the addition of agonist. Agonists, or the appropriate vehicle controls were finally added in 10 µl of buffer and the incubation terminated after the appropriate length of time by the addition of 50 µl of 10 M HCl to each well which led to cell lysis.

In some experiments it was necessary to analyse intra- and extracellular cyclic AMP content independently. These assays were performed as above. However, before the termination of each agonist-stimulated incubation, supernatant buffer was carefully removed from each well for the analysis of extracellular [³H]-cyclic AMP content (as described below). An identical volume of fresh Hanks/HEPES buffer (1 ml) was then rapidly applied to each well followed immediately by cell lysis in the normal way, thereby enabling intracellular [³H]-cyclic AMP content to be determined.

[³H]-cyclic AMP was isolated by sequential Dowex-alumina chromatography (Donaldson *et al.*, 1988). [³H]-cyclic AMP, in 0.95 ml aliquots of the supernatant layers, and [¹⁴C]-cyclic AMP (100 µl), which was used as a tracer, were applied to Dowex 50 ion exchange resin (0.6 ml) in plastic Econo columns (Bio-Rad), previously treated with 5 ml 1 M HCl and 20 ml distilled water. Columns were washed with 3 ml distilled water and were then placed above similar plastic columns containing 0.6 g neutral alumina (previously washed with 20 ml of 0.1 M imidazole before use). [³H]-cyclic AMP was eluted from Dowex onto the alumina columns with distilled water (4–5 ml). The alumina columns were then placed directly above scintillation vials and [³H]-cyclic AMP was eluted in 5 ml of 0.1 M imidazole. Radioactivity was determined by liquid scintillation counting. Recovery of cyclic AMP from the columns was routinely 70–80%. Furthermore, additional 50 µl aliquots were removed from the supernatant and used to determine the total radioactivity present in each sample; these 'totals' were then used to correct for variations in the number of cells present in each well.

In order to confirm the separation of cyclic AMP from other adenine nucleotides by this procedure, 1 ml aliquots of 150 µM solutions of ATP, ADP, cyclic AMP, AMP, adenosine and adenine were subjected to Dowex 50 chromatography. Sequential 1 ml aliquots of water were applied to each column and the eluate optical density was measured at 260 nm. Triplicate determinations were made for each compound. ATP and ADP were completely eluted within the first three 1 ml fractions. Cyclic AMP was eluted during fractions 4 to 6 and AMP (17%) began to elute, after the normal cyclic AMP collection period, during fractions 8 to 10. Adenine and adenosine were completely retained on the columns for the full 10 fraction collection period.

Data analysis

Accumulation of [³H]-cyclic AMP was expressed as either radioactivity in d.p.m. (individual experiments) or as a percentage of the maximal stimulation by an agonist (maximal response minus basal). Agonist concentration-response curves were fitted to a logistic equation using the non-linear

regression programme GraphPAD. The equation fitted was:

$$\% \text{ of maximal response} = \frac{E_{\max} \times X^n}{(EC_{50})^n + X^n}$$

where $E_{\max}$ is the maximal response (100%), X is the agonist concentration, EC_{50} is the concentration of agonist producing half maximal response and n is the slope parameter.

Antagonist inhibition curves were analysed using the same programme according to the expression:

$$\% \text{ of maximal response} = 100 - \frac{100 \times A^n}{(IC_{50})^n + A^n}$$

where IC_{50} is the concentration producing half maximal inhibition of the response to 1 µM agonist and A is the antagonist concentration.

Apparent antagonist dissociation constants (K_D) were calculated assuming competitive antagonism using a modification of the null method first described by Lazareno & Roberts (1987). Briefly, a concentration-response curve to agonist was generated and a concentration (X^1 ; normally 1 µM) of agonist was chosen which gave a response greater than 50% of the maximum agonist response. The concentration of antagonist (IC_{50}) required to reduce the response to this concentration of agonist by 50% was then determined. From the agonist concentration-response curve obtained in the absence of antagonist, the concentration of agonist (X^0) that yielded a response equivalent to 50% of that produced by X^1 (in the absence of antagonist) was also identified. The apparent K_D was then calculated from the expression:

$$\frac{X^1}{X^0} = \frac{IC_{50}}{K_D} + 1$$

where X^1/X^0 is equivalent to the agonist dose-ratio that would be used in the analysis of parallel dose-response curves obtained in the presence and absence of an IC_{50} concentration of antagonist.

The dissociation constant for the partial agonist (K_p) salmeterol was determined by a method described by Stephenson (1956). The concentration of isoprenaline (D_1) that produced the same cyclic AMP response as that to 1 µM salmeterol was first determined. Concentration-response curves for isoprenaline were then constructed in the presence and absence of a fixed concentration of salmeterol (1 µM) termed P . The concentrations of isoprenaline that produced equivalent cyclic AMP responses in the absence (D_2 ; $D_2 > D_1$) and presence (D_3) of salmeterol were calculated. The dissociation constant of the partial agonist was determined from the expression:

$$\frac{D_3}{(D_2 - D_1)} = 1 + \frac{P}{K_p}$$

Values were expressed as means $\pm$ s.e.mean and n in the text refers to the number of separate experiments. In individual experiments, data points were calculated from the mean ($\pm$ s.e.mean) of 4 determinations. Statistical analysis was performed by use of unpaired Student's t tests.

Chemicals

2,8-[³H]-adenine (specific activity 31 Ci mmol⁻¹) and 8-[¹⁴C]-cyclic AMP (specific activity 306 mCi mmol⁻¹) were purchased from New England Nuclear (Herts.) and Amersham International (Bucks.), respectively. Dowex 50W, H⁺-form (200–400 mesh), neutral alumina (type WN-3), imadazole, ($\pm$)-isoprenaline hydrochloride and atenolol were obtained from Sigma (Dorset). DMEM, FCS and horse serum were purchased from Northumbria Biologicals (Northumberland) and glutamine from Flow Laboratories (Herts.). The gifts of salmeterol free base (Glaxo, Herts.), ICI 118551 hydrochloride (erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropyl-aminobutane-2-ol; ICI Pharmaceuticals, Cheshire) and rolipram (Schering, Berlin, Germany) are gratefully acknowledged.

Stock solutions of drugs were prepared in Hanks/HEPES buffer except for ICI 118551 (Bilski *et al.*, 1983) which was dissolved in ethanol and salmeterol which was prepared as stock solution of 10 mM in Hanks/HEPES buffer with the addition of one drop of glacial acetic acid per ml.

Results

Comparison of salmeterol- and isoprenaline-stimulated cyclic AMP accumulation

Previous investigations have shown that the onset of the action of salmeterol is delayed compared with other β -adrenoceptor agonists in isolated airway smooth muscle (Ball *et al.*, 1991; Dougall *et al.*, 1991). This is probably due to the lipophilic nature of this molecule. However, 1 μ M salmeterol stimulated a rapid increase in [3 H]-cyclic AMP accumulation in B50 cell monolayers reaching a steady-state level within 5 min; this response appeared to be maintained for at least 20 min (Figure 1). The mean $t_{1/2}$ obtained in three separate experiments was 1.13 ± 0.09 min. A rapid onset of cyclic AMP production was also produced by 1 μ M isoprenaline ($t_{1/2}$ 0.44 ± 0.03 min, $n = 4$).

B50 cells treated with 10 μ M salmeterol only produced a 1.7 ± 0.1 fold increase in [3 H]-cyclic AMP over basal levels ($n = 4$). However, in the presence of rolipram (0.1 mM) the response to salmeterol was enhanced (2.9 ± 0.2 fold over basal levels, $n = 4$). Rolipram was therefore included in all subsequent experiments. Salmeterol stimulated the formation of [3 H]-cyclic AMP in B50 cells in a concentration-dependent manner (Figure 2a). Concentration-response analysis yielded an EC_{50} value of 36.7 ± 18.4 nM ($n = 4$) and a slope parameter of 0.64 ± 0.07 ($n = 4$). Accumulation of cyclic AMP induced by isoprenaline (10 min incubation period) was much greater than that stimulated by salmeterol (Figure 3). Treatment with 10 μ M isoprenaline led to a 7.9 ± 0.9 fold rise in second messenger formation ($n = 22$) compared to basal levels whereas the same concentration of salmeterol gave only $46.0 \pm 4.2\%$ ($n = 6$) of this response. The EC_{50} value and slope parameter for isoprenaline were 294 ± 84 nM and 0.89 ± 0.04 respectively ($n = 22$).

Effect of β -adrenoceptor antagonists on salmeterol-stimulated cyclic AMP accumulation

Preincubation of B50 cell monolayers with various concentrations of the β_2 -selective antagonist, ICI 118551, for 30 min attenuated cyclic AMP accumulation when cells were stimulated by 1 μ M salmeterol for a further 10 min period

Figure 1 Time course showing the accumulation of total [3 H]-cyclic AMP in response to 1 μ M salmeterol in B50 cell monolayers. Data were obtained in the presence (●) or absence (□) of agonist. Each data point represents the mean $\pm$ s.e.mean of quadruplicate determinations in a single experiment. This experiment was repeated twice yielding similar results.

Figure 2 (a) Salmeterol-stimulated accumulation of [3 H]-cyclic AMP in the presence of 0.1 mM rolipram. The basal accumulation of total cyclic AMP is represented by the column labelled C. (b) Concentration-response curve for the inhibition of salmeterol-induced [3 H]-cyclic AMP accumulation by the β_2 -selective antagonist, ICI 118551. The response to 1 μ M salmeterol is shown by the cross-hatched column marked S. Cell monolayers were preincubated with ICI 118551 for 30 min before stimulation with 1 μ M salmeterol for a further 10 min. The results of (a) and (b) were obtained in the same experiment. Data represent the means $\pm$ s.e.mean of results from 4 incubations in a single experiment. Where not shown, s.e.means were within the size of the symbol. Similar results were obtained in 2 other experiments.

(Figure 2b). Increasing doses of ICI 118551 also inhibited cyclic AMP formation in B50 cells treated with isoprenaline (1 μ M). The mean apparent dissociation constants (K_D) obtained for ICI 118551 were 2.2 ± 0.7 nM ($n = 3$) and 1.6 ± 0.5 nM ($n = 3$) in salmeterol- and isoprenaline-stimulated cells respectively.

Inclusion of the β_1 -selective antagonist, atenolol, in the assay system did not alter salmeterol- and isoprenaline-stimulated (1 μ M) [3 H]-cyclic AMP production at concentrations up to 100 μ M atenolol (results not shown; $n = 3$).

Investigation of full/partial agonist interactions

In this series of experiments the ability of salmeterol to antagonize the effects of a more efficacious agonist (isoprenaline) was investigated. B50 cell monolayers were

Figure 3 Comparison of concentration-response curves for [3 H]-cyclic AMP accumulation stimulated by isoprenaline (●) and salmeterol (□) for a 10 min period. Responses are expressed as a percentage (after subtraction of basal values) of the maximum response to isoprenaline ($10 \mu\text{M}$) which was measured in each experiment. Data represent the combined mean $\pm$ s.e. mean of quadruplicate determinations obtained in each of 6 experiments. Where not shown, s.e. means were within the size of the symbol.

Figure 4 The effect of salmeterol on the [3 H]-cyclic AMP response to various concentrations of isoprenaline. Both drugs were added simultaneously to the assay system and the incubation continued for 10 min. The concentration-response curve for isoprenaline is shown in the absence (●) and presence (□) of $1 \mu\text{M}$ salmeterol. The basal accumulation of cyclic AMP and the response to $1 \mu\text{M}$ salmeterol are shown by the columns labelled C and S respectively. Each data point represents the mean $\pm$ s.e. mean of quadruplicate determinations. Where not shown, s.e. means were within the size of the symbol. This experiment was repeated 3 times with similar results.

simultaneously stimulated with various concentrations of isoprenaline in the presence or absence of $1 \mu\text{M}$ salmeterol (Figure 4). In the presence of salmeterol ($1 \mu\text{M}$), the response to high concentrations of isoprenaline ($>0.1 \mu\text{M}$) was attenuated by salmeterol. Analysis of these data yielded a value of $55.6 \pm 28.2 \text{ nM}$ ($n=4$) for the dissociation constant (K_D) of the partial agonist, salmeterol.

Efflux of cyclic AMP from B50 cells

Whilst investigating the long term effects of salmeterol on cyclic AMP accumulation it became apparent that the second messenger accumulated in the extracellular medium. To investigate this phenomenon more closely, time courses for isoprenaline- and salmeterol-stimulated cyclic AMP accumulation were re-examined by analysing both extra- and intracellular [3 H]-cyclic AMP content obtained from supernatant layers and lysed cells respectively.

Figure 5 shows the time courses for the intracellular accumulation, excretion and total levels (intra- and extracellular) of cyclic AMP from B50 cells in the presence of isoprenaline ($1 \mu\text{M}$) or salmeterol ($1 \mu\text{M}$). In each case cyclic AMP accumulated rapidly within the cells, but after a variable lag period [3 H]-cyclic AMP levels in the extracellular fluid began to increase significantly. The basal accumulation of [3 H]-cyclic AMP was also monitored in all experiments at $t=0$ and $t=120 \text{ min}$. During each 2 h assay period, the basal accumulation of both intra- and extracellular cyclic AMP was found to increase (1.3 ± 0.1 fold ($n=3$) and 1.6 ± 0.2 fold ($n=3$) respectively) due to the presence of rolipram in the incubation medium.

In B50 cells, after 5 min incubation with $1 \mu\text{M}$ isoprenaline, the total accumulation of cyclic AMP appeared to rise to a high steady-state level (Figure 5a). However, intracellular levels of cyclic AMP rose initially (7.0 ± 1.0 fold over basal levels, $n=3$) but thereafter declined. After a 60 min incubation period for example, intracellular levels of the second messenger were only 14.7% of maximal levels (Figure 5a) and within 2 h levels of the second messenger had decreased to 6.2% of those measured at the peak of the response (Figure 5a). Profiles of time courses from several other experiments also showed this decrease in intracellular levels of cyclic AMP after the initial peak response. After 60 min and 120 min, intracellular levels of the second messenger were only $37.4 \pm 11.4\%$ and $17.4 \pm 6.0\%$ ($n=3$) of those

Figure 5 Time courses for the appearance of intracellular (●), extracellular (□) and total (intra- and extracellular) (○) [3 H]-cyclic AMP accumulation after stimulation of B50 cells with $1 \mu\text{M}$ isoprenaline (a) or $1 \mu\text{M}$ salmeterol (b) (drugs were added at $t=0$). Extracellular cyclic AMP represents the [3 H]-cyclic AMP present in 1 ml of the incubation medium. The intracellular level of [3 H]-cyclic AMP was monitored by lysing the cells in an equal volume of freshly applied Hanks/HEPES medium (after removal of the incubation medium). The basal accumulation of intra- and extracellular [3 H]-cyclic AMP at $t=0$ and $t=120 \text{ min}$ are represented by the cross-hatched and open columns respectively. Data in each graph represent means $\pm$ s.e. mean of quadruplicate determinations in a single experiment. Where not shown, s.e. means were within the size of the symbol. All assays were repeated on at least 2 further occasions and similar results were obtained within each experiment.

measured at the peak of the response. Furthermore, the accumulation of intracellular cyclic AMP had fallen to 1.7 ± 0.4 fold over time-matched basal levels ($n = 3$) by the end of this incubation period. In contrast, after this 2 h incubation period extracellular levels of cyclic AMP were 5.0 ± 0.3 fold higher than time-matched basal values ($n = 3$) (Figure 5a).

A simple assay was then performed in order to determine whether the decrease in intracellular levels of [3 H]-cyclic AMP during these time course experiments was a consequence of uptake of isoprenaline into cells, metabolism of the drug or receptor desensitization. Cells were preincubated with $1 \mu\text{M}$ isoprenaline for 30 min. Cell monolayers were then washed twice with fresh prewarmed buffer before the cells were rechallenged with isoprenaline ($1 \mu\text{M}$) at $t = 40$ min. The accumulation of intra- and extracellular [3 H]-cyclic AMP was then assessed as before. Figure 6 shows that the removal of isoprenaline from the incubation medium led to a rapid decrease in both intra- and extracellular levels of cyclic AMP. After isoprenaline ($1 \mu\text{M}$) was re-introduced, the accumulation of cyclic AMP began to increase again both intra- and extracellularly. However, intracellular accumulation of cyclic AMP did not attain the initial peak value observed after the original application of isoprenaline. Instead, the profile of cyclic AMP accumulation mirrored that in which isoprenaline had not been washed out of the incubation medium (Figures 6 and 5a). These data support the idea that isoprenaline induces β_2 -adrenoceptor desensitization in B50 cells.

In cell monolayers treated with salmeterol ($1 \mu\text{M}$), the total accumulation of cyclic AMP appeared to rise rapidly and increase in a linear manner during the 2 h assay period (Figure 5b). Intracellular levels of cyclic AMP also rapidly increased over a 5 min incubation period (3.3 ± 0.6 fold over basal levels, $n = 3$) (Figure 5b). However, the decline of intracellular cyclic AMP was less pronounced than that observed in isoprenaline-stimulated cells over longer periods of incubation (Figure 5b). After 60 min and 120 min, intracellular levels of cyclic AMP had decreased to $62.2 \pm 4.5\%$ and $72.6 \pm 20.3\%$ ($n = 3$) of those measured at the peak of the response. In addition, after this 2 h incubation period, levels of the second messenger remained 2.1 ± 0.3 fold higher than time-matched basal values ($n = 3$) (Figure 5b). Furthermore, the extracellular levels of cyclic AMP increased in a linear fashion and were 3.6 ± 0.3 fold higher ($n = 3$) than the equivalent control values 2 h after the application of salmeterol to these cells.

Figure 6 Influence of isoprenaline rechallenge on the time course of [3 H]-cyclic AMP accumulation. B50 cell monolayers were incubated with $1 \mu\text{M}$ isoprenaline for 30 min, washed twice with Hanks/HEPES buffer and re-stimulated with $1 \mu\text{M}$ isoprenaline at $t = 40$ min (as shown by the arrows). Intracellular ($\bullet$) and extracellular ($\square$) levels of cyclic AMP were measured throughout the 2 h incubation period. Basal levels of intra- and extracellular [3 H]-cyclic AMP at $t = 0$ and $t = 120$ min are represented by the cross-hatched and open columns respectively. Each point represents the mean $\pm$ s.e.mean of 4 determinations in a single experiment.

The long duration of action of salmeterol

B50 cell monolayers were incubated with either $1 \mu\text{M}$ isoprenaline or salmeterol for 5 min before the drug-containing buffer was removed, the cells washed twice and drug-free buffer was replaced in each well. As before, levels of intracellular, extracellular and basal cyclic AMP were determined during the assay period. Isoprenaline-stimulated cells showed a rapid decrease in total [3 H]-cyclic AMP accumulation after the removal of drug-containing media with levels of the second messenger returning to basal values within 5 min. Furthermore, intracellular levels of cyclic AMP also rapidly decreased over the same period and cyclic AMP extrusion was not observed during the 60 min assay period (Figure 7a).

However, in salmeterol-stimulated cells, removal of drug-containing media did not appear to alter the total accumulation of cyclic AMP (Figure 7b) which remained at a high level during the 2 h incubation period (Figure 7b). Profiles of the time courses showing intra- and extracellular [3 H]-cyclic AMP production were similar to those obtained when the cell monolayers had not been washed with drug-free buffer (Figures 7b and 5b). Levels of intracellular cyclic AMP measured after 60 min and 120 min were $48.8 \pm 3.7\%$ and $48.1 \pm 1.1\%$ respectively of those observed after the initial peak response ($n = 3$). At the end of the incubation period, intracellular levels of the second messenger were still 1.6 ± 0.2 fold higher than time-matched basal levels ($n = 3$).

Figure 7 The effect of removing isoprenaline- and salmeterol-containing medium from the assay system on [3 H]-cyclic AMP accumulation. Isoprenaline (Iso, $1 \mu\text{M}$) (a) or salmeterol (Sal, $1 \mu\text{M}$) (b) was removed from the assay medium after a 5 min incubation (as shown by the arrow) by replacing the existing buffer with fresh prewarmed medium that contained rolipram (0.1 mM) but not salmeterol. Intracellular ($\bullet$) and extracellular ($\square$) levels of [3 H]-cyclic AMP were measured throughout the appropriate assay period as described in the Methods section. Basal levels of [3 H]-cyclic AMP measured at $t = 0$ and at the end of the appropriate assay period are represented by cross-hatched (intracellular) and open (extracellular) columns respectively. Data represent the means $\pm$ s.e.mean of quadruplicate determinations in a single experiment. Where not shown, s.e.means were within the size of the symbol. Similar results were obtained in 2 additional experiments.

Figure 8 Influence of ICI 118551 challenge on the time course of $[^3\text{H}]$ -cyclic AMP accumulation. Salmeterol (Sal, 1 μM)-containing buffer was removed from the assay medium after a 5 min incubation (as shown by the arrow) by washing twice with prewarmed Hanks/HEPES buffer (which contained 0.1 mM rolipram). ICI 118551 (1 μM) was then applied to each well at (a) $t=15$ min or (b) $t=60$ min (see arrows). Both intra- (●) and extracellular (□) levels of $[^3\text{H}]$ -cyclic AMP were measured throughout the appropriate assay period. The basal accumulation of $[^3\text{H}]$ -cyclic AMP measured at $t=0$ and at the end of each assay period is represented by cross-hatched (intracellular) and open (extracellular) columns respectively. The results of (a) and (b) were obtained in the same experiment. Each point represents the mean $\pm$ s.e. mean of 4 determinations in a single experiment. Where not shown, s.e. means were within the size of the symbol. Similar results were obtained in 2 additional experiments.

(Figure 7b). A substantial portion of cyclic AMP was observed in the extracellular fluid. At the end of the 2 h incubation period cyclic AMP accumulation had risen to 2.9 ± 0.3 fold above equivalent basal levels ($n=3$) (Figure 7b).

In a further series of experiments, cells were incubated with salmeterol (1 μM) for 5 min before being washed twice with fresh prewarmed buffer. The β_2 -selective antagonist ICI 118551 (1 μM) was then added to each well at either $t=15$ min or $t=60$ min (Figure 8). In cells treated with 1 μM ICI 118551 at $t=15$ min, intracellular levels of $[^3\text{H}]$ -cyclic AMP rapidly returned to basal values and extracellular levels of $[^3\text{H}]$ -cyclic AMP remained low (Figure 8a). Figure 8b shows that the response to salmeterol was well maintained following washout of drug-containing buffer until the administration of ICI 118551 at $t=60$ min. At this point, intracellular levels of $[^3\text{H}]$ -cyclic AMP rapidly fell towards basal values whereas the extracellular accumulation of cyclic AMP appeared to plateau, indicating that extracellular cyclic AMP was not rapidly metabolized by phosphodiesterases.

Discussion

Previous pharmacological studies have shown that salmeterol is a potent and highly selective β_2 -adrenoceptor agonist with

a long duration of action in guinea-pig (Ball *et al.*, 1991; Dougall *et al.*, 1991) and human (Ullman & Svedmyr, 1988a,b) airway smooth muscle. In this investigation we have examined some general features of salmeterol-stimulated cyclic AMP accumulation in the rat neuroblastoma B50 cell line (Schubert *et al.*, 1974). Monolayer cultures of this cell line provide a simple model system in which to investigate the effect of this long-acting agonist on β_2 -adrenoceptor-stimulated cyclic AMP accumulation (Ruck *et al.*, 1990).

In the presence of rolipram (0.1 mM), an inhibitor of the cyclic AMP selective (type IV) phosphodiesterase isoenzyme (Reeves *et al.*, 1987; Donaldson *et al.*, 1988; Nicholson *et al.*, 1991), salmeterol potently stimulated cyclic AMP accumulation in B50 cell monolayers. The slope of the concentration-response curve to salmeterol was similar to that obtained with isoprenaline. However, salmeterol (EC_{50} 37 nM) was found to be 8 fold more potent than isoprenaline (EC_{50} 294 nM). The $[^3\text{H}]$ -cyclic AMP response to salmeterol was potentially antagonized by the β_2 -selective antagonist, ICI 118551 (O'Donnell & Wanstall, 1980; Bilski *et al.*, 1983; Molenaar & Summers, 1987). The apparent K_D value for ICI 118551 (2.2 ± 0.7 nM), calculated assuming competitive antagonism, was similar to the value obtained for antagonism of the cyclic AMP response elicited by isoprenaline (K_D 1.6 ± 0.5 nM) which suggests that both drugs activate identical receptor populations. This K_D value agrees well with other β_2 -adrenoceptor K_D values (0.5 nM in guinea-pig uterus: Bilski *et al.*, 1983; 0.6 nM in bovine trachea: Hall & Hill, 1988). In the presence of the β_1 -selective antagonist, atenolol, the cyclic AMP response to both drugs was not altered. Taken together these results provide strong evidence that the cyclic AMP response to salmeterol is mediated via β_2 -adrenoceptors.

It has previously been shown that salmeterol has a slower onset of action on airway smooth muscle than other β -adrenoceptor agonists including isoprenaline, salbutamol, clenbuterol and formoterol (Ball *et al.*, 1991; Dougall *et al.*, 1991) which is thought to be due to the lipophilicity of this large molecule. In B50 cells, however, maximal accumulation of $[^3\text{H}]$ -cyclic AMP was rapid in response to both salmeterol ($t_{1/2}$ 1.13 min) and isoprenaline ($t_{1/2}$ 0.44 min). This rapid onset of action by salmeterol in B50 cells is likely to be a consequence of the use of cell monolayers which significantly reduces the diffusion barriers that exist when a drug is introduced into a tissue environment.

A direct comparison of concentration-response curves to both drugs indicated that salmeterol was capable of generating only 46% of the cyclic AMP response elicited by a maximal concentration of isoprenaline. This suggests that salmeterol behaves as a partial agonist in this system. This finding agrees well with a previous investigation that also showed that salmeterol has a lower efficacy than isoprenaline for β_2 -adrenoceptors in guinea-pig tracheal strips (Ball *et al.*, 1991; Dougall *et al.*, 1991). Since salmeterol is also more potent than isoprenaline in stimulating cyclic AMP accumulation in B50 cells, salmeterol appears to have a higher affinity than isoprenaline for the β_2 -adrenoceptor. In order to determine the affinity of this partial agonist for the β_2 -adrenoceptor, full/partial agonist interactions were employed. The pattern obtained was as expected for a partial agonist. Our estimate from this interaction of the apparent dissociation constant (K_p) for salmeterol (55.6 nM) was similar to that estimated by Coleman *et al.* (1990) and Dougall *et al.* (1991).

Whilst investigating the duration of action of salmeterol in B50 cells it was noted that a substantial portion of the cyclic AMP produced by these monolayers in response to both salmeterol and isoprenaline accumulated in the extracellular medium. Cyclic AMP efflux systems have been observed in some bacteria and a variety of cultured animal cells (Makman & Sutherland, 1965; King & Mayer, 1974; Mawe *et al.*, 1974; Rindler *et al.*, 1978; Marley *et al.*, 1991). In B50 cells stimulated by either agonist there was a rapid elevation of intracellular cyclic AMP but extracellular levels of this

second messenger appeared only after a short lag period (5–10 min). After the initial increase in intracellular cyclic AMP in isoprenaline-stimulated cells (10 min incubation period), levels of the second messenger began to decrease in a linear fashion over a 2 h interval whereas extracellular levels of cyclic AMP continued to rise over the same period. In intact cellular systems, intracellular cyclic AMP would normally be expected to achieve a plateau level (even in the presence of a competitive phosphodiesterase inhibitor such as rolipram) at which a steady state is reached between cyclic AMP synthesis and cyclic AMP breakdown via phosphodiesterase (Donaldson *et al.*, 1988). Consequently, this decrease in intracellular cyclic AMP levels following β_2 -adrenoceptor stimulation may be due to reduced cyclic AMP synthesis as a consequence of ongoing receptor desensitization (Leftkowitz *et al.*, 1990), uptake of isoprenaline into B50 cells and metabolism of the drug during the 2 h assay period or it may be secondary to the activation of cyclic AMP extrusion mechanisms. In this latter case, a new steady state level of cyclic AMP would be established within B50 cells following the development of an additional cyclic AMP removal mechanism.

The idea that receptor desensitization is being observed is supported by data from an experiment in which isoprenaline was removed from the incubation medium but cells were then rechallenged with the same dose of β -agonist (1 μ M). After cell monolayers were exposed to a second stimulation by isoprenaline, intracellular [3 H]-cyclic AMP accumulation did increase but not to the levels observed after the initial challenge of isoprenaline. Instead, levels of cyclic AMP were similar to the accumulation of cyclic AMP measured in cells that had not undergone a washing process and renewed stimulation with isoprenaline, suggesting that receptor desensitization mechanisms are operating in B50 cells following isoprenaline stimulation.

β_2 -Adrenoceptors in B50 monolayers stimulated by salmeterol did not appear to undergo such rapid desensitization since intracellular levels of cyclic AMP were maintained above basal values throughout the assay period although some reduction in cyclic AMP accumulation was observed during the 2 h incubation period. Furthermore, the level of extracellular cyclic AMP (which does not appear to be broken down rapidly by phosphodiesterases; cf. Figure 8) continues to increase almost linearly (indicative of substantial on-going cyclic AMP synthesis) between 30 and 120 min following salmeterol administration. This reduced degree of desensitization (compared to isoprenaline) may be a consequence of the lower efficacy of salmeterol. Alternatively, it is possible that the lipophilic nature of this β_2 -agonist might alter the conformation of the agonist-receptor complex and thus sterically hinder phosphorylation of particular amino acid residues on the β_2 -adrenoceptor by protein kinase A and β -adrenoceptor kinase or inhibit the putative functioning of β -arrestin in uncoupling the receptor from G_s (Leftkowitz *et al.*, 1990).

References

- BALL, D.I., BRITTAI, R.T., COLEMAN, R.A., DENYER, L.H., JACK, D., JOHNSON, M., LUNTS, L.H.C., NIALS, A.T., SHELDRIK, K.E. & SKIDMORE, I.F. (1991). Salmeterol, a novel, long-acting β_2 -adrenoceptor agonist: characterization of pharmacological activity *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **104**, 665–671.
- BARNES, P.J. (1986). Bronchodilator mechanisms. In *Asthma: Clinical Pharmacology and Therapeutic Progress*. ed. Kay, A.B. pp. 146–160. Oxford: Blackwell.
- BARNES, P.J., IND, P.W. & DOLLERY, C.T. (1984). Beta-adrenoceptors in asthma and their responses to agonists. In *Asthma: Physiology, Immunopharmacology and Treatment*. ed. Kay, A.B., Austen, K.F. & Lichtenstein, L.M. pp. 339–358. London: Academic Press.
- BILSKI, A.J., HALLIDAY, S.E., FITZGERALD, J.D. & WALE, J.C. (1983). The pharmacology of a β_2 -selective adrenoceptor antagonist ICI 118,551. *J. Cardiovasc. Res.*, **5**, 430–437.
- BIRNBAUMER, L. (1990). G proteins in signal transduction. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 675–705.
- BRADSHAW, J., BRITTAI, R.T., COLEMAN, R.A., JACK, D., KENNEDY, I., LUNTS, L.H.C. & SKIDMORE, I.F. (1987). The design of salmeterol, a long-acting selective β_2 -adrenoceptor agonist. *Br. J. Pharmacol.*, **92**, 590P.
- BRITTAI, R.T. (1990). Approaches to a long-acting, selective β_2 -adrenoceptor stimulant. *Lung*, **168**, 111–114.
- CARRÉ, I.A. & EDMUNDS, L.N. Jr. (1992). cAMP-dependent kinases in the algal flagellate *Euglena gracilis*. *J. Biol. Chem.*, **267**, 2135–2137.
- COLEMAN, R.A., JOHNSON, M., NIALS, A.T. & SUMNER, M.J. (1990). Salmeterol, but not formoterol, persists at β_2 -adrenoceptors *in vitro*. *Br. J. Pharmacol.*, **99**, 121P.
- It is possible that the extrusion phenomenon serves, along with phosphodiesterase activity, to maintain low intracellular concentrations of cyclic AMP. Furthermore, extracellular cyclic AMP may have an important physiological role. Receptors for cyclic AMP are thought to be located on the external surfaces of plasma membranes in *Euglena* (Carré & Edmonds, 1992) and four cyclic AMP receptor subtypes (cAR1-4) have been cloned and sequenced in *Dictyostelium* (Klein *et al.*, 1988; Saxe *et al.*, 1991a,b). The efflux process appears to involve an active transport mechanism which depends on a chemical source of energy such as ATP (Doore *et al.*, 1975). Rindler *et al.* (1978) have shown that inhibitors of mitochondrial function and glycolysis (e.g. oligomycin, valinomycin and FCCP) reduced ATP concentrations in C6 rat glioma cells and avian erythrocytes and cyclic AMP efflux was inhibited by a corresponding degree.
- Although cyclic AMP efflux exists in B50 cells, it is still possible to study the long term effects of salmeterol in these monolayers by measuring intracellular cyclic AMP accumulation. In isoprenaline-treated cells that had been washed with drug-free medium after a 5 min stimulatory period, intracellular cyclic AMP levels rapidly decreased towards basal values. Furthermore, cyclic AMP efflux from these cells was negligible indicating that isoprenaline is easily removed from its site of action by this simple washing process. When the same procedure was performed on salmeterol-stimulated cells, intracellular levels of cyclic AMP were sustained above basal values and extrusion of cyclic AMP from B50 cells was observed. Overall the profile of cyclic AMP production and excretion was similar to that in which salmeterol stimulation of cells had been maintained. These data are consistent with the hypothesis (Jack, 1991; Ball *et al.*, 1991) that the lipophilic nature of this molecule allows it to anchor at the putative 'exo-site' within the β_2 -adrenoceptor protein and from which it cannot be displaced by washing with drug-free medium, thereby allowing salmeterol to persist at its site of action. However, as has been observed in guinea-pig trachea (Ball *et al.*, 1991), the action of salmeterol can nevertheless be reversed by the administration of a β_2 -selective antagonist (ICI 118551).
- In conclusion, salmeterol appears to be a potent and selective β_2 -adrenoceptor agonist with a long duration of action in B50 cells *in vitro*. It behaves as a partial agonist in this system and since it is more potent than isoprenaline in stimulating cyclic AMP accumulation in these cells, this suggests that salmeterol has a higher affinity than isoprenaline for the β_2 -adrenoceptor. However, it is intriguing that there is no obvious sign of receptor desensitization over a 120 min period of stimulation despite the continued presence of salmeterol at its site of action.

We thank Malcolm Johnson for helpful discussions and Glaxo Group Research Ltd. for financial support.

- DONALDSON, J. BROWN, A.M. & HILL, S.J. (1988). Influence of rolipram on the cyclic 3',5'-adenosine monophosphate response to histamine and adenosine in slices of guinea-pig cerebral cortex. *Biochem. Pharmacol.*, **37**, 715–723.
- DOORE, B.J., BASHER, M.M., SPITZER, N., MAWE, R.C. & SAIER, M.H. Jr. (1975). Regulation of adenosine 3':5'-monophosphate efflux from rat glioma cells in culture. *J. Biol. Chem.*, **250**, 4371–4372.
- DOUGALL, I.G., HARPER, D., JACKSON, D.M. & LEFF, P. (1991). Estimation of the efficacy and affinity of the β_2 -adrenoceptor agonist salmeterol in guinea-pig trachea. *Br. J. Pharmacol.*, **104**, 1057–1061.
- GILMAN, A.G. (1987). G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.*, **56**, 615–649.
- HALL, I.P. & HILL, S.J. (1988). β -adrenoceptor stimulation inhibits histamine-stimulated inositol phospholipid hydrolysis in bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **95**, 1204–1212.
- JACK, D. (1991). A way of looking at agonism and antagonism: lessons from salbutamol, salmeterol and other β -adrenoceptor agonists. *Br. J. Clin. Pharmacol.*, **31**, 501–514.
- KING, C.D. & MAYER, S.E. (1974). Inhibition of egress of adenosine 3',5'-monophosphate from pigeon erythrocytes. *Mol. Pharmacol.*, **10**, 941–953.
- KLEIN, P., SUN, J.J., SAXE, C.L., KIMMEL, A.R., JOHNSON, R.L. & DEVREOTES, P.N. (1988). A chemoattractant receptor controls development in *Dictyostelium discoideum*. *Science*, **241**, 1467–1472.
- KUME, H., TAKAI, A., TOKUNO, H. & TOMITA, T. (1989). Regulation of Ca^{2+} dependent K^+ channel activity in tracheal myocytes by phosphorylation. *Nature*, **341**, 152–154.
- LAZERENO, S. & ROBERTS, F.F. (1987). Measuring muscarinic antagonist potency using stimulated phosphoinositide breakdown in rat cortex slices. *Br. J. Pharmacol.*, **92**, 677P.
- LEFKOWITZ, R.J., HAUSDORFF, W.P. & CARON, M.G. (1990). Role of phosphorylation in desensitization of the β -adrenoceptor. *Trends Pharmacol. Sci.*, **11**, 190–194.
- MAKMAN, R.S. & SUTHERLAND, E.W. (1965). Adenosine 3',5'-cyclic monophosphate in *E. coli*. *J. Biol. Chem.*, **240**, 1309–1314.
- MARLEY, P.D., THOMSON, K.A., JACHNO, K. & JOHNSTON, M.J. (1991). Histamine-induced increases in cyclic AMP levels in bovine adrenal medullary cells. *Br. J. Pharmacol.*, **104**, 839–846.
- MAWE, R., DOORE, B., MCCAMAN, M., FEUCHT, B. & SAIER, M. (1974). Regulation of cyclic AMP excretion in bacteria and cultured animal cells. *J. Cell Biol.*, **63**, 211a.
- MCCREA, K.E., JOHNSON, M. & HILL, S.J. (1992). The effect of salmeterol on cyclic AMP accumulation in a neuronal cell line. *Br. J. Pharmacol.*, **104**, 249P.
- MOLENAAR, P. & SUMMERS, R.J. (1987). Characterization of β_1 and β_2 adrenoceptors in guinea-pig atrium: functional and receptor binding studies. *J. Pharmacol. Exp. Ther.*, **241**, 1041–1047.
- NICHOLSON, C.D., CHALLISS, R.A.J. & SHAHID, M. (1991). Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci.*, **12**, 19–27.
- O'DONNELL, S.R. & WANSTALL, J.C. (1980). Evidence that ICI 118,551 is a potent highly β_2 -selective adrenoceptor antagonist and can be used to characterize β_2 -adrenoceptor populations in tissues. *Life Sci.*, **27**, 671–677.
- REEVES, M.L., LEIGH, B.K. & ENGLAND, P.J. (1987). The identification of a new cyclic nucleotide phosphodiesterase in human and guinea-pig cardiac ventricle. *Biochem. J.*, **241**, 537–543.
- RINDLER, M.J., BASHOR, M.M., SPITZER, N. & SAIER, M.H. Jr. (1978). Regulation of adenosine 3',5'-monophosphate efflux from animal cells. *J. Biol. Chem.*, **253**, 5431–5436.
- RUCK, A., MILLNS, P., KENDALL, D.A. & HILL, S.J. (1990). Expression of β_2 -adrenoceptors mediating cyclic AMP accumulation in astroglial and neuronal cell lines derived from the rat CNS. *Biochem. Pharmacol.*, **40**, 2371–2375.
- SAXE, C.L., JOHNSON, R.L., DEVREOTES, P.N. & KIMMEL, A.R. (1991a). Multiple genes for cell surface cAMP receptors in *Dictyostelium discoideum*. *Dev. Genet.*, **12**, 6–13.
- SAXE, C.L., JOHNSON, R.L., DEVREOTES, P.N. & KIMMEL, A.R. (1991b). Expression of a cAMP receptor gene of *Dictyostelium* and evidence for a multi gene family. *Genes Dev.*, **5**, 1–8.
- SCHUBERT, D., HEINEMANN, S., CARLISLE, W., TARIKAS, H., KIMES, B., PATRICK, J., STEINBACH, J.H., CULP, W. & BRANDT, B.L. (1974). Clonal cell lines from the rat central nervous system. *Nature*, **249**, 224–227.
- STEPHENSON, R.P. (1956). A modification of receptor theory. *Br. J. Pharmacol. Chemother.*, **11**, 379–393.
- ULLMAN, A. & SVEDMYR, N. (1988a). Salmeterol, a new long acting inhaled β_2 -adrenoceptor agonist: comparison with salbutamol in adult asthmatic patients. *Thorax*, **43**, 674–678.
- ULLMAN, A. & SVEDMYR, N. (1988b). Inhaled salmeterol a new β_2 -adrenoceptor agonist produces sustained bronchodilation in asthmatic patients without causing tachyphylaxis. *Am. Rev. Resp. Disease*, **137**, 32.

(Received March 15, 1993

Revised May 17, 1993

Accepted June 2, 1993)