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Correlation of cyclic AMP accumulation and relaxant actions of salmeterol and salbutamol in bovine tracheal smooth muscle

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1 The ability of salmeterol to stimulate cyclic AMP accumulation and relaxation has been compared with that of salbutamol in bovine tracheal smooth muscle. In addition, the anti-spasmogenic effects of these agents and their abilities to modulate histamine-stimulated [^{3}H]-inositol phosphate accumulation have also been investigated.

2 In tissue strips, a close temporal correlation was found to exist between salmeterol (0.1 μM)-induced relaxation of methacholine (500 nM)-induced tone and cyclic AMP accumulation, both maximal reversal of induced tone (26.2 $\pm$ 6.0%) and maximal levels of cyclic AMP accumulation being achieved after 30-40 min. In contrast to salmeterol, salbutamol exerted greater and more rapid effects on both parameters. Maximal reversal of methacholine-induced tone (79.3 $\pm$ 14.0%) and maximal levels of cyclic AMP accumulation were produced within 5 min.

3 Salmeterol-induced cyclic AMP accumulation (EC_{50} = 5.3 [1.8-15.2] nM) and inhibition of histamine (0.1 mM)-stimulated [^{3}H]-inositol phosphate accumulation (IC_{50} = 1.4 [0.3-6.3] nM) were both more potent than those induced by salbutamol (EC_{50} = 169 [99-290] nM; IC_{50} = 13.8 [7.0-27.4] nM). However, maximal effects exerted by each of these agents were similar in magnitude.

4 Anti-spasmogenic effects were examined by β-adrenoceptor agonist application to tissue strips prior to construction of spasmogen concentration-effect curves. Both salmeterol and salbutamol exerted more marked inhibition of the contractile response induced by histamine than that induced by methacholine, salmeterol being the more potent agent, while salbutamol produced a greater maximal inhibitory effect.

5 The results demonstrate that salmeterol is a more potent agent than salbutamol and have highlighted a close temporal correlation between promotion of cyclic AMP accumulation and tissue relaxation stimulated by each agent when both parameters are measured under identical conditions.

Keywords: β-Adrenoceptor agonists; salmeterol; salbutamol; cyclic AMP accumulation; smooth muscle relaxation; phosphoinositide turnover

Introduction

Salmeterol (Salm) is a potent, highly selective and long acting β_2-adrenoceptor agonist which has been specifically developed for bronchodilator therapy in the treatment of asthma (Ball *et al.*, 1991; Johnson, 1991; Nials *et al.*, 1993a,b; Rabe *et al.*, 1993). Structurally, Salm contains an identical saligenin moiety to salbutamol (Salb), but differs from the latter in possessing a lipophilic N-substituent phenylalkyloxyalkyl side chain, which is thought to endow Salm with its longevity of action (Jack, 1991; Nials *et al.*, 1993b).

At present the precise mechanism underlying the extended duration of action of Salm remains contentious. The so-called 'exo-site' hypothesis suggests that the lipophilic side chain of Salm anchors the molecule at, or close to, its site of action (Jack, 1991; Rhodes *et al.*, 1992), although it is unclear whether the lipophilic side chain of Salm associates with the phospholipid bilayer or a lipophilic domain within the β-adrenoceptor itself. Whatever the physical nature of the 'exo-site', the receptor-Salm association must be suitably flexible to allow the saligenin headgroup to associate with and dissociate from, the active site of the β-adrenoceptor, whilst remaining firmly attached within the vicinity of the binding pocket. This requirement arises from the observation that although the relaxant action of Salm can be reversed by β-adrenoceptor antagonists, relaxation is re-established following wash-out of the antagonist without the need for Salm re-addition (Ball *et al.*, 1991; Nials *et al.*, 1993a). An alternative explanation for the extended duration of action of Salm, and other long-acting β-adrenoceptor agonists such as formoterol, has been put forward by Anderson and colleagues (1994). These workers have proposed a model based on 'microkinetic diffusion', where the membrane acts as a depot, slowly releasing lipophilic β-adrenoceptor agonist (Anderson *et al.*, 1994).

Although considerable work has now been carried out to characterize the effects of Salm in animal and human airways smooth muscle *in vitro* (Ball *et al.*, 1991; Dougall *et al.*, 1991; Nials *et al.*, 1993a,b; Källström *et al.*, 1994; Naline *et al.*, 1994) and *in vivo* (Ullman & Svedmyr, 1988; Ball *et al.*, 1991; Malo *et al.*, 1992; Rabe *et al.*, 1993), surprisingly little work has been carried out to establish the action of this agent at the level of signal transduction pathways in airways smooth muscle.

In the present study, the abilities of Salm to stimulate adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation and to cause relaxation have been correlated in bovine tracheal smooth muscle (BTSM) preparations and compared with the effects of Salb. In addition, the anti-spasmogenic actions of Salm and Salb and their abilities to modulate spasmogen-stimulated second messenger-generating pathways has also studied. Preliminary accounts of some of this work have been presented to the British Pharmacological Society (Ellis *et al.*, 1994a,b).

Methods

Tissue preparation

Bovine trachealis was obtained from a local abbatoir, transported to the laboratory in Krebs-Henseleit buffer (KHB;

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composition in mM: NaCl 120, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25, HEPES 5, glucose 10, 95% O_2/5% CO_2) at 4°C and then cleaned of epithelium and connective tissue. The tracheal smooth muscle was either chopped into slices (300 × 300 μm) with a McIlwain tissue chopper or cut into strips (10 × 10 × 2 mm). Slices were incubated at 37°C in KHB for 60 min with 4 changes of buffer. Tissue slices which were not used immediately were maintained for 24 h in minimum essential medium (Gibco BRL) supplemented with penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37°C in a 5% CO_2 incubator. Tissue strips were either used immediately or were maintained overnight in KHB at 4°C.

Tension measurements

BTSM strips were mounted for isometric tension recording in 7 ml tissue baths containing KHB (37°C). The tissues were suspended under 1 g of tension and allowed to equilibrate for 60 min with 3 changes of KHB. Tension was readjusted to 1 g after the first 2 changes of KHB and had stabilized to 0.5–1 g before the start of the experiments.

Anti-spasmogenic experiments

Methacholine (MCh; 1 μM) or histamine (Hist; 1 mM) was added to the tissue to induce tone. Tissues were then washed at 10 min intervals over a period of at least 30 min, until tone had returned to basal levels. Anti-spasmogenic effects of β_2-adrenoceptor agonists were investigated by pretreatment of the tissue with Salm or Salb (range; 0.1 nM–1 μM) for 20 and 5 min respectively, these time periods having been previously established as being sufficient to allow the β_2-adrenoceptor agonists to reach equilibrium. After this pre-exposure period, Hist (1–1000 μM) or MCh (1–30 000 nM) concentration-effect curves were constructed by cumulative addition to the tissue baths at 3 min intervals, a time sufficient for each response to reach a plateau level. Tissues were then washed at 10 min intervals over a period of at least 30 min, until basal tone was re-achieved. A higher concentration of β_2-adrenoceptor agonist was then applied and spasmogen curves were reconstructed. Time-matched control response curves were constructed for all tissues. Results were expressed as a % of the maximum contractile response achieved in the corresponding control spasmogen-contraction curves.

Relaxation time-course studies

Tissues were pre-contracted with MCh (0.5 μM). When the contractile response had reached a plateau (approximately 20 min) a single, maximal concentration of Salm (0.1 μM) or Salb (1 μM) was added to the tissue bath. Reversal of MCh-evoked tone was recorded over a period of 2 h. All tissues were control-matched to assess deterioration of spasmogen tone over the experimental period.

Measurement of β_2-adrenoceptor agonist-induced cyclic AMP accumulation

Aliquots (75 μl) of packed slices were transferred into vials containing 400 μl of KHB in a shaking water-bath maintained at 37°C. Slices were incubated for 30 min in the absence or presence of 0.1 mM 3-isobutyl-1-methylxanthine (IBMX), at which point β_2-adrenoceptor agonists were added at the concentrations and for the time periods indicated in the Results section. Incubations were terminated by the addition of ice-cold perchloric acid (PCA, 2 M). Cyclic AMP was measured in neutralized tissue extracts by the method of Brown *et al.*, (1971). In brief, 50 μl of sample or standard (concentration range: 0.125–10 pmol per 50 μl) were added to 100 μl [^{3}H]-cyclic AMP (approximately 75 000 d.p.m.; prepared in 50 mM Tris/HCl/4 mM EDTA, pH 7.5;) and 150 μl of bovine adrenal binding protein (also prepared in the assay buffer, see Brown *et al.*, 1971). Samples were incubated at 4°C for at least 90 min. Free and bound cyclic AMP were separated by charcoal-precipitation in assay buffer which contained 0.1% bovine serum albumin. Radioactivity in the supernatants was determined by liquid-scintillation counting.

In a separate series of experiments, tissue strips were incubated under tension (approximately 1 g) in KHB with maximally-effective concentrations of Salm (0.1 μM) or Salb (10 μM) for increasing time periods (range 1–60 min). Incubations were terminated by freeze-clamping tissues using a liquid N_2-cooled clamping device. Tissues were ground with a liquid N_2-cooled pestle and mortar and cyclic AMP was extracted with ice-cold PCA (2 M). After thawing, tissues were homogenized and samples were centrifuged (4000 *g*, 4°C, 20 min). Aliquots of supernatant were removed and neutralized with $KHCO_3$ (1.5 M). Determination of β_2-adrenoceptor agonist-stimulated cyclic AMP accumulation was as described for BTSM slices.

Measurement of β-adrenoceptor agonist-induced inhibition of histamine-stimulated [^{3}H]-InsP accumulation

Tissue slices were pre-labelled (24 h) with *myo*-[^{3}H]-inositol and Hist-stimulated [^{3}H]-InsP accumulation was measured as previously described (Challiss *et al.*, 1992). Briefly, labelled BTSM slices were washed and aliquots (75 μl) transferred to vials containing KHB, 0.8 μCi [^{3}H]-inositol and 5 mM LiCl. After 30 min incubation, slices were exposed to Salm (0.1–1000 nM), Salb (1–10 000 nM) or isoprenaline (Iso; 10 μM) for 20 min prior to the addition of Hist (0.1 mM). Following a 30 min spasmogen-exposure period, incubations were terminated by the addition of PCA (2 M) and aliquots of neutralized tissue extracts were then assayed for total [^{3}H]-InsP accumulation.

In all experiments where accumulation of cyclic AMP or inhibition of total [^{3}H]-InsP production were measured, tissue pellets were solubilized with 2 M NaOH and protein content was determined by the method of Lowry *et al.* (1951). Results of cyclic AMP assays are expressed as pmol cyclic AMP mg^{-1} protein and results of experiments measuring the inhibition of Hist-stimulated [^{3}H]-InsP production are expressed as d.p.m. mg^{-1} protein.

Materials

[^{3}H]-adenosine 3′:5′-cyclic monophosphate (ammonium salt) was purchased from New England Nuclear and *myo*-[^{3}H]-inositol was purchased from Amersham. Isoprenaline (bitartrate salt), histamine dihydrochloride, acetyl-β-methylcholine chloride and 3-isobutyl-1-methylxanthine were purchased from Sigma. Dowex 1 × 8 (100–200 mesh, Cl^-form) was purchased from BioRad Laboratories Ltd. Salmeterol and salbutamol were gifts from Glaxo Group Research Ltd.

Data analysis

All data are expressed as mean ± s.e.mean for *n* separate experiments. EC_{50}/IC_{50} values are expressed as geometrically-derived means with 95% confidence limits. Where possible the data have been fitted to the equation:

$$F_x = \frac{(X/E)^n}{1 + (X/E)^n}$$

where E = 50% of the maximum response and n = slope factor, using a commercial curve-fitting programme. The solution to this equation was used to produce the best-fit curves illustrated in many of the figures. Significant differences between means were assessed by Student's unpaired *t* test, the null hypothesis being rejected when *P* is less than 0.05.

Results

Time-course of relaxation of MCh-maintained contraction of BTSM strips by Salm and Salb

Tissue tone was induced by the addition of MCh (0.5 μM, mean tension generated 16.4±1.2 g; $n=10$). After the contractile response had reached a stable value, single concentrations of Salm (0.1 μM) were applied and inhibition of MCh-induced tone was recorded for a period of 2 h. Results from the time-matched control tissues demonstrated that the MCh-induced contractile response was well-maintained over this experimental period. Each of the β_2-adrenoceptor agonists produced contrasting profiles of BTSM relaxation (Figure 1). Salm-induced relaxation was slowly developing, approached a maximal level at > 20 min and achieved 26.2±6.0% suppression of MCh-induced tone at 33 min. This maximal level of relaxation was well-maintained over the remainder of the experiment. Results of a separate series of experiments found that a higher concentration of Salm (1 μM) produced no greater relaxant effect. Salb caused a greater and much more rapid reversal of MCh-maintained contraction, maximal inhibition of tone being 79.3±14.0% at 5 min. After this peak relaxant effect had been achieved, the spasmolytic effect of Salb was gradually reversed to 25.0±12.8% at 2 h, a relaxation level which did not differ significantly from the relaxation produced by Salm at 2 h (24.4±8.2%)

Time-course of Salm- and Salb-induced cyclic AMP accumulation in BTSM strips: correlation with relaxation data

In order to compare directly the time-courses of Salm (0.1 μM)-induced cyclic AMP accumulation and relaxation of BTSM, we measured both parameters in the same tissue strip preparation. Figure 2 illustrates that a good temporal relationship exists between these two parameters. A significant increase in cyclic AMP levels above basal was first observed at 10 min (basal, 5.2±0.4; +Salm, 7.0±0.3 pmol mg^{-1} protein; $P<0.001$; data not illustrated) and maximum levels of cyclic AMP were achieved between 30 and 40 min after Salm addition, being 8.8±0.9 pmol mg^{-1} protein at 40 min. This time-course corresponds with that for the relaxation response achieved by application of Salm to pre-contracted BTSM strips, with a maximum after approximately 30 min, (Figure 2). Levels of cyclic AMP were not significantly reduced at a later time point (7.9±0.7 pmol mg^{-1} protein at 60 min). The results of a separate series of experiments showed that a higher concentration of Salm (1 μM) did not produce greater increases in cyclic AMP accumulation. In contrast, Salb (10 μM) stimulated a more rapid and significantly greater ($P<0.01$) accumulation of cyclic AMP to a maximal level of 12.6±0.7 pmol mg^{-1} protein within 5 min. Thus, a good temporal correlation between second messenger accumulation and relaxation was also seen for Salb in BTSM strips.

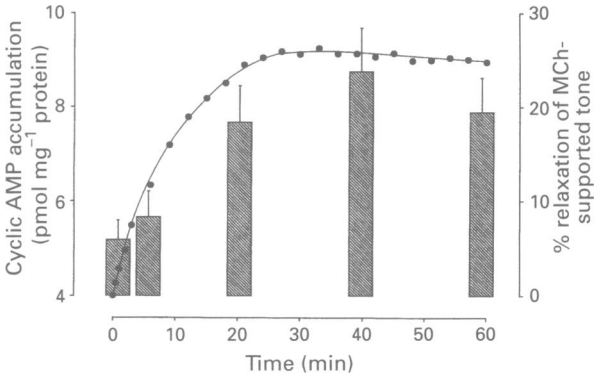

Figure 2 Time-courses of Salm (0.1 μM)-induced relaxation (●) of MCh (0.5 μM)-supported tone and cyclic AMP accumulation (columns) in BTSM strips. Determinations of cyclic AMP levels were made after exposing tissue strips to Salm for the times indicated. Each column represents the mean±s.e.mean measurement from 12 separate tissues. Each data point on the relaxation line-graph represent the mean of 4 separate experiments; error bars have been omitted for clarity.

Time-course of Salm-, Salb- and Iso-induced cyclic AMP accumulation in BTSM slices

Tissue slices were stimulated with single concentrations of Salm (0.1 μM), Salb (10 μM) and Iso (10 μM) for increasing time periods up to 20 min in the absence or presence of 0.1 mM IBMX (to inhibit phosphodiesterase activity and so amplify cyclic AMP accumulation). In agreement with the results achieved in the strip preparation, the onset of cyclic AMP accumulation induced by Salm application was slower than that observed for other β_2-adrenoceptor agonists used and was similar in the absence and presence of phosphodiesterase inhibition. Figure 3 shows comparative time-course data for Salm, Salb and Iso in the presence of 0.1 mM IBMX. Significant increases over basal cyclic AMP levels (11.3±0.4 pmol mg^{-1} protein) were first observed after 1 min upon application of Salm (25.4±3.5; $P<0.01$) and 15 s after application of both Salb (34.2±2.1; $P<0.001$) and Iso (33.2±3.7 pmol mg^{-1} protein; $P<0.001$). Salm-induced cyclic AMP accumulation approached maximum levels after

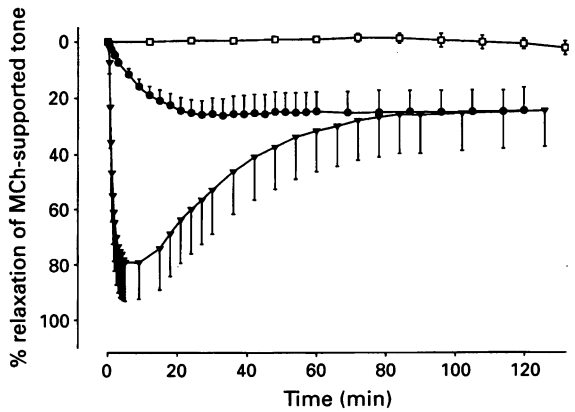

Figure 1 Time-courses of relaxation of MCh (0.5 μM)-maintained contraction of BTSM strips by 0.1 μM Salm (●) and 1 μM Salb (▼). Results from time-matched control tissues to which no addition of β_2-adrenoceptor agonist was made are also shown (□). Each data point represents the mean±s.e.mean of 4 separate experiments.

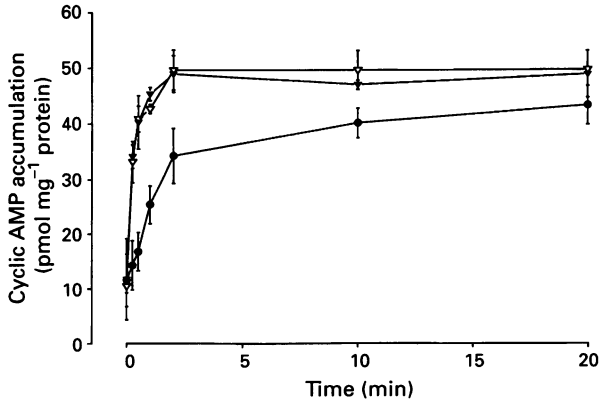

Figure 3 Time-courses of β-adrenoceptor agonist-induced cyclic AMP accumulation in BTSM slices. Tissues were pre-exposed to IBMX (0.1 mM) for 30 min prior to the addition of 0.1 μM Salm (●), 10 μM Salb (▼) or 10 μM Iso (▽) for the time-periods indicated. Each data point represents the mean±s.e.mean from 3 separate experiments, each performed in triplicate.

10 min, with the maximum response being achieved after 20 min (43.4±3.5 pmol mg^{-1} protein). In contrast, Salb and Iso both caused maximum accumulation of cyclic AMP after 2 min, values being 49.0±3.5 and 49.7±3.6 pmol mg^{-1} protein for Salb and Iso respectively. There was no significant decrease from maximal cyclic AMP levels stimulated by any of the β-adrenoceptor agonists over the period of the experiments.

Concentration-dependence of β-adrenoceptor agonist-induced cyclic AMP accumulation

Salm and Salb both caused concentration-dependent increases of cyclic AMP accumulation (Figure 4a), Salm being the more potent of the two agents (EC_{50} values; 5.3 [1.8–15.2] and 169 [99–290] nM for Salm and Salb respectively). Maximally-effective concentrations of Iso (10 μM) and Salb (10 μM) stimulated cyclic AMP accumulation which did not significantly differ from each other (Figure 4b). Cyclic AMP levels were increased from a basal level of 3.6±0.3 to 18.9±2.0, 20.2±2.2 and 14.8±2.4 pmol mg^{-1} protein by Iso, Salb and Salm respectively.

Comparison of the anti-spasmogenic effects of Salm and Salb

In order to examine the anti-spasmogenic effects of Salm and Salb, experiments involving the addition of increasing concentrations (0.1–1000 nM) of each β_2-adrenoceptor agonist to isolated BTSM strips prior to the construction of MCh and Hist-induced concentration-effect curves were carried out. Increasing concentrations of Salm and Salb shifted MCh-induced contraction curves to the right without reducing the maximal effect produced by MCh (30 μM) (Figures 5a and b).

At a concentration of 0.1 μM, each agent caused an approximate 5 fold increase in EC_{50} from a control value of 127 [42–393] to 672 [462–978] and 714 [300–1688] nM in the presence of Salm and Salb respectively. Both Salm and Salb exhibited considerably greater inhibitory effects on Hist than on MCh-induced spasm (Figures 5c and d). In addition to shifting contraction curves to the right, increasing concentrations of β_2-agonist also reduced the maximum response to Hist (1 mM).

Of the two β_2-adrenoceptor agonists, Salb caused a more marked inhibition of Hist-induced contraction, a concentration of 10 nM causing an increase in EC_{50} from 21.3 [10.2–44.7] to 46.4 [17.7–121] μM, while 0.1 μM Salb shifted the curve further and caused a 71.4±8.8% suppression of Hist-induced tone. In comparison to Salb, Salm caused a more potent inhibition of Hist-induced spasm, with 1 nM shifting the EC_{50} from a control value of 15.4 [1.9–125] to 40.2 [26.5–60.9] μM. A maximum shift of EC_{50} to 105 [39–282] μM was achieved at a concentration of 10 nM Salm. This concentration of Salm also caused a 33.7±7.6% suppression of the Hist-induced maximal response.

Inhibitory effects of β_2-adrenoceptor agonists on Hist-induced [^{3}H]-InsP accumulation

In order to examine intracellular events occurring during the anti-spasmogenic actions of β_2-adrenoceptor agonists, we carried out experiments involving the addition of Salm, Salb and Iso to BTSM slices (labelled for 24 h with [^{3}H]-inositol) 20 min prior to stimulation with Hist (0.1 mM). Addition of Salm (0.1 μM), Salb (1 μM) or Iso (10 μM) had no effect on basal accumulations, but all caused inhibitions of Hist-induced [^{3}H]-InsP accumulation to levels which did not significantly differ from each other (Table 1). Experiments carried out to compare the concentration-dependent inhibition of Hist-stimulated [^{3}H]-InsP accumulation of Salm (0.1–100 nM) and Sale (1–10 000 nM) demonstrated Salm to be the more potent of the two agents (IC_{50} values were 1.4 [0.3–6.3] and 13.8 [7.0–27.4] nM for Salm and Salb respectively; see Figure 6).

Discussion

Previous studies have established that Salm is a potent and selective β_2-adrenoceptor agonist capable of causing prolonged relaxation of airways smooth muscle both *in vitro* and *in vivo* (Ullman & Svedmyr, 1988; Ball *et al.*, 1991; Nials *et al.*, 1993a,b; Naline *et al.*, 1994). The present study was undertaken to correlate changes in cyclic AMP accumulation and relaxant activities of Salm and Salb, to compare spasmolytic and anti-spasmogenic actions of these agents and to assess the abilities of Salm and Salb to inhibit Hist-stimulated [^{3}H]-InsP accumulation in bovine tracheal smooth muscle.

A striking feature arising from previous contractile studies was the slow onset of spasmolytic action of Salm relative to other β-adrenoceptor agonists such as Salb and Iso in airways

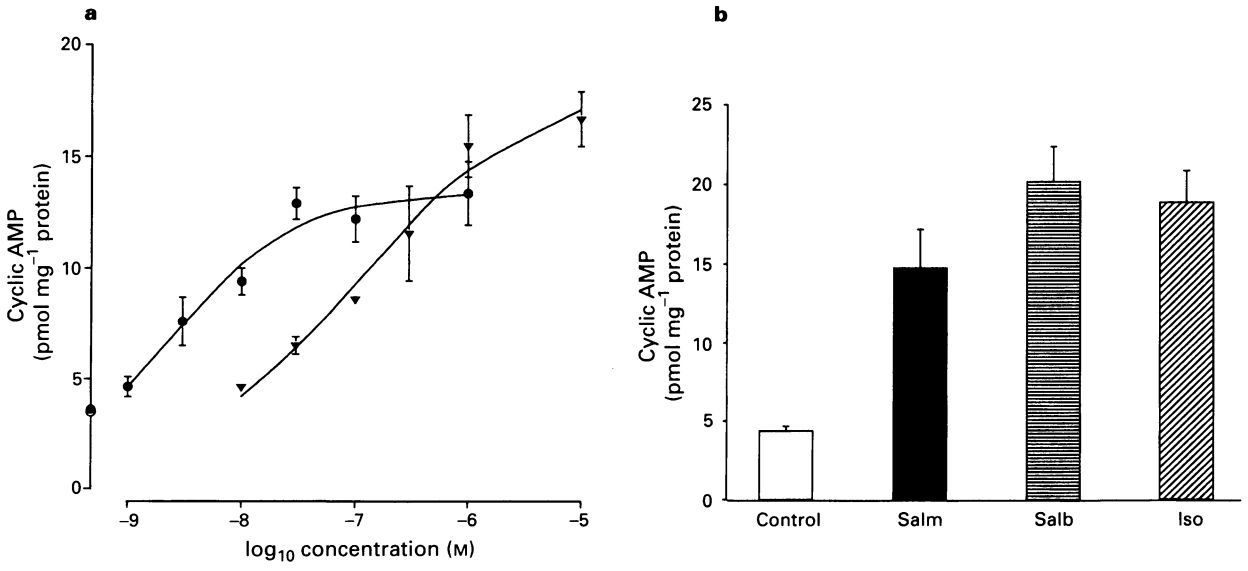

Figure 4 Concentration-dependence of Salm- and Salb-stimulated cyclic AMP accumulation in BTSM slices. Slices were incubated in the presence of (a) increasing concentrations of Salm (●) or Salb (▼), or (b) single concentrations of Salm (0.1 μM), Salb (10 μM) and Iso (10 μ) for periods of 20 min prior to the determination of cyclic AMP levels. Each data point or bar represents the mean±s.e.mean for 3 separate experiments, each performed in triplicate.

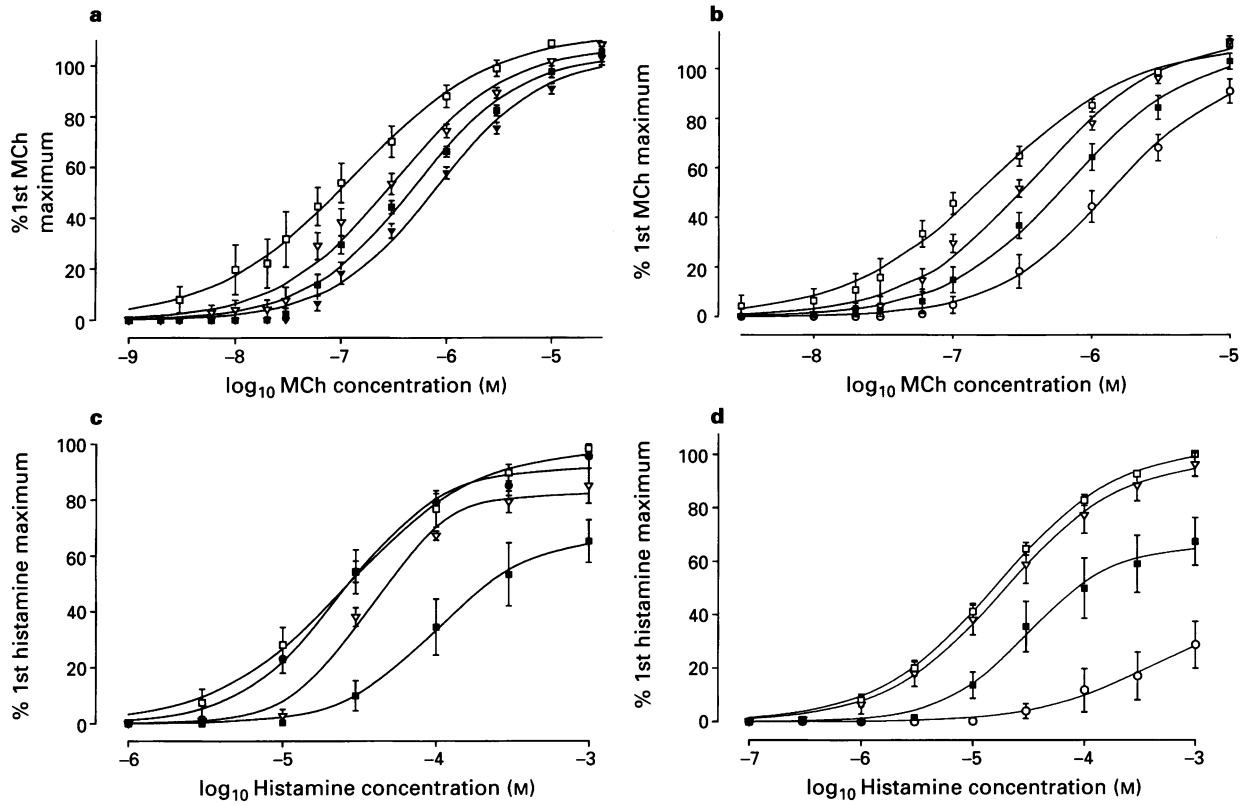

Figure 5 Anti-spasmogenic effects of Salm and Salb on Hist- and MCh-induced contraction of BTSM strips. Tissues were pre-exposed to increasing concentrations (0.1 ●; 1 ▽; 10 ■; 100 ○; 1000 nM ▼) of Salm (a and c 20 min) or Salb (b and d; 5 min) prior to the construction of MCh (a and b) or Hist (a and c) cumulative concentration-effect curves. Some of the concentration-effect curves produced from these experiments have been omitted from the figures for clarity. Control spasmogen-induced concentration-effect curves which were constructed in the absence of β_2-adrenoceptor agonist are also shown (□). Each data point represents the mean ± s.e.mean from 8 (a), 4 (b), 7 (c) and 8 (d) separate tissues.

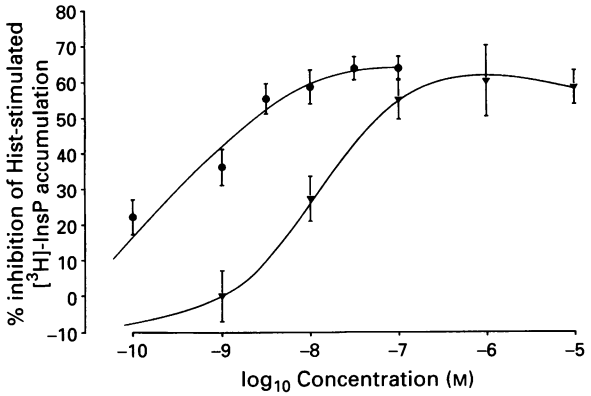

Figure 6 Concentration-dependent inhibitory effects of Salm and Salb on Hist-stimulated [^{3}H]-InsP accumulation in BTSM slices. Tissues were pre-incubated in the presence of increasing concentrations of Salm (●) or Salb (▼) for 20 min prior to the addition of Hist (0.1 mM) for a further 30 min. Each data point represents the mean ± s.e.mean of 3 separate experiments each performed in triplicate.

smooth muscle preparations (Ball *et al.*, 1991; Dougall *et al.*, 1991; Ullman *et al.*, 1992; Nials *et al.*, 1993a,b). Here we have confirmed the differences between Salm and Salb with respect to the time-courses over which relaxation develops in MCh-contracted BTSM strips. Furthermore, in parallel experiments, the time-course of Salm-stimulated cyclic AMP accumulation was also assessed. A close temporal correlation could be demonstrated between the time-courses of cyclic AMP accumulation and the development of relaxation in BTSM strips. Thus, maximal increases in cyclic AMP accumulation and relaxant activity were only observed to occur 30–40 min after Salm addition, whereas Salb evoked second messenger and functional responses within 5 min of addition to strips.

Although the rates of cyclic AMP accumulation and the relaxant action of Salm and Salb were well-correlated, the maximal relaxant effect of Salb (79.3 ± 14.0% at 5 min after Salb addition) was substantially greater than that induced by Salm (26.2 ± 6.0% at 33 min after Salm addition). In the strip preparation the peak increase in cyclic AMP accumulation was greater than that observed in the presence of Salm; however, it is also possible that, at least with respect to spasmolytic actions, the magnitude of the initial relaxant response may be governed, not only by the extent to which cellular cyclic AMP increases, but also by the rate at which such an increase occurs. The idea that the rate of generation of a second messenger (or indeed an increased flux without a detectable change in second messenger concentration (Deeg *et al.*, 1988), can encode information has been hypothesized (Levitzki & Barsinai, 1991). Studies comparing agents such as Salm and Salb, which differ markedly in the times taken for onset of action, might prove particularly useful in exploring whether 'kinetic' as well as amplitude encoding of signalling information occurs with respect to the adenylyl cyclase/cyclic AMP system.

The time-courses and concentration dependencies of cyclic AMP accumulation stimulated by Salm, Salb and Iso were also investigated in BTSM slices. Although a relatively slow onset of action of Salm was also observed in this preparation, the discrepancy between Salm, Salb and Iso was noticeably less than that observed in tissue strips. These data, taken together with the observation of McCrea & Hill (1993) that the rate at which Salm causes an increase in cyclic AMP accumulation in neuroblastoma cell monolayers approaches that of Iso, suggest that the onset of Salm action depends to a considerable extent on the tissue preparation being examined. Thus, it is likely that

Table 1 Inhibition of histamine (Hist)-stimulated accumulation of [^{3}H]-InsP by β-adrenoceptor agonists in BTSM slices

β-Agonist	Concentration (M)	Accumulation of [^{3}H]-InsP (d.p.m. mg^{-1} protein) −Hist	+Hist	% inhibition of Hist-stimulated response
Control	–	1820 ± 240	10929 ± 1286	–
Salm	10^{-7}	1821 ± 649	5186 ± 328	63.7 ± 3.2
Salb	10^{-6}	2085 ± 374	5839 ± 920	60.2 ± 9.9
Iso	10^{-5}	2124 ± 528	4249 ± 509	71.5 ± 5.9

[^{3}H]-inositol pre-labelled tissue was incubated for 20 min in the absence or presence of β-adrenoceptor agonist prior to a 30 min incubation with 0.1 mM Hist. Values represent mean ± s.e. mean for 3 separate experiments each performed in triplicate.

the onset of Salm-evoked actions will be highly dependent on the structural complexity of the system under investigation, with onset times for Salm in simple systems (e.g. cell monolayers or suspensions of single cells) approaching those of other β-adrenoceptor agonists.

Previous functional studies have shown that Salm relaxes electrically, or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced tone in guinea-pig isolated trachealis (Ball *et al.*, 1991; Nials *et al.*, 1993b) and inherent or $PGF_{2\alpha}$-induced tone in human bronchial smooth muscle (Ball *et al.*, 1991; Nials *et al.*, 1993a) more potently than either Salb or Iso, whilst generally exhibiting a lower efficacy. The partial agonist activity of Salm relative to Iso, has been illustrated in a number of other studies (Dougall *et al.*, 1991; Chiu *et al.*, 1993; Källström *et al.*, 1994). Here we have shown that relative to Salb, Salm is more potent in stimulating a concentration-dependent increase in cyclic AMP accumulation in BTSM (EC_{50}s: Salm 5.3 [1.8–15.2]; Salb 169 [99–290] nM). Although a maximally-effective concentration of Salm consistently caused a slightly smaller increase in cyclic AMP accumulation compared with that caused by either Salb or Iso, the differences were not statistically significant. This latter finding differs from that of McCrea & Hill (1993) who demonstrated that, whilst Salm was more potent than Iso on cyclic AMP accumulation in B50 neuroblastoma cells, maximally-effective concentrations of Salm elicited an increase in cyclic AMP accumulation which was only 46% of that stimulated by Iso. Previous studies, notably by Lemoine and colleagues, have demonstrated that activation of a small fraction of the β-adrenoceptor population is sufficient to elicit a maximal cyclic AMP response in calf trachealis (Lemoine *et al.*, 1989). Thus, it is perhaps not surprising that Iso, Salb and Salm can elicit similar maximal increases in cyclic AMP accumulation.

In addition to comparing the spasmolytic effects of Salm and Salb, we have also investigated the anti-spasmogenic actions of these agents. Thus, BTSM strips were equilibrated with different concentrations of Salm or Salb before the contractile response to addition of incremental concentrations of MCh or Hist was assessed. Both agents were more effective against contractile responses to Hist, with increasing concentrations of Salm or Salb-progressively shifting concentration-effect curves to the right and suppressing the degree of BTSM contraction at high concentrations of Hist (1 mM). In general, the anti-spasmogenic actions of Salm and Salb are consistent with their more widely reported spasmolytic effects. Thus, 1 nM Salm significantly affected the Hist concentration-effect curve and a maximal anti-spasmogenic action was approached at 10 nM Salm; in contrast, 1 nM Salb was without effect and 100 nM Salb was required to suppress maximally Hist-stimulated contraction. Despite clear indication that Salm is a more potent anti-spasmogenic agent than Salb, the latter exerted a much greater effect on Hist-induced contraction at a maximally effective concentration, suggesting efficacy differences between Salm and Salb with respect to anti-spasmogenic activity.

Multiple mechanisms have been proposed to explain how β-adrenoceptor agonist-stimulated increases in cyclic AMP concentration cause relaxation of airways smooth muscle (Giembycz & Raeburn, 1991). One mechanism of potential significance is the ability of β-adrenoceptor agonists (and other agents which increase cyclic AMP levels) to inhibit Hist-stimulated inositol phospholipid hydrolysis (Hall & Hill, 1988; Madison & Brown, 1988; Hall *et al.*, 1989). These initial reports hypothesized that inhibition of inositol phospholipid turnover might be extrapolated to infer an effect on the concentration of inositol 1,4,5-trisphosphate, a second messenger generally acknowledged to be responsible for the initiation of pharmacomechanical coupling in airways smooth muscle (Chilvers *et al.*, 1994a). Although more recent studies have not supported this idea (see Challiss *et al.*, 1993), it is likely that the ability of β-adrenoceptor agonists to inhibit a spasmogen-stimulated signal transduction pathway will contribute, to a greater or lesser degree, to the relaxant activity of these agents.

In the present study, we have shown that Salm, Salb and Iso can each inhibit Hist-stimulated [^{3}H]-InsP accumulation to approximately similar extents. Salm was about 10 fold more potent than Salb in evoking this response and concentration-effect curves for inhibition of Hist-stimulated [^{3}H]-InsP accumulation by both Salm and Salb were to the left relative to the concentration-dependencies of these agents to stimulate cyclic AMP accumulation in BTSM, suggesting that sub-maximal increases in cyclic AMP are sufficient to inhibit maximally Hist-stimulated [^{3}H]-InsP accumulation.

A substantial component of the Hist-stimulated phosphoinositide-specific phospholipase C (PI-PLC) activity is lost on removal of extracellular Ca^{2+} (Chilvers *et al.*, 1994b). This suggests that the enhancement of PI-PLC activity by Hist is dependent on both a direct receptor-G protein-mediated activation of PI-PLC activity by increased sarcoplasmic Ca^{2+} brought about by direct or indirect activation of Ca^{2+}-influx pathway(s) by Hist. It has been suggested that the ability of β-adrenoceptor agonists to inhibit Hist-stimulated [^{3}H]-InsP accumulation arises as a consequence of the membrane hyperpolarizing action of these agents which reduce voltage-sensitive Ca^{2+}-influx in this tissue (Challiss *et al.*, 1993). Thus in common with K^+-channel openers, the inhibitory effects of β-adrenoceptor agonists on Hist-stimulated [^{3}H]-InsP accumulation can be completely negated by preventing hyperpolarization (Challiss, 1992; Challiss *et al.*, 1992). It is interesting to note that recent studies have provided evidence that whilst Salb and Iso can cause membrane hyperpolarization of guinea-pig trachealis (Cook *et al.*, 1993) and increased Rb^+-efflux from bovine trachealis (Chiu *et al.*, 1993), Salm evokes neither of these actions. Thus, whether Salm causes inhibition of Hist-stimulated [^{3}H]-InsP accumulation by a different mechanism to that of Salb and Iso remains to be elucidated.

In summary, the present study has demonstrated that the slow onset of Salm-induced relaxation of MCh-supported tone is closely correlated with a similarly slow increase in cellular cyclic AMP levels in bovine trachealis. In this tissue, Salm appears to be more potent in relaxing MCh-supported tone (spasmolytic activity) and opposing contraction caused by either MCh or Hist (anti-spasmogenic activity) than Salb. However, in common with previous studies into the relative spasmolytic activities of Salm and Salb (Ball *et al.*, 1991; Nials *et al.*, 1993a,b), Salb evoked greater maximal effects on con-

tractile responses to MCh and Hist compared to those of Salm. These data lend further support to the interpretation that Salm is a potent β_2-adrenoceptor agonist exhibiting a lower intrinsic efficacy than Salb (Dougall *et al.*, 1991).

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