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SHORT COMMUNICATION

Dissociation Between β-Adrenoceptor-Mediated Cyclic AMP Accumulation and Inhibition of Histamine-Stimulated Phosphoinositide Metabolism in Airways Smooth Muscle

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ABSTRACT. Spasmogen-stimulated phosphoinositide hydrolysis represents one of the major signalling pathways mediating pharmacomechanical coupling in airways smooth muscle (ASM), and cyclic AMP-induced inhibition of phosphoinositidase C has been proposed as an important mechanism underlying the bronchodilator properties of β_2-adrenoceptor agonists. To examine this hypothesis in more detail we have undertaken a direct comparison of the effects of salbutamol and salmeterol, short- and long-acting β_2-adrenoceptor agonists respectively, on cyclic AMP accumulation and histamine-stimulated [^{3}H]-inositol phospholipid hydrolysis in bovine tracheal smooth muscle (BTSM) slices. Although salmeterol displayed a similarly greater potency over salbutamol for both stimulation of cyclic AMP, and inhibition of [^{3}H]-inositol phosphate accumulation, there was a clear disparity between these agents with respect to both their efficacies and the duration of their effects. Hence while salmeterol caused a more protracted, but initially smaller increase in cyclic AMP accumulation compared to salbutamol, the inhibition of histamine-stimulated [^{3}H]-inositol phosphate accumulation observed with salmeterol was of identical duration to salbutamol and was more marked than that of salbutamol at early time points. These data suggest that cyclic AMP accumulation is not the sole mechanism responsible for β_2-adrenoceptor-induced inhibition of phosphoinositide turnover in BTSM, and would support a recent proposal that cyclic AMP-dependent inhibition of agonist-stimulated Ca^{2+} mobilization in ASM may be mediated by factors independent of inositol phosphate generation. BIOCHEM PHARMACOL 53;10:1565-1568, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Airways smooth muscle; β_2-adrenoceptors; H_1-histamine receptors; salmeterol; salbutamol; cyclic AMP; inositol phosphates

β_2-adrenoceptor agonists remain one of the most important groups of drugs used in the management of asthma and other forms of chronic airflow obstruction. While the precise mechanism(s) underlying the bronchodilator properties of these agents remains unknown [1], it has been suggested that the ability of these agents, together with membrane permeant cyclic AMP analogues and cyclic nucleotide phosphodiesterase inhibitors, to attenuate agonist-stimulated phosphoinositidase C (PIC) activation may be an important factor. However, the bronchoconstrictor agonist selectivity of this effect with, for example, muscarinic cholinoceptor-mediated PIC responses being largely resistant to such inhibition [2, 3], and recent data implicating cyclic AMP-dependent and -independent modulation of K_{Ca} channel activity in the bronchodilator action of β_2-adrenoceptor agonists [see 1], has led to a need to reassess the importance of cyclic AMP/cyclic AMP-dependent protein kinase-PIC interactions in airways smooth muscle (ASM).

Salmeterol has been developed as a highly selective and long-acting β_2-adrenoceptor agonist which, in contrast to short-acting agents such as salbutamol and terbutaline, induces a persistent non-desensitizing relaxation of ASM *in vitro* and long-lasting (>12 hr) bronchodilatation *in vivo* [4]. In radioligand binding studies, salmeterol displays non-competitive and high-affinity binding, and as a consequence of possessing a long lipophilic side-chain, does not readily dissociate from its 'exosite' binding domain [4]. In this study we have exploited the differences in efficacy and duration of action of salmeterol and salbutamol to assess the importance of cyclic AMP-induced inhibition of agonist-stimulated phosphoinositide hydrolysis to the relaxant effects of β_2-adrenoceptor agonists.

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Abbreviations: ASM, airways smooth muscle; PIC, phosphoinositidase C; BTSM, bovine tracheal smooth muscle; $InsP_x$, total inositol phosphate pool; cAMP, adenosine cyclic-3',5'-monophosphate; TCA, trichloroacetic acid.

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MATERIALS AND METHODS

Materials

Myo-[^{3}H]-inositol (12–20 Ci/mmol) and [^{3}H]-cyclic AMP (ammonium salt) (25–40 Ci/mmol) were purchased from DuPont (UK) Ltd., (Stevenage, UK). Dowex AG1-X8 (200–400 mesh, formate form) was obtained from BioRad Laboratories Ltd., (Watford, UK). Tissue culture supplies and M199 medium were obtained from Life Technologies (Paisley, UK). Histamine dihydrochloride, carbachol, 3-isobutyl-1-methylxanthine, tri-n-octylamine and 1,1,2-trichlorotrifluorethane were purchased from Sigma Chemical Co., (Poole, UK). Salmeterol and salbutamol were gifts from Dr. M. Johnson, Glaxo–Wellcome Research and Development Ltd., (Middlesex, UK). All other reagents were obtained from commercial sources and were of analytical grade.

Tissue Preparation and [^{3}H]-Inositol Labelling

BTSM slices (300 × 300 μm) were prepared using a McIlwain tissue-chopper as detailed previously [5] and incubated for 60 min at 37°C in oxygenated Krebs-Henseleit buffer (KHB, in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.2, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.7). The slices were then washed in M199 tissue culture medium and labelled with *myo*-[^{3}H]-inositol by adding 50 μl aliquots of gravity-packed slices to 930 μl M199 containing 1 μCi [^{3}H]-inositol in 24 well tissue culture plates for 24 hr in a 5% CO_2 incubator. This protocol permits near steady-state radiolabelling of the agonist-sensitive phosphoinositide pool [5].

β_2-Agonist-Induced Inhibition of Histamine-Stimulated [^{3}H]-InsP$_x$ Accumulation

Following a 24 hr incubation with [^{3}H]-inositol, LiCl (final concentration 10 mM) was added and the BTSM slices exposed to salmeterol (1 pM–100 nM), salbutamol (1 nM–1 μM) or vehicle for 30 min prior to addition of histamine (1 mM). After a 30 min spasmogen-treatment period, incubations were terminated by the addition of 200 μl 3 M ice-cold trichloroacetic acid (TCA). Samples were placed on ice for 20 min, the contents transferred to plastic insert vials, vortexed and centrifuged (2000 × *g*, 20 min, 4°C). Samples were neutralized using EDTA/1,1,2-trichlorotrifluoroethane/tri-n-octylamine as previously detailed [5] and total [^{3}H]-InsP$_x$ (comprising [^{3}H]-InsP$_{1-4}$) separated by anion-exchange chromatography [5].

β_2-Agonist-Induced Cyclic AMP Accumulation

BTSM slices were prepared, pre-incubated in KHB, and incubated overnight in M199 prior to incubation with salmeterol (100 nM), salbutamol (5 μM) or vehicle for the times shown. Reactions were stopped at the times indicated using TCA and neutralized extracts prepared as detailed above. Histamine was added 30 min and, where indicated, IBMX (100 μM) was added 20 min before termination. Cyclic AMP was measured by the method of Brown *et al.* [6] with tissue pellets solubilized in 2 M NaOH to allow the protein content to be determined.

In experiments examining the duration of the effect of salmeterol and salbutamol on [^{3}H]-InsP$_x$ and cAMP accumulation, BTSM were incubated in the presence ([^{3}H]-InsP$_x$ samples) or absence (cyclic AMP) of [^{3}H]-inositol for 24 hr and then treated with buffer, salmeterol (100 nM) or salbutamol (5 μM) under identical conditions for 0.5, 1, 3, 6, 12 or 24 hr before determining cyclic AMP levels and histamine-stimulated [^{3}H]-InsP$_x$ accumulation.

Data Analysis

[^{3}H]-InsP$_x$ accumulations were calculated as dpm/50 μl BTSM slices and cyclic AMP values as pmol/mg protein. IC_{50} values are expressed as geometrically-derived means. All values are presented as means ± SEM for duplicate or triplicate determinations performed in at least $N = 3$ separate experiments. Statistical comparisons of values were performed using the Student's *t*-test for unpaired observations with $P < 0.05$ considered to be significant.

RESULTS AND DISCUSSION

Our previous studies in BTSM have demonstrated that salmeterol causes a concentration-dependent increase in cyclic AMP accumulation with a potency (EC_{50} 5.3 nM) that is 30-fold greater than salbutamol (EC_{50} 169 nM) [7]. In the current study we demonstrate that salmeterol displays a similar potency differential over salbutamol for inhibition of histamine-stimulated [^{3}H]-InsP$_x$ accumulation ($-\log EC_{50}$ (M) 9.717 ± 0.4 (0.29 nM) and 7.998 ± 0.191 (10.8 nM) respectively; see Fig. 1). At this early time-point (30 min), salmeterol also causes a significantly greater maximal effect in inhibiting [^{3}H]-InsP$_x$ accumulation compared to salbutamol (61.3 ± 2.7 and 47.3 ± 1.0% inhibitions, respectively; $P < 0.05$; Fig. 1). However, examination of the effects of these agents on cyclic AMP accumulation at 30 min demonstrates a greater efficacy of salbutamol over salmeterol (Fig. 2a, see also [7]). Furthermore, whereas cyclic AMP returns to basal levels by 3 hr in response to salbutamol, cyclic AMP levels remain elevated for >6 hr post salmeterol addition (Fig. 2a). However, despite this clear difference in the longevity of the cyclic AMP response (which correlates closely with the duration of the relaxant properties of these β_2-agonists [7]), no difference was observed in the time-course of salmeterol and salbutamol-induced inhibition of histamine-stimulated [^{3}H]-InsP$_x$ accumulation (Fig. 2b). Thus, at 6 hr after salbutamol or salmeterol addition, significant 25–30% inhibitions of the agonist-stimulated phosphoinositide response were observed.

In a further series of experiments, BTSM slices were incubated with 100 nM salmeterol or 5 μM salbutamol for 0.5–12 hr, with 100 μM IBMX added 20 min prior to

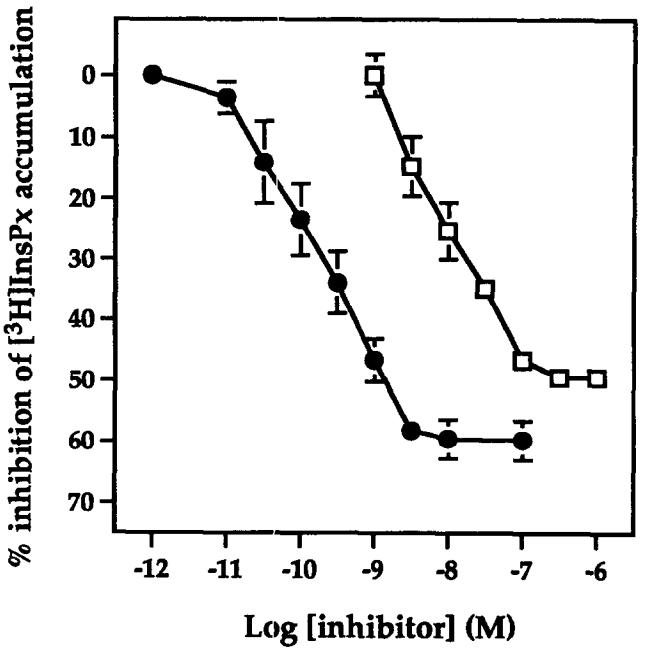
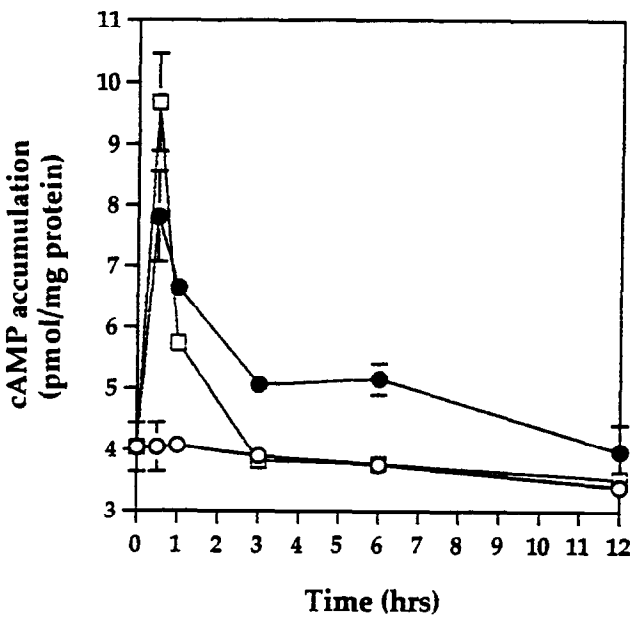


FIG. 1. Concentration-dependence of salmeterol and salbutamol inhibition of histamine-stimulated [^{3}H]-InsP$_x$ accumulation in BTSM slices. BTSM slices were preincubated in oxygenated KH buffer for 60 min and 50 µl portions transferred to 24 well tissue culture plates containing 1 mL M199 and 1 µCi [^{3}H]-inositol. After a 24 hr incubation at 37°C, 10 mM LiCl plus salmeterol (1 pM–100 nM, filled circles), salbutamol (1 nM–1 µM, open squares) or buffer were added followed after 15 min by 1 mM histamine. After 30 min the reactions were terminated and [^{3}H]-InsP$_x$ separated as detailed in the text. Data are expressed as % inhibition of histamine-stimulated [^{3}H]-InsP$_x$ accumulation measured in the absence of β_2-agonists (Control, 5703; +histamine, 133568 dpm/50 µl BTSM slices). Values represent means ± SEM for 5 separate experiments, each performed in triplicate.



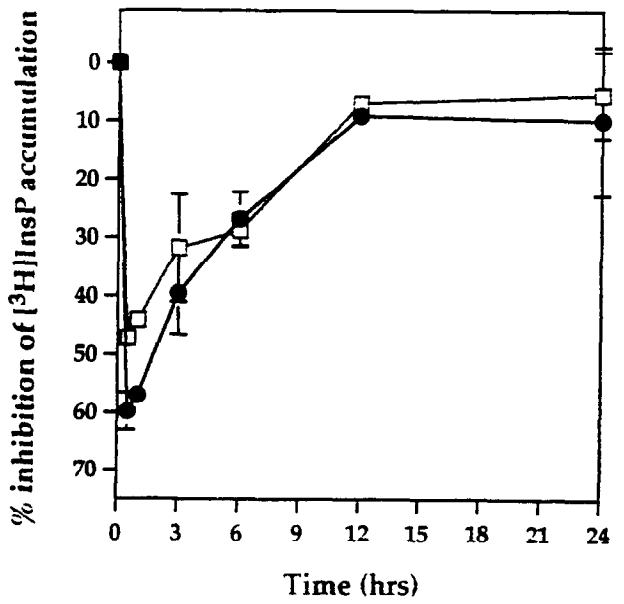


FIG. 2. Time-course for salmeterol- and salbutamol-stimulated cyclic AMP accumulation and inhibition of histamine-stimulated [^{3}H]-InsP$_x$ accumulation in BTSM slices. BTSM slices were incubated in 1 mL M199 in the presence or absence of 2.5 µCi [^{3}H]-inositol for 24 hr prior to the addition of 100 nM salmeterol (filled circles), 5 µM salbutamol (open squares) or vehicle (open circles) for 0.5–24 hr. At the time points indicated, reactions were either terminated for determination of cyclic AMP levels (a), or the BTSM slices stimulated for 30 min with histamine (1 mM) in the presence of 10 mM LiCl prior to extraction and quantification of [^{3}H]-InsP$_x$ accumulation (b) (expressed as % inhibition of histamine-stimulated [^{3}H]-InsP$_x$ accumulation over control values measured at each time point in the absence of β_2-agonist). Values represent means ± SEM for 3 experiments, each performed in triplicate.

measurement of cyclic AMP levels. The addition of IBMX caused a significant increase in cyclic AMP accumulation in control, salmeterol and salbutamol samples at all times, however the increases in cyclic AMP levels observed at 3 and 6 hr with salbutamol were not significantly different to control samples (Table 1); this contrasts with the effect of salmeterol where addition of IBMX at 3 or 6 hr caused a significant increase in cyclic AMP levels over vehicle-control values (Table 1). This indicates that the return of cyclic AMP levels to control values 3 hr post salbutamol addition (Fig. 2a) reflects essentially complete loss of salbutamol-stimulated cyclic AMP formation, rather than continuing formation with enhanced metabolism and increased cyclic AMP flux. The similar time-courses of salmeterol and salbutamol-induced inhibition of histamine-stimulated [^{3}H]-InsP$_x$ accumulation are also unlikely to be explained by the requirement for a threshold level of cyclic AMP to observe such an effect, since both salbutamol and salmeterol display considerably greater potency with respect to inhibition of [^{3}H]-InsP$_x$ accumulation compared to cyclic AMP elevation.

The argument for cyclic AMP-mediated inhibition of [^{3}H]-InsP$_x$ accumulation being a central mechanism for β_2-adrenoceptor-mediated relaxation of ASM is weakened further by the observation that muscarinic cholinoceptor-

TABLE 1. 12 hr time-course of cyclic AMP-stimulatory effects of salmeterol and salbutamol in the absence and presence of acute (20 min) phosphodiesterase inhibition by IBMX

Time (h)	± IBMX	Cyclic AMP (pmol/mg protein) Vehicle	+salbutamol (5 μM)	+salmeterol (0.1 μM)
0.5	−	3.8 ± 0.3	9.6 ± 1.0§	8.5 ± 0.8§
	+	13.4 ± 1.7†	41.6 ± 7.0*,‡	35.1 ± 3.7†,§
3	−	3.9 ± 0.6	4.8 ± 1.2	6.4 ± 0.8‡
	+	14.9 ± 2.0†	20.4 ± 3.6*	25.8 ± 3.2†,‡
6	−	3.6 ± 0.5	3.7 ± 1.1	5.5 ± 1.0‡
	+	15.1 ± 1.3†	14.7 ± 2.0*	23.4 ± 3.0†,‡
12	−	4.1 ± 1.0	4.2 ± 0.8	4.7 ± 1.2
	+	13.1 ± 1.7†	12.7 ± 2.1*	15.3 ± 3.9*

BSTM slices were prepared and maintained overnight in a CO_2 incubator at 37°C (see Materials and Methods Section) before challenge with either salmeterol, salbutamol or vehicle for the time indicated. Incubations were stopped at 0.5, 3, 6 or 12 hr. Histamine (100 μM) was added at 30 min, and where indicated IBMX (100 μM) was added at 20 min, before termination. Data are shown as means ± SE mean for 4 separate experiments performed in duplicate or triplicate. Statistical significance (Student's *t*-test) is indicated as: * significantly different +IBMX vs −IBMX, (* $P < 0.05$; † $P < 0.01$); ‡ Significantly different from vehicle control, (‡ $P < 0.05$; § $P < 0.01$).

stimulated phosphoinositide turnover is largely resistant to inhibition by cyclic AMP-elevating agents including salbutamol [2, 3], an effect which cannot be explained solely on the basis of muscarinic receptor reserve [8]. In this study, salmeterol likewise failed to inhibit [^{3}H]-$InsP_x$ accumulation elicited by the muscarinic agonist carbachol ([^{3}H]-$InsP_x$, dpm/50 μl BTSM slices/30 min: control 2932 ± 472100 nM, salmeterol 2677 ± 127100 nM, carbachol 185467 ± 29550, carbachol + salmeterol 183615 ± 34095; $N = 4$). Hence we have been able to dissociate the cyclic AMP-elevating properties of β_2-adrenoceptor agonists from their ability to inhibit phosphoinositide hydrolysis on the basis of differences in potency, efficacy, duration of action and contractile agonist sensitivity. While cyclic AMP activation of cyclic AMP-dependent protein kinase can undoubtedly cause phosphorylation and inhibition of PIC activity, these data support the view of Hoiting *et al.* [9] that β_2-adrenoceptor-induced relaxation in ASM, and in particular the prolonged spasmolytic effect of salmeterol, is not due to cyclic AMP-dependent inhibition of agonist-stimulated phosphoinositide hydrolysis.

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