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IN VITRO ACTION OF COMBINED SALBUTAMOL AND THEOPHYLLINE ON ANAPHYLACTIC CONTRACTIONS, MEDIATOR RELEASE AND CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE IN LUNG PARENCHYMA

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The mechanisms involved in the anti-asthmatic action of combined bronchodilator therapy was studied by determining the effects of combined salbutamol and theophylline on anaphylactic contractions, histamine release, prostaglandin (PG)$F_{2\alpha}$ release, cyclic 3′,5′-adenosine monophosphate (c-AMP) and smooth muscle tone in guinea-pig peripheral airways in vitro. Combined, salbutamol (3×10^{-8} M) and theophylline (3×10^{-4} M) markedly inhibited anaphylactic contractions (85.8%) in lung strips. The inhibition of anaphylactic contractions was significantly greater than inhibition of histamine and $PGF_{2\alpha}$-induced contractions. Histamine release was reduced by 66.1% but $PGF_{2\alpha}$ was not significantly altered. Increased c-AMP was observed with combined salbutamol (3×10^{-8} M) and theophylline (3×10^{-4} M) in the absence of antigen. The combined effect of salbutamol and theophylline was always greater than the sum of their individual effects. The results demonstrate that in peripheral airways theophylline potentiates the action of salbutamol both directly via smooth muscle relaxation and indirectly via inhibition in mediator release.

c-AMP Anaphylaxis Bronchodilators Lung strips Mediator release

1. Introduction

In asthma airway calibre is determined largely by smooth muscle tone, which in turn is under the influence of chemical mediators released from mast cells during anaphylaxis. β-Adrenoceptor agonists and phosphodiesterase inhibitors relax tracheal and bronchial smooth muscle and they inhibit anaphylactic mediator release from leucocytes (Lichtenstein and Margolis, 1968; Bourne et al., 1972), peritoneal cells (Koopman et al., 1970) and lung (Schild 1936; Assem and Schild, 1971; Orange et al., 1971; Ishizaka et al., 1972). The action of these drugs on muscle tone and mediator release is associated with an increase in cyclic 3′,5′-adenosine-monophosphate (c-AMP) in the cells (Orange et al., 1971; Bourne et al., 1972; Katsuku and Murad, 1977; Triner et al., 1977).

Theophylline is known to potentiate the effect of β-adrenoceptor agonists on tracheal smooth muscle tone and on anaphylactic mediator release from parenchyma (Koopman et al., 1970; Orange et al., 1971; Lefcoe et al., 1975) presumably by increasing intracellular levels of c-AMP. Moreover, theophylline may potentiate the clinical effectiveness of terbutaline and isoprenaline in asthma therapy (Campbell et al., 1977; Wolfe et al., 1978). It has not been possible, however, to convincingly demonstrate the mechanisms of action of combined drug treatment because in vitro tension and mediator release have not been measured in the one preparation of airways. In this study some of the

factors controlling the tone of peripheral airways smooth muscle have been investigated in guinea-pig lung parenchyma. The effect of combined salbutamol and theophylline on anaphylactic contractions in lung parenchymal strips has been measured. Also the effect of the drugs on the release of two endogenous mediators, histamine and prostaglandin (PG)$F_{2\alpha}$, smooth muscle tone and c-AMP levels in the lung parenchyma has been concurrently determined.

2. Materials and methods

2.1. Measurement of tension in superfused lung strips from sensitised guinea-pigs

Isolated lung parenchymal strips were prepared and superfused with Krebs solution for recording tension as described by Mitchell and Denborough (1979). Guinea-pigs weighing between 440 and 770 g were used. All animals were sensitised to bovine serum albumin (BSA) in Freund's complete adjuvant. The animals were boosted after 7 days and used 3 to 4 weeks after initial sensitisation.

2.2. Measurement of histamine and prostaglandin (PG)$F_{2\alpha}$ release

Anaphylactic mediator release was measured from the lung parenchyma which was not used for lung strips (see above). The lung was chopped, then incubated in gassed (95%O_2/5%CO_2) Krebs solution for 1 h. The incubation solution was withdrawn by pipette, then replaced with fresh solution, every 15 min until the drug or BSA additions. The final incubation solution containing the released mediators was divided for histamine (Mitchell and Denborough, 1979) and $PGF_{2\alpha}$ assay.

$PGF_{2\alpha}$ was assayed by radioimmunoassay. Goat anti-$PGF_{2\alpha}$ serum was generously supplied by Dr. K.T. Kirton, Upjohn Company. Rabbit anti-goat serum was raised by immunising rabbits s.c. with 1 ml of 5 mg/ml aqueous solution of goat anti-$PGF_{2\alpha}$ emulsified in Freund's complete adjuvant. Booster injections of 1 mg were given at weekly intervals. The rabbits were bled at 7 weeks. The assay procedure was as follows — 0.6 ml Tris—HCl buffer (pH 8.0) was added to duplicate Eppendorf tubes along with 0.2 ml of the sample to be assayed. 0.1 ml 100 pg/ml labelled $PGF_{2\alpha}$ ({5,6,8,11,12,14,15,(n)-^{3}H}-$PGF_{2\alpha}$) was added and this was followed by 0.1 ml goat anti-$PGF_{2\alpha}$ (final dilution 1 : 1000). After 1 h at 20—25°C 0.1 ml rabbit anti-goat serum was added (final dilution 1 : 50). After 16 h at 4°C the tubes were centrifuged (8000 × *g*, 2 min) and the radioactivity (cpm) in 0.1 ml supernatant counted in a Packard Tri-Carb Liquid Scintillation counter. Bray's scintillant (Bray, 1960) was used. Standards containing known amounts of $PGF_{2\alpha}$ were prepared with each assay. The cpm in the samples and standards were expressed as a percentage of the cpm in blanks (containing no goat anti-$PGF_{2\alpha}$ serum). The percent labelled $PGF_{2\alpha}$ bound was then calculated:

$$1 - \frac{\text{cpm in sample or standard}}{\text{cpm in blank}} \times 100.$$

The non-specific binding of labelled $PGF_{2\alpha}$ to serum from control, non-immunised rabbits was measured in each assay and did not exceed 2.5%. The threshold sensitivity (Dighe et al., 1975) of the assay averaged 90 pg $PGF_{2\alpha}$. The relative cross-reactivity of other prostaglandins for the goat anti-$PGF_{2\alpha}$ serum is as follows: $PGF_{2\alpha}$, 100%; $PGF_{1\alpha}$, 54%; 15-keto $PGF_{2\alpha}$, 0.2%; PGE_1, <0.04%; PGE_2, <0.04%.

2.3. Measurement of c-AMP

The c-AMP content of the incubated lung tissue was determined using a kit (The Radiochemical Centre, Amersham) which was based on competitive protein binding. Immediately after the incubation period the lung tissue was placed in an ice bath and then the lung was homogenized in 0.5 ml ice-cold

trichloracetic acid (5%) using a Duall 5-ml glass tissue grinder. The homogenate was centrifuged (8000 × *g*, 2 min) and the pellet was saved for protein estimation. The supernatant was washed 6 times with 5 ml water-saturated ether to remove the trichloracetic acid. The samples, containing c-AMP were lyophilised and then resuspended in 0.5 ml Tris—EDTA buffer (pH 7.5) prior to assay.

2.4. Measurement of protein

The pellet (see above) was digested in 5% NaOH and the protein estimated by the method of Lowry et al. (1951). Histamine and $PGF_{2\alpha}$ release and the c-AMP contents were expressed as units per mg protein.

2.5. Drugs and treatment of data

The drugs used were: salbutamol sulphate (Glaxo Australia), theophylline (Sigma), acetylcholine chloride (Sigma), propranolol hydrochloride (Sigma), indomethacin (Sigma), BSA (Fr V, Armour Pharmaceuticals), Freund's complete adjuvant (Commonwealth Serum Laboratories), histamine acid phosphate (Sigma), $PGF_{2\alpha}$ (Upjohn), {5,6,8,11,12,14,15, (n)-^{3}H} $PGF_{2\alpha}$, 100 μCi/ml (The Radiochemical Centre, Amersham).

In section 3.1 each experiment was done using two lung samples or two lung strips in three animals. Where chemical or radioimmunological tests were carried out each measurement was made in duplicate or triplicate and then meaned. Statistical analyses were undertaken with Student's *t*-test.

3. Results

3.1. Effect of salbutamol and theophylline on anaphylactic contractions, mediator release and c-AMP in lung parenchyma

Salbutamol and theophylline were given 15 min before, and during, challenge with BSA because this time was found to be sufficient for maximal relaxation of drug-induced contractile responses in lung strips.

Salbutamol (3×10^{-8} M) partially inhibited anaphylactic contractions (1 mg/ml BSA for 5 min) in lung parenchymal strips whereas theophylline (3×10^{-4} M) did not. When salbutamol and theophylline were combined the effect of salbutamol was significantly potentiated ($P < 0.02$), (table 1, fig. 1). The inhibitory effect of salbutamol was blocked by 10^{-7} M propranolol.

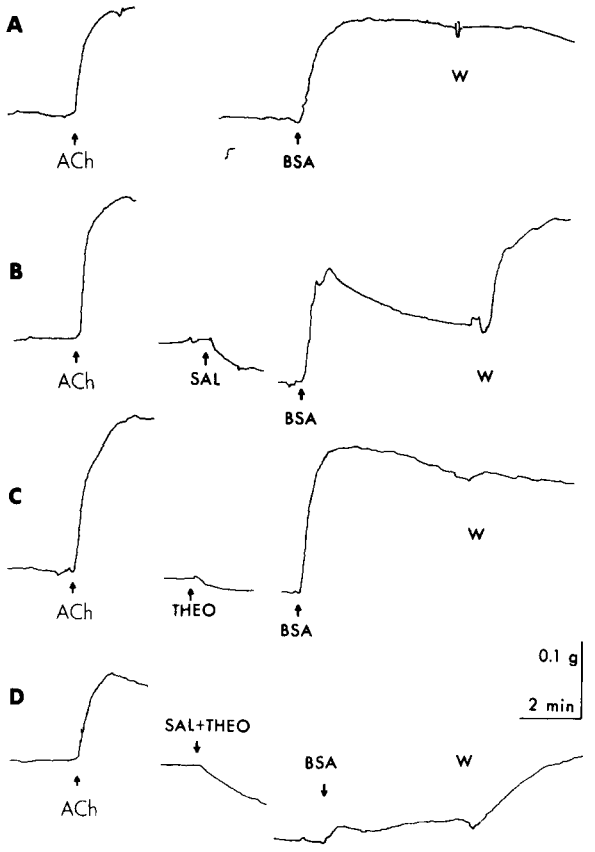

Fig. 1. Example tracings showing the effect of salbutamol and theophylline on anaphylactic contractions in four guinea-pig superfused lung strips. A, control anaphylactic response to 1 mg/ml BSA (BSA); B, effect of 3×10^{-8} M salbutamol (SAL); C, effect of 3×10^{-4} M theophylline (THEO); D, effect of combined salbutamol and theophylline (SAL + THEO). The lung strips were superfused with fresh Krebs solution at W. The effect of a maximal concentration of acetylcholine (ACh, 10^{-3} M) was first tested in each preparation.

TABLE 1

Effect of relaxant drugs on tension, histamine release, $PGF_{2\alpha}$ release, and c-AMP, in lung parenchyma.

Treatment	Tension [1]	Histamine release [2]	$PGF_{2\alpha}$ release [3]	c-AMP [4]
None			53.9 ± 14.4	3.54 ± 0.30 *
Control BSA	78.8 ± 3.8	13.0 ± 2.0	90.5 ± 18.1	6.36 ± 0.90
BSA + salbutamol (3×10^{-8} M)	49.4 ± 11.0 *	9.1 ± 1.6	45.6 ± 8.3	6.70 ± 1.15
BSA + theophylline (3×10^{-4} M)	79.2 ± 10.2	10.3 ± 2.5	52.5 ± 10.6	6.48 ± 0.79
BSA + salbutamol + theophylline	11.1 ± 5.4 ***	4.4 ± 2.1 *	42.5 ± 8.0	6.42 ± 0.85

[1] Percentage maximal acetylcholine (10^{-3} M) response.
[2] ng/mg protein.
[3] pg/mg protein.
[4] pmol/mg protein.
Means ± S.E., n = 6.
* $P < 0.05$
*** $P < 0.001$ } compared to 'control BSA'.

Concurrent with the anaphylactic contractions BSA (1 mg/ml for 10 min) caused an increase ($P < 0.01$) in histamine release (from 14.2 ± 1.7 to 27.2 ± 2.9 ng/mg protein) from chopped lung parenchyma and a small increase in $PGF_{2\alpha}$ release but this was not significant ($P > 0.1$) (table 1). Combined salbutamol and theophylline inhibited histamine release by about 66%. Separately the drugs did not significantly alter histamine release. The small increase in $PGF_{2\alpha}$ release with BSA was not seen when the relaxant drugs were present (table 1).

The c-AMP content of the lung tissue was doubled ($P < 0.05$) by BSA (table 1) but there was no change in the level when salbutamol and theophylline were included with the BSA. An experiment was done omitting BSA from the medium in order to determine whether the rise in c-AMP elicited by BSA masked drug-induced changes in c-AMP. The basal c-AMP level was 3.91 ± 0.27 pmol/mg protein (n = 8). Neither salbutamol nor theophylline significantly altered these levels (3.60 ± 0.34 and 4.77 ± 0.40 pmol/mg protein, respectively; n = 8); however when

TABLE 2

Effect of relaxant drugs and indomethacin on tension, c-AMP, and $PGF_{2\alpha}$ release in lung parenchyma.

Treatment	Tension [1]	c-AMP [2]	$PGF_{2\alpha}$ release [3]
None		5.20 ± 0.79	92.3 ± 30.3
Control BSA	70.4 ± 10.5	5.11 ± 0.90	89.2 ± 13.2
BSA + salbutamol (10^{-7} M)	8.1 ± 3.8 ***	6.42 ± 0.73	—
BSA + theophylline (10^{-3} M)	25.3 ± 13.1 *	7.87 ± 1.06	—
BSA + salbutamol + theophylline	6.5 ± 4.0 ***	9.00 ± 0.84 **	—
BSA + indomethacin (10^{-6} M)	—	4.25 ± 0.64	38.8 ± 8.3 **

[1] Percentage maximal acetylcholine (10^{-3} M) response.
[2] pmol/mg protein.
[3] pg/mg protein.
Means ± S.E., n = 6.
* $P < 0.05$
** $P < 0.02$
*** $P < 0.001$ } compared to 'control BSA'.

TABLE 3

Effect of relaxant drugs on anaphylactic, histamine and $PGF_{2\alpha}$-induced contractions in lung strips.

Contractile stimulus [1]	% Inhibition caused by		
	Salbutamol (3×10^{-8} M)	Theophylline (3×10^{-4} M)	Salbutamol + theophylline
Anaphylactic (78.8%)(n = 6)	37.8 ± 14.0	12.9 ± 6.7	85.8 ± 6.8
Histamine (~70%)(n = 8)	0 **	10.7 ± 3.3	29.0 ± 5.0 ***
$PGF_{2\alpha}$ (~50%)(n = 8)	0 **	18.5 ± 4.2	62.7 ± 4.8 *

[1] Means ± S.E., numbers in parentheses denote the size of control contractions relative to the maximal acetylcholine (10^{-3} M) response.

* $P < 0.05$
** $P < 0.02$
*** $P < 0.001$
} compared to inhibition of anaphylactic contractions.

combined the drugs increased the c-AMP content to 5.91 ± 0.61 pmol/mg protein ($P < 0.02$, n = 8). The effect of higher concentrations of salbutamol (10^{-7} M) and theophylline (10^{-3} M) were tested on anaphylactic tension and c-AMP. With higher concentrations all three drug treatments inhibited anaphylactic contractions. However, only when the drugs were combined was there a significant increase in c-AMP (table 2).

Prostaglandins have been shown to increase c-AMP in leucocytes (Bourne et al., 1972). Since $PGF_{2\alpha}$ was released from the lung parenchyma the effect of indomethacin (10^{-6} M) on $PGF_{2\alpha}$ release and c-AMP was tested. Indomethacin inhibited $PGF_{2\alpha}$ release from the lung ($P < 0.02$) but it had no significant effect on c-AMP (table 2).

3.2. Comparison of inhibitory effect of salbutamol and theophylline on anaphylactic and drug-induced contractions in lung strips

Identical experimental conditions were used to compare inhibition of anaphylactic contractions and histamine- (10^{-5} M) and $PGF_{2\alpha}$- (3×10^{-5} M) induced contractions. The concentrations of histamine and $PGF_{2\alpha}$ used gave contractions of approximately 70% and 50%, respectively, of the maximum response to acetylcholine (10^{-3} M).

Salbutamol (3×10^{-8} M) caused a significantly greater percentage inhibition of the anaphylactic contractions compared with the histamine and $PGF_{2\alpha}$-induced responses (table 3). Theophylline (3×10^{-4} M) had a small effect on all three stimuli but in contrast with salbutamol there were no significant differences ($P > 0.5$) in the inhibition of the anaphylactic and the histamine and $PGF_{2\alpha}$-induced contractions. Potentiation was observed on all three stimuli when salbutamol and theophylline were combined. Combined the relaxant drugs caused only partial inhibition of the histamine and $PGF_{2\alpha}$-induced contractions whereas the anaphylactic responses were almost abolished (table 3).

4. Discussion

In this study we have compared the effects of salbutamol and theophylline on anaphylactic contractions in peripheral airways with their effects on some mediator-induced contractions, c-AMP levels and the release of two endogenous spasmogens, histamine and $PGF_{2\alpha}$, in the lung.

Theophylline potentiated the bronchodilating action of salbutamol in lung strips in response to antigen-challenge. Salbutamol on its own partially inhibited anaphylactic

contractions whereas the concentration of theophylline used had no overall effect. In recent clinical trials theophylline has been found to either enhance or potentiate the effectiveness of terbutaline and isoprenaline in asthma therapy (Wolfe et al., 1978) possibly by interacting on the peripheral airways (Campbell et al., 1977). Phosphodiesterase inhibitors potentiate β-adrenoceptor mediated increases in c-AMP levels in airways smooth muscle (Lefcoe et al., 1975; Katsuki and Murad, 1977; Triner et al., 1977) and lung parenchyma (Kaliner et al., 1971; Orange et al., 1971). The synergism observed between salbutamol and theophylline on anaphylactic contractions in lung strips, then, maybe due to potentiation in their effects on smooth muscle tone, mediator release, or both.

Individually, salbutamol (3×10^{-8} M) and theophylline (3×10^{-4} M) did not significantly lower histamine release; combined though, they inhibited histamine release by about 66%. The reduction in $PGF_{2\alpha}$ release with the drugs was not significant though Mathé (1976) has demonstrated inhibition in PG release from perfused guinea-pig lungs with adrenaline. Moreover, the possibility that other potent bronchoconstrictor metabolites of arachiodonic acid, such as thromboxane A_2, might be inhibited by salbutamol and theophylline cannot be excluded. Slow-reacting substance (SRS-A) is an important mediatior of anaphylaxis in guinea-pig lung (Mitchell and Denborough, 1979) but it was not practicable to assay its release in these experiments. The inhibitory effect of β-adrenoceptor agonists on histamine and SRS-A release is quantitatively similar (Orange et al., 1971) so it is probable that SRS-A release was also reduced by combined salbutamol and theophylline in the guinea-pig lung.

The effect of the bronchodilators on histamine and $PGF_{2\alpha}$-induced contractions in lung strips was studied in order to estimate the contribution made by smooth muscle relaxation to the overall antianaphylactic effect of salbutamol and theophylline. The effect of salbutamol plus theophylline was substantially greater on anaphylactic contractions than on histamine and $PGF_{2\alpha}$-induced contractions. No differences were observed regarding the slight relaxation responses to theophylline. Combined, the bronchodilators caused between 29% and 62.7% relaxation of histamine and $PGF_{2\alpha}$-induced contractions, respectively. It is more pertinent to compare inhibition of anaphylactic contractions with inhibition of the histamine-induced contractions because the size of the control responses to BSA and histamine were similar (70 ~ 80% maximum acetylcholine-induced contraction). A substantial part then, of the antianaphylactic effect of combined salbutamol and theophylline treatment appears to be due to inhibition in mediator release. Moreover, although 3×10^{-8} M salbutamol had no inhibitory effect on histamine and $PGF_{2\alpha}$-induced contractions it significantly reduced anaphylactic contractions. The antianaphylactic effect of salbutamol at this concentration may be due entirely to inhibition of mediator release. The extent of blockade of SRS-A-induced tone in the airways needs determining however before the exact proportions can be estimated.

On its own salbutamol did not raise c-AMP although it did inhibit anaphylactic contractions in lung strips. Similar observations have been made by Triner et al. (1977) who report that low concentrations of catecholamines relax tracheal smooth muscle without causing an increase in the c-AMP content of the tissue. Without BSA, combined salbutamol and theophylline increased c-AMP by 51%. This increase was not observed in the presence of BSA (table 1) probably because the basal level of c-AMP was already elevated by antigen-challenge. This observation suggests that smooth muscle tone might not be determined by total c-AMP content per se but is perhaps also dependent on levels in different tissue compartments. Alternatively, some of the bronchodilator action of β-adrenoceptor stimulants and phosphodiesterase inhibitors may be independent of c-AMP and be mediated via changes in Ca^{2+} metabolism (Kol-

beck et al., 1978). Many different cell types are represented in lung parenchyma so the possibility remains that the observed changes in c-AMP may not accurately reflect levels in mast cells and smooth muscle. When high concentrations of salbutamol (10^{-7} M) and theophylline (10^{-3} M) were used with BSA there was marked inhibition in contractions and a significant rise in c-AMP when the drugs were used in combination. The c-AMP content in the lung may be partly due to PG release. PGE_1 and PGE_2 increase c-AMP in leucocytes (Bourne et al., 1972) and PGE_2 and $PGF_{2\alpha}$ are released from lung. In the present experiments, indomethacin halved $PGF_{2\alpha}$ release but only slightly inhibited c-AMP. Concurrent inhibition in PG release and c-AMP has been demonstrated (Mathé, 1976) with indomethacin in the perfused guinea-pig lung.

The principal finding of this investigation is that theophylline can potentiate the anti-anaphylactic action of salbutamol in lung parenchyma both directly via inhibition of smooth muscle tone and indirectly via inhibition of mediator release. The desirability of conducting clinical trials with theophylline in combination with aerosolised β-adrenoceptor stimulants in asthma has been discussed by Paterson and Yellin (1978). Results from the present investigation provide further rationale for using doses of theophylline which may potentiate the clinical effectiveness of drugs such as salbutamol in asthma.

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