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Effects of phosphodiesterase inhibitors on normal and chemically-skinned isolated airway smooth muscle

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1 The effects of three phosphodiesterase inhibitors (papaverine, isobutyl methyl xanthine (IBMX) and SKF 94120) were examined on tension responses and cyclic nucleotide content (both cyclic AMP and cyclic GMP) of normal and Triton X-100 skinned isolated trachealis of the guinea-pig.

2 The three inhibitors were approximately equipotent in eliciting concentration-dependent relaxation of histamine-induced contractions of the trachealis.

3 Papaverine-induced relaxation was associated with concentration-related increases in the levels of both cyclic nucleotides.

4 IBMX at low concentrations (1 μmol l^{-1}) produced significant relaxation (36%) of histamine-contracted trachealis without changing cyclic nucleotide levels. At a ten fold higher concentration IBMX-induced relaxation (95%) was associated with a selective increase in tissue cyclic GMP levels. Only at the highest concentration tested (100 μmol l^{-1}) did IBMX increase cyclic AMP levels significantly.

5 SKF 94120 (1 μmol l^{-1}) elicited a 23% relaxation of the contracted trachealis without altering the tissue content of either cyclic nucleotide. At the two higher concentrations tested (10 and 100 μmol l^{-1}), SKF 94120-induced relaxation was accompanied by a selective increase in the levels of cyclic AMP.

6 In the skinned trachealis Ca^{2+} (10 and 20 μmol l^{-1})-induced contractions were significantly inhibited by the calmodulin antagonist calmidazolium (10 μmol l^{-1}) and by cyclic AMP (10 μmol l^{-1}), the catalytic subunit of cyclic AMP-dependent protein kinase (0.1 μmol l^{-1}) and cyclic GMP (10 μmol l^{-1}).

7 Papaverine (100 μmol l^{-1}) significantly inhibited (31 $\pm$ 6%) the Ca^{2+}-induced contractions of the skinned trachealis. Both IBMX and SKF 94120 were without effect.

8 It is concluded that cyclic nucleotide-dependent mechanisms have an inhibitory action on the biochemical processes that lead to contraction of the guinea-pig trachealis. The results suggest that a functional sarcoplasmic reticular and/or plasma membrane is essential for the expression of IBMX- and SKF 94120-induced relaxation. This is not the case for papaverine. The results also highlight the fact that significant relaxant responses of airway smooth muscle can be produced by phosphodiesterase-inhibiting drugs without concomitant elevations in tissue cyclic nucleotide content.

Introduction

It is widely accepted that increased levels of cyclic nucleotides (both cyclic AMP and cyclic GMP) in many smooth muscles, including airway smooth muscle, accompany relaxation induced by a variety of different agents (Bar, 1974; Katsuki & Murad, 1977; Triner *et al.*, 1977; Diamond, 1978; Lau & Lum, 1983; Bergstrand, 1985; Murad, 1985; Rodger, 1986). However, in common with results obtained using other muscle types, a direct cause and effect relationship between concentrations of either adenosine 3′:5′-cyclic monophosphate (cyclic AMP) or guanosine 3′:5′-cyclic monophosphate (cyclic GMP) and tension changes has not been demonstrated.

Cyclic AMP is regarded as exerting its effects on smooth muscle cells by first activating a protein kinase enzyme which in turn phosphorylates a variety of different proteins within the cell. This cyclic AMP-dependent protein kinase may act at several different sites, within airway smooth muscle cells, to promote relaxation via different mechanisms (see Rodger, 1985; 1986). The possibilities include: phosphorylation of Ca^{2+} channels to inhibit entry of Ca^{2+} into the cell, phosphorylation of myosin light chain kinase to inhibit the interaction of actin and myosin, augmenta-

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tion of Ca^{2+} uptake into intracellular stores such as the sarcoplasmic reticulum so reducing activator Ca^{2+} for contraction, stimulation of Ca^{2+} extrusion from the cell and stimulation of the plasmalemmal Na^+, K^+-ATPase which may enhance Na^+-Ca^{2+} exchange (for references see Rodger, 1986). In contrast to that which is known for cyclic AMP-mediated effects little is known about how cyclic GMP brings about relaxation, apart from the fact that it too operates via activation of a specific protein kinase.

It has been proposed that drugs such as the methylxanthines and papaverine exert their bronchodilator effect via inhibition of cyclic nucleotide phosphodiesterase and consequent increases in the levels of either cyclic AMP or cyclic GMP, or both (see for example; Katsuki & Murad, 1977; Polson *et al.*, 1978; 1982; Selvig & Bjerve, 1982; Bergstrand, 1985; Murad, 1985). At therapeutic plasma concentrations, however, drugs such as theophylline exert a minimal inhibitory effect upon cyclic nucleotide phosphodiesterases (see Bergstrand, 1985 for references). Several other mechanisms have, therefore, been proposed to explain how methylxanthines exert their bronchodilator effect. These include antagonism of adenosine receptors (Fredholm, 1980), increased secretion of endogenous catecholamines (Higbee *et al.*, 1982), inhibition of constrictor prostanoid formation (Horrobin *et al.*, 1977) and a reduction in intracellular Ca^{2+} concentrations (Kolbeck *et al.*, 1979). To date, none of these proposed mechanisms of action is, in its own right, wholly satisfactory (Persson, 1985).

The aims of this study were two fold. First, to examine further the relationship between intracellular concentrations of both cyclic AMP or cyclic GMP and relaxation of guinea-pig isolated airway smooth muscle. To do this we have used three drugs which inhibit cyclic AMP and/or cyclic GMP phosphodiesterase, namely papaverine, 3-isobutyl-1-methylxanthine (IBMX) and SKF 94120 (Gristwood *et al.*, 1985; Reeves *et al.*, 1987). Second, to examine the effects of these two drugs on Ca^{2+}-induced contractions of chemically-skinned preparations of guinea-pig trachealis. In such preparations tension development can be studied under conditions in which the ionic environment surrounding the contractile apparatus can be carefully controlled. It is possible, therefore, to examine whether the phosphodiesterase-inhibiting drugs exert an inhibitory effect on airway smooth muscle via suppression of the Ca^{2+} sensitivity of the contractile apparatus. Furthermore, since the plasma membrane is disrupted by the skinning procedure, it is also possible to study directly the effects of drugs and chemicals, for example cyclic AMP and cyclic GMP, which would not readily penetrate the cell membrane in intact cells.

A preliminary account of these findings has been presented to the British Pharmacological Society (Bryson & Rodger, 1987).

Methods

Intact tracheal smooth muscle

Tissue preparation Male, Dunkin-Hartley guinea-pigs were killed by stunning and bleeding. The trachea was rapidly excised and placed in Krebs-Henseleit solution (KHS) of the following composition (in mmol l^{-1}): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.7. The trachea was dissected free of extraneous connective tissue and cut into rings by sectioning between adjacent cartilage bands. A silk suture was tied to the cartilage on either side of the smooth muscle. The rings were then anchored in water-jacketed 10 ml tissue baths containing KHS at 37°C and bubbled with a gaseous mixture containing 95% O_2 and 5% CO_2. Having ensured that the smooth muscle fibres were correctly orientated in the vertical plane, the tissues were connected via silk sutures to isometric force-displacement transducers (Grass FTO3C; Quincy, Mass.). Changes in isometric tension were recorded on an ink-writing curvilinear polygraph (Grass, model 7, Quincy, Mass.).

Before the commencement of each experiment the tissues were equilibrated for at least 60 min under an initial resting tension of 2 g. During this period the bathing medium was changed three times. Following equilibration, the tissues were treated with flurbiprofen (1 μmol l^{-1}) in order to inhibit the generation of cyclo-oxygenase products (Rome & Lands, 1975), e.g., prostaglandin E_2 (PGE_2), PGI_2 and thromboxane A_2, known to be produced during tissue contraction induced by some agonists (Orehek *et al.*, 1973; Weichman *et al.*, 1982). Flurbiprofen remained in the tissue bath for the duration of each experiment.

Construction of cumulative concentration-effect curves Sixty minutes after administration of flurbiprofen tissues were contracted with an EC_{75} concentration of histamine (10 μmol l^{-1}; calculated from preliminary experiments). Once the tonic phase of the contraction was established (after approximately 10 min) increasing concentrations of either papaverine, IBMX or SKF 94120 were administered to the tissue bath in a cumulative fashion, in accordance with the method of van Rossum (1963). Only one cumulative concentration-effect curve was obtained from each tissue. The relaxant effect obtained is expressed as a percentage reversal of the histamine-induced contraction. Thus, relaxation to a level below the baseline tension, present before the addition of histamine, is indicated by a value in excess of 100%.

Cyclic nucleotide measurements In the experiments in

which cyclic nucleotide levels were measured, three concentrations (1, 10 and 100 μmol l^{-1}) of papaverine, IBMX and SKF 94120 were selected for study. In these studies the drug under test was administered as a bolus and, at the peak of the induced relaxant effect, the tissues were freeze-clamped using tongs pre-cooled in liquid nitrogen. The individual deep frozen rings were then weighed before being placed in 2 ml capacity Teflon vials (pre-cooled in liquid nitrogen) containing 1 ml of 6% trichloro-acetic acid (TCA) and a 0.9 mm stainless steel grinding ball. Tissues were then microdismembranated for 30 s (Braun Mikro-dismembrator II). The pulverised material was transferred to a 5 ml polypropylene centrifugation tube, residual material being removed from the Teflon vial with a further 1 ml of TCA. Each tube was then centrifuged at 8000 *g* for 15 min at 4°C. After centrifugation the supernatant was decanted off into a large Pyrex tube and extracted six times with 10 ml of cold (4°C) water-saturated diethyl ether to ensure removal of the TCA and lipids present in the extract. Following each addition of diethyl ether the contents of the tube were shaken manually for 30 s. When the layers had separated (after standing briefly) the upper ether layer was carefully removed using a Pasteur pipette attached to a suction aspirator. Residual traces of ether were evaporated by heat (60°C for 5 min) and the samples stored at − 20°C until required for assay. Storage at − 20°C is sufficient to prevent breakdown of the cyclic nucleotides after deproteinization and ether washing.

Before assaying for cyclic nucleotide content each sample extract (450 μl for cyclic AMP and 600 μl for cyclic GMP) was freeze dried (Edwards, EF4 Modulyo) in 1.5 ml polypropylene vials (Eppendorf). The freeze-dried material was reconstituted in sufficient buffer to allow duplicate measurements of both the cyclic nucleotides to be made.

For cyclic AMP measurements the commercially available protein binding kit (TRK 432) was used (Amersham International). For each assay a standard curve was constructed and tritium radioactivity was counted in a liquid scintillation spectrometer (tri Carb 460CD Packard) after the addition of 1 ml of the scintillation mixture (Picofluor 30, Packard). The detection limit and sensitivity of each assay was arbitrarily defined as that amount of unlabelled cyclic AMP required to inhibit the binding of tritiated cyclic AMP to the binding protein by 15% (IC_{15}) and 50% (IC_{50}), respectively, when compared with the binding for the zero standard.

Tissue cyclic GMP content was measured using a modification of the radioimmunoassay method described by Brooker *et al.* (1979). Briefly, 100 μl of either a known amount of cyclic GMP standard (from RIA kit TRK 500, Amersham International; 0–2 pmol in 50 mmol l^{-1} sodium acetate, pH 6.2) or of an unknown reconstituted sample was placed in an Eppendorf assay tube. Cyclic GMP antiserum (100 μl of a 1:3000 dilution in 0.1% w/v bovine serum albumin) was added to each tube followed by 25 μl of guanosine 3', 5'-cyclic phosphoric acid 2'-0-succinyl 3-[^{125}I]-iodotyrosine methyl ester (2000 d.p.m. ml^{-1} in 0.1% w/v bovine serum albumin). The tubes were then sealed, vortex mixed and allowed to achieve equilibrium by incubating at 4°C overnight. Separation of protein-bound cyclic GMP from the unbound nucleotide was achieved by the adsorption of the free cyclic GMP onto activated charcoal (10 mg ml^{-1} in 100 mmol l^{-1} K_2HPO_4 buffer; 0.25% w/v bovine serum albumin) followed by centrifugation at 1200 *g* (MSE Micro Centaur) for 2 min. The radioactivity associated with each sample was then counted in a Panax gamma-counter for 100 s. A calibration curve was constructed in terms of the radioactivity bound against the concentrations of cyclic GMP in the standards. Amounts of cyclic GMP in the unknown samples were then determined by reference to the calibration curve.

Skinned tracheal smooth muscle

Male Dunkin-Hartley guinea-pigs (400–500 g) were killed by stunning and bleeding. The trachea was rapidly removed, placed in ice-cold KHS and cleaned of adherent connective tissue. Tracheal rings were cut and skinned of their plasma membranes according to the method described by Sparrow *et al.* (1984). In brief, rings were incubated for 4 h, at 4°C, in a 'skinning' solution of the following composition (in mmol l^{-1}): KCl 50, sucrose 150, imidazole 20 (pH 7.4), dithioerythritol (DTE) 0.5, ethyleneglycol-*bis* (β-aminoethylether)-N,N'-tetraacetic acid (EGTA) 5, containing 1% v/v Triton X-100. At the conclusion of this period in the 'skinning' solution the tissues were rinsed for 15 min in the same solution but with the Triton X-100 omitted. Tissues were then stored at − 20°C, for up to six days, in a 'storage' solution of the following composition (in mmol l^{-1}): imidazole 20 (pH 7.4), DTE 0.5, EGTA 4, $MgCl_2$ 10, ATP 7.5, $NaNO_3$ 1 containing 50% w/v glycerol.

For isometric recording of tension changes tissues were set up as described above. The preparations were bathed in a 'relaxing' solution at 20°C under an applied resting tension of 0.5 g. The 'relaxing' solution contained (in mmol l^{-1}): imidazole 20, EGTA 4, $MgCl_2$ 10, ATP 7.5, $NaNO_3$ 1 and KH_2PO_4 6. The pH of the solution was adjusted carefully to 6.7 using KOH. The 'relaxing' solution did not contain any added calmodulin. The 'contracting' solution had an identical composition to the 'relaxing' solution except that instead of EGTA (4 mmol l^{-1}) it contained Ca-EGTA (4 mmol l^{-1}). The free calcium ion concentration ($[Ca^{2+}]$) in the 'contracting' solution was increased by mixing appropriately the 'relaxing' and 'contracting' solutions. The free $[Ca^{2+}]$ in the solution

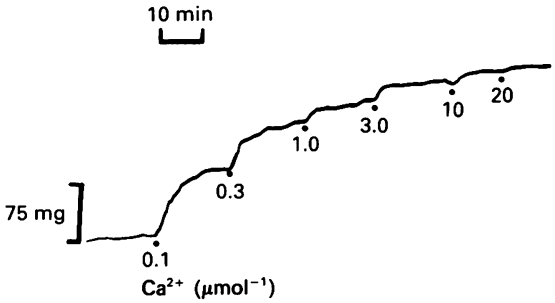

Figure 1 Typical tension recording of a guinea-pig Triton X-100-skinned, isolated trachealis contracted with Ca^{2+}. Increasing concentrations of Ca^{2+} were added at the points indicated.

was calculated using the apparent stability constant for EGTA of 1.2×10^6 at 20°C and pH 6.7 (Portzehl *et al.*, 1964).

In initial experiments cumulative concentration-effect curves were constructed, to assess the viability of the preparations, by increasing the concentration of free Ca^{2+} in the 'contracting' solution. A typical example of the tension record obtained is shown in Figure 1.

It was a feature of the skinned preparations that the magnitude of the contractions, elicited by the same concentration of Ca^{2+} given at 40–60 min intervals, diminished with time. Thus in order to examine the effect of different drugs/agents on the Ca^{2+}-induced contractions the following protocol was adopted. Preparations were arranged into two groups (control and test) which were run in parallel. Initially, preparations in each group were contracted using 20 μmol l^{-1} Ca^{2+}. Normally, peak contraction was achieved after 15–20 min. Tissues were then washed repeatedly over a 30 min period with the 'relaxing' solution. Once baseline tension levels had been re-established, the drug under test was administered to the tissue baths containing the test preparations, with the parallel control preparations receiving an equivalent amount of the vehicle in which the drug was dissolved. Drugs were incubated for 10 min before raising the Ca^{2+} concentration to 20 μmol l^{-1}, and remained in contact with the tissue throughout the second Ca^{2+}-induced contraction. Once peak contraction had been reached the tissues were again washed with the relaxant solution until baseline tension was re-established. A third Ca^{2+}-induced contraction was elicited 60 min after the commencement of the second contraction. No drugs were present during this contraction. After washout and recovery from the third Ca^{2+}-induced contraction each preparation in the two groups received methacholine (100 μmol l^{-1}). Failure of methacholine to elicit a contraction of the skinned tissues was taken as an indication that the skinning procedure had been successful (Ito & Itoh, 1984). Addition of Ca^{2+} in place of methacholine always induced a contraction of the preparations, indicating that the failure to respond was not simply due to deterioration of the preparations with time.

Drugs and solutions

The following drugs and chemicals were used: acetyl-β-methylcholine chloride (methacholine, Sigma), adenosine 3′:5′-cyclic monophosphate (cyclic AMP, sodium salt, Sigma), adenosine-5′-triphosphate (ATP, disodium salt, Sigma), bovine serum albumin (Sigma), calmidazolium (Sigma), cyclic AMP-dependent protein kinase (catalytic subunit, Sigma), dithioerythritol (DTE, Sigma), ethylene-*bis*-(βamino-ethylether)-N,N′-tetraacetic acid (EGTA, Sigma), guanosine 3′:5′-cyclic phosphoric acid 2′-0-succinyl 3-[^{125}I]-iodotyrosine methyl ester (Amersham), guanosine 3′:5′ cyclic monophosphate (cyclic GMP, sodium salt, Sigma), histamine acid phosphate (Sigma), imidazole (Sigma), 3-isobutyl-1-methylxanthine (IBMX, Aldrich), papaverine hydrochloride (Sigma), sodium flurbiprofen (Boots), Triton X-100 (BDH). SKF 94120 (5-(4-acetamidophenyl) pyrazin-2[1H]-one) was a gift from Smith, Kline & French. All drugs were dissolved in distilled water with the exception of IBMX and SKF 94120, which were made up in 25% propylene glycol, and calmidazolium, which was dissolved in 100% dimethyl sulphoxide. Stock solutions were diluted with distilled water immediately before use to give the desired test concentration.

Statistical analysis

Results in the text are expressed as the mean ± s.e.mean. Results were analysed non-parametrically by use of the Mann Whitney U test. A value of $P < 0.05$ was considered significant.

Results

Intact tracheal smooth muscle

Effect of papaverine, IBMX and SKF 94120 on histamine-induced tone Papaverine (1 nmol l^{-1} to 100 μmol l^{-1}) and IBMX (1 nmol l^{-1} to 100 μmol l^{-1}) produced concentration-dependent relaxation of the histamine-contracted tracheal preparations. The relaxations induced by each drug were slow, although similar in time course, requiring approximately 10 min to achieve peak relaxant effect after each drug addition. At concentrations greater than 5 μmol l^{-1} both papaverine and IBMX elicited greater than 100% relaxation of the trachealis (see Methods for explana-

Table 1 Effects of papaverine, isobutyl methylxanthine (IBMX) and SKF 94120 on cyclic AMP and cyclic GMP levels in guinea-pig isolated trachealis

Treatment	*Conc.* (μM)	*Relaxation* (%)	*Cyclic AMP* (pmol mg^{-1})	*Cyclic GMP* (pmol mg^{-1})
Papaverine	Vehicle	0	0.18 ± 0.002	0.19 ± 0.03
	1	35 ± 5	0.28 ± 0.03*	0.32 ± 0.02*
	10	90 ± 5	0.37 ± 0.05*	0.38 ± 0.01*
	100	110 ± 3	1.26 ± 0.02*	0.55 ± 0.09*
IBMX	Vehicle	0	0.13 ± 0.04	0.11 ± 0.003
	1	36 ± 4	0.15 ± 0.02	0.21 ± 0.06
	10	95 ± 6	0.18 ± 0.05	0.27 ± 0.03*
	100	111 ± 2	0.49 ± 0.04*	0.33 ± 0.01*
SKF 94120	Vehicle	0	0.18 ± 0.002	0.25 ± 0.04
	1	23 ± 4	0.25 ± 0.05	0.17 ± 0.05
	10	41 ± 5	0.27 ± 0.02*	0.26 ± 0.07
	100	120 ± 3	1.43 ± 0.38*	0.29 ± 0.05

Values are the mean ± s.e.mean for 4–7 separate experiments.
*Indicates significant difference from control value ($P < 0.05$).

tion). SKF 94120 (1 nmol l^{-1} to 100 μmol l^{-1}), a selective type III (cyclic AMP) phosphodiesterase inhibitor (Reeves *et al.*, 1987), produced essentially similar effects to those elicited by IBMX and papaverine. The mean EC_{50} values (± s.e.mean) for the three drugs were; papaverine 2.75 ± 0.1 μmol l^{-1}, IBMX 0.95 ± 0.3 μmol l^{-1} and SKF 94120 2.08 ± 0.4 μmol l^{-1}.

Effect of papaverine, IBMX and SKF 94120 on cyclic nucleotide levels Papaverine, at the three concentrations tested (1, 10 and 100 μmol l^{-1}), induced significant, concentration-related increases in the levels of both cyclic AMP and cyclic GMP (Table 1). These increases in the cyclic nucleotide content of the tissue were associated with concentration-dependent relaxations of the preparations. In contrast, IBMX at the lowest concentration used (1 μmol l^{-1}) failed to increase either cyclic AMP or cyclic GMP levels despite eliciting a similar (36 ± 4%) relaxation of the preparation to that produced by papaverine (Table 1). At 10 μmol l^{-1} IBMX significantly increased cyclic GMP levels in the tissue but was without significant effect on the content of cyclic AMP. Only at the highest concentration used (100 μmol l^{-1}) did IBMX produce a significant elevation in the levels of cyclic AMP (Table 1); cyclic GMP content was further elevated by IBMX at this concentration (Table 1). SKF 94120 resembled IBMX in its effects in that it relaxed the airway preparations (23 ± 4%) at the lowest concentration tested without affecting the levels of either cyclic nucleotide. At the intermediate and highest concentrations used, SKF 94120 selectively increased cyclic AMP levels (Table 1).

Skinned tracheal smooth muscle

Effects of calmidazolium, cyclic AMP, cyclic AMP kinase and cyclic GMP Calmidazolium (10 μmol l^{-1}), a compound that inhibits the binding of Ca^{2+} to the calcium-binding protein calmodulin (Gietzen *et al.*, 1981), inhibited the Ca^{2+}-induced contractions of the skinned trachealis by 20 ± 7% (Table 2). When a lower concentration of Ca^{2+} (10 μmol l^{-1}) was used to induce contraction of the skinned trachealis, calmidazolium exerted a significantly greater inhibitory effect (39 ± 6%, $n = 5$).

Incubation of the skinned trachealis for 10 min with either cyclic AMP (10 μmol l^{-1}) or the catalytic

Table 2 Inhibitory effects of various drug treatments on Ca-induced contraction of chemically-skinned trachealis of the guinea-pig

Treatment	*Conc.* (μM)	*Inhibition* (%)
Calmidazolium	10	20 ± 7*
Cyclic AMP	10	60 ± 8*
A-kinase (catalytic sub-unit)	0.1	40 ± 3*
Cyclic GMP	10	31 ± 5*
Papaverine	100	31 ± 6*
IBMX	100	0
SKF 94120	100	0

Values shown are the mean ± s.e.mean for 5 experiments.
*Indicates significant difference from time-matched

IBMX = isobutyl methylxanthine

subunit of its dependent protein kinase (0.1 μmol l^{-1}) inhibited the Ca^{2+}-induced (20 μmol l^{-1}) contractions by 60 ± 8% and 40 ± 3%, respectively (Table 2).

Cyclic GMP (10 μmol l^{-1}) exerted a similar effect to that observed with cyclic AMP, inhibiting the contractions induced by Ca^{2+} (20 μmol l^{-1}) by 31 ± 5% (Table 2).

Effects of papaverine, IBMX and SKF94120 The effects of papaverine, IBMX and SKF 94120 were tested at only one concentration (100 μmol l^{-1}), which caused maximum relaxation in the intact preparations whilst simultaneously elevating, significantly, the levels of one or both cyclic nucleotides (see above). Papaverine produced a significant (31 ± 6%) inhibition of the Ca^{2+}-induced contractions (Table 2). In contrast, both IBMX and SKF 94120 were without effect on the Ca^{2+}-induced contractions (Table 2).

Discussion

Since the early observations of Sutherland and his coworkers (Sutherland & Rall, 1960; Butcher & Sutherland, 1962), that theophylline inhibited cyclic nucleotide phosphodiesterase, numerous attempts have been made to try and reconcile this effect with the smooth muscle relaxant effects of the compound. To date, however, the precise molecular mechanism(s) underlying the bronchodilator effect of the methylxanthines has not been clearly established (for references see Introduction). The results of the experiments described here, using intact trachealis preparations, show that papaverine and IBMX are equipotent as relaxants of histamine-contracted airway smooth muscle of the guinea-pig. Another point in common is the similarity in the time course of relaxation induced by both drugs. However, consideration of their effects on cyclic nucleotide levels in the tissue clearly separates the two drugs with regard to the mechanisms underlying their relaxant actions. To all intents and purposes papaverine possesses a profile of activity that is consistent with it being a non-selective inhibitor of cyclic nucleotide phosphodiesterases (Katsuki & Murad, 1977; Katsuki *et al.*, 1977). Thus, the relaxant effects of papaverine are closely paralleled by increases in both cyclic AMP and cyclic GMP levels within the tissue. IBMX, on the other hand, clearly elicits relaxation of the trachealis at low concentrations that cause no detectable alteration in the levels of cyclic nucleotides in the tissue. It is only at concentrations producing about 90% relaxation that IBMX begins to exert a significant inhibitory effect upon phosphodiesterase but to elevate only cyclic GMP levels. Still higher (five to ten fold) concentrations are necessary before cyclic AMP levels are elevated significantly. Thus, it is unreasonable to ascribe the relaxant effect of IBMX solely to an inhibitory effect on phosphodiesterase(s). These data with IBMX are in good agreement with those previously found for the related xanthine, theophylline (Lohman *et al.*, 1977; Kolbeck *et al.*, 1979; Polson *et al.*, 1979; Bergstrand, 1980; 1985; Taylor & Downes, 1982). Further, the preferential effect of IBMX for increasing cyclic GMP levels is consistent with published data that show a five fold selectivity factor for its inhibition of cyclic GMP phosphodiesterase (Davis & Kuo, 1978).

SKF 94120 is a recently described selective inhibitor of the 'low K_m' phosphodiesterase enzyme (PDE III) in guinea-pig and human tissues (Gristwood *et al.*, 1985; Reeves *et al.*, 1987). The results obtained in the present experiments are entirely consistent with SKF 94120 acting as a selective inhibitor of cyclic AMP phosphodiesterase in that, even at the highest concentration tested, it failed to alter the levels of cyclic GMP. Like IBMX, however, SKF 94120 elicited relaxation at concentrations that did not significantly elevate cyclic AMP levels. Thus, like IBMX, the relaxant effects of SKF 94120 cannot be wholly attributed to inhibition of cyclic AMP phosphodiesterase.

In recent years numerous investigators have employed chemical skinning procedures in order to make smooth muscle cells permeable. This technique enables the intracellular environment to be manipulated directly and the effects on the contractile machinery of the cell to be studied without the intervening influence of the plasma membrane and, under certain conditions, the sarcoplasmic reticulum. Several different methods for chemically skinning airway smooth muscle have been described recently (Ito & Itoh, 1984; Sparrow *et al.*, 1984; Hashimoto *et al.*, 1985; Allen *et al.*, 1986a, b; Cortijo *et al.*, 1987).

In our study we adopted the method of Sparrow *et al.* (1984) who used Triton X-100 as the skinning agent. Using this technique we found it was unnecessary to add exogenous calmodulin in order to generate a contraction to Ca^{2+}. In this regard we confirm the observations of Sparrow *et al.* (1984) and Cortijo *et al.* (1987). However, we found that addition of calmodulin (1 μmol l^{-1}) failed to potentiate the Ca^{2+}-induced contractions in our skinned trachealis preparations (data not shown). This finding is contrary to that observed by Sparrow *et al.* (1984) but is in complete agreement with the recent observations of Cortijo *et al.* (1987).

The Ca^{2+}-induced contraction of the skinned trachealis is mediated, at least in part, by a calmodulin sensitive mechanism. This conclusion is supported by the finding that calmidazolium, a known inhibitor of calmodulin (Gietzen *et al.*, 1981) reduced the size of the Ca^{2+}-induced contractions. Furthermore, the magnitude of the inhibition was greater the lower the $[Ca^{2+}]$ used to induce contraction, although complete inhibition could not be achieved. This inability of

calmidazolium to inhibit fully the contractions induced by Ca^{2+} is indicative of a non-calmodulin-mediated component of contraction, perhaps mediated via a direct action of Ca^{2+} on the contractile proteins.

Cyclic AMP and the catalytic component of its dependent protein kinase both inhibited the Ca^{2+}-induced contractions of the skinned preparations. A similar inhibition, in skinned trachealis, with the catalytic subunit of cyclic AMP-dependent protein kinase has been demonstrated by Sparrow *et al.* (1984). Since the plasma membrane is markedly disrupted by the skinning treatment (Cortijo *et al.*, 1987), these effects of cyclic AMP and its kinase cannot be ascribed to either a stimulant effect upon ATP-dependent Ca^{2+} extrusion mechanisms or an inhibitory effect upon the influx of extracellular Ca^{2+} through membrane calcium channels. In other smooth muscles Triton X-100 is found to destroy the functional integrity of both the sarcoplasmic reticulum and the mitochondria (Endo *et al.*, 1982; Meisheri & Ruegg, 1983; Stout & Diecke, 1983). If this effect of Triton is also manifest in airway smooth muscle then the observed effects of both cyclic AMP and its kinase cannot be attributed to uptake of Ca^{2+} from the cytosol into either the sarcoplasmic reticulum or mitochondria. In all likelihood, the inhibitory effects are mediated via a phosphorylation mechanism at the level of the contractile apparatus. It has been proposed that cyclic AMP-dependent phosphorylation of myosin light chain kinase (MLCK) markedly reduces its sensitivity to activation by calmodulin so downgrading its catalytic activity and impeding the development of tension (Adelstein & Hathaway, 1979; Conti & Adelstein, 1981; Ruegg *et al.*, 1983; see also Rodger, 1986).

Cyclic GMP exerted similar inhibitory effects in the skinned trachealis to those that we observed with cyclic AMP. These findings are similar to those described by Pfitzer *et al.* (1984) who used cyclic GMP in porcine Triton-skinned coronary arteries. Since cyclic GMP is thought to exert its effects via activation of a cyclic GMP-dependent protein kinase it would have been interesting to test the purified catalytic subunit of this enzyme. However, this was not possible. There are several reports of drugs which selectively increase cyclic GMP levels producing relaxation of airway smooth muscle (Jamieson & Taylor, 1979; Murad, 1985; Suzuki *et al.*, 1986). Such circumstantial evidence does suggest, therefore, that cyclic GMP has a functional role to play in the relaxation process. However, at the present time there is insufficient detailed information, concerning the role of cyclic GMP in excitation-contraction coupling in smooth muscle, to permit an explanation for the effects observed in the skinned trachealis fibres to be advanced.

In the skinned trachealis experiments the effects of papaverine, on the one hand, and IBMX and SKF 94120, on the other, were even more sharply differentiated than in the studies using intact tissues. Papaverine significantly inhibited the Ca^{2+}-induced contractions; IBMX, and SKF 94120, were without effect. A similar lack of effect of aminophylline in skinned trachealis has been found by Allen *et al.* (1986a). The simplest, and most likely, explanation of these results is that during the skinning process the site(s) of action of IBMX and SKF 94120 was lost whereas that for papaverine remained functionally intact. As mentioned above, Triton X-100 is thought to disrupt not only the plasma membrane of smooth muscle cells but also the sarcoplasmic reticulum. This being the case then it is conceivable that for IBMX and SKF 94120 to express their inhibitory/relaxant effects they require either an intact cell membrane or sarcoplasmic reticulum, or perhaps both. Papaverine, on the other hand, has no such requirements. With hindsight, saponin, which disrupts the plasmalemma but leaves both the sarcoplasmic reticulum and contractile apparatus intact (Saida & Nonamura, 1978; Itoh *et al.*, 1981; Endo *et al.*, 1982), might have been a better choice of chemical skinning agent for our experiments. Its more selective skinning effect could possibly have enabled us to differentiate between the sarcoplasmic reticulum and plasmalemma as the specific site of action of IBMX and SKF 94120.

An alternative explanation is that in the skinned trachealis preparations no phosphodiesterase was present to be inhibited by any of the drugs used. Such an explanation would mean that for papaverine to exert an effect it would have had to act directly upon the contractile proteins to inhibit their interaction. Clearly, this is not a mechanism of action applicable to either IBMX or SKF 94120. In an attempt to assess this possibility, we tried to measure the levels of cyclic nucleotides in the skinned preparations before and after treatment with papaverine and IBMX. Although we did achieve limited success in measuring the cyclic nucleotide content, the results were so inconsistent that we were unable to draw any firm conclusions from them.

In conclusion, therefore, we have shown that cyclic nucleotide-dependent mechanisms have an inhibitory action on the biochemical processes that lead to contraction of the guinea-pig trachealis. Furthermore, the results suggest that a functional sarcoplasmic reticular and/or plasma membrane is essential for the expression of IBMX- and SKF 94120-induced relaxation. This is not the case for papaverine. The results further highlight the fact that relaxant responses of airway smooth muscle can be elicited by phosphodiesterase-inhibiting drugs without concomitant elevations in cyclic nucleotides.

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