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CORRELATION BETWEEN INHIBITION OF A CYCLIC GMP PHOSPHODIESTERASE AND RELAXATION OF CANINE TRACHEAL SMOOTH MUSCLE

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Abstract—Inhibition of partially purified cyclic nucleotide phosphodiesterase activity as well as pharmacologically induced relaxation of respiratory airways smooth muscle was examined to determine whether any correlation between these two effects could be found. The phosphodiesterase in extracts of canine tracheal smooth muscle was chromatographed on a DEAE Bio-Gel A column and eluted with a sodium chloride gradient. The peak I activity hydrolyzed cGMP at a higher rate than cAMP although the apparent K_m values for these two cyclic nucleotides were relatively close. Comparison of the K_i values for alkylxanthine inhibitors of peak I activity correlated remarkably well with the EC_{50} values of the same compounds as relaxants of canine tracheal smooth muscle strips. It is concluded that inhibition of the peak I enzyme may cause accumulation of an intracellular pool of cyclic nucleotide and thus produce or contribute to the muscle relaxant effects that were observed.

Methylxanthines have become important pharmacological agents in the therapy of reversible airways obstruction, apparently deriving their beneficial effects from their abilities to relieve contractile spasms of respiratory airways smooth muscle. The molecular mechanism of action of these pharmacological agents remains unresolved at the present time. In the late 1950s and early 1960s, Sutherland and colleagues [1, 2] suggested that the methylxanthines produce their biological effects by inhibition of cyclic nucleotide phosphodiesterase (ED 3.1.4.17), the enzyme that promotes the hydrolytic inactivation of cAMP† in cells.

To examine this hypothesis we separated different fractions of phosphodiesterase activity from canine tracheal smooth muscle using column chromatography. At least five peaks of activity were eluted from the column. Comparison of the pharmacological inhibition of one of these peaks (V), which contained a high affinity cAMP-specific enzyme, revealed a strong correlation with the relaxation of tracheal smooth muscle strips produced by the same compounds. This correlation was the subject of a previous report [3]. Another peak of treacheal smooth muscle–phosphodiesterase activity (I) appeared to hydrolyze cGMP much better than cAMP, and we considered it of interest to determine whether inhibition of this cGMP enzyme might also show a correlation with tracheal smooth muscle relaxation. The results are presented in this report.

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† Abbreviations: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; EC_{50}, molar concentration producing 50% relaxation of canine tracheal smooth muscle; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*′-tetraacetic acid; K_i, inhibitor constant; K_m, Michaelis constant; and V_{max}, maximum velocity.

MATERIALS AND METHODS

Materials

3-Methylxanthine was purchased from Vega Biochemical (Tuscon, AZ). Bio-Rad AG 1-X8 and DEAE Bio-Gel A were purchased from Bio-Rad (Richmond, CA). Cyclic AMP[2, 8-^{3}H] (36.4 Ci/mmole) was from the New England Nuclear Corp. (Boston, MA), and cyclic GMP[8-^{3}H] (18.3 Ci/mmole) was from Amersham (Arlington Heights, IL). Other chemicals, enzymes, reagents and calmodulin were purchased from the Sigma Chemical Co. (St. Louis, MO). Buffer A [mM]: Tris–HCl (pH 7.5), 40; 2-mercaptoethanol, 3.74; $MgCl_2$, 5; and $CaCl_2$, 0.01. Krebs–Ringer buffer [mM]: NaCl, 117; KCl, 4; $NaHCO_3$, 25; $MgSO_4$, 2.4; NaH_2PO_4, 1.2; $CaCl_2$, 2.5; and dextrose, 11.

Methods

The methods employed in this study have been described previously [3] and are summarized here briefly.

Tissues. Canine tracheal smooth muscle strips were dissected from the tracheas of dogs (mixed breed and sex) anesthetized with 30 mg/kg of pentobarbital sodium given via the intravenous route. Some of the strips were mounted in isolated tissue baths for contractile force experiments as described below. Other strips were stored frozen (−70°) until homogenization in a Waring blender and extraction into buffer A. The 105,000 *g* (1 hr) supernatant fraction from tracheal smooth muscle extracts was applied to a DEAE Bio-Gel A column, and five

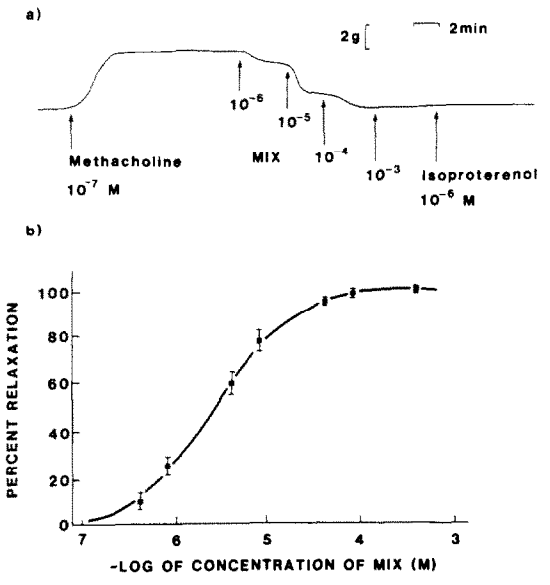

Fig. 1. (a) Response of an isolated canine tracheal smooth muscle strip to the effects of 1-methyl, 3-isobutylxanthine (MIX). Pharmacological compounds were added at the points indicated, and the contractile tension was recorded as described in the text (see Methods). (b) Mean dose–response curve illustrating the relaxant effects of 1-methyl, 3-isobutylxanthine on seven isolated strips. Bars represent standard errors.

peaks of activity were eluted with a linear NaCl gradient. The peak I fraction was concentrated using an Amicon TCF2 system with a YMIO filter and then lyophilized and stored at −70° until assayed.

PDE assay. The procedure described by Thompson and Appleman (1971) was employed with slight modification [3]. [^{3}H]cAMP or [^{3}H]cGMP was used as substrated in 100 μl volumes of buffer A containing 0.05% bovine serum albumin and 0.12 units of alkaline phosphatase (Sigma No. P-4252) [4]. After incubation for 10 min at 37°, an aliquot (1 ml) of Bio-Rad AG 1-X8 resin suspended in a mixture of isopropanol and water (1:1) was added to bind the unreacted substrate. The tritium-labeled nucleoside-reaction product remained unbound and was counted by liquid scintillation spectrometry. Protein content was measured as described by Schaffner and Weissmann [5].

Mechanical activity. Tracheal smooth muscle strips were mounted in tissue baths containing Krebs–Ringer buffer (see Materials for composition) oxygenated with 95% O_2 and 5% CO_2 and maintained at 37°. Isometric tension was measured using a Grass model 7B polygraph to record the output signals from FTO3C force-displacement transducers. Following an equilibration period of 30 min or more, each strip was contracted with 0.1 μM methacholine (acetyl-β-methylcholine chloride) and the relaxant effects of alkylxanthines were determined by constructing dose–response curves according to the method of Van Rossum [6]. A typical tracing showing a smooth muscle response is shown in Fig. 1a. The amount of methacholine utilized to place strips in contractile tension (0.1 μM) produced about 20% of the maximum obtainable contraction. At the end of each dose–response curve for alkylxanthines, maximal (100%) relaxation was determined by the addition of 1 μM isoproterenol hydrochloride. The mean dose–response curve for 1-methyl, 3-isobutylxanthine is shown in Fig. 1b as an example of the dose–response curves obtained by this method. EC_{50} Values were calculated according to the method of Fleming *et al.* [7].

RESULTS

Peak I phosphodiesterase.

The peak I enzyme was prepared from the soluble fraction of tracheal smooth muscle extracts by chromatography on DEAE Bio-Gel A as described previously [3]. The resulting preparation hydrolyzed both cAMP and cGMP although relatively greater activity was found toward cGMP as substrate. Further analysis revealed non-linear Michaelis–Menten

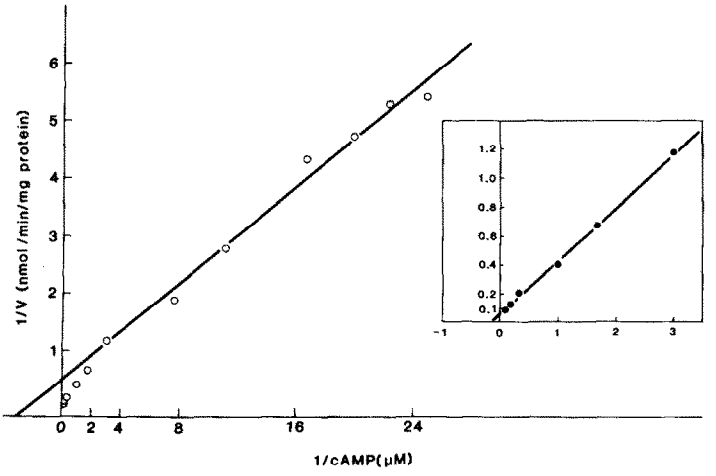

Fig. 2. Lineweaver–Burk plot of peak I catalyzed reaction velocities with various levels of cAMP as substrate. Each point represents the mean of six to eight determinations. Inset shows the data obtained with cAMP levels at the high end of the range employed.

Table 1. Apparent Michaelis constants and maximum velocities determined for peak I tracheal smooth muscle-phosphodiesterase activity

	Low K_m*	Low V_{max}†	High K_m*	High V_{max}†
Cyclic AMP‡	0.5 ± 0.1	1.9 ± 0.1	6.6 ± 0.5	15.9 ± 0.6
Cyclic GMP§	0.3 ± 0.1	19.6 ± 2.5‖	4.1 ± 0.6¶	82.1 ± 12.1‖

* Expressed in micromolar concentrations.
† Expressed in nmoles·min^{-1}·(mg protein)$^{-1}$.
‡ Values for cyclic AMP are means ± S.E.M. from three experiments similar to the experiment shown in Fig. 2.
§ Values for cyclic GMP are means ± S.E.M. from five experiments similar to the experiment shown in Fig. 3.
‖ Different from cAMP ($P < 0.005$).
¶ Different from cAMP ($P < 0.02$).

kinetics when substrate levels were varied between 0.04 and 10 μM, and two apparently linear segments were identified in the Lineweaver–Burk plot for each of the two cyclic nucleotides studied (Figs. 2 and 3). Accordingly, two apparent K_m values were determined for cAMP and two for cGMP (Table 1). The low K_m for cGMP was not significantly different from the low K_m for cAMP, but the low V_{max} was 10.3 times as large, indicating a much more rapid hydrolysis of the guanine nucleotide. The high K_m was slightly lower for cGMP than cAMP and the high V_{max} was five times as large for cGMP as cAMP. Thus, of the two nucleoside cyclic phosphates tested, cGMP seemed to be the better substrate for the peak I enzyme.

Calmodulin

Activation by calmodulin was examined using cGMP at 1.4 and 22 μM levels. Addition of 23 units of calmodulin (Sigma P-0270) to 100 μl reaction mixtures (containing 100 μM $CaCl_2$) had no effect on peak I activity nor was any change observed after the addition of 400 μM EGTA. Therefore, it was concluded that calmodulin did not produce calcium-dependent activation of the enzyme.

Correlation between peak I phosphodiesterase inhibition and tracheal smooth muscle relaxation

The inhibitory effects of four alkylxanthines were examined using the method of Dixon [8] to determine inhibitor constants. For this purpose cGMP was used as substrate at levels (0.045 and 0.06 μM) that detected the low apparent K_m activity. Figure 4 shows a plot of -log K_i for enzyme inhibition versus -log EC_{50} for tracheal smooth muscle relaxation for each of the four alkylxanthines tested. A remarkably high correlation coefficient [9] was found for these data ($r = 0.9996$), indicating a highly significant correlation ($P < 0.001$).

A major criticism of the hypothesis that methylxanthines produce their smooth muscle relaxant effects as a result of phosphodiesterase inhibition stems from observations that higher concentrations of methylxanthines are required for phosphodiesterase inhibition than are necessary for pharmacological effectiveness [10–12]. In the present study, we calculated the ratio K_i/EC_{50} for each of the four compounds tested, and these are shown in Table 2. All ratios were approximately one or less than one, indicating that phosphodiesterase inhibition was accomplished with concentrations no greater than were required for smooth muscle relaxation.

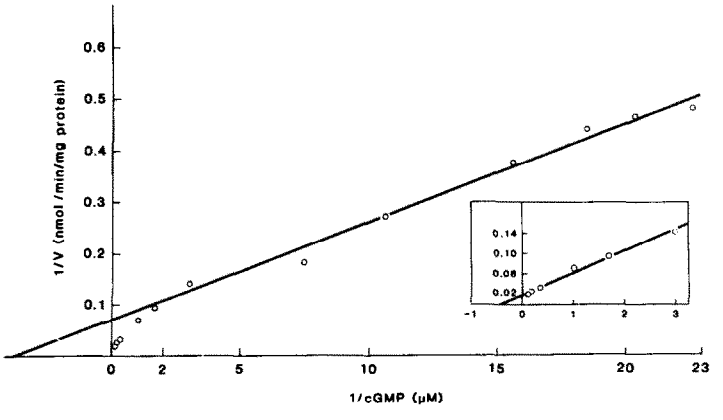

Fig. 3. Lineweaver–Burk plot of peak I catalyzed reaction velocities with various levels of cGMP as substrate. Each point represents the mean of six to eight determinations. Inset shows the data obtained with cGMP levels at the high end of the range employed.

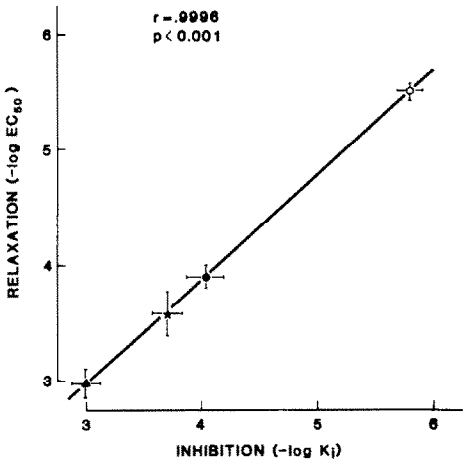

Fig. 4. Correlation between inhibition of peak I phosphodiesterase activity and pharmacological relaxation of tracheal smooth muscle strips by four alkylxanthines. Means ± S.E.M. are illustrated for three or four determinations of -log K_i and from seven to sixteen determinations of -log EC_{50} for each agent. The correlation coefficient (r) was calculated by standard statistical procedure [9]. Key: (▲) caffeine, (★) 3-methylxanthine, (●) theophylline, and (○) 1-methyl, 3-isobutylxanthine.

DISCUSSION

The hypothesis that methylxanthines produce their biological effects as the result of phosphodiesterase inhibition was put forward by Sutherland and coworkers in the late 1950s and early 1960s [1, 2]. Because of the importance of methylxanthines in the treatment of reversible obstructive airways disease, this hypothesis is of particular interest as a possible explanation for the pharmacological relaxation of airways smooth muscle. However, the hypothesis of Sutherland *et al.* lost credence in recent years largely due to the existence of fairly large discrepancies between the levels of methylxanthines that produce bronchodilitation and the higher levels that are required for phosphodiesterase inhibition [10–12]. In addition, two other hypotheses for the mechanism of action of methylxanthines were introduced recently, i.e. (1) they act by altering permeability to or binding of calcium in intracellular organelles [11, 12], and (2) they act by blocking adenosine receptors [12–14]. The former hypothesis is subject to the criticism that the discrepancies between levels of methylxanthines required for pharmacological effectiveness and those needed to affect calcium metabolism are even higher than the discrepancies that have been reported for phosphodiesterase inhibition [11, 12]. The latter (adenosine-receptor) hypothesis appears to be inconsistent with recent findings reported by Persson [15, 16] showing that methylxanthines which inhibit phosphodiesterase, but which are devoid of adenosine-receptor blocking activity, produce relaxation of guinea pig airways smooth muscle. In addition, studies in our laboratory have revealed that adenosine does not produce contraction of tracheal smooth muscle strips nor is the relaxant effect of theophylline on tracheal smooth muscle affected by the presence or absence of adenosine [17]. Therefore, it appears that neither of these two alternative hypotheses offers a superior explanation for methylxanthine action in airways smooth muscle.

Table 2. Ratio of K_i for peak I phosphodiesterase inhibition to EC_{50} for tracheal smooth muscle relaxation

Alkylated xanthine	K_i/EC_{50}
Caffeine	1.1
Theophylline	0.9
3-Methylxanthine	0.8
1-Methyl, 3-isobutylxanthine	0.6

The objective of our investigation was to re-examine the hypothesis of Sutherland *et al.* by determining whether levels of alkylxanthines required for inhibition of one or more of the isolated forms of phosphodiesterase from tracheal smooth muscle might correspond more closely with levels required for smooth muscle relaxation than was found when total soluble (unpurified) phosphodiesterase was studied. We have reported previously [3] that inhibition of one of the separated peaks of tracheal smooth muscle-phosphodiesterase manifesting a high affinity for cAMP (peak V) shows a high correlation with tracheal smooth muscle relaxation ($P < 0.001$). Furthermore, levels of xanthines required for peak V phosphodiesterase inhibition are in the same range as levels required to produce tracheal smooth muscle relaxation. We now report that inhibition of a second peak of tracheal smooth muscle-phosphodiesterase (peak I) also showed a remarkably strong positive correlation with relaxation ($P < 0.001$), and that levels of xanthines that inhibited the peak I enzyme were essentially the same as those that produced relaxation of tracheal smooth muscle strips (Table 2). This peak I enzyme differed from peak V in terms of chromatographic properties, enzyme kinetics, and apparent substrate specificity.

Because peak I activity hydrolyzes cGMP much more rapidly than cAMP, the question of the role of cGMP in the control of smooth muscle tone is relevant to the findings presented herein. Early reports suggested that cGMP could be a "second messenger" mediating the contractile effects of some pharmacological agents [18, 19]. Numerous more recent investigations suggest that cGMP may be involved in the smooth muscle relaxant effects of sodium azide, sodium nitrite, and sodium nitroprusside [20–29] while still other observations seem to deny a simple "second messenger" role for cGMP in the regulation of smooth muscle tone [30–32].

Our finding of a strong correlation between peak I-PDE inhibition and tracheal smooth muscle relaxation would seem, on first consideration, to corroborate a role for cGMP in smooth muscle relaxation, but, although peak I activity hydrolyzes cGMP faster than cAMP *in vitro*, we have not yet demonstrated that cGMP is the substrate for this enzyme in the intact cells where compartmentalization of cyclic nucleotide pools could be a factor. Therefore, an unambiguous role for cGMP in the regulation of

smooth muscle tone is not yet established by these data.

We have now demonstrated that pharmacological inhibitions of two partially purified forms of cyclic nucleotide phosphodiesterase show strong correlations with smooth muscle relaxation. These correlations are better than were observed using total soluble (unpurified) phosphodiesterase extracts. One possible explanation for this finding is that different pools of cyclic nucleotides exist in cells because of subcellular compartmentalization, and that these pools are regulated by different forms of phosphodiesterase represented by the different chromatographic peaks of enzyme activity. It may be further speculated that the pools of cyclic nucleotides regulated by peak I and/or peak V enzymes are involved in the control of tracheal smooth muscle relaxation whereas cyclic nucleotide pools regulated by other forms of the enzyme may not be involved. In this view, the original hypothesis of Sutherland *et al.* is seen as the correct explanation for methylxanthine-induced smooth muscle relaxation, but with the modification that only certain forms of smooth muscle phosphodiesterase represent the pharmacologically important forms of the enzyme.

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