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Studies on the inhibition of phosphodiesterase-catalyzed cyclic AMP and cyclic GMP breakdown and relaxation of canine tracheal smooth muscle

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Several pharmacological agents are known which inhibit cyclic 3′,5′-nucleotide phosphodiesterase(s)(EC 3.1.4.17) and also produce bronchodilation [1–7]. The present investigation was undertaken to determine whether a quantitative correlation could be found in respiratory smooth muscle between the phosphodiesterase (PDE) inhibition and muscular relaxation produced by some of these agents. Our findings support the concept [8, 9] that relaxation is produced as a result of pharmacological inhibition of cyclic-3′,5′-adenosine monophosphate (cAMP) breakdown and the subsequent accumulation of cAMP in cells. Inhibition of cyclic-3′,5′-guanosine monophosphate (cGMP), a tissue constituent that has been implicated as a stimulator of smooth muscle contraction, can also be correlated with relaxation, although in most cases inhibition of cGMP is not as great as for cAMP.

Theophylline, caffeine, acetyl-β-methylcholine Cl (methacholine), *l*-isoproterenol-*d*-bitartrate, cAMP and

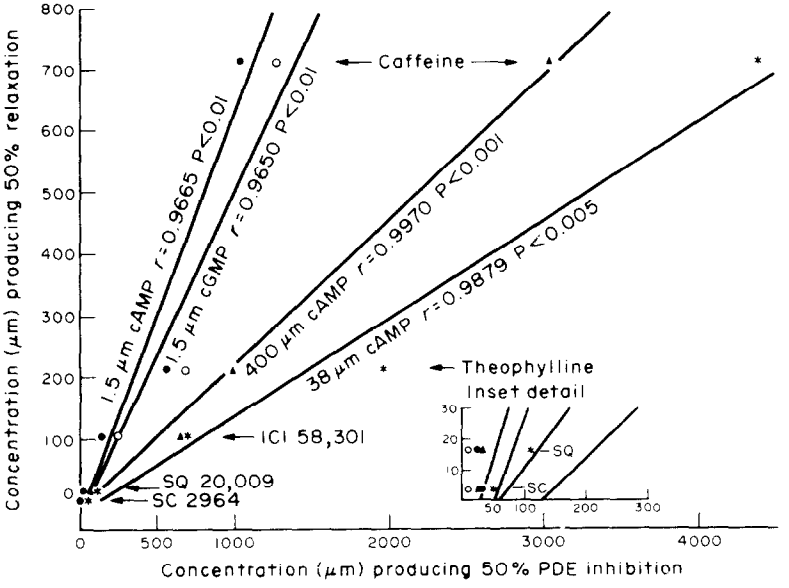

Fig. 1. Correlations between drug concentrations producing 50 per cent tracheal smooth muscle relaxation vs concentrations producing 50 per cent inhibition of phosphodiesterase under four different substrate conditions. At the 50 per cent relaxing concentration indicated for each drug are four points corresponding to the 50 per cent PDE-inhibiting concentrations of the same drug with 1.5 μM cGMP (○), or 1.5 (●), 38(*) or 400 (▲) μM cAMP as substrate. Data represent geometric means derived from four to seven relaxation curves or three to four inhibition experiments and calculated after Fleming *et al.* [12]. Standard errors ranged from 1.1 to 2.1 μM. Regression lines were drawn by the method of least squares. P indicates the probability that the linear correlation coefficient, *r*, is zero. Points clustered in the lower left corner are shown in greater detail in the inset.

cGMP were purchased from Sigma Chemical Co. 3-Acetamido-6-methyl-8-*n*-propyl-*s*-triazolo [4,3-a] pyrazine (ICI 58,301), 1-methyl-3-isobutylxanthine (SC 2964) and 1-ethyl-4-(isopropyl-idenehydrazino)-1 H-pyrazolo(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, HCl (SQ 20,009) were gifts from Stuart Pharmaceuticals, G. D. Searle, and Squibb & Sons respectively. cAMP[^{3}H-G] (38.4 Ci/m-mole) and cGMP[8-^{3}H] (10.2 Ci/m-mole) were purchased from New England Nuclear and ICN Pharmaceuticals respectively.

Smooth muscle was obtained from tracheas of 43 female dogs anesthetized with 35 mg/kg of pentobarbital sodium. This was either homogenized (Broeck tissue grinders) in 50 mM Tris–HCl (pH 7.5) containing 0.25 M sucrose for phosphodiesterase assay, or cut into strips to mount in baths for contractile force measurements. For assay, homogenates were centrifuged at 105,000 *g* for 1 hr, and supernatant fluids from 12 dogs pooled. The assay was a modification of the procedure of Thompson and Appleman [10] inasmuch as it measured the conversion at 37°C of [^{3}H]cAMP or [^{3}H]cGMP to labeled products in the presence of excess alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1., Sigma Type 3 from *Escherichia coli*) [11]. Also, the products were separated from substrates by the addition of 1 ml Bio Rad AG1 X8 resin (1:3 suspension in 1 mM HCl) to 100-μl reaction mixtures containing tissue extract, alkaline phosphatase, 50 mM Tris–HCl (pH 7.5) and 5 mM $MgCl_2$ with or without inhibitors.

For relaxation studies, muscle strips were equilibrated in gassed (95% O_2, 5% CO_2) Krebs bicarbonate solution (37°) for 45 min, then placed "in tone" by the addition of 10^{-7} M methacholine. Test drugs were added in serially increasing concentrations to generate concentration–response curves. As a reference standard, 100 per cent relaxation was produced by the addition of a supramaximal concentration (1 μM) of isoproterenol at the end of each experiment.

The five agents tested produced concentration-dependent inhibition of phosphodiesterase and relaxation of smooth muscle. The relaxation was time dependent, reaching a maximum in 5–10 min and sometimes was preceded by a transient contractile spike, especially immediately after the addition of drug concentrations at the upper end of the dose–response curves. The nature of these early contractions is not yet known. Inhibition of phosphodiesterase activity was substrate dependent, and was generally strongest at the lowest (1.5 μM) level of cAMP or cGMP employed. Figure 1 shows that good correlations were found between concentrations of drugs producing 50 per cent PDE inhibition with these substrates vs 50 per cent tracheal relaxation. Inhibition at higher (38 and 400 μM) cAMP levels was weaker, but produced even closer linear correlations (Fig. 1). Although not shown in Fig. 1, when high levels of cGMP were used, none (400 μM) or only some (38 μM) of the drugs were capable of producing 50 per cent PDE inhibition. It was also noted that, regardless of substrate, higher drug concentrations were required to produce 50 per cent enzyme inhibition than 50 per cent muscular relaxation; the ratios expressing these relationships ranged from approximately 1.5:1 (1.5 μM cAMP) to 6.3:1 (38 μM cAMP) for the curves shown.

It has been proposed that drugs which inhibit cAMP breakdown in smooth muscle produce relaxation as a result of cAMP accumulation in cells [8, 9]. One means of testing this hypothesis is to determine whether a quantitative correlation can be found between phosphodiesterase inhibition and the relaxation produced by a series of pharmacological agents. This criterion was successfully applied by Pöch and Umfahrer [13] who found a positive linear correlation using eleven agents that produced both inhibition and relaxation of guinea pig colon. A similar correlation can be shown to exist for the effects of five agents [theophylline, papaverine, eupaverine, 6,7-dimethoxy-1-(3',4'-dimethoxyphenyl) isoquinoleine-chlorhydrate, and 6,7-dimethoxy-4-parachloro-benzylisoquinoleine-bromhydrate] studied in rat aorta by Lugnier *et al.* [14], although Collins and Sutter [15] were unable to find a consistent relationship between the inhibitory and relaxing effects of three agents (papaverine, SC 2964 and RA 233) in rabbit mesenteric-portal vein.

Because of the importance of phosphodiesterase inhibitors used as bronchodilators in the treatment of asthma and other reversible bronchospastic lung disease, the present investigation was directed toward respiratory smooth muscle. Vulliemoz *et al.* [1, 2] have found that concentrations of papaverine and theophylline that relax canine bronchi also inhibit bronchial phosphodiesterase. Pöch [6] showed that amounts of papaverine, eupaverine and aminophylline which produced 57–96 per cent relaxation of bovine tracheal smooth muscle also significantly inhibited phosphodiesterase (46–89 per cent) and increased tissue cAMP by 30–50 per cent during relaxation. Additionally papaverine the theophylline have been found to increase cAMP levels in guinea pig tracheal smooth muscle and tracheal ring incubations, respectively [16, 17], although relaxant effects were not reported. Our investigation showed a correlation between inhibition of phosphodiesterase-catalyzed cAMP breakdown and muscular relaxation, and thus further supports the hypothesis that phosphodiesterase inhibition leads to relaxation in smooth muscle of the respiratory tree, i.e. no correlation would be expected in the absence of a biological connection. Our current finding that, in general, larger drug concentrations are necessary for enzyme inhibition than for muscular relaxation suggests the possible contribution of amplification mechanisms in the sequence of reactions between cAMP and its ultimate effect on contractile elements [18], although possible contributions of as yet unidentified actions of the tested drugs have not been ruled out.

Of further major significance is the fact that our studies herein reported were extended to include cGMP because this chemical analog of cAMP has been implicated as a stimulator of smooth muscle contraction [16, 19–24] and because the pharmacological agents of interest also inhibit cGMP breakdown in addition to that of cAMP [25, 26]. The relationship between cGMP and muscular relaxation is not as clear as for cAMP. Thus, while breakdown of high cGMP levels was not inhibited or only weakly inhibited relative to cAMP, at its lowest level (1.5 μM), inhibition of cGMP was nearly comparable to, although in most cases still weaker than, that of cAMP. Of further potential importance is the fact that a significant positive correlation was found between inhibition of 1.5 μM cGMP breakdown and muscular relaxation. This lower cGMP level would seem to be the most meaningful with respect to events in intact cells, since papaverine and methylxanthines appear to raise tissue cGMP levels nearly as much or more than cAMP in guinea pig tracheal smooth muscle [16, 17], rat ductus deferens [21] and small intestine [27]. Thus, it would appear that pharmacologically elevated levels of cGMP cannot be involved in stimulation of smooth muscle contraction, since the predominant effect of the pharmacological inhibitors of cGMP breakdown was relaxation. However, the possibility cannot as yet be ruled out that contractile effects of cGMP are specifically antagonized by simultaneously rising cAMP levels [28], or that there are different tissue compartments [15] for containing phosphodiesterase with different pharmacological susceptibilities [29]. More experimentation, therefore, will be necessary before final conclusions on the role of cGMP in respiratory smooth muscle can be drawn.

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