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ANALYSIS OF THE RELATIONSHIP BETWEEN PHARMACOLOGICAL INHIBITION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE AND RELAXATION OF CANINE TRACHEAL SMOOTH MUSCLE

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Abstract—Dose–response curves for muscular relaxation produced by five phosphodiesterase inhibitors were compared to curves for inhibition of phosphodiesterase-catalyzed breakdown of cyclic AMP or cyclic GMP. The rank-order of potency of the agents as muscular relaxants was similar to their order of potency as phosphodiesterase inhibitors. When enzyme activity was measured with 1.5, 38 or 400 μM cyclic AMP or 1.5 μM cyclic GMP as substrate, it was found that two agents, caffeine and SQ 20,009 [1-ethyl-4-(isopropylidenehydrazino)-1H-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, HCl], inhibited activity in concentrations equal to or slightly less than were required for muscular relaxation. The remaining three agents, theophylline, MIX (1-methyl-3-isobutylxanthine), and ICI 58,301 (3-acetamido-6-methyl-8-*n*-propyl-*s*-triazolo[4,3-a]pyrazine), were required in 2.4- to 10.3-fold higher concentrations for enzyme inhibition than for muscular relaxation. It was concluded that, although these findings are generally consistent with the hypothesis that phosphodiesterase inhibition is important in the mechanism of action of the drugs tested, it may be necessary to take into account additional, presently unknown factors to explain fully the relaxant effects of these drugs on respiratory smooth muscle.

It has been proposed that certain pharmacological agents produce relaxation of smooth muscle as a result of their inhibition of cellular cyclic nucleotide phosphodiesterase (EC 3.1.4.17)[1, 2]. One useful approach to the examination of this question has been to determine whether correlations can be found between the potencies of some of these agents as smooth muscle relaxants and their potencies as phosphodiesterase inhibitors. At the present time, positive correlations of this kind in some mammalian nonrespiratory [3–5] and respiratory [6–12] smooth muscles have been reported. However, it was felt that a continuing and more comprehensive analysis of the apparent relationship between pharmacological inhibition of phosphodiesterase and respiratory smooth muscle relaxation should be undertaken. The present paper represents a continuing effort in this direction.

MATERIALS AND METHODS

Materials. Theophylline (TPH), caffeine (CAF), acetyl-β-methyl choline Cl (methacholine), *l*-isoproterenol-*d*-bitartrate, cyclic 3′,5′-adenosine monophosphate (cAMP), cyclic 3′,5′-guanosine monophosphate (cGMP) and alkaline phosphatase (type III from *Escherichia coli*) were purchased from the Sigma Chemical Co. (St. Louis, MO). 3-Acetamido-6-methyl-8-*n*-propyl-*s*-triazolo[4,3-a]pyrazine (ICI 58,301), 1-methyl-3-isobutylxanthine (MIX) and 1-ethyl-4-(isopropylidenehydrazino)-1H-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, HCl (SQ 20,009) were gifts obtained through ICI America, Inc. (Wilmington, DE), G. D. Searle & Co. (Chicago, IL), and Squibb & Sons (Princeton, NJ), respectively. cAMP[^{3}H-G] (38.4 Ci/m-mole) and cGMP[8-^{3}H] (10.2 Ci/m-mole) were obtained from the New England Nuclear Corp. and the International Chemical and Nuclear Corp. respectively.

Trachealis smooth muscle. Smooth muscle was obtained from more than fifty female dogs weighing 15–25 kg. The dogs were anesthetized with 35 mg/kg of pentobarbital sodium, and their tracheas were removed surgically and placed in ice-cold buffer solution containing 50 mM Tris–HCl (pH 7.5). Smooth muscle in the non-cartilagenous region of each trachea was removed and the mucosal layer and visible connective tissue were dissected away. Further preparations for mechanical activity or phosphodiesterase experiments were carried out immediately.

Mechanical activity. Freshly excised muscle was cut into approximately 1 × 3 × 15 mm strips which were tied at each end with suture silk and mounted in a 10-ml tissue bath. Krebs–bicarbonate buffer of the following composition (mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 24.9; glucose, 11.1; and $CaCl_2$, 2.5 (pH 7.5) was maintained at 37° and aerated with 95% O_2, 5% CO_2. Tension was measured using a Grass FTO3C force-displacement transducer and was recorded on a Grass model 7 polygraph. Tension was adjusted to 0.5 g and, after 45-min equilibration time, contraction (7–9 g) was induced by 10^{-7} M methacholine. Relaxation was produced by addition of test drugs in serially increasing concentrations using only one test drug per strip. At the end of individual experiments, the maximal (100 per cent) relaxation was determined by the addition of 10^{-6} M isoproterenol.

Phosphodiesterase. Freshly dissected smooth muscle was minced with scissors and then homogenized (approximately 1 min) in 6–7 vol. of the Tris buffer (0°) using Broeck tissue grinders. The homogenate was centrifuged at 800 *g* for 30 min and the supernatant

fluids were recentrifuged at 105,000 *g* for 1 hr. Phosphodiesterase activity was assayed immediately in the supernatant and particulate fractions and aliquots of the 105,000 *g* supernatant fluids were pooled and stored at −90° until needed for enzyme studies.

Phosphodiesterase activity was determined by the method of Thompson and Appleman [13] as modified by Koltz *et al.* [14] and Schwartz and Passonneau [15]. The standard procedure measured conversion at 37° of [^{3}H]cAMP or [^{3}H]cGMP to labeled products in 100-μl volumes containing the centrifuged tissue extract, excess alkaline phosphatase, 50 mM Tris–HCl (pH 7.5) and 5 mM $MgCl_2$ with or without the addition of inhibitors. None of the inhibitors affected the alkaline phosphatase-catalyzed step. Reactions were stopped by the addition of 1-ml volumes of a 1:3 (v/v) mixture of BioRad AG 1 × 8 resin in 1 mM HCl. Labeled products remaining in the supernatant fluid after sedimentation of the resin were counted by liquid scintillation spectrometry. Protein was determined by the method of Lowry *et al.* [16].

RESULTS

Tracheal smooth muscle phosphodiesterase. The major objective of the investigation was to compare pharmacological inhibition of canine tracheal smooth muscle phosphodiesterase with muscular relaxation. To aid in the selection of appropriate conditions for the analysis of drug effects on phosphodiesterase, certain properties of the smooth muscle enzyme activity were examined.

Preliminary experiments showed that a large majority (83–114 per cent) of the activity found in the whole homogenates of the smooth muscle preparation was recovered in the 105,000 *g* supernatant fractions whether assayed with 1.5 or 400 μM cAMP or cGMP as substrate. Also, addition of 5 mM $MgCl_2$ was found to be optimal for phosphodiesterase activity at these substrate levels.

Activity measured with substrate levels ranging from 0.08 to 520 μM, when graphed as Lineweaver–Burk plots was non-linear except near the high and low ends

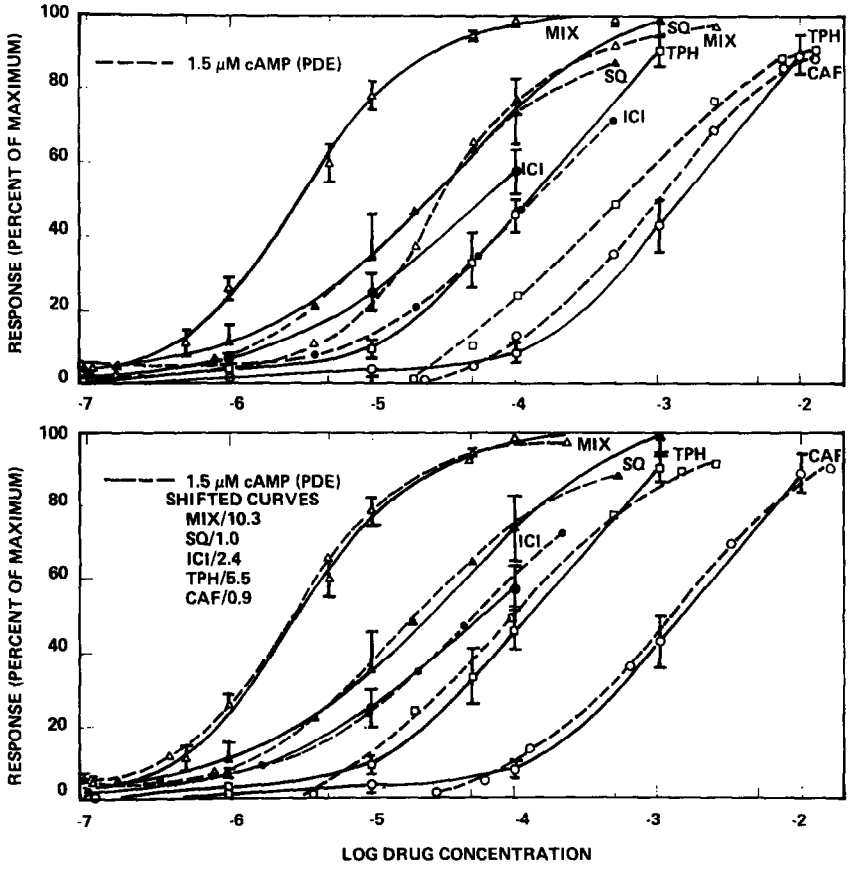

Fig. 1. Comparison of dose–response curves for tracheal smooth muscle relaxation (solid lines) with inhibition of the phosphodiesterase-catalyzed breakdown of 1.5 μM cAMP (broken lines) produced by five pharmacological agents. The top panel shows the data plotted without adjustment for the differences between concentrations required for muscular relaxation versus enzyme inhibition. The bottom panel shows the inhibition curves replotted after adjustment was made according to the indicated ratios for the concentration required for phosphodiesterase inhibition/concentration required for muscular relaxation. Standard errors are shown for relaxation experiments with three to sixteen muscles at each drug concentration. Each point for enzyme inhibition represents the mean derived from 8 to 28 determinations on the pooled soluble enzyme fractions from fourteen dogs. Points on the steepest portion of the curves are those derived from the largest numbers of measurements. Complete dose–response curves for ICI were not determined because of the low solubility of this compound.

of the cAMP and cGMP concentrations examined. However, the apparently linear portions of these plots were extrapolated to give close approximations of the highest and lowest apparent K_m values, as described by Lin and Cheung [17]. These were 2.5 and 308 μM for cAMP and 0.73 and 39 μM for cGMP, and were used as approximate points of reference for selection of substrate levels (1.5, 38 and 400 μM) for subsequent inhibitor studies.

Phosphodiesterase inhibition. At the lowest level of cAMP (1.5 μM) examined for drug effects, hydrolysis was inhibited in a concentration-dependent manner by all five compounds studied. As shown in Fig. 1, these results were plotted together with dose–response curves for muscular relaxation such that enzyme inhibition and muscular relaxation, as percentages of their maximum obtainable responses, were on the same scale (ordinate). Plotted in this way, the inhibition curve for each drug was approximately parallel to its corresponding relaxation curve, but these corresponding curves were not always aligned with respect to drug concentration (Fig. 1, top panel). By adjusting for the difference in drug concentration (abscissa), it was possible to align each inhibition curve with its corresponding relaxation curve as shown in Fig. 1, bottom panel. By this process, a factor was determined, as shown in the figure, which represents the ratio of drug concentration required for inhibition to the concentration required for relaxation. For example, MIX was required in 10.3 times greater concentrations to produce phosphodiesterase inhibition than to produce equivalent muscular relaxation, each effect being expressed as a percentage of the maximum obtainable response. In contrast, the ratio for SQ 20,009 was 1.0, indicating that amounts of this drug that were required for inhibition were the same as required for equivalent relative relaxation.

Among the drugs tested, three (MIX, ICI and TPH) were required in relatively higher concentrations for phosphodiesterase inhibition than for muscular relaxation and two (SQ and CAF) were required in approximately equal amounts for both effects.

Similar results were obtained when 1.5 μM cGMP was substituted for cAMP as substrate. Figure 2 shows the alignment of concentration–effect curves that was obtained by adjusting the inhibition curves for the differences in drug concentration required for inhibition compared to relaxation. The inhibition versus relaxation concentration ratios thus derived show that MIX, ICI 58,301 and theophylline were needed in relatively higher concentrations for enzyme inhibition than for muscular relaxation. Caffeine was needed in about equal concentrations and SQ 20,009 in relatively lower concentrations for the biochemical versus mechanical effects.

When the levels of cAMP were raised to 38 or 400 μM, markedly different results were obtained. The dose–response curves for enzyme inhibition were not parallel to corresponding curves for muscular relaxation when these effects were plotted together using the same relative scales. Note that in Fig. 3, the data for enzyme inhibition are plotted using a scale (right-hand side) that is 1.4 times larger than for muscular relaxation (left-hand side). This use of different scales was necessary in order for corresponding biochemical and mechanical curves to be approximately parallel, and they could then be aligned by adjustment for different inhibitory versus relaxant drug concentrations (abscissa) as before. These different scales for biochemical and mechanical responses were adopted in view of the fact that there is no apparent reason for assuming that a given percentage of phosphodiesterase inhibition should produce numerically the same percentage of smooth muscle relaxation. Using this method of analysis, the same three drugs that were required in relatively greater concentrations for inhibition versus relaxation with 1.5 μM levels of cAMP (Fig. 1) were also required in relatively greater amounts at 38 and 400 μM cAMP (see ratios, Fig. 3). Furthermore, as with the lower cAMP level, caffeine and SQ 20,009 showed approximately equal or slightly greater (SQ; 400 μM cAMP) biochemical versus mechanical potency.

It is of further potential interest to note that, when 38

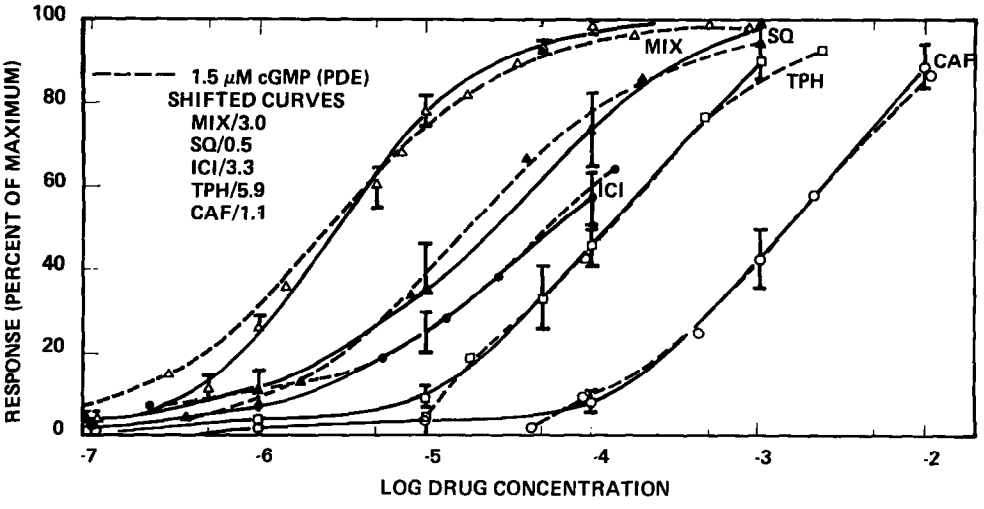

Fig. 2. Comparison of dose–response curves for tracheal smooth muscle relaxation (solid lines) with inhibition of phosphodiesterase-catalyzed breakdown of 1.5 μM cGMP (broken lines) produced by five pharmacological agents. The inhibition curves have been plotted after adjustment was made according to the indicated ratios for the concentration required for phosphodiesterase inhibition/concentration required for muscular relaxation. Other procedures are identical with those presented in the legend to Fig. 1.

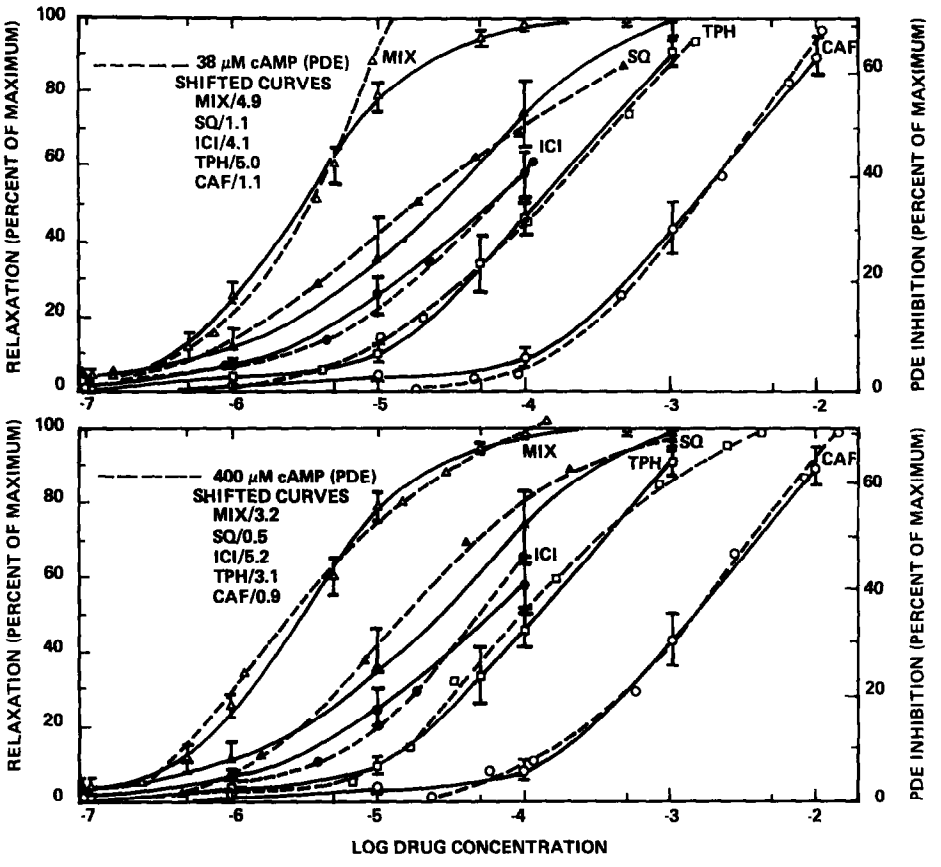

Fig. 3. Comparison of dose–response curves for tracheal smooth muscle relaxation (solid lines) with inhibition of phosphodiesterase-catalyzed breakdown of 38 and 400 μM cAMP (broken lines) produced by five pharmacological agents. The inhibition curves have been plotted after adjustment was made according to the indicated ratios for the concentration required for phosphodiesterase inhibition/concentration required for muscular relaxation. Other procedures are identical with those presented in the legend to Fig. 1.

or 400 μM cGMP was used as substrate in place of cAMP, the phosphodiesterase inhibition was so low, and the resultant concentration–effect curves were so divergent in slope (curves not shown), that they were not aligned with curves for smooth muscle relaxation using the methods presented above.

DISCUSSION

Kukovetz *et al.* [9] have shown an apparent correlation between the ED_{50} values (50 per cent effective doses) of five agents as relaxants of bovine tracheal smooth muscle and their respective K_i values (inhibition constants) for phosphodiesterase inhibition. The pattern of these data derived from tracheal muscle corresponded closely with the pattern of similar data derived from bovine artery and guinea pig colon preparations. Triner *et al.* [10] found that the ED_{50} values for relaxation of canine bronchi by papaverine and theophylline were relatively close to the respective K_i values for phosphodiesterase inhibition. Newman *et al.* [11] reported a linear relationship between the ED_{50} concentrations of eight agents as relaxants of guinea pig tracheal muscle and the ID_{50} (50 per cent inhibitory) concentrations for inhibition of "low affinity" phosphodiesterase, and we have reported that positive linear correlations ($P < 0.05$) could be found between the ED_{50} values of five drugs as relaxants of canine tracheal smooth muscle and the ID_{50} values for phosphodiesterase inhibition [12]. These correlations are considered to support the hypothesis that pharmacological inhibition of phosphodiesterase is the major mechanism of the relaxant effects of these compounds [11, 12].

The alignment of concentration–effect curves for phosphodiesterase inhibition with those for mechanical relaxation was undertaken with the objective of analyzing further the apparent relationship between the observed biochemical and mechanical effects of five pharmacological agents. At 1.5 μM substrate levels, relative inhibition of phosphodiesterase appeared to be comparable to relative muscular relaxation, inasmuch as the concentration–effect curves for inhibition were parallel to those for relaxation when the same scale (ordinate) was used for both effects. However, when enzyme inhibition was studied with 38 or 400 μM levels of cAMP, inhibition and relaxation curves were parallel only when the scale for relative enzyme inhibition was 1.4 times larger than for muscular relaxation. There is no way to predict at the present time what the numerical relationship between relative inhibition and muscular relaxation might be in a particular tissue, inasmuch as several amplification steps may be interposed between the biochemical and mechanical actions [18]. Therefore, in effect, a factor of 1.4, determined by visual

examination, was applied to the slopes of the high substrate dose–response curves for all drugs. Thus plotted, these curves were approximately parallel to their corresponding mechanical dose–response curves. In this way, it was possible to examine the ratio of each drug concentration required for enzyme inhibition relative to muscular relaxation at high substrate levels in a manner similar to the comparison used for 1.5 μM levels.

The hypothesis that the drugs under examination owe their muscular relaxant action entirely to inhibition of cAMP breakdown in cells and that their action would be influenced only by passive distribution into cellular compartments requires that all test drugs inhibit cAMP phosphodiesterase activity in intact cells in concentrations equal to or less than required for muscular relaxation, i.e. inhibition/relaxation concentration ratios of 1.0 or less. None of the experimental conditions examined, comparing inhibition *in vitro* with relaxation in intact cells, resulted in this or any other uniform ratio for all drugs tested. Three drugs (MIX, ICI and TPH) were required in larger amounts for inhibition than for relaxation (ratios from 2.4 to 10.3) regardless of whether cAMP was at the 1.5, 38 or 400 μM level. The other two drugs (SQ and CAF) were required in about equimolar or somewhat smaller amounts for enzyme inhibition compared to muscular relaxation (ratios from 0.5 to 1.1). Possible explanations for these differences are as follows. First, *in vitro* conditions (such as concentrations of the enzyme, ions, or endogenous modulators in the diluted extracts) for measuring phosphodiesterase inhibition might not reflect accurately the presumed drug–enzyme interaction in intact cells. Second, in intact cell preparations, compartments may exist which contain (a) species of phosphodiesterase that have different sensitivities to inhibitors than total soluble phosphodiesterase [19] and/or (b) accumulations of certain inhibitors that are different from concentrations in the bathing medium. Third, actions not involving phosphodiesterase may contribute to the mechanical effects of some or all of the drugs [20].

The biological importance of the apparent relationship between pharmacological inhibition of the breakdown of low levels (1.5 μM) of cGMP and muscular relaxation is not clear at the present time. However, accumulating evidence appears to indicate that there is no simple relationship between tissue cGMP metabolism and the contractile state of smooth muscle [21, 22], and, therefore, this finding with cGMP tends to detract from the significance of similar findings with cAMP. It should also be pointed out that, in contrast to the effects of inhibitors measured directly on phosphodiesterase activity, correlations have not always been found between tissue levels of cAMP and the relaxation of smooth muscle produced by phosphodiesterase inhibitors [9, 23, 24].

It is concluded that the comparisons of dose–response curves presented here are consistent with the hypothesis that phosphodiesterase inhibition is important in the mechanism of action of the pharmacological agents examined on airway smooth muscle. However, the finding that three of the agents require higher relative concentrations for muscular relaxation (compared to enzyme inhibition) than the other two suggests, but does not prove, that additional factors should be considered to explain fully the smooth muscle relaxant effects of these drugs.

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