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INHIBITION OF A HIGH AFFINITY CYCLIC AMP PHOSPHODIESTERASE AND RELAXATION OF CANINE TRACHEAL SMOOTH MUSCLE

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Abstract—The cyclic nucleotide phosphodiesterase (PDE) activity of canine tracheal smooth muscle (CTSM) was examined. Column chromatography of soluble CTSM–PDE revealed five peaks of activity. One of these peaks (V) was examined further in this study and showed a high affinity for adenosine 3′,5′-cyclic monophosphate (K_m = 0.63 μM). Seven pharmacological PDE inhibitors were tested for their abilities to inhibit the peak V enzyme and also for their abilities to cause mechanical relaxation of CTSM strips in isolated tissue baths. A strong correlation ($P < 0.001$) between peak V PDE inhibition ($-\log K_i$) and airway muscle relaxation ($-\log ED_{50}$) was found.

Methylxanthines have been found to be useful in the treatment of bronchial asthma because of their ability to relax the smooth muscle surrounding respiratory airways. However, the molecular mechanism by which methylxanthines produce their smooth muscle relaxant effect has not yet been established. One molecular site of action that has been suggested is cyclic nucleotide phosphodiesterase (PDE, EC 3.1.4.17), an enzyme that catalyzes the hydrolytic inactivation of adenosine 3′,5′-cyclic monophosphate (cAMP) in cells [1, 2]. As part of our investigation into the mechanism of action of methylxanthines, we have examined canine tracheal smooth muscle (CTSM) extracts chromatographically for the presence of different forms of PDE that might be differently related to the muscle relaxant effects of methylxanthines. At least five peaks of PDE activity were found. An analysis of the relationship between pharmacological inhibition of one of these peaks of CTSM–PDE activity and CTSM relaxation is included in this report.

MATERIALS AND METHODS

Materials. 1-Methylxanthine, 3-methylxanthine and 7-methylxanthine were purchased from Vega Biochemicals (Tucson, AZ). 1-Ethyl-4-(isopropylidenehydrazino)-1*H*-pyrazolo (3,4-*b*)-pyridine-5-carboxylic acid, ethyl ester, HCl (SQ 20,009) was a gift from Squibb & Sons (Princeton, NJ). Bio-Rad AG 1-X8, DEAE Bio-Gel A and prepacked TSK columns 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] (6.7 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: Tris–HCl (pH 7.5), 40 mM; 2-mercaptoethanol, 3.75 mM; $MgCl_2$, 5 mM; and $CaCl_2$, 10 μM. Buffer B: 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.5), 20 mM; 2-mercaptoethanol, 3.75 mM; and $Ca(OH)_2$, 50 μM. Krebs–Ringer solution: NaCl, 117.0 mM; KCl, 4.0 mM; $NaHCO_3$, 25 mM; $MgSO_4$, 2.4 mM; NaH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; and dextrose, 11.0 mM.

Tissues. Smooth muscle was obtained from tracheas of dogs of mixed breed and random sex, weighing about 20 kg and anesthetized with 30 mg/kg of pentobarbital sodium (intravenous). After removal of the cartilagenous material and mucosal layer, the tracheal muscle was stored frozen (−70°) until homogenization and extraction for PDE experiments, or it was cut into strips and mounted in isolated tissue baths for contractile force measurements. For PDE experiments, 10–20 *g* of tissue was homogenized (3 × 60 sec) in 2 vol. of buffer A at 4° in a Waring blender fitted with an Eberbach semimicro container. After centrifugation of the homogenate at 1,500 *g* (30 min), the supernatant fraction was recentrifuged at 105,000 *g* (1 hr). The final supernatant material was applied to a DEAE Bio-Gel A column, and the fractions that were collected from the column were stored frozen (−70°) until assayed.

Phosphodiesterase assay. The assay procedure was a modification of the method described by Thompson and Appleman [3]. The reaction is based on the PDE-catalyzed conversion of [^{3}H]cAMP or [^{3}H]cGMP to the corresponding labeled nucleoside 5′-monophosphate which is subsequently dephosphorylated by alkaline phosphatase [4]. Each assay was started by addition of a suitably diluted amount

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of PDE and was carried out for 10 min at 37° in 100 μl volumes of buffer A containing 0.05% bovine serum albumin (BSA) and 0.12 units of alkaline phosphatase (Sigma P-4252). Each reaction was stopped by addition of a 1 ml volume of a slurry (1:3) of Bio-Rad AG 1-X8 resin in a mixture of equal volumes of H_2O and isopropyl alcohol. The resin bound unreacted cyclic nucleotides, leaving the dephosphorylated reaction products in the supernatant fluid. The supernatant fluid was sampled and counted by liquid scintillation spectrometry. Protein was measured by the method of Lowry *et al.* [5] or that of Schaffner and Weissman [6] using BSA as standard.

Mechanical relaxation of smooth muscle. Strips of CTSM (about 1 mm × 2.5 mm × 18 mm) were suspended in Krebs–Ringer solution oxygenated with 95% O_2/5% CO_2 and maintained at 37°. Tension was measured with Grass FTO3C force-displacement transducers and recorded on a Grass model 7B polygraph. Following at least 30 min of equilibration time, after which the resting tension was about 1 g, each strip was contracted by addition of 0.1 μM methacholine (acetyl-β-methylcholine Cl), and a cumulative dose–response curve for one of the relaxant drugs was constructed according to the method of Van Rossum [7]. A dose–response curve could be constructed in about 15 min using this procedure. Total (100%) relaxation (i.e. to baseline) was determined at the end of each experiment by addition of 1 μM isoproterenol. All PDE inhibitors used were capable of producing 100% relaxation. ED_{50} values (geometric means) were calculated according to the method of Fleming *et al.* [8].

RESULTS

DEAE Bio-Gel A. To examine CTSM extracts for the presence of different forms of PDE, the 105,000 *g* supernatant fraction was applied to a DEAE Bio-Gel A column (1.6 cm × 30 cm). The column was eluted with buffer A containing a NaCl gradient (0.015 to 0.15 M) resulting in the appearance of five peaks of activity as illustrated in Fig. 1. Peaks I and IV seemed to be relatively specific for cGMP; peaks II and III hydrolyzed both of the cyclic nucleotides tested, and peak V seemed to be relatively specific for cAMP. The latter peak appeared to be well separated from other PDE activities by one pass over the DEAE Bio-Gel A column.

Peak V PDE. A Lineweaver–Burk plot of initial velocities versus cAMP levels (0.1 to 6.0 μM) for the peak V enzyme is shown in Fig. 2. The apparent K_m for cAMP determined from three similar experiments on different preparations of peak V enzyme was 0.63 ± 0.09 μM. Addition of calmodulin (Sigma P-0270) up to 19 units/100 μl (in the presence of 100 μM $CaCl_2$ and 6 μM cAMP) showed no activation of peak V activity (not illustrated). Addition of 500 μM ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA) in place of $CaCl_2$ did not reveal any decrease in activity. The apparent K_m for cGMP was 3.0 ± 0.3 μM (mean ± S.E.M., N = 3).

High pressure liquid chromatography of the peak V material on a Bio-Sil TSK-400 (gel-filtration) column gave a single peak of activity (not illustrated) corresponding to an estimated molecular size of 63,000–84,000.

Correlation with CTSM relaxation. Inhibition of peak V PDE activity was determined by the method of Dixon [9] for seven pharmacological agents of which six were methylxanthines. The one non-methylxanthine inhibitor used (SQ 20,009) has been found to produce CTSM relaxation similar to the methylxanthines [10]. The inhibitor constants (K_i) for all seven pharmacological agents were compared with the ED_{50} values for the same agents used as relaxants of CTSM strips in isolated tissue baths. Figure 3

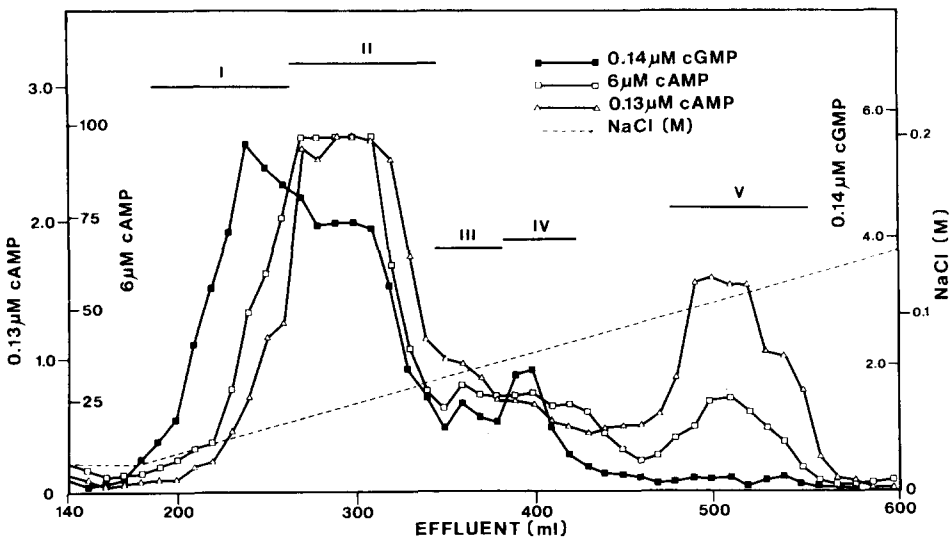

Fig. 1. Elution of soluble canine tracheal smooth muscle phosphodiesterase activity from a DEAE Bio-Gel A column. The column was eluted with a NaCl gradient in buffer A (Materials and Methods). Fractions of approximately 10 ml were collected and assayed in duplicate for enzyme activity according to the standard procedure. Activity is expressed as nmoles of product formed per ml per 10 min.

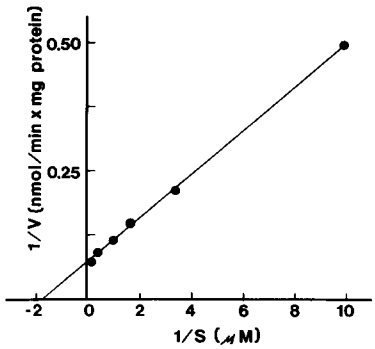

Fig. 2. Hydrolysis of cAMP (0.1 to 6 μM) by peak V PDE activity. Each point is the average of six assays.

shows a plot of $-\log K_i$ versus $-\log ED_{50}$ which reveals a highly significant correlation between K_i and ED_{50} ($r = 0.972$, $P < 0.001$). When only methylxanthines were included in the calculations, the correlation was still highly significant ($r = 0.970$, $P < 0.005$).

The molar ratio of the concentration of inhibitor required for PDE inhibition relative to the concentration required for muscle relaxation (K_i/ED_{50}) would be expected to be near unity if PDE inhibition produced a proportional amount of CTSM relaxation. This ratio was calculated for each of the inhibitors used in this study, and the mean (± S.E.M.) of these ratios was found to be 1.10 (± 0.16) for the six methylxanthines and 1.14 (± 0.18) for all seven agents. Therefore, the K_i/ED_{50} ratio calculated for these drugs did not appear to differ significantly from unity.

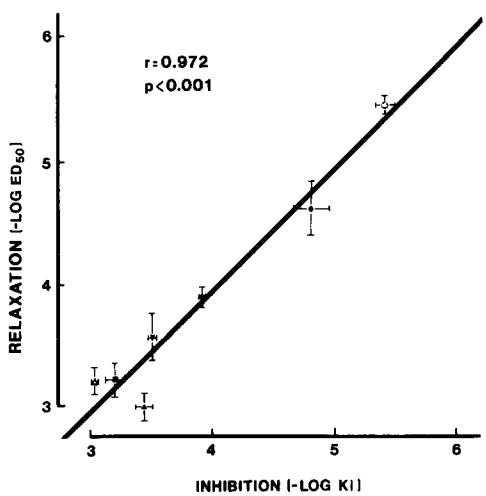

Fig. 3. Correlation between inhibition of peak V PDE and relaxation of isolated CTSM strips. Each K_i was determined by the method of Dixon [9] on three different preparations of peak V enzyme. Each ED_{50} was derived from experiments on six to sixteen isolated CTSM strips. Data points (and bars) represent means (and S.E.M.) of the logs of K_i and ED_{50} in micromoles/liter. Key: (☆) 7-methylxanthine, (★) 3-methylxanthine, (■) 1-methylxanthine, (▲) caffeine, (◆) SQ 20,009, (●) theophylline, and (○) 1-methyl, 3-isobutylxanthine.

DISCUSSION

At the present time, the molecular mechanism of action of methylxanthines on respiratory smooth muscle has not been clearly demonstrated despite being the subject of considerable pharmacological interest in recent years. One molecular site of action that has been suggested is PDE [1, 2] because inhibition of PDE by methylxanthines could conceivably lead to an elevation of cellular cAMP levels and thereby cause relaxation of the muscle. One approach used to examine this possibility has been to compare pharmacological inhibition of PDE activity with smooth muscle relaxation [11–23]. Statistically significant correlations have been reported for pharmacological inhibition of PDE and the relaxation of various types of smooth muscle [14, 19–23] but, for studies that were limited to only respiratory smooth muscle, the correlations reported thus far have been of only borderline significance when plotted as logarithms of drug concentrations [23] as was done in the present study. A further difficulty is that several-fold higher concentrations of some of the methylxanthines have been required to produce PDE inhibition than are needed to produce corresponding amounts of relaxation of respiratory smooth muscle [10].

Studies that have been published up to the present time dealing with correlations between PDE inhibition and smooth muscle relaxation have been carried out using unpurified (total soluble) PDE activity [14, 16, 20–23] or PDE purified from a different tissue and animal species than was used in the muscle relaxation experiments [16, 19]. It is well known that the total soluble PDE activity from mammalian pulmonary tissues contains a mixture of different forms of PDE that respond differently to pharmacological inhibitors [24, 25]. Therefore, the possibility was raised that a better correlation between pharmacological PDE inhibition and airway smooth muscle relaxation might be found if various forms of PDE were studied after isolation.

Our findings showed that at least five peaks of PDE activity were consistently present in soluble CTSM extracts. One of these activities (peak V) was examined in further detail and showed a high affinity for cAMP. This activity also appeared to be insensitive to activation by calmodulin-Ca^{2+}. Pharmacological inhibition of the peak V enzyme produced results that support the view that mechanical relaxation is linked to, and is possibly the result of, PDE inhibition, i.e. pharmacological inhibition of peak V PDE showed a strong statistical correlation with muscle relaxation. Also, the K_i/ED_{50} ratio was found to be reasonably close to unity, indicating a 1:1 ratio between concentrations required for PDE inhibition and those required for CTSM relaxation. These findings suggest that previously reported discrepancies (referred to in the first paragraph of this Discussion) between inhibition of total soluble PDE and airway muscle relaxation may be resolved if the pharmacologically relevant PDE activity could be examined in isolation from other PDE activities.

We have not yet examined isolated CTSM–PDE activity in peaks other than peak V in sufficient detail to know whether additional correlations may be

found. Although the correlation between peak V PDE inhibition and muscle relaxation lends support to the concept that PDE inhibition can produce bronchodilation, the question of whether PDE inhibition produces the bronchodilation associated with administration of methylxanthines to patients is complex and open to further investigation. The issues surrounding this question have been reviewed recently by Bergstrand [26].

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