0026-895X/90/020206-09$02.00/0

MOLECULAR PHARMACOLOGY, 37:206-214

Characterization and Selective Inhibition of Cyclic Nucleotide Phosphodiesterase Isozymes in Canine Tracheal Smooth Muscle

THEODORE J. TORPHY and LENORA B. CIESLINSKI

Department of Pharmacology, Smith Kline and French Laboratories, King of Prussia, Pennsylvania 19406-0939

Received May 13, 1989; Accepted November 2, 1989

SUMMARY

Cyclic nucleotide phosphodiesterases (PDEs) from canine trachealis were characterized with respect to their kinetic properties, sensitivity to selective inhibitors, and subcellular distribution. Extracts from whole tissue homogenates were applied to DEAE-Sepharose anion exchange columns and eluted with a linear sodium acetate gradient. Three major peaks of PDE activity were resolved. The first (PDE I), which eluted at 0.2 M sodium acetate, was applied to a calmodulin (CaM)-Sepharose affinity column and resolved into CaM-insensitive and CaM-sensitive PDEs. The CaM-insensitive isozyme (PDE Ia) had apparent K_m values of 135 μM (cAMP) and 4 μM (cGMP) and was potently inhibited by zaprinast (K_i = 0.1 μM). The CaM-sensitive isozyme (PDE Ic) had apparent K_m values of 1 μM (cAMP) and 2 μM (cGMP) and was inhibited by zaprinast with an apparent K_i of 35 μM. The second peak of activity (PDE II) from the anion exchange column eluted at 0.3 M sodium acetate and had apparent K_m values of 93 μM (cAMP) and 60 μM (cGMP). The enzyme displayed positive cooperativity with respect to the hydrolysis of cAMP (n_H = 1.7). Low concentrations of cGMP (0.1–1 μM) reduced cooperativity (n_H = 1.1) and increased the hydrolysis of 1 μM cAMP. The third peak of activity from the anion exchange column eluted at 0.6 M sodium acetate and displayed anomalous kinetics that suggested the presence of two isozymes. This was supported by the observation that enzyme activity was only partially inhibited by SK&F 94120 or Ro 20-1724 but was abolished by the combination of the two PDE inhibitors. Subsequent studies confirmed the existence of two isozymes. The first, PDE III, had apparent K_m values of 0.3 μM (cAMP) and 8 μM (cGMP) and was inhibited by cGMP (IC_{50} = 0.1 μM), SK&F 94120 (K_i = 7.8 μM), and SK&F 94836 (K_i = 0.4 μM). The second, PDE IV, had apparent K_m values of 4 μM (cAMP) and 40 μM (cGMP) and was inhibited by Ro 20-1724 (K_i = 5.2 μM) and rolipram (K_i = 0.5 μM) but not by cGMP. Assessment of the 100,000 × *g* soluble and particulate PDE activity revealed that all five isozymes were present in the soluble fraction, but only four isozymes (PDEs Ia, Ic, III, and IV) were present in the particulate fraction. These results indicate that five distinct PDE isozyme exist in canine trachealis and that these isozymes differ in their kinetic characteristics, sensitivity to activators and inhibitors, and subcellular distribution.

cAMP and cGMP are thought to be important intracellular regulators of airway smooth muscle tone (1). Increases in either cAMP or cGMP are associated with agonist-induced relaxation of bovine (2), canine (3–5), guinea pig (6), and opossum (7) tracheal smooth muscle. The physiologic actions of these second messengers are mediated by cAMP- or cGMP-dependent protein kinase, enzymes that phosphorylate and consequently alter the activity of physiologically relevant substrates. In airway smooth muscle, activation of either cyclic nucleotide cascade system induces relaxation by reducing the cytosolic Ca^{2+} concentration (8), directly interfering with the activity of contractile proteins (9), or both.

Intracellular concentrations of cyclic nucleotides are determined by their relative rates of formation and degradation. Cyclic nucleotides are degraded by a family of enzymes, termed PDEs, that inactivate cAMP or cGMP by catalyzing the hydrolysis of the 3′-phosphoester bond to form the corresponding inactive 5′-nucleotide. The various PDE isoforms differ with respect to their physical characteristics, substrate (cAMP or cGMP) specificity, kinetic characteristics, tissue distribution, subcellular location, and endogenous activators and inhibitors (10–13). Recently, interest in PDEs as drug targets has undergone a marked resurgence, triggered primarily by two factors, 1) the demonstration that individual PDE isozymes are distributed heterogeneously among different tissues (10) and 2) the synthesis of isozyme-selective PDE inhibitors (14). Taken together, these factors raise the prospect that a high degree of tissue selectivity can be bred into new generations of PDE inhibitors. Indeed, this prospect has been fulfilled by the development of various inhibitors of the low K_m cAMP PDE (15) found in the myocardium (16, 17) and vascular smooth muscle (18, 19). These compounds possess a marked selectivity for cardiac and vascular tissue and are currently under clinical investigation as novel inotropic/vasodilator agents (20, 21).

Another potential target for isozyme-selective PDE inhibitors is airway smooth muscle. Because both cAMP and cGMP

ABBREVIATIONS: PDE, phosphodiesterase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

are thought to mediate airway smooth muscle relaxation, compounds that inhibit the hydrolysis of either cyclic nucleotide may possess bronchodilator activity. In view of the considerations outlined above, the present study was conducted to identify PDE isozymes in canine trachealis and to evaluate the activity of purported isozyme-selective inhibitors against the various enzymes. This information will serve as a foundation for future studies directed toward defining the role of various PDE isozymes in regulating cyclic nucleotide content in intact airway smooth muscle.

Materials and Methods

Tissue preparation. Canine tracheal smooth muscle was removed, prepared, frozen, and stored as described previously (5). Before homogenization, 4 g of frozen muscle were pulverized under liquid nitrogen using a Spex freezer mill. All subsequent operations were conducted at 4°. The pulverized tissue was homogenized with a Brinkman PT 10/35 Polytron (two 10-sec bursts at setting 6) in 10 volumes of buffer (pH 6.5) containing 20 mM bis-Tris, 2.5 mM dithiothreitol, 10 mM EDTA, and 0.1% Triton X-100 plus a protease inhibitor mixture of 2 mM benzamidine, 20 μg/ml soybean trypsin inhibitor, 100 μg/ml bacitracin, 100 μM *p*-tosyl-L-lycine chloromethyl ketone, and 50 μM phenylmethylsulphonyl fluoride. The homogenate was then sonicated (10 sec/ml; setting 4) using a Branson Sonifier Cell Disruptor 200 and centrifuged at 20,000 × *g* for 30 min. The supernatant was filtered through four layers of gauze and applied to a DEAE-Sepharose (Pharmacia) column for separation of PDE activities.

DEAE-Sepharose chromatography. PDE isozyme separation was performed on a DEAE-Sepharose column (1.6-cm i.d.), with a 10-ml bed volume, equilibrated with the homogenization buffer. The entire 20,000 × *g* supernatant from a tissue homogenate was applied to the column. The column was then washed with 200 ml of homogenization buffer. No detectable PDE activity was found in this initial wash. The PDE isozymes were then eluted with 400 ml of a linear (0–1.0 M) sodium acetate gradient in the homogenization buffer, at a flow rate of 100 ml/hr. Eight-milliliter fractions were collected over the entire gradient. To help stabilize the enzyme, bovine serum albumin was added to each fraction to a final concentration of 0.1%. PDE activity was assayed in each fraction using 1 μM [^{3}H]cAMP or 1 μM [^{3}H]cGMP as substrate (see below for assay method). All fractions were assayed in the absence and presence of 10 μM cGMP, an activator of cGMP-stimulated PDE and an inhibitor of cGMP-inhibited PDE, or calmodulin (50 units of calmodulin plus 2 mM Ca^{2+} per assay tube), an activator of Ca^{2+}·calmodulin-stimulated PDE. At least 80% of the total homogenate PDE activity was recovered from the anion exchange column. Fractions within a peak of specific PDE activity were combined and concentrated for further characterization, using an Amicon YM10 Diaflo ultrafilter. Less than 10% of the total activity was lost to the Amicon filter. Aliquots of fractions were stored at −20°. Frozen fractions could be kept for at least 1 year without loss of activity.

Calmodulin-Sepharose affinity chromatography. A 10-ml (1.0-cm i.d.) calmodulin-Sepharose (Pharmacia) affinity column was prepared and equilibrated with a pH 7.5 buffer containing 20 mM Tris, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 mM EDTA, 0.25 M sucrose, 2.5 mM dithiothreitol, and the protease inhibitor mixture described above. To further purify peak I and peak II PDEs isolated from the DEAE-Sepharose columns, they were applied to an equilibrated calmodulin affinity column in the presence of excess Ca^{2+} (2 mM). The unbound calmodulin-insensitive protein was eluted from the column with 75–100 ml of the equilibration buffer. The calmodulin-bound activity was eluted from the affinity column with 100 ml of the equilibration buffer containing 2.5 mM EGTA in the absence of $CaCl_2$. Flow rate was set at 15 ml/hr. Two and a half-milliliter fractions were collected and assayed for PDE activity using cAMP and cGMP in the presence and absence of Ca^{2+}·calmodulin. Of the total PDE activity applied to the calmodulin affinity columns, 90% was routinely recovered in the eluate. Fractions containing the PDE activity were combined, concentrated with an Amicon YMIO Diaflo ultrafilter, and stored as described above for further characterization.

Separation of particulate and soluble PDEs. Particulate and soluble PDEs were separated by a modification of the method described by Weishaar and co-workers (22). Tracheal smooth muscle was gently homogenized in 10 volumes of 250 mM sucrose, 10 mM Tris·HCl (pH 7.8), 5 mM $MgCl_2$, 0.2 mM EGTA plus the previously described protease inhibition cocktail, using a Potter-Elvehjem homogenizer with a serrated Teflon pestle. The homogenate was then centrifuged at 100,000 × *g* for 60 min.

The pellet was washed twice by resuspension in 10 volumes of the homogenization buffer and centrifuged as above. The pellet was then resuspended in the homogenization buffer containing 0.4 M NaCl, 1.0% Triton X-100, and 0.1% Brij 30 (polyoxyethylene 4-lauryl ether) and was incubated overnight at 4°. This detergent extraction procedure solubilized 50% of the PDE activity present in the pellet. The detergent-extracted proteins were then centrifuged and the supernatant was dialyzed for 5 hr against the ion exchange equilibration buffer containing the protease inhibitors. Between 10 and 20% of the total enzyme was lost routinely during the dialysis procedure. The dialyzed supernatant was then applied to a DEAE ion exchange column and eluted as previously described.

PDE assay. PDE activity was assayed using a modification of the method of Davis and Daly (23). The reaction was conducted in 0.1 ml of standard mixture containing (final concentrations): 50 mM Tris·HCl buffer (pH 7.5), 5 mM $MgCl_2$, 50 μM [^{14}C]5′-AMP (approximately 400 dpm/nmol) as carrier and for recovery of product, 1 μM [^{3}H]cAMP (approximately 2000 dpm/pmol), and enzyme. The reaction was initiated with either enzyme or substrate and incubated at 30°. Incubation time was varied depending on the amount of enzyme activity. The reaction was terminated by placing reaction vessels in a 100° heating block for 1 min before transfer to an ice bath. To separate cyclic nucleotide substrates from 5′-nucleotide products, 0.5 ml of 0.1 M HEPES buffer (pH 8.5) containing 0.1 M NaCl was first added to each sample. The entire sample was then applied to a polyacrylamide-boronate gel column (0.5 g of Biorad Affi-gel 601 in a 0.7 × 10 cm Bio-Rad Econo-column), which had been equilibrated with the 0.1 M HEPES/0.1 M NaCl buffer (pH 8.5). The unreacted cyclic nucleotides were eluted with 8 ml of equilibration buffer. The 5′-monophosphate products were eluted with 10 ml of 0.25 M acetic acid into a scintillation vial containing 10 ml of Beckman Ready-Solv-MP scintillation cocktail. Radioactivity was measured via scintillation counting.

Recovery of [^{3}H]5′-AMP, as determined with the [^{14}C]5′-AMP carrier, was 80–90%. All assays were conducted in the linear range of the reaction, where less than 20% of the initial substrate is hydrolyzed. Kinetic assays were controlled so as to limit substrate hydrolysis to no greater than 10%.

Cyclic GMP hydrolysis was assayed using a protocol identical to the one described above, with [^{3}H]cGMP as the substrate.

Determination of kinetic parameters. For determination of values of V_{max}, K_{max}, and n_H (Hill coefficient), the concentration of cAMP or cGMP was varied while the amount of [^{3}H]cyclic nucleotide per assay tube was kept constant. Appropriate corrections were made for the changes in specific activity of the substrate. Enzymes exhibiting simple kinetics were analyzed with computer programs described by Cleland (24), using a nonlinear least squares regression analysis. When complex kinetics were evident, the data were evaluated using an iterative analysis for two enzymes acting on the same substrate (25).

Determination of inhibition constants was carried out using substrate and inhibitor concentrations that bracketed the K_m and estimated K_i. The data were fit to various inhibition (i.e., competitive, noncompetitive, uncompetitive) equations using computer programs described by Cleland (24). The appropriate equation was selected by analysis of the variance and residuals.

Materials. Concentrated (1 mM) stock solutions of SK&F 94120 [5-(4-acetamidophenyl)-2-(1H)-pyrazinone] and zaprinast were pre-

pared with 0.1 N NaOH. Stock solutions (1 mM) of SK&F 94836 [2-cyano-1-methyl-3-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridine-3-yl)phenyl]guanidine], SK&F 95654 [5-methyl-6-[4-4-oxo-1,4-dihydropyridin-1-yl)phenyl]-4,5-dihydro-3(2H)-pyridazinone], rolipram, and Ro 20-1724 were prepared by dissolving the compounds in a small amount of dimethyl sulfoxide before diluting them with an appropriate volume of H_2O. SK&F 94120, SK&F 94836, SK&F 95654, and zaprinast were synthesized by Dr. William Coates and colleagues, Smith Kline and French Laboratories, Ltd. (Welwyn, England). Rolipram was synthesized by Dr. Sigfried Christensen, Smith Kline and French Laboratories (King of Prussia, PA). Ro 20-1724 was obtained from Hoffmann-LaRoche (Nutley, NJ). 8-Methoxymethyl-1-isobutyl-3-methylxanthine was a generous gift from Dr. Jack Wells, Vanderbilt University (Nashville, TN). Calmodulin (P-2277), dipyridamole, and theophylline were obtained from Sigma (St. Louis, MO) and 3-isobutyl-1-methylxanthine was obtained from Aldrich (Milwaukee, WI).

Results

DEAE-Sepharose chromatography. The typical elution profile of canine tracheal PDEs from DEAE-Sepharose anion exchange columns is shown in Fig. 1. Three major peaks of activity were identified. These peaks eluted at 0.2, 0.3, and 0.6 M sodium acetate and were tentatively designated as PDE I, II, and III, respectively, according to the nomenclature of Reeves and co-workers (16). In the absence of activators, peak III preferred cAMP (1 μM) as a substrate, whereas peaks II preferred cGMP (1 μM) (Fig. 1A). Peak I hydrolyzed cAMP and cGMP to roughly the same extent (Fig. 1A). The hydrolysis of cAMP by fractions from peak I was increased approximately 2-fold by the addition of Ca^{2+}·calmodulin (Fig. 1B), whereas 10 μM cGMP (Fig. 1C) increased the enzyme activity of peak II severalfold and partially inhibited the activity of peak III.

Calmodulin-Sepharose affinity chromatography. The ability of Ca^{2+}·calmodulin to stimulate enzyme activity in peak I suggested that these fractions contained a Ca^{2+}·calmodulin-activated PDE. To further purify the PDE(s) from peak I, fractions 5 through 10 were pooled, concentrated, applied to a calmodulin-Sepharose affinity column, and eluted with buffer containing 1 mM Ca^{2+} (Fig. 2). Theoretically, Ca^{2+}·calmodulin-activated PDE should be retained by the column in the presence of Ca^{2+}, whereas PDEs not dependent on calmodulin should elute with the void volume. As shown in Fig. 2, a peak of PDE activity eluted in the presence of Ca^{2+}. This peak of activity, designated as PDE Ia, hydrolyzed cGMP to a much greater

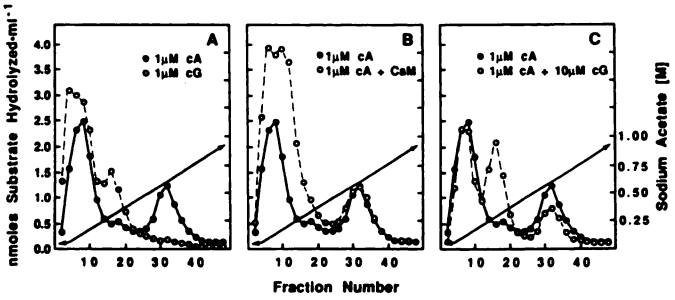

Fig. 1. Elution profile of canine tracheal smooth muscle PDE activity from DEAE-Sepharose anion exchange columns. Crude enzyme preparations from tissue homogenates were applied to the column and eluted with a linear sodium acetate gradient. A, PDE activity in the presence of 1 μM cAMP (*cA*) or 1 μM cGMP (*cG*). B, cAMP (1 μM) hydrolysis in the presence and absence of Ca^{2+}·calmodulin (*CaM*). C, the hydrolysis of 1 μM cAMP in the presence and absence of 10 μM cGMP. The data are representative of eight separate experiments.

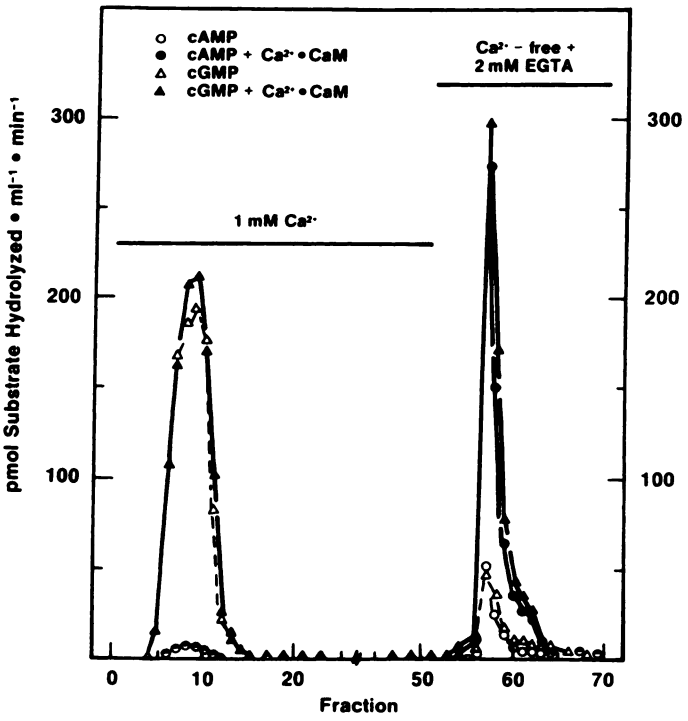

Fig. 2. Calmodulin (*CaM*)-Sepharose affinity chromatogram of PDE peak I activity isolated from canine tracheal smooth muscle. Fractions 6 through 10 collected from the anion exchange column (Fig. 1) were pooled, concentrated, and applied to the affinity column. The elution buffer contained 1 mM Ca^{2+} until fraction 52, at which point the buffer was changed to one containing 2 mM EGTA without Ca^{2+}. Enzyme activity was measured in the presence of 1 μM cAMP or cGMP and in the presence and absence of Ca^{2+}·calmodulin. The data are representative of four separate experiments.

extent than cAMP when activity was measured in the presence of 1 μM substrate. This enzyme was not stimulated by Ca^{2+}·calmodulin. After the collection of 52 fractions, the Ca^{2+}-containing elution buffer was changed to one containing 2 mM EGTA and no Ca^{2+}. Under these conditions, a second peak of activity eluted that hydrolyzed cAMP and cGMP to an equal extent and was stimulated 6-fold by the addition of Ca^{2+}·calmodulin (Fig. 2). This enzyme was designated PDE Ic.[1]

Kinetic analysis of peak I activities. To further characterize PDEs Ia and Ic, PDE-containing fractions from the calmodulin affinity column were pooled, concentrated, dialyzed, and subjected to kinetic analyses. PDE Ia possessed a considerable selectivity for cGMP, having an apparent K_m for cGMP hydrolysis of 4.2 μM versus an apparent K_m for cAMP hydrolysis of 135 μM (Fig. 3; Table 1). The relative V_{max} for cGMP hydrolysis was also 4-fold greater than that for cAMP hydrolysis.

A kinetic analysis of PDE Ic is shown in Fig. 4. This enzyme had an equal affinity (apparent K_m = 1–2 μM) and V_{max} for both cAMP and cGMP (Table 1). The activity of the enzyme was increased by the addition of Ca^{2+}·calmodulin, which produced a 2-fold decrease in the apparent K_m and a 4-fold increase in the V_{max} (Fig. 4).

Kinetic analysis of peak II activity. Fractions from the

[1] We have previously identified a Ca^{2+}·calmodulin-stimulated PDE from human trachealis (26) that has kinetic characteristics slightly different from those described here. Because the human PDE was termed PDE Ib, the slightly different enzyme identified in the present study has been designated PDE Ic.

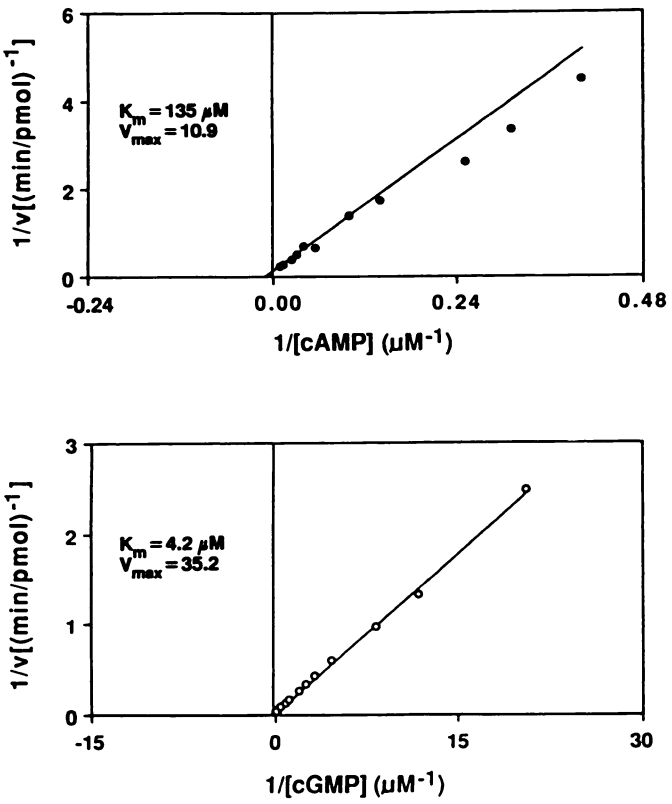

Fig. 3. Kinetic analysis of PDE Ia. The data represent initial velocities of cAMP (*top*) and cGMP (*bottom*) hydrolysis mediated by the calmodulin-insensitive enzyme isolated from the calmodulin-Sepharose affinity column (Fig. 2). The *lines* represent nonlinear least squares regression analyses of the untransformed data.

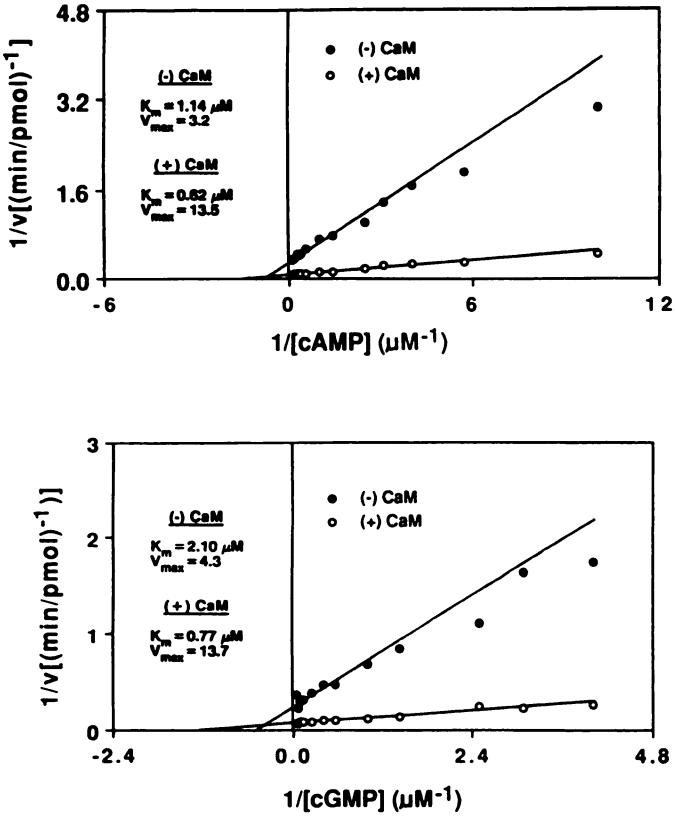

Fig. 4. Kinetic analysis of PDE Ic. The data represent initial velocities of cAMP (*top*) and cGMP (*bottom*) hydrolysis mediated by the calmodulin-stimulated enzyme isolated from the calmodulin-Sepharose affinity column (Fig. 2). Assays were conducted in the presence and absence of Ca^{2+}·calmodulin (*CaM*). The *lines* represent nonlinear least squares regression analyses of the untransformed data.

TABLE 1

Kinetic characteristics of canine tracheal PDE isozymes

Values were obtained in the absence of endogenous activators (e.g., cGMP, Ca^{2+}·calmodulin) and are representative of two to five determinations from different preparations.

PDE	Isozyme[a]	HbFe(II)O		Relative V_{max}
		cAMP	cGMP	cAMP/cGMP
		μM		
Ia	cGMP-specific	135	4	0.25
Ic	Ca^{2+}·calmodulin-stimulated	1	2	1
II	cGMP-stimulated	93[b]	60[b]	0.6
III	cGMP-inhibited[c]	0.3	8	ND[d]
IV	Ro 20-1724-inhibited[e]	4	40	2

[a] Isozyme nomenclature is the same as that used by Beavo (11).
[b] PDE II displayed positive cooperativity with respect to the hydrolysis of both cAMP (n_H = 1.7) and cGMP (n_H = 1.3).
[c] Kinetic parameters for PDE III were determined in the presence of 10 μM rolipram to limit the contribution of PDE IV to total cyclic nucleotide hydrolysis.
[d] ND, not determined.
[e] Kinetic parameters for PDE IV were determined in the presence of 100 μM SK&F 94120 to limit the contribution of PDE III to total cyclic nucleotide hydrolysis.

anion exchange column (Fig. 1) containing cGMP-stimulated PDE activity (fractions 14–18) were pooled, concentrated, and applied to the calmodulin-Sepharose affinity column. The PDE that eluted with the void volume, termed PDE II, was stimulated by cGMP and was retained for kinetic analysis. Kinetic analysis of the enzyme retained by the affinity column indicated that it represented PDE Ic (data not shown). A kinetic analysis of PDE II revealed an apparent K_m for cAMP of 93 μM (Fig. 5; Table 1). In the absence of cGMP, the kinetics of cAMP

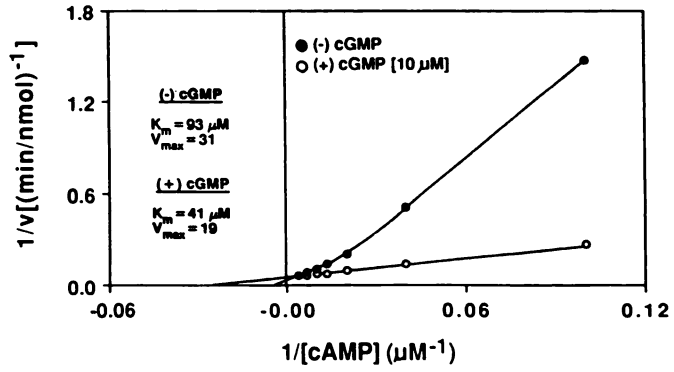

Fig. 5. Kinetic analysis of PDE II. Fractions 14 through 18 of the anion exchange column (Fig. 1) were pooled, concentrated, and applied to the calmodulin-Sepharose affinity column. The data represent the initial velocities of cAMP hydrolysis mediated by the calmodulin-insensitive enzyme isolated from the affinity column.

hydrolysis displayed positive cooperativity, with a Hill coefficient (n_H) of 1.7. The addition of 10 μM cGMP reduced the degree of cooperativity (n_H = 1.1), resulting in a substantial increase in the rate of cAMP hydrolysis (Fig. 5). The K_{act} (concentration necessary to produce half-maximal activation) for cGMP was approximately 0.5 μM. The kinetics of cGMP hydrolysis displayed less cooperativity (n_H = 1.3) than did cAMP hydrolysis. The apparent K_m for cGMP was 60 μM (Table 1).

Kinetic analysis of peak III activity. The kinetics of

cAMP hydrolysis produced by fractions comprising peak III PDE activity were complex (Fig. 6A). The curvilinear Lineweaver-Burk plot suggested the presence of more than one enzyme. Indeed, kinetic data subjected to an iterative analysis best fit a two-site model, one enzyme with an apparent K_m for cAMP of 0.25 μM and a second enzyme with an apparent K_m for cAMP of 8.8 μM (Fig. 6A).

Isozyme-selective PDE inhibitors were employed to support the contention that two enzymes were present in peak III PDE. Using PDEs isolated from human and guinea pig cardiac tissue, Reeves and co-workers (16) demonstrated that SK&F 94120 selectively inhibits a low K_m cGMP-inhibited PDE (designated PDE III), whereas rolipram selectively inhibits a PDE (designated PDE IV) that is not inhibited by low concentrations of cGMP and has a slightly higher K_m for cAMP. In the present study, inclusion of rolipram (10 μM) to minimize the contribution of PDE IV to cAMP hydrolysis in kinetic studies resulted in a nearly linear Lineweaver-Burk plot that defined an apparent K_m of 0.3 μM (Fig. 6B; Table 1). In a series of experiments in which SK&F 94120 (100 μM) was included to reduce the activity of PDE III, cAMP hydrolysis displayed linear kinetics, with an apparent K_m of 3.9 μM (Fig. 6C; Table 1). The apparent K_m values for cAMP determined in these studies, 0.38 μM for PDE III and 3.9 μM for PDE IV, are very similar to those obtained from the analogous isozyme isolated from cardiac tissue (16). Using the same approach, PDE III hydrolyzed cGMP with an apparent K_m of 8 μM and PDE IV hydrolyzed cGMP with an apparent K_m of 40 μM (Table 1). Cyclic AMP hydrolysis (1 μM) by PDE III was inhibited by cGMP, with an apparent K_i of 0.12 μM (Fig. 7).

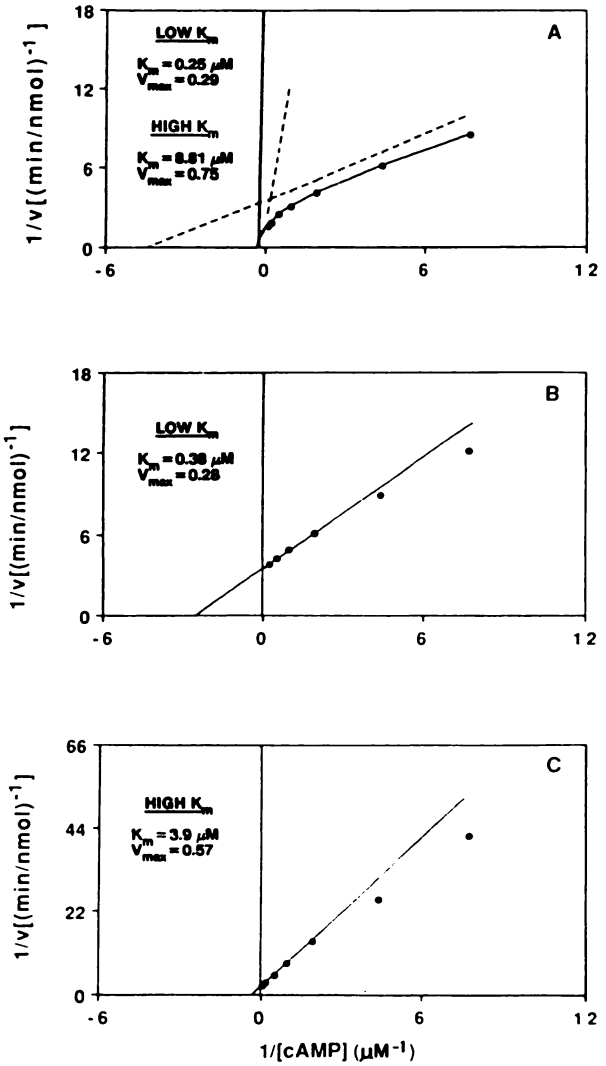

Fig. 6. Kinetic analysis of PDE III and PDE IV activity. The data represent initial velocities of cAMP hydrolysis mediated by the third peak of PDE activity (fractions 28 through 35) isolated from the anion exchange column (Fig. 1). Cyclic AMP hydrolysis was measured in the absence of inhibitors (A) and in the presence of 10 μM rolipram (B) or 100 μM SK&F 94120 (C) to inhibit the activity of contaminating PDE IV and PDE III, respectively. The *dashed lines* (A) represent the results of an iterative analysis modeled after two enzymes acting on the same substrate. The *solid lines* (B and C) represent nonlinear least squares regression analyses of the untransformed data.

Effects of PDE inhibitors. The kinetic properties of the canine tracheal PDEs in the presence of several compounds previously identified as isozyme-selective PDE inhibitors were determined. Zaprinast, a selective PDE I inhibitor (14), inhibited both PDE Ia and PDE Ic in a competitive manner, with apparent K_i values of 0.12 and 35 μM, respectively (Fig. 8).

The apparent K_i values for SK&F 94120 and SK&F 94836, selective inhibitors of PDE III (16, 27), were determined in the mixed PDE III/PDE IV preparations in the presence of 10 μM rolipram to inhibit contaminating PDE IV activity (Fig. 9, A and B). Under these conditions, SK&F 94120 and SK&F 94836 inhibited PDE III in a competitive manner with apparent K_i values of 7.8 and 0.5 μM, respectively (Fig. 9, A and B). In the presence of 100 μM SK&F 94120 to inhibit PDE III activity, Ro 20-1724 inhibited PDE IV with an apparent K_i of 5.2 μM and rolipram inhibited PDE IV with an apparent K_i of 0.5 μM (Fig. 9, C and D).

When assayed against other isozymes, zaprinast, SK&F 94120, SK&F 94836, Ro 20-1724, and rolipram displayed a considerable degree of selectivity (Table 2). SK&F 95654 was the most potent (IC_{50} = 0.7 μM) and selective inhibitor of PDE III tested (Table 2). 8-Methoxymethyl-1-isobutyl-3-methylxanthine was most active against PDE Ia, although it also inhibited PDEs Ic and II with reasonable potency. Dipyridamole inhibited PDEs Ia and IV. The inhibitory activity of theophylline

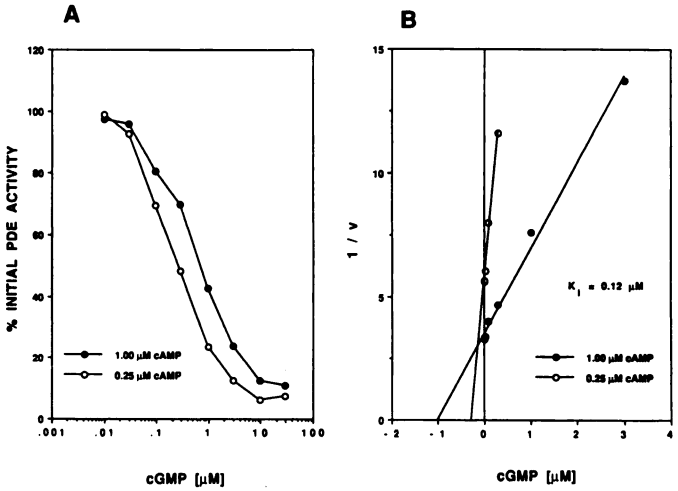

Fig. 7. Inhibitory effect of cGMP on PDE III activity. Assays were conducted in the presence of 10 μM rolipram to limit cAMP hydrolysis mediated by contaminating PDE IV. Activity was measured in the presence of 0.25 or 1.0 μM [^{3}H]cAMP (A). A Dixon plot of the data appears in B. The data are representative of two separate experiments.

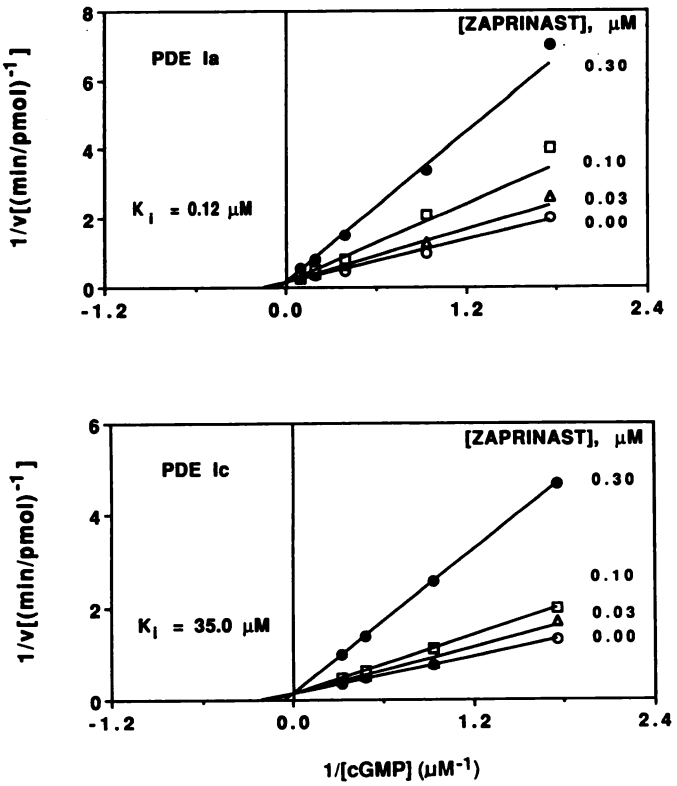

Fig. 8. Effect of zaprinast on cGMP hydrolysis mediated by PDE Ia (*top*) and PDE Ic (*bottom*). The *lines* represent results from nonlinear regression analyses of the original untransformed data fit to the equation for competitive inhibition. Experiments were conducted in the absence of Ca^{2+}·calmodulin.

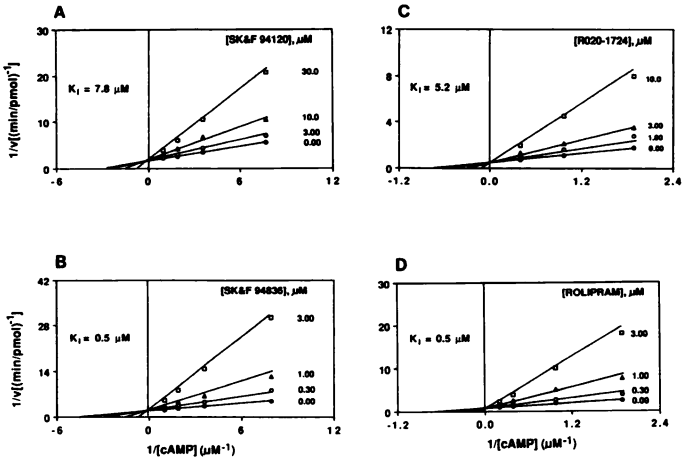

Fig. 9. A and B, Effect of SK&F 94120 (A) and SK&F 94836 (B) on PDE III activity. Assays were conducted in the presence of 10 μM rolipram to limit the hydrolysis of cAMP mediated by contaminating PDE IV. The *lines* represent results from nonlinear regression analyses of the original untransformed data fit to the equation for competitive inhibition. C and D, Effect of Ro 20-1724 (C) and rolipram (D) on PDE IV activity. Assays were conducted in the presence of 100 μM SK&F 94120 to limit the hydrolysis of cAMP mediated by contaminating PDE IV. The *lines* represent results from nonlinear regression analyses of the original untransformed data fit to the equation for competitive inhibition.

and 1-isobutyl-3-methylxanthine, nonselective PDE inhibitors, also was assessed for comparison (Table 2).

Subcellular localization of PDE isozymes. Soluble and particulate PDE isozymes were extracted as described in Materials and Methods. Of the total PDE activity in the canine trachealis, 80–90% was recovered in the soluble fraction (100,000 × *g* supernatant). The DEAE-Sepharose elution profile of the soluble enzymes was virtually identical to that of the whole tissue PDEs shown in Fig. 1. All five isozymes were present in the soluble fraction from whole trachealis.

TABLE 2

Effect of various inhibitors on the activity of canine trachealis PDE isozymes

The values represent mean IC_{50} values from two preparations. PDE activity was assessed in the presence of 1 μM [^{3}H]cGMP (PDEs Ia and Ic) or 1 μM [^{3}H]cAMP (PDEs II, III, and IV). Activity of PDE III was assessed in the presence of 10 μM rolipram and the activity of PDE IV was assessed in the presence of 100 μM SK&F 94120.

Inhibitor	IC_{50}				
	Ia	Ic	II	III	IV
			μM		
Zaprinast	0.1	27	66	>100	>100
SK&F 94120	>100	>100	>100	14	>100
SK&F 94836	31	>100	>100	1.4	>100
SK&F 95654	>100	>100	>100	0.7	>100
Ro 20-1724	75	>100	>100	>100	3
Rolipram	50	>100	>100	>100	0.6
Dipyridamole	2.5	>300	50	85	6
8-Methoxymethyl-1-isobutyl-3-methylxanthine	7.5	20	19	>100	72
Theophylline	42	>100	>100	>100	>100
Isobutyl-1-methylxanthine	0.8	23	27	11	21

The detergent extraction procedure solubilized 50–60% of the total PDE activity in the 100,000 × *g* pellet. The profile of the detergent-extracted particulate PDEs was different from that of the soluble fraction (Fig. 10). The first peak of particulate PDE activity that eluted from the anion exchange column hydrolyzed cAMP and cGMP to the same extent (Fig. 10A), was stimulated severalfold by Ca^{2+}·calmodulin (Fig. 10B), and was inhibited weakly (IC_{50} = 70 μM) by zaprinast. Calmodulin-Sepharose affinity chromatography revealed this peak to be composed almost exclusively of PDE Ic. Cyclic GMP (10 μM) failed to stimulate PDE activity in any fraction, suggesting the absence of PDE II, but strongly inhibited the broad peak of cAMP-selective PDE activity eluting between fractions 30 and 45 (Fig. 10C). SK&F 94836 (30 μM) inhibited this PDE activity to the same degree as cGMP, whereas rolipram (30 μM) had only a modest inhibitory effect (Fig. 10D). These data suggest that, compared with the PDE activity in the soluble fraction, the activity of the particulate PDE is enriched in PDE III relative to PDE IV.

The 40–50% of the total particulate PDE activity that was not extracted from the 100,000 × *g* pellet could not be identified with certainty. However, cAMP hydrolysis in this preparation was strongly inhibited by SK&F 94836 (30 μM) and cGMP (10 μM) but not by rolipram (30 μM). In contrast, cGMP hydrolysis was nearly abolished by 30 μM zaprinast. Neither cAMP or cGMP PDE activity in this fraction was enhanced by Ca^{2+}·calmodulin. These results suggest that the activity remaining in the detergent-extracted 100,000 × *g* pellet represented primarily PDE Ia and PDE III.

Discussion

Because of the purported roles of cAMP and cGMP as second messengers mediating relaxation of airway smooth muscle (1), it is of interest to determine the relative functional importance

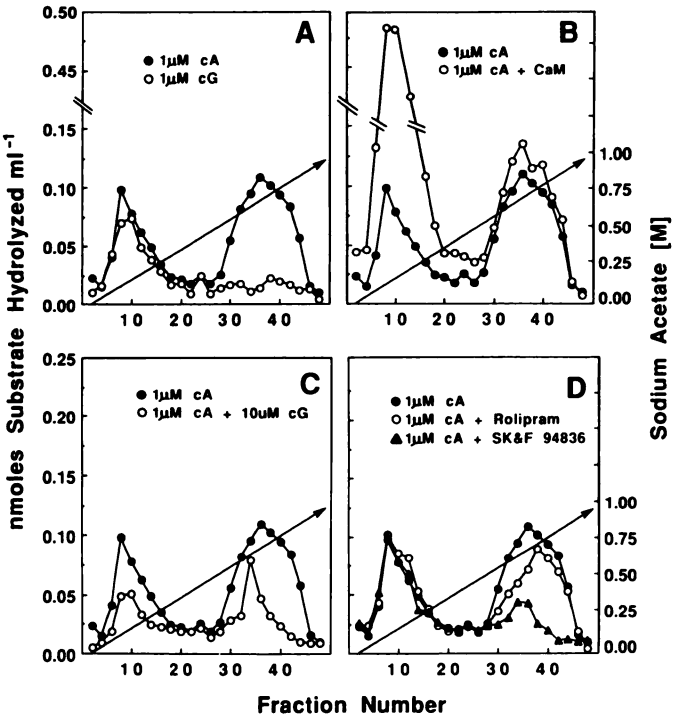

Fig. 10. Elution profile of trachealis particulate PDE activity from DEAE-Sepharose anion exchange columns. Enzymes from the 100,000 × *g* particulate fraction of trachealis homogenates were applied to the column and eluted with a linear sodium acetate gradient. A, PDE activity in the presence of 1 μM cAMP (*cA*) or 1 μM cGMP (*cG*). B, cAMP (1 μM) hydrolysis in the presence and absence of Ca^{2+}·calmodulin (*CaM*). C, cAMP (1 μM) hydrolysis in the presence and absence of 10 μM cGMP. D, cAMP (1 μM) hydrolysis in the presence of 30 μM SK&F 94836 or 30 μM rolipram. The data are representative of three separate experiments.

of various PDE isozymes in this tissue. Accomplishing this necessitates the completion of two distinct series of experiments. First, PDE isozymes from the target tissue should be isolated, characterized, and identified. Second, because the mere presence of an isozyme in tissue homogenates does not confirm its functional significance, the role of individual isozymes in regulating cyclic nucleotide content in the intact tissue should be assessed. The purpose of the present study was to fulfill the first requirement using the canine trachealis as a model of airway smooth muscle.

The first peak of PDE activity eluting from DEAE-Sepharose anion exchange contained two distinct isozymes, a cGMP-selective isozyme (PDE Ia) and a Ca^{2+}·calmodulin-stimulated enzyme (PDE Ic). The cGMP-selective PDE had a 35-fold greater affinity for cGMP than cAMP. The kinetic characteristics of this enzyme resembled those of the cGMP-selective PDE isolated from human platelets (28) and lung (29). The Ca^{2+}·calmodulin-stimulated enzyme in the canine trachealis had apparent K_m values of 1–2 μM for both cGMP and cAMP. These values are virtually identical to those obtained from the Ca^{2+}·calmodulin-stimulated PDE from rat testis (30) but different from those of the Ca^{2+}·calmodulin-stimulated enzyme from bovine lung (31) or brain (32), which hydrolyze cGMP with an apparent K_m of 1–3 μM and cAMP with a 10-fold greater apparent K_m of 30–40 μM. Activation of the tracheal enzyme by Ca^{2+}·calmodulin increased the hydrolysis of both cAMP and cGMP by decreasing the apparent K_m 2-fold and increasing the V_{max} 4-fold. This corresponds to an 8-fold overall increase in catalytic efficiency (V_{max}/K_m). Both the PDE Ia and Ic isolated from canine trachealis were inhibited competitively by zaprinast (M&B 22,948), a compound previously identified as a selective peak I PDE inhibitor (33), although its apparent K_i against the cGMP-selective enzyme (0.12 μM) was nearly 300-fold greater than against the Ca^{2+}·calmodulin-stimulated PDE (35 μM). A recent study of canine tracheal peak I PDE that had not been separated into cGMP-selective and Ca^{2+}·calmodulin-stimulated components revealed an IC_{50} for zaprinast of 0.5 μM (19), a value between that for PDE Ia and PDE Ic.

The PDE II identified in canine trachealis had kinetic properties very similar to those reported for cGMP-stimulated PDE isolated from bovine heart (34) and rat liver (35). As reported previously, the activity of this enzyme displayed positive cooperativity with respect to substrate, and the hydrolysis of 1 μM cAMP was increased severalfold by the addition of cGMP. Presumably, cGMP increases the activity of the enzyme binding to an allosteric site (34). Because of the ability of low concentrations of cGMP (0.1–1 μM) to increase cAMP hydrolysis by PDE II, it is tempting to speculate that the cyclic AMP content of airway smooth muscle may be reduced by bronchorelaxants such as nitroglycerin and sodium nitroprusside, agents that stimulate large increases in cGMP content (2, 4).

Results from this study suggest that the canine trachealis contains two cAMP-selective PDEs. One, PDE III, has an apparent K_m for cAMP of 0.3 μM and the hydrolysis of cAMP is inhibited by cGMP with an IC_{50} of 0.2 μM. This, along with the high potency of inhibitors such as SK&F 94120 and SK&F 94836, suggests that the PDE III in canine trachealis is similar to a membrane-bound PDE in cardiac tissue that serves as the molecular target for inotropes (15, 16, 36). It should be noted, however, that the apparent K_i for SK&F 94120 is nearly 10-fold lower against cardiac PDE III (16) than airway PDE III (present study). This apparent discrepancy could stem from species or tissue differences. Alternatively, the use of a mixed PDE III/PDE IV preparation and the inclusion of rolipram in the present kinetic studies may have obscured the true K_i. The second cAMP-selective PDE, PDE IV, has a somewhat higher apparent K_m for cAMP (4 μM) and is not inhibited by low concentrations of cGMP. This enzyme is inhibited by Ro 20-1724 and rolipram but not by the inotropes SK&F 94120 and SK&F 94836. These kinetic characteristics are similar to those of the soluble cardiac cAMP-selective PDE (22, 36), recently termed the Ro 20-1724-inhibited PDE (11).

The kinetic characteristics of the two cAMP-selective enzymes must be viewed with caution, inasmuch as they were derived from a mixed PDE preparation. Our attempts to resolve the two enzymes from canine trachealis have not been successful, although these isozymes have been separated using human myocardium as the tissue source (16). Regardless of the precision of the kinetic parameters obtained in this study, the results strongly suggest the presence of both low K_m cAMP PDEs.

The isozyme profile of the 100,000 × *g* particulate fraction differed from that of the soluble fraction. The soluble fraction contained all five PDE isozymes, whereas the particulate apparently lacked PDE II and thus contained only four of the isozymes. It also appeared that the relative activity of PDE III versus PDE IV was greater in the particulate fraction than in the soluble fraction. The functional significance of the subcellular distribution of PDE isozymes in the trachealis is unknown. In cardiac tissue, however, Weishaar and co-workers

(22) have proposed that inhibition of particulate but not soluble PDE III results in an increase in contractility. Moreover, inhibition of cardiac PDE IV, which is a soluble enzyme, does not result in a functional response (22). This information suggests that a distinct subcellular distribution of PDE isozymes in canine trachealis may indeed influence the mechanical response of the tissue to isozyme-selective PDE inhibitors.

The possibility that one or more of the PDE isozymes identified in this study originates from cells other than smooth muscle cells cannot be eliminated with certainty. This seems unlikely, however, inasmuch as the overwhelming proportion of the total cell population of the canine trachealis is composed of smooth muscle (37).

What role do the various PDE isozymes have in the regulation of cyclic nucleotide content in intact canine trachealis? Preliminary studies addressing this question have been conducted using isozyme-selective PDE inhibitors. Relaxation of the canine trachealis following the administration of SK&F 94836 is accompanied by an increase in cAMP content and activation of cAMP-dependent protein kinase (38). Moreover, SK&F 94836 potentiates isoproterenol-induced relaxation and cAMP accumulation but has no effect on the mechanical or biochemical responses to sodium nitroprusside (38), an agent thought to relax airway smooth muscle by a cGMP-mediated mechanism (4). These results suggest that PDE III is important for the hydrolysis of cAMP, but not cGMP, in intact canine trachealis. Recently, a similar strategy was used in an attempt to define the roles of PDEs Ia, Ic, and IV in regulating cyclic nucleotide content in canine trachealis (39). In these studies, zaprinast potentiated relaxation and cGMP accumulation in response to sodium nitroprusside but had no effect on the responses to isoproterenol. In contrast, Ro 20-1724 potentiated the biochemical and mechanical effects of isoproterenol but not the responses to sodium nitroprusside. These results suggest that PDE Ia and perhaps PDE Ic regulate cGMP content in the intact tissue, whereas PDE IV, like PDE III, regulates cAMP content. Obviously, however, additional studies are required before the biologic function of the individual isozymes can be defined with certainty.

In summary, the canine trachealis contains five distinct PDE isozymes. These isozymes differ in their kinetic characteristics, endogenous regulators, subcellular location, and sensitivity to various enzyme inhibitors. These results should provide a foundation for future studies concerning the role and regulation of PDE isozymes in airway smooth muscle.

Acknowledgments

The authors wish to thank Drs. Paul England, Mark Levy, and Martin Reeves for their helpful suggestions. The careful preparation of this manuscript by Ms. Dotti Lavan is also gratefully acknowledged.

References

1. Torphy, T. J., and W. T. Gerthoffer. Biochemical mechanisms of airway smooth muscle contraction and relaxation, in *Current Topics in Pulmonary Pharmacology and Toxicology* (M. A. Hollinger, ed.) Vol. 1. Elsevier, New York, 23–56 (1986).
2. Katsuki, S., and F. Murad. Regulation of adenosine cyclic 3′,5′-monophosphate levels and contractility in bovine tracheal smooth muscle. *Mol. Pharmacol.* **13**:330–341 (1977).
3. DeLanerolle, P. cAMP, myosin phosphorylation, and isometric relaxation of airway smooth muscle. *J. Appl. Physiol.* **64**:705–709 (1988).
4. Fiscus, R. R., T. J. Torphy, and S. E. Mayer. Cyclic GMP-dependent protein kinase activation in canine tracheal smooth muscle by methacholine and sodium nitroprusside. *Biochim. Biophys. Acta* **805**:382–392 (1984).
5. Torphy, T. J., C. Zheng, S. M. Peterson, R. R. Fiscus, G. A. Rinard, and S. E. Mayer. The inhibitory effect of methacholine on drug-induced relaxation, cAMP accumulation, and cAMP-dependent protein kinase activation in canine tracheal smooth muscle. *J. Pharmacol. Exp. Ther.* **233**:409–417 (1985).
6. Murad, F., and H. Kimura. Cyclic nucleotide levels in incubations of guinea pig trachea. *Biochim. Biophys. Acta* **343**:275–286 (1974).
7. Torphy, T. J., M. Burman, L. M. Schwartz, and M. A. Wasserman. Differential effects of methacholine and leukotriene D_4 on cyclic nucleotide content and isoproterenol-induced relaxation in the opossum trachea. *J. Pharmacol. Exp. Ther.* **237**:332–340 (1986).
8. Gunst, S. J., and J. Q. Stropp. Effect of Na-K adenosinetriphosphatase activity on relaxation of canine tracheal smooth muscle. *J. Appl. Physiol.* **64**:635–641 (1988).
9. Sparrow, M. P., M. Pfitzer, M. Gagelmann, and J. C. Rüegg. Effect of calmodulin, Ca^{2+}, and cAMP protein kinase on skinned tracheal smooth muscle. *Am. J. Physiol.* **246**:C308–C314 (1984).
10. Appleman, M. M., M. A. Ariano, D. J. Takemoto, and R. H. Whitson. Cyclic nucleotide phosphodiesterase, in *Handbook of Experimental Pharmacology* (J. A. Nathanson and J. W. Kebabian, eds.), Vol. 58/1. Springer-Verlag, Berlin, 262–300 (1982).
11. Beavo, J. A. Multiple isozymes of cyclic nucleotide phosphodiesterase. *Adv. Second Messenger Phosphoprotein Res.* **22**:1–38 (1988).
12. Weishaar, R. E. Multiple molecular forms of phosphodiesterase: an overview. *J. Cyclic Nucleotide Res.* **11**:463–472 (1987).
13. Wells, J. N., and J. G. Hardman. Cyclic nucleotide phosphodiesterase. *Adv. Cyclic Nucleotide Res.* **8**:119–143 (1977).
14. Weishaar, R. E., M. H. Cain, and J. A. Bristol. A new generation of phosphodiesterase inhibitors: multiple molecular forms of phosphodiesterase and the potential for drug selectivity. *J. Med. Chem.* **28**:537–545 (1985).
15. Harrison, S. A., D. H. Reifsynder, B. Gallis, G. G. Cadd, and J. A. Beavo. Isolation and characterization of bovine cardiac muscle cGMP-inhibited phosphodiesterase: a receptor for new cardiotonic drugs. *Mol. Pharmacol.* **29**:506–514 (1986).
16. Reeves, M. L., B. K. Leigh, and P. J. England. The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. *Biochem. J.* **241**:535–541 (1987).
17. Weishaar, R. E., D. C. Kobylarz-Singer, M. M. Quade, R. P. Steffen, and H. R. Kaplan. Multiple molecular forms of phosphodiesterase and the regulation of cardiac muscle contractility. *J. Cyclic Nucleotide Res.* **11**:513–527 (1987).
18. Lugnier, C., P. Schoeffter, A. Le Bec, E. Strouthou, and J. C. Stoclet. Selective inhibition of cyclic nucleotide phosphodiesterases of human bovine and rat aorta. *Biochem. Pharmacol.* **35**: 1743–1751 (1986).
19. Silver, P. J., L. T. Hamel, M. H. Perrone, R. G. Bentley, C. R. Bushover, and D. B. Evans. Differential pharmacologic sensitivity of cyclic nucleotide phosphodiesterase isozymes isolated from cardiac muscle, arterial and airway smooth muscle. *Eur. J. Pharmacol.* **15**:85–94 (1988).
20. Colucci, W. S., R. F. Wright, and E. Braunwald. New positive inotropic agents in the treatment of congestive heart failure: mechanisms of action and recent clinical developments. *N. Engl. J. Med.* **314**: 349–358 (1986).
21. Erhardt, P. W. In search of the digitalis replacement. *J. Med. Chem.* **30**:231–237 (1987).
22. Weishaar, R. W., D. C. Kobylarz-Singer, and H. R. Kaplan. Subclasses of cyclic AMP phosphodiesterase in cardiac muscle. *J. Mol. Cell. Cardiol.* **19**:1025–1036 (1987).
23. Davis, C. W., and J. W. Daly. A simple direct assay of 3′,5′-cyclic nucleotide phosphodiesterase activity based on the use of polyacrylamide-boronate affinity gel chromatography. *J. Cyclic Nucleotide Res.* **5**:65–74 (1979).
24. Cleland, W. W. Statistical analysis of enzyme kinetic data. *Methods Enzymol.* **63**:103–138 (1979).
25. Spears, G., J. G. T. Sneyd, and E. G. Loten. A method for deriving kinetic constants for two enzymes acting on the same substrate. *Biochem. J.* **125**:1149–1151 (1971).
26. Cieslinski, L. B., M. L. Reeves, and T. J. Torphy. Cyclic nucleotide phosphodiesterases (PDEs) in canine and human tracheal smooth muscle. *FASEB J.* **2**:A1065 (1988).
27. Reeves, M. L., R. W. Gristwood, B. K. Leigh, and P. J. England. SK&F 94836, a potent inotropic agent, is a selective inhibitor of type III phosphodiesterase activity from several tissues. *Br. J. Pharmacol.* **92**:773A (1987).
28. Hidaka, H., and T. Asano. Human blood platelet 3′,5′-cyclic nucleotide phosphodiesterase. *Biochim. Biophys. Acta* **429**:485–497 (1976).
29. Bergstrand, H., B. Lundquist, and A. Schurmann. Cyclic nucleotide phosphodiesterase: partial purification and characterization of a high affinity enzyme activity from human lung tissue. *J. Biol. Chem.* **253**:1881–1891 (1978).
30. Purvis, K., A. Olsen, and V. Hansson. Calmodulin-dependent cyclic nucleotide phosphodiesterase in immature rat testis. *J. Biol. Chem.* **256**:11434–11441 (1981).
31. Sharma, R. K., and J. H. Wang. Purification and characterization of bovine lung calmodulin-dependent cyclic nucleotide phosphodiesterase: an enzyme containing calmodulin as a subunit. *J. Biol. Chem.* **261**:14160–14166 (1986).
32. Sharma, R. K., T. H. Wang, E. Wirch, and J. H. Wang. Purification and properties of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase. *J. Biol. Chem.* **255**:5916–5923 (1980).
33. Weishaar, R. E., S. D. Burrows, D. C. Kobylarz, M. M. Quade, and D. B. Evans. Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and platelets: isolation, characterization and

effects of various reference phosphodiesterase inhibitors and cardiotonic agents. *Biochem. Pharmacol.* **35**:787–800 (1986).
34. Martins, T. J., M. C. Mumbey, and J. A. Beavo. Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. *J. Biol. Chem.* **257**:1973–1979 (1982).
35. Pyne, N. J., M. E. Cooper, and M. D. Houslay. Identification and characterization of both the cytosolic and particulate forms of cyclic GMP-stimulated cyclic AMP phosphodiesterase from rat liver. *Biochem. J.* **234**:325–334 (1986).
36. Kithas, P. A., M. Artman, W. J. Thompson, and S. J. Strada. Subcellular distribution of high-affinity type IV cyclic AMP phosphodiesterase activity in rabbit ventricular myocardium: relation to the effects of cardiotonic drugs. *Circ. Res.* **62**:782–789 (1988).
37. Stephens, N. L. Airway smooth muscle, in *Lung Cells in Disease* (A. Bouhuys, ed.). Elsevier, New York, 113–138 (1976).
38. Torphy, T. J., M. Burman, L. B. F. Huang, and S. S. Tucker. Inhibition of the low K_m cyclic AMP phosphodiesterase in intact canine trachealis by SK&F 94836: mechanical and biochemical responses. *J. Pharmacol. Exp. Ther.* **246**:843–850 (1988).
39. Torphy, T. J., M. Burman, and L. B. F. Huang. Role of phosphodiesterase (PDE) isozymes in regulating cyclic nucleotide content in canine trachealis. *FASEB J.* **2**:A1066 (1988).

Send reprint requests to: Theodore J. Torphy, Ph.D., Department of Pharmacology, L-532, Smith Kline and French Laboratories, P.O. Box 1539, King of Prussia, PA 19406-0939.