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Role of Cyclic Nucleotide Phosphodiesterase Isozymes in Intact Canine Trachealis

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SUMMARY

The role of individual cyclic nucleotide phosphodiesterase (PDE) isozymes in regulating cAMP and cGMP content in intact canine trachealis was examined using isozyme-selective and nonselective PDE inhibitors. The inhibitors used in this study were characterized previously [*Mol. Pharmacol.* **37**:206–214 (1990)] and included: 1) zaprinast, an inhibitor (K_i = 0.1 μM) of the cGMP-specific PDE (cAMP K_m = 135 μM; cGMP K_m = 4 μM); 2) SK&F 94120, an inhibitor (K_i = 7 μM) of the cGMP-inhibited PDE (cAMP K_m = 0.3 μM; cGMP K_m = 8 μM); 3) Ro 20-1724, an inhibitor (K_i = 5 μM) of the cAMP-specific PDE (cAMP K_m = 4 μM; cGMP K_m = 40 μM); and 4) 3-isobutyl-1-methylxanthine (IBMX), a nonselective PDE inhibitor (IC_{50} = 1–30 μM). In addition to the aforementioned isozymes, canine trachealis contains a Ca^{2+}/calmodulin-stimulated PDE (cAMP K_m = 1 μM; cGMP K_m = 2 μM) and a GMP-stimulated PDE (cAMP K_m = 93 μM; cGMP K_m = 60 μM), for which selective inhibitors are not available. Isolated canine trachealis strips were contracted with methacholine and exposed to various concentrations of PDE inhibitors, before being relaxed by the cumulative addition of isoproterenol, an adenylate cyclase activator, or sodium nitroprusside, a guanylate cyclase activator. At the completion of the concentration-response studies, tissues were flash-frozen and assayed for cyclic nucleotide content. Neither isoproterenol-induced relaxation nor cAMP accumulation was altered by zaprinast, but both of these responses were potentiated by pretreatment of tissues with either SK&F 94120 or Ro 20-1724. The effects of SK&F 94120 and Ro 20-1724 were additive, and the combination of SK&F 94120, Ro 20-1724, and IBMX had no greater effect on the responses to isoproterenol than did either IBMX alone or the combination of SK&F 94120 plus Ro 20-1724. In contrast, zaprinast potentiated sodium nitroprusside-induced relaxation and cGMP accumulation, whereas neither SK&F 94120 nor Ro 20-1724 altered these responses. IBMX produced a greater potentiation than did zaprinast, and the combination of zaprinast and IBMX had a greater effect than either agent alone. The results of this study suggest that the cGMP-inhibited and cAMP-specific PDEs are responsible for cAMP hydrolysis in intact canine trachealis, whereas cGMP hydrolysis is mediated by the cGMP-specific PDE as well as the Ca^{2+}/calmodulin-stimulated PDE and/or the cGMP-stimulated PDE.

Relaxation of airway smooth muscle can be mediated by increases in the cellular concentration of cAMP or cGMP (1, 2). These second messengers induce relaxation by activating cyclic nucleotide-dependent protein kinases (3, 4), which, in turn, alter the activity of functionally relevant proteins through phosphorylation cascade pathways (2).

In intact tissues, absolute cellular concentrations of cAMP and cGMP are determined by the relative rate of cyclic nucleotide formation versus the rates of cyclic nucleotide degradation and removal. The major cellular mechanism by which cyclic nucleotides are inactivated is through the action of PDEs, enzymes that catalyze the hydrolysis of the 3′-phosphoester bond of cAMP or cGMP to form the corresponding inactive 5′-nucleotide metabolite. PDEs are a diverse family of isozymes that differ in their primary protein sequence, physical and kinetic characteristics, substrate selectivity, tissue distribution, subcellular location, and endogenous activators and inhibitors (5–8). Recently, selective inhibitors of several of the isozymes have been identified (6, 9, 10). As described below, such inhibitors provide important tools for determining the role of PDEs in intact cells.

A potential consequence of the diversity among PDEs is that different isozymes may subserve distinct functions *in vivo*. For example, certain isozymes may mediate cAMP hydrolysis, whereas the action of other PDEs may be directed against cGMP. With regard to modulation of the cellular content of one or the other cyclic nucleotide, one PDE may regulate basal levels, whereas another may regulate cyclic nucleotide levels under conditions in which the activity of adenylate or guanylate cyclase is elevated (11).

Canine trachealis homogenates contain five distinct PDE isozymes (12). The purpose of the present study was to determine the role of these isozymes in regulating cyclic nucleotide content in intact canine trachealis. Similar to the approach

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ABBREVIATIONS: PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine.

taken by Lorenz and Wells (13) to describe the role of PDE isozymes in the bovine coronary artery, we used isozyme-selective PDE inhibitors to potentiate relaxation and cyclic nucleotide accumulation in response to either isoproterenol, an activator of adenylate cyclase, or sodium nitroprusside, an activator of guanylate cyclase. The results of these experiments provide evidence to suggest that, in canine trachealis, the PDE isozymes responsible for hydrolyzing cAMP are distinct from those responsible for hydrolyzing cGMP. A preliminary account of some of these data has appeared previously (14).

Materials and Methods

Tissue preparation. Mongrel dogs (15–25 kg) of either sex were anesthetized with an intravenous injection of pentobarbital. Tracheae were excised and placed in aerated (95% O_2/5% CO_2) cold (0°) Krebs-Henseleit buffer (pH 7.4) of the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 10; and ascorbic acid, 1.0. The trachea from a single animal was cut into as many as 12 individual rings. Thin trachealis strips (2–3-mm wide × 0.5-mm thick; 30–40 mg) were obtained from the rings by separation of the smooth muscle layer from mucosa, epithelium, and cartilage. The strips were mounted on plastic tissue clips, fastened to stainless steel tissue hooks, transferred to water-jacketed quick-release tissue baths, and incubated at 37° for 45 min in the aerated buffer. During this stabilization period, the trachealis strips were maintained at 4 g of resting tension and washed with fresh buffer every 5 min for the first 15 min and then at 15-min intervals before the addition of drugs. Mechanical responses were recorded on a Grass model RPS7C polygraph or a Beckman Dynograph model R-612 polygraph, via Grass force displacement transducers (FTO3C or FT03D).

Drug additions. After the 45-min incubation period, all tissues were treated with 1 μM meclofenamic acid to prevent prostanoid formation. Fifteen minutes after the addition of meclofenamic acid, trachealis strips were contracted with 1 or 3 μM methacholine. The contraction induced by methacholine reached 90–95% of plateau within 5 min of agonist addition. At that time, tissues were treated with vehicle or various concentrations of PDE inhibitors. When tissues were treated with combinations of PDE inhibitors, the compounds were added simultaneously. Ten minutes after the addition of PDE inhibitors, trachealis strips were relaxed by the cumulative addition of isoproterenol or sodium nitroprusside. Because the cumulative concentration-response studies took approximately 30 min to complete, PDE inhibitors were in contact with the tissue for a total of 40 min by the end of the experiment. In each experiment, methacholine-contracted trachealis strips exposed to vehicle alone, PDE inhibitor alone, or relaxant alone served as time-matched controls.

Control and drug-treated tissues were clamp-frozen with Wollenburger clamps (precooled in liquid N_2) when maximum relaxation was attained at the end of cumulative concentration-response studies. Frozen tissues were stored at −70° before being assayed for cyclic nucleotide content.

Contractions induced by methacholine remained stable over the entire 40- to 45-min observation period. None of the PDE inhibitors, used alone or in combination, relaxed trachealis strips that were contracted by 1 or 3 μM methacholine. Consequently, relaxation was induced only by the addition of isoproterenol or sodium nitroprusside. The ability to detect and quantify potentiation of the responses to isoproterenol or sodium nitroprusside in the presence of PDE inhibitors was, thus, greatly simplified.

Cyclic nucleotide measurement. One milliliter of cold (0°) 10% trichloroacetic acid, containing 4000 cpm of [^{3}H]cAMP or [^{3}H]cGMP added as a tracer, was added to a cold ground glass homogenizing tube containing approximately 10 mg of frozen tissue. The tissue was homogenized and transferred to 12- × 75-mm glass test tubes, and precipitated protein was separated from the soluble extract by centrifugation at 3000 × *g* for 10 min. Trichloroacetic acid was removed from the sample with five successive extractions with water-saturated ether (15). cAMP and cGMP were acetylated (15) and their amounts were determined using commercially available radioimmunoassay kits (New England Nuclear, Boston, MA). Cyclic nucleotide content was corrected for percentage of recovery (85–95%) and expressed as pmol of cyclic nucleotide/mg of protein. Protein content was determined by the method of Lowry *et al.* (16), using bovine serum albumin as a standard.

Control experiments were conducted to determine the cross-reactivity of the various compounds used in this study with the cAMP and cGMP antibodies. All compounds were subjected to the same acetylation procedure used when tissue cAMP or cGMP levels were measured. Various concentrations of the compounds were then incubated with the cAMP or cGMP antiserum and the appropriate radiolabeled cyclic nucleotide tracer. SK&F 94120, Ro 20-1724, rolipram, zaprinast, IBMX, isoproterenol, and sodium nitroprusside were at least 10^7-fold less potent than acetylated cAMP or cGMP in competing with the radiolabeled tracer for binding.

Data analysis and statistical evaluation. Data are expressed as the mean ± standard error. To determine EC_{50} values, responses to an agonist were calculated as a percentage of the maximum response to that agonist. EC_{50} values were calculated from linear regression analyses of probit-transformed data and expressed as a negative logarithm. Statistical differences between two means were determined by Student's *t* test for paired or unpaired samples. Differences among the means of more than two groups were analyzed by the multiple comparison method of Williams (17). Differences among means were accepted as significant at $p < 0.05$.

Drugs. Kinetic constants for the isozyme-selective PDE inhibitors used in this study, along with the kinetic characteristics of PDE isozymes in canine trachealis, were determined previously (12) and are shown in Table 1. All inhibitors used possessed at least a 25-fold selectivity for the corresponding isozyme (12). SK&F 94120 [5-(4-acetamidophenyl)-pyrazin-2-(1*H*)-one] and zaprinast (M&B 22,948) were synthesized by Dr. William Coates and colleagues (SmithKline Beecham Pharmaceuticals, Welwyn, England). Stock solutions (10 mM) of these compounds were prepared in saline by adjustment of the pH to 9 with 0.1 N NaOH. Ro 20-1724 was obtained as a gift from Hofmann-LaRoche (Nutley, NJ). Stock solutions (10 mM) of Ro 20-1724 were prepared by dissolving the compound in a small volume of dimethyl sulfoxide before diluting it with an appropriate volume of H_2O. IBMX was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Regulation of isoproterenol-stimulated cAMP accumulation and relaxation. The effect of inhibition of the cGMP-specific PDE on isoproterenol-induced relaxation and cAMP accumulation is shown in Fig. 1. Zaprinast had no effect

TABLE 1

Kinetic characteristics of canine tracheal PDEs and isozyme-selective inhibitors

Each inhibitor possesses at least a 25-fold selectivity for the appropriate isozyme (see Ref. 12). Values were taken from Ref. 12.

Isozyme[a]	$K_{m_{app}}$ cAMP	$K_{m_{app}}$ cGMP	Inhibitor	K_i
	μM			μM
cGMP-specific	135	4	Zaprinast	0.1
Ca^{2+}/calmodulin-stimulated	1	2	NA[b]	
cGMP-stimulated	93	60	NA	
cGMP-inhibited	0.3	8	SK&F 94120	7
cAMP-specific	4	40	Ro 20-1724	5

[a] Isozyme nomenclature taken from Beavo and Reifsnyder (5).
[b] NA, not available.

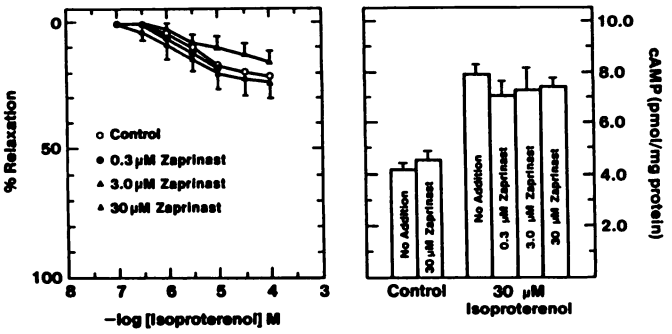

Fig. 1. Effect of zaprinast on isoproterenol-induced relaxation (*left*) and cAMP accumulation (*right*) in canine trachealis (n = 6). Tissues were contracted with 1 μM methacholine, treated with vehicle (O) or various concentrations of zaprinast (●, 0.3 μM; △, 3 μM; ▲, 30 μM), and relaxed by the cumulative addition of isoproterenol. At the end of the concentration-response studies, control (no isoproterenol) and isoproterenol-treated tissues were flash-frozen and assayed for cAMP content.

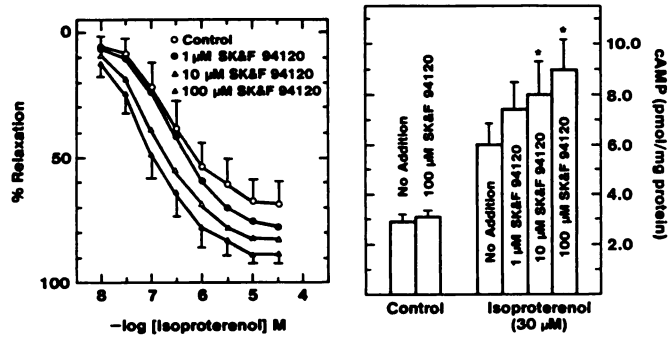

Fig. 2. Effect of SK&F 94120 on isoproterenol-induced relaxation (*left*) and cAMP accumulation (*right*) in canine trachealis (n = 6). Tissues were contracted with 1 μM methacholine, treated with vehicle (O) or various concentrations of SK&F 94120 (●, 1 μM; △, 10 μM; ▲, 100 μM), and relaxed by the cumulative addition of isoproterenol. At the end of the concentration-response studies, control (no isoproterenol) and isoproterenol-treated tissues were flash-frozen and assayed for cAMP content. *, Greater than value in the presence of isoproterenol alone ($p < 0.05$).

on either parameter, even when used at a concentration (30 μM) 300-fold above its K_i. This suggests that the cGMP-specific PDE does not participate in cAMP hydrolysis in the intact trachealis.

Unlike the results with zaprinast, SK&F 94120 produced a concentration-dependent potentiation of isoproterenol-induced relaxation and cAMP accumulation (Fig. 2). The highest concentration of SK&F 94120 used, 100 μM, increased the maximum relaxation in response to isoproterenol from 70 ± 8% (control) to 91 ± 3% ($p < 0.05$) and produced a 3.7-fold leftward shift of the concentration-response curve (−log EC_{50} = 6.45 ± 0.23 in control versus 7.02 ± 0.24 with SK&F 94120; $p < 0.05$).

Inhibition of the cAMP-specific enzyme also potentiated isoproterenol-induced relaxation and cAMP accumulation (Fig. 3). Maximum isoproterenol-induced relaxation was increased from 35 ± 5% to 82 ± 3% ($p < 0.05$) by 100 μM Ro 20-1724, and the EC_{50} was decreased 10-fold (−log EC_{50} = 5.68 ± 0.22 in control versus 6.72 ± 0.18 with Ro 20-1724; $p < 0.05$).

The effect of a combination of 100 μM SK&F 94120 and 100 μM Ro 20-1724 is shown in Fig. 4. As shown before, the responses to isoproterenol were potentiated by using either of these two PDE inhibitors. When used in combination, the effects were additive. Maximum isoproterenol-induced relaxation was increased from 34 ± 4% (control) to approximately

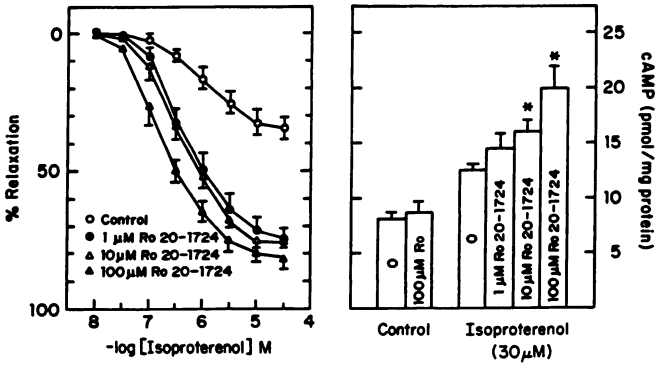

Fig. 3. Effect of Ro 20-1724 on isoproterenol-induced relaxation (*left*) and cAMP accumulation (*right*) in canine trachealis (n = 6). Tissues were contracted with 1 μM methacholine, treated with vehicle (O) or various concentrations of Ro 20-1724 (●, 1 μM; △, 10 μM; ▲, 100 μM), and relaxed by the cumulative addition of isoproterenol. At the end of the concentration-response studies, control (no isoproterenol) and isoproterenol-treated tissues were flash-frozen and assayed for cAMP content. The data were presented previously in Ref 14. *, Greater than value in the presence of isoproterenol alone ($p < 0.05$).

50% in tissues treated with either SK&F 94120 or Ro 20-1724 alone and to 77 ± 6% in tissues treated with the combination (Fig. 4). Compared with control isoproterenol EC_{50} values (−log EC_{50} = 5.32 ± 0.17), those for tissues treated with either Ro 20-1724 or SK&F 94120 alone were decreased 4- or 8-fold, respectively, whereas those for tissues treated with a combination were decreased 16-fold. The effects of SK&F 94120 and Ro 20-1724 on the isoproterenol-induced increment in cAMP accumulation also were additive, with the individual agents each producing an increase of 4 pmol of cAMP/mg of protein over the value obtained with isoproterenol alone and the combination of PDE inhibitors producing an increment of 9 pmol of cAMP/mg of protein (Fig. 4).

It is noteworthy that, in the absence of isoproterenol, neither SK&F 94120 nor Ro 20-1724 had an effect on basal cAMP content in methacholine-contracted trachealis strips (Figs. 2, 3, and 4). The combination of the two PDE inhibitors, however, did produce a small but significant increase in cAMP accumulation under the same conditions (Fig. 4). This increase in cAMP content was not accompanied by relaxation.

The effect of 100 μM IBMX, a compound that inhibits all PDE isozymes with IC_{50} values ranging from 1 to 30 μM (12), is shown in Fig. 5. IBMX produced a pattern of potentiation that was identical to that observed with the combination of SK&F 94120 and Ro 20-1724 (Fig. 4). Maximum relaxation in response to isoproterenol was increased by IBMX from 33 ± 3% to 72 ± 7%, and the EC_{50} for isoproterenol was decreased by 20-fold. Coincident with its effects on relaxation, 100 μM IBMX increased isoproterenol-induced cAMP accumulation from 8.6 ± 1.1 pmol/mg of protein to 20.5 ± 3.4 pmol/mg of protein. This increase, 2.4-fold, was nearly identical to the 2.2-fold increase observed with the combination of SK&F 94120 and Ro 20-1724 (Fig. 4).

As with the combination of Ro 20-1724 and SK&F 94120, 100 mM IBMX produced a small increase in basal (no isoproterenol) cAMP content in methacholine-contracted tissues that was not accompanied by relaxation (Fig. 5).

In another study, the effect of a combination of IBMX, Ro 20-1724, and SK&F 94120 on the mechanical and biochemical responses to isoproterenol was examined (Fig. 6). In these

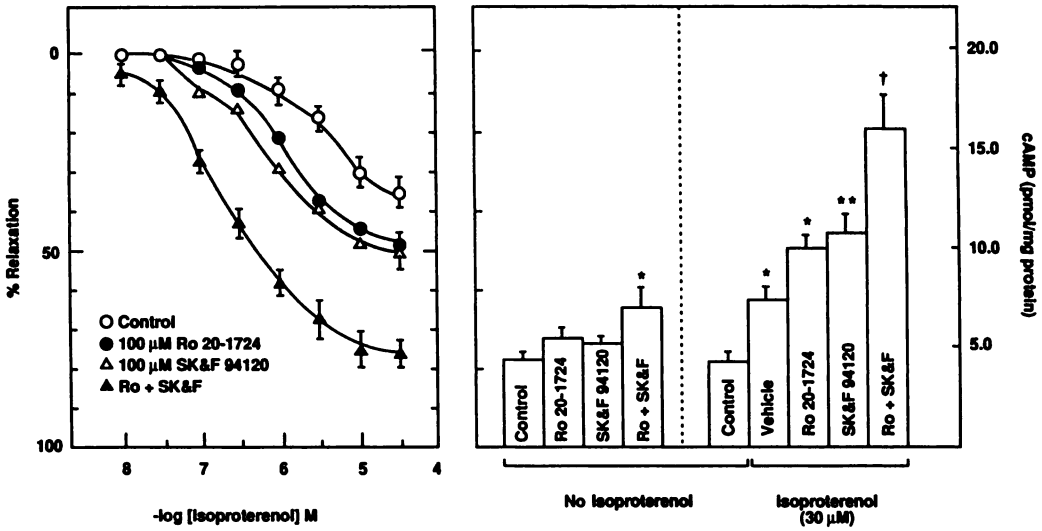

Fig. 4. Effect of SK&F 94120 (100 μM) and Ro 20-1724 (100 μM), alone and in combination, on isoproterenol-induced relaxation (*left*) and cAMP accumulation (*right*) in canine trachealis (n = 6). After induction of contraction with 3 μM methacholine, tissues were treated with vehicle (O), Ro 20-1724 (●), SK&F 94120 (△), or the combination (▲), before being relaxed by the cumulative addition of isoproterenol. At the end of the concentration-response studies, tissues were flash-frozen and assayed for cAMP content. *, Greater than control value at $p < 0.05$; **, $p < 0.01$. †, Greater than value in the presence of Ro 20-1724 or SK&F 94120 alone ($p < 0.01$).

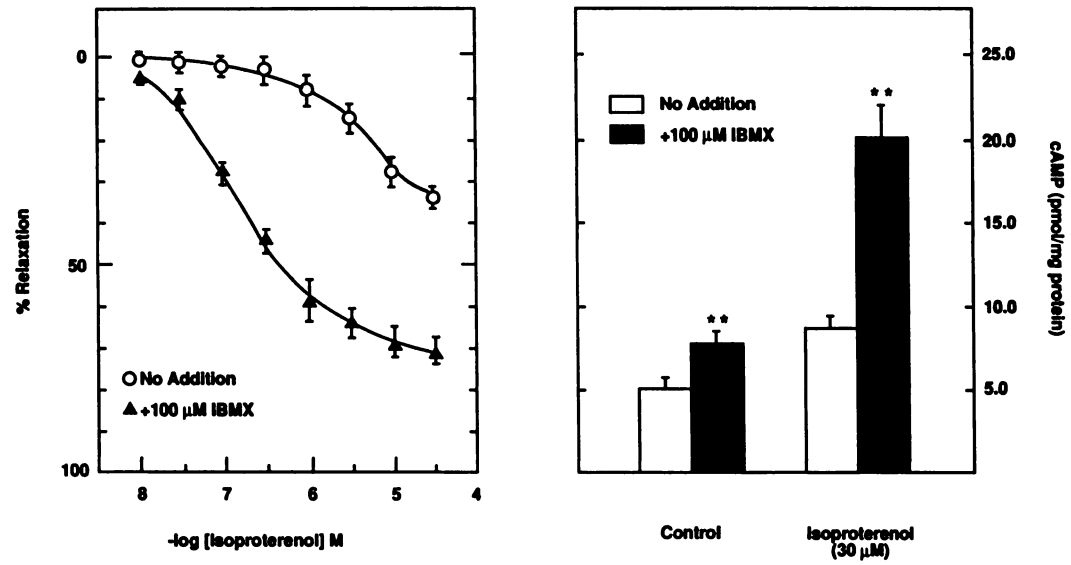

Fig. 5. Effect of IBMX (100 μM) on isoproterenol-induced relaxation (*left*) and cAMP accumulation (*right*) in canine trachealis (n = 6). Tissues were contracted with 3 μM methacholine, treated with vehicle (O) or IBMX (▲), and relaxed by the cumulative addition of isoproterenol. At the end of concentration-response studies, control (no isoproterenol) and isoproterenol-treated tissues were flash-frozen and assayed for cAMP content. **, Greater than value in the absence of IBMX ($p < 0.01$).

experiments, the combination of IBMX with Ro 20-1724 and SK&F 94120 produced no greater effect on isoproterenol-induced relaxation or cAMP accumulation than did IBMX alone or the combination of Ro 20-1724 and SK&F 94120 (Fig. 6). These results suggest that no isozymes other than the cGMP-inhibited PDE and the cAMP-specific PDE are substantial contributors to cAMP hydrolysis in the intact trachealis.

Regulation of basal cAMP content. The effect of various PDE inhibitors on basal cAMP content in noncontracted trachealis strips is shown in Table 2. As observed with methacholine-contracted tissues, neither SK&F 94120 (100 μM) nor Ro 20-1724 (100 μM) altered cAMP content when used alone. However, cAMP accumulation was increased 3-fold when these agents were used in combination. IBMX (100 μM) elicited the same increase as the combination of SK&F 94120 and Ro 20-1720, and a slight additional increment in cAMP accumulation was observed when all three PDE inhibitors (SK&F 94120, Ro 20-1720, and IBMX) were used simultaneously.

Effect of adenosine on cAMP accumulation. Large amounts of adenosine are known to be released from smooth muscle during contraction (18). Moreover, certain PDE inhibitors, especially xanthines, can act as adenosine receptor antagonists. Because of the possibility that the PDE inhibitors used in this study were altering cAMP content by antagonizing the actions of endogenously released adenosine, it was of interest to determine the effect of this purine on basal and isoproterenol-stimulated cAMP content in trachealis strips (Table 3). Adenosine neither increased basal cAMP content nor decreased hormone-stimulated cAMP accumulation, indicating the absence of functional A_1 and A_2 purinoceptors in this preparation.

Regulation of sodium nitroprusside-stimulated cGMP accumulation and relaxation. Zaprinast potentiated sodium nitroprusside-induced relaxation and cGMP accumulation, in a concentration-dependent manner (Fig. 7). The highest concentration of zaprinast used, 30 μM, increased the maximum relaxation in response to sodium nitroprusside from 16 ± 3% (control) to 46 ± 4% but did not alter the EC_{50} ($-\log EC_{50}$ = 4.75 ± 0.19 in control versus 4.82 ± 0.29 with 30 μM zaprinast). Zaprinast (30 μM) also produced a small increase in basal (no sodium nitroprusside) cGMP content (Fig. 7) but did not relax tissues in the absence of sodium nitroprusside. These results suggest that, in contrast to its apparent lack of activity against

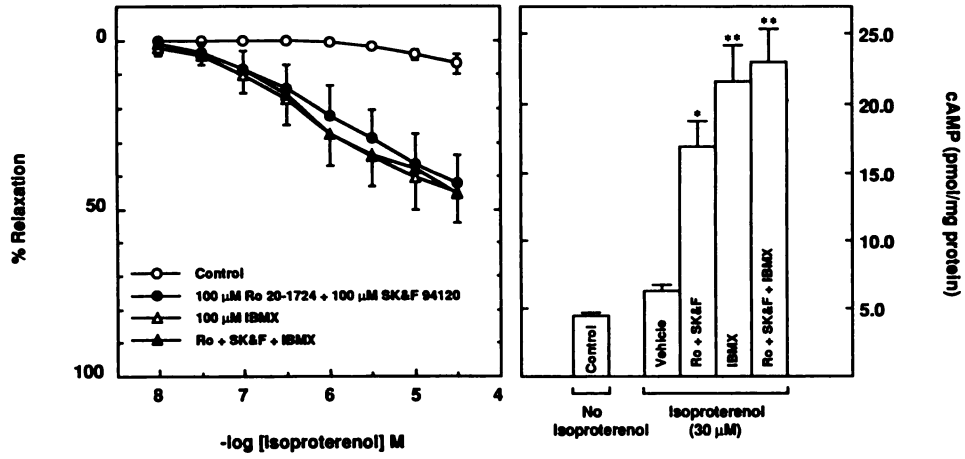

Fig. 6. Effect of SK&F 94120 (100 μM), Ro 20-1724 (100 μM), and IBMX (100 μM) on isoproterenol-induced relaxation (*left*) and cAMP accumulation (*right*) in canine trachealis (n = 10). After induction of contraction with 3 μM methacholine, tissues were treated with vehicle (○), Ro 20-1724 plus SK&F 94120 (●), IBMX (△), or a combination of all three agents (▲), before being relaxed by the cumulative addition of isoproterenol. Tissues were flash-frozen at the end of the concentration-response studies and assayed for cAMP content. *, Greater than value in the presence of isoproterenol alone at $p < 0.05$; **, $p < 0.01$.

TABLE 2

Effect of PDE inhibitors on basal cAMP accumulation in canine trachealis (n = 6)

Trachealis strips were prepared and incubated as described in Materials and Methods, except that they were not contracted with methacholine. Tissues were exposed to SK&F 94120 (100 μM), Ro 20-1724 (100 μM), and IBMX (100 μM), alone or in combination, for 40 min before being assayed for cAMP accumulation.

Treatment	cAMP content
	pmol/mg of protein
Vehicle	5.0 ± 0.8
SK&F 94120	6.3 ± 1.0
Ro 20-1724	5.8 ± 0.6
SK&F 94120 + Ro 20-1724	15.1 ± 3.7[a]
IBMX	14.1 ± 0.9[a]
SK&F 94120 + Ro 20-1724 + IBMX	20.1 ± 0.9[b]

[a] Greater than value in vehicle-treated control tissues ($p < 0.01$).
[b] Greater than values in tissues treated with IBMX alone or the combination of SK&F 94120 and Ro 20-1724 ($p < 0.05$).

TABLE 3

Effect of adenosine on basal and isoproterenol-stimulated cAMP accumulation in canine trachealis

Trachealis strips were prepared and incubated as described in Materials and Methods but were not contracted with methacholine. Tissues were treated with vehicle or adenosine (100 μM) for 2 min, before being treated with vehicle or isoproterenol (30 μM) for an additional 2 min.

Treatment	cAMP content
	pmol/mg of protein
Vehicle	6.3 ± 0.7
Adenosine	5.4 ± 0.8
Isoproterenol	10.7 ± 1.2[a]
Isoproterenol + adenosine	9.0 ± 0.9[a]

[a] Value greater than vehicle-treated control ($p < 0.05$).

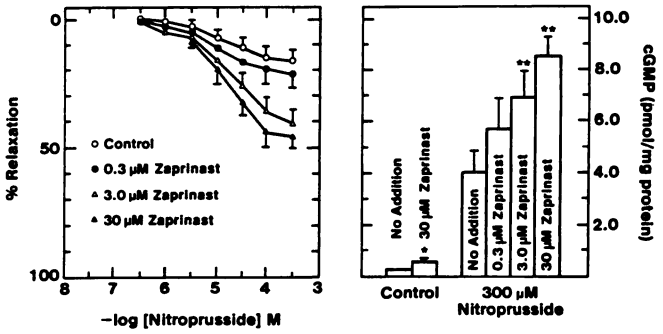

Fig. 7. Effect of zaprinast on sodium nitroprusside-induced relaxation (*left*) and cGMP accumulation (*right*) in canine trachealis (n = 6). Tissues were contracted with 1 μM methacholine, treated with vehicle (○) or various concentrations of zaprinast (●, 0.3 μM; △, 3 μM; ▲, 30 μM), and relaxed by the cumulative addition of sodium nitroprusside. At the end of the concentration-response studies, control (no sodium nitroprusside) and sodium nitroprusside-treated tissues were flash-frozen and assayed for cGMP content. The data were presented previously in Ref. 14. *, Greater than control value in the absence of zaprinast ($p < 0.05$). **, Greater than value in the presence of sodium nitroprusside alone ($p < 0.01$).

cAMP (Fig. 1), the cGMP-specific PDE hydrolyzes cGMP in the intact canine trachealis.

Neither SK&F 94120 (100 μM) nor Ro 20-1724 (100 μM), used alone or in combination, altered the responses to sodium nitroprusside (Fig. 8). This suggests that neither the cGMP-inhibited PDE nor the cAMP-specific PDE hydrolyzes cGMP in the intact tissue.

The effect of 100 μM IBMX on responses to sodium nitroprusside (Fig. 9) was similar to that produced by 30 μM zaprinast (Fig. 7). Maximum sodium nitroprusside-induced relaxation was increased from 19 ± 4% (control) to 53 ± 8% by IBMX, but the sodium nitroprusside EC_{50} was unaltered. Sodium nitroprusside-induced cGMP accumulation was increased from 3.7 ± 0.4 pmol/mg of protein to 9.4 ± 0.8 pmol/mg of protein in the presence of IBMX (Fig. 9). Like zaprinast, IBMX also produced a small but significant increase in cGMP content in the absence of sodium nitroprusside (Fig. 9).

The effect of zaprinast (30 μM) and IBMX (100 μM), alone and in combination, on sodium nitroprusside-induced relaxation and cGMP accumulation is shown in Fig. 10. In this series of experiments, maximum relaxation in response to sodium nitroprusside was enhanced to the same degree by either zaprinast (30 μM) or IBMX (100 μM), although IBMX appeared to have a greater effect than did zaprinast on relaxation in response to low concentrations of sodium nitroprusside. Interestingly, IBMX had a greater effect than zaprinast on cGMP accumulation induced by 300 μM sodium nitroprusside (Fig. 10). Moreover, the combination of zaprinast and IBMX enhanced sodium nitroprusside-induced cGMP accumulation to a greater extent than did either PDE inhibitor when used alone. The combination of zaprinast and IBMX also produced an additional, albeit small, increment in sodium nitroprusside-induced relaxation. It is noteworthy that the combination of sodium nitroprusside (300 μM), zaprinast (30 μM), and IBMX (100 μM) produced a 34-fold increase in cGMP accumulation, elevating the basal content from 0.6 ± 0.1 pmol/mg of protein to 20.5 ± 2.2 pmol/mg of protein.

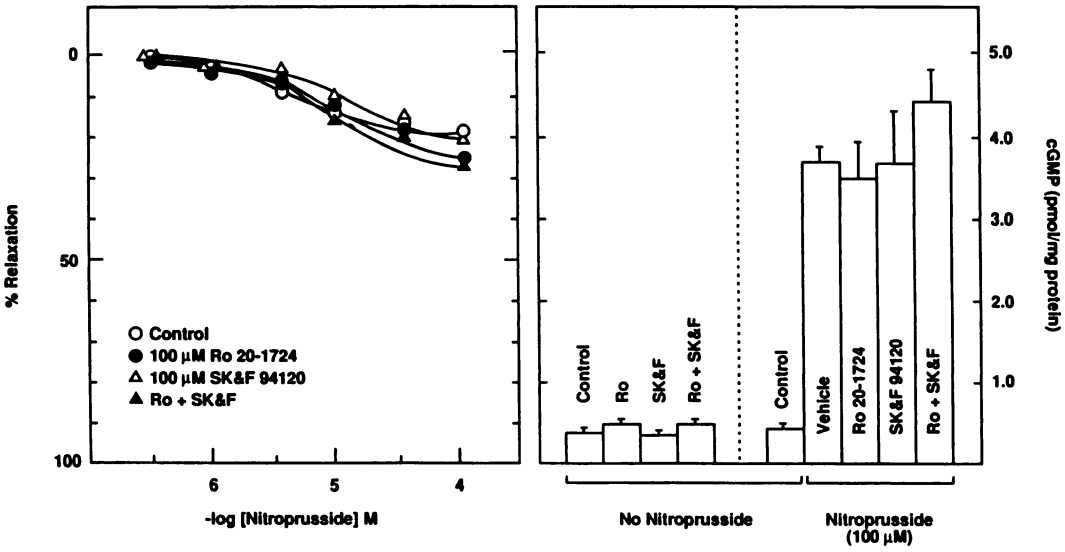

Fig. 8. Effect of, SK&F 94120 (100 μM) and Ro 20-1724 (100 μM), alone and in combination, on sodium nitroprusside-induced relaxation (*left*) and cGMP accumulation (*right*) in canine trachealis (n = 6). Tissues were contracted with 3 μM methacholine, treated with vehicle (○), Ro 20-1724 (●), SK&F 94120 (△), or the combination (▲), and relaxed by the cumulative addition of sodium nitroprusside. At the end of the concentration-response studies, control (no sodium nitroprusside) and sodium nitroprusside-treated tissues were frozen and assayed for cGMP content. Standard errors were omitted from relaxation data for clarity.

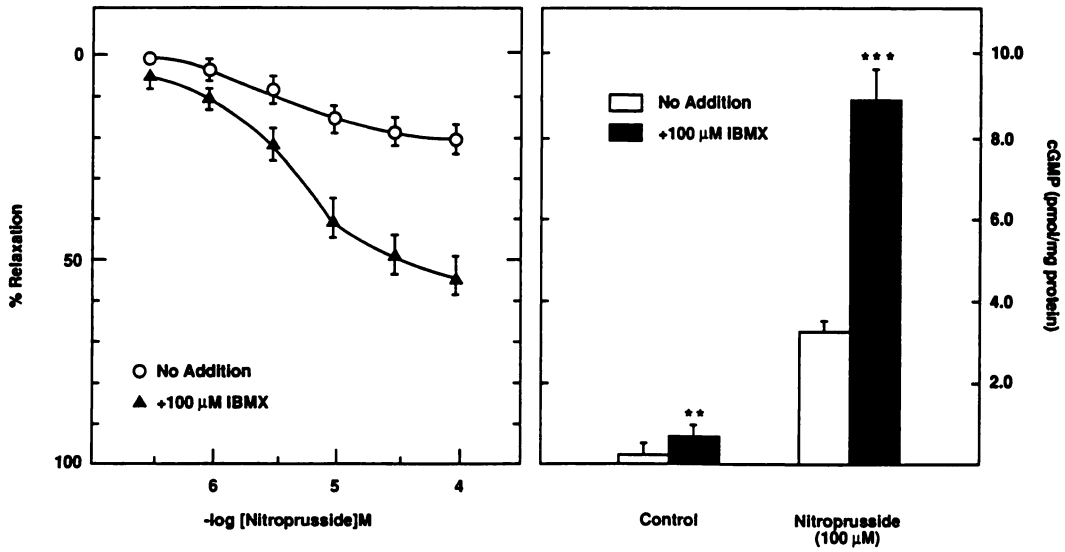

Fig. 9. Effect of IBMX (100 μM) on sodium nitroprusside-induced relaxation (*left*) and cGMP accumulation (*right*) in canine trachealis (n = 6). Tissues were contracted with 1 μM methacholine, treated with vehicle (○) or IBMX (▲), and relaxed by the cumulative addition of sodium nitroprusside. At the end of the concentration-response studies, control (no sodium nitroprusside) and sodium nitroprusside-treated tissues were frozen and assayed for cGMP content. **, Greater than corresponding value in the absence of IBMX at $p < 0.01$; ***, $p < 0.001$.

Discussion

For over 2 decades, inhibition of cyclic nucleotide PDE activity has been recognized as a mechanism by which various compounds induce smooth muscle relaxation. Thus, it is not surprising that several previous studies have attempted to correlate the PDE-inhibitory activity of various compounds with their ability to relax airway smooth muscle (19–23). Although results from some of these studies support a link between PDE inhibition and relaxation (22, 23), the results of other studies are equivocal (19–21). In many cases, correlations between PDE-inhibitory activity and relaxation of smooth muscle were established using nonselective PDE inhibitors. This complicates the interpretation of experimental results in two ways. First, it is likely that relaxation of smooth muscle induced by nonselective PDE inhibitors results from simultaneous increases in the accumulation of both cAMP and cGMP. Attempting to associate relaxation with increases in tissue content of one or the other cyclic nucleotide is thus made problematical. Second, the use of nonselective inhibitors does not provide information about the role of specific PDE isozymes in regulating cyclic nucleotide content in intact tissues.

Recently, the experimental limitations imposed by nonselective PDE inhibitors have been overcome by the advent of a new generation of compounds that show a marked degree of selectivity for various isozymes (6, 9, 10). In fact, several studies using such inhibitors have provided compelling evidence supporting a role for the low K_m, cGMP-inhibited PDE in regulating cAMP content in airway smooth muscle (24–26). In the present study, we used isozyme-selective PDE inhibitors in an attempt to define the roles of various PDEs in regulating cAMP and cGMP content in canine trachealis. This tissue contains five distinct PDE isozymes, possessing various degrees of preference for either cAMP or cGMP as a substrate (12). Nonetheless, each isozyme is capable of hydrolyzing both cyclic nucleotides *in vitro*, albeit often with substantially different velocities (12). Consequently, the activity and substrate preference of individual PDEs *in vivo* are impossible to predict solely from the kinetic characteristics of isolated isozymes. In the present study, three isozyme-selective PDE inhibitors were used as pharmacological probes to define the activity of various PDEs in intact canine trachealis. These inhibitors included: 1) zaprinast, an inhibitor (K_i = 0.1 μM) of the cGMP-specific PDE; 2)

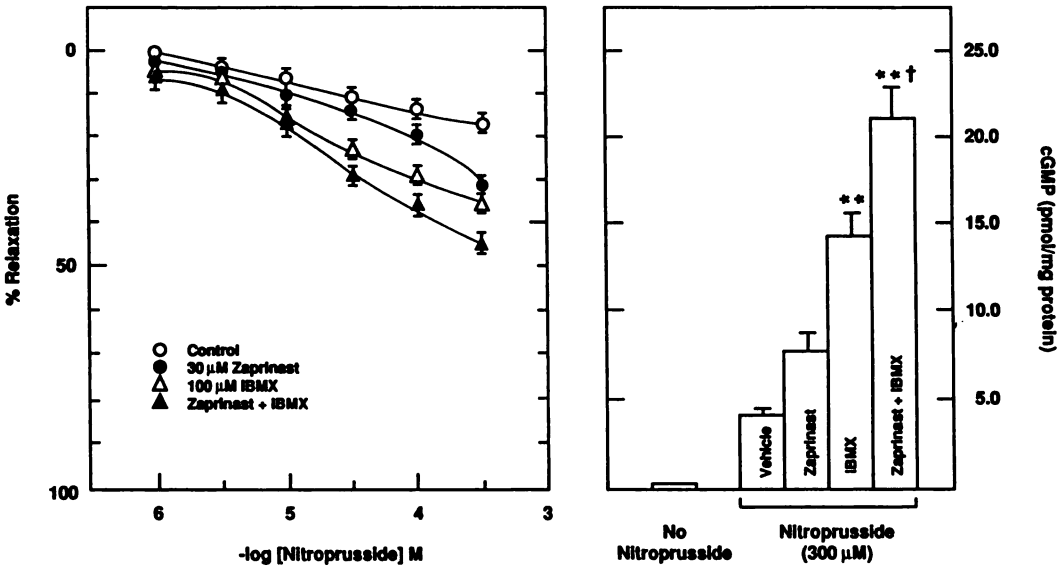

Fig. 10. Effect of zaprinast (30 μM) and IBMX (100 μM), alone and in combination, on sodium nitroprusside-induced relaxation (*left*) and cGMP accumulation (*right*) in canine trachealis (n = 6). Tissues were contracted with 3 μM methacholine, treated with vehicle (○) or zaprinast (●), IBMX (△), or the combination (▲), and relaxed by the cumulative addition of sodium nitroprusside. At the end of the concentration-response studies, control (no sodium nitroprusside) and sodium nitroprusside-treated tissues were frozen and assayed for cGMP content. **, Greater than value in the presence of sodium nitroprusside alone ($p < 0.01$). †, Greater than value in the presence of sodium nitroprusside plus IBMX ($p < 0.05$).

SK&F 94120, an inhibitor (K_i = 7 μM) of the low K_m, cGMP-inhibited cAMP PDE; and 3) Ro 20-1724, an inhibitor (K_i = 5 μM) of a second low K_m cAMP PDE, termed the cAMP-specific PDE (5, 12). At concentrations up to 100 μM, the highest concentration used in previous enzymatic studies with the various isozymes isolated from canine trachealis, none of these compounds significantly inhibited PDEs other than the specific isozyme being targeted (12). A fourth inhibitor, IBMX, was employed to nonselectivity inhibit all PDE activity. The ability of these agents to potentiate the mechanical (i.e., relaxation) and biochemical (i.e., cyclic nucleotide accumulation) responses to isoproterenol or sodium nitroprusside was used as evidence to support an involvement of the individual isozymes in the hydrolysis of cAMP or cGMP in the intact trachealis.

In this study, isoproterenol-induced relaxation and cAMP accumulation were potentiated in a concentration-dependent manner by either SK&F 94120 or Ro 20-1724. In contrast, zaprinast had no effect on the responses to isoproterenol. These data indicate that both the cGMP-inhibited PDE and the cAMP-specific PDE, but not the cGMP-specific PDE, regulate hormone-stimulated cAMP accumulation in intact trachealis. The roles of the Ca^{2+}/calmodulin-stimulated and cGMP-stimulated PDEs are difficult to assess because of the lack of potent and selective inhibitors of these enzymes. Although vinpocetine has been reported to be a selective, albeit not particularly potent (K_i = 15 μM), inhibitor of the Ca^{2+}/calmodulin-stimulated PDEs isolated from rat (27) and rabbit (28) aorta, it failed to inhibit (IC_{50} > 100 μM) the Ca^{2+}/calmodulin-stimulated PDE in the canine trachealis.[2] Nonetheless, two observations argue against an important function of either the cGMP-stimulated PDE or the Ca^{2+}/calmodulin-stimulated PDE in regulating cAMP content in the canine trachealis. First, the synergistic effect of the combination of SK&F 94120 and Ro 20-1724 on isoproterenol-induced relaxation and cAMP accumulation was identical to that of IBMX, an agent that inhibits all five PDEs in the canine trachealis with IC_{50} values between 1 and 30 μM (12). Second, the simultaneous use of all three inhibitors, IBMX, SK&F 94120, and Ro 20-1724, produced no greater potentiation of isoproterenol-induced responses than did either IBMX alone or the combination of SK&F 94120 and Ro 20-1724. Thus, inhibition by IBMX of the Ca^{2+}/calmodulin-stimulated PDE and the cGMP-stimulated PDE did not appear to alter cAMP metabolism, suggesting that neither of these isozymes is a major regulator of cAMP content in the canine trachealis.

Although the data tend to rule out a major role for the Ca^{2+}/calmodulin-stimulated PDE as a contributor to cAMP metabolism in the canine trachealis, this conclusion must be viewed cautiously. In the bovine coronary artery, this PDE is activated upon contraction, presumably as a result of a transient increase in cytosolic Ca^{2+} content (29). Consequently, it is possible that the Ca^{2+}/calmodulin-stimulated enzyme in the canine trachealis is activated in a similar manner, immediately after the addition of methacholine. Cyclic nucleotide content was not determined until 40 min after methacholine addition, a time at which cytosolic Ca^{2+} concentrations were likely to have dropped to near basal levels (30). Consequently, it is possible that the contribution of the Ca^{2+}/calmodulin-stimulated PDE was not assessed when the isozyme was in a fully active state.

Hypothetically, the effect of PDE inhibitors on cyclic nucleotide levels in intact tissues should be related in part to the rate of cyclic nucleotide synthesis. That is, PDE inhibitors should have a greater effect on cAMP content when adenylate cyclase activity is elevated. The results of this study indeed support this postulate. When used alone, neither SK&F 94120 nor Ro 20-1724 elevated cAMP content under basal conditions (i.e., absence of isoproterenol), but both agents potentiated isoproterenol-induced cAMP accumulation. Interestingly, however, basal cAMP content was increased when tissues were treated with IBMX or the combination of SK&F 94120 and Ro 20-1724. This observation suggests that the activity of either the cGMP-inhibited PDE or the cAMP-specific PDE is sufficient to maintain basal cAMP content within a narrow range. This control mechanism appears to be overwhelmed, however, when both isozymes are inhibited simultaneously. Under these conditions, basal cAMP levels increase substantially.

Basal cAMP content was elevated by IBMX or the combination of SK&F 94120 and Ro 20-1724 regardless of whether

[2] Torphy and Cieslinski, unpublished observations.

trachealis strips were exposed to methacholine. However, the cAMP increment was substantially greater in noncontracted tissues than in methacholine-contracted tissues. This observation can be explained by results of previous studies indicating that muscarinic agonists inhibit adenylate cyclase activity in airway smooth muscle (31). This, in turn, decreases the ability of both PDE inhibitors (26) and activators of adenylate cyclase (4, 32) to enhance cAMP accumulation in this tissue.

Sodium nitroprusside-induced relaxation and cGMP accumulation were potentiated by zaprinast but not by SK&F 94120 or Ro 20-1724. This suggests a role for the cGMP-specific PDE in regulating cGMP metabolism but not a role for either the cGMP-inhibited or the cAMP-specific PDE. Interestingly, the combination of IBMX and zaprinast produced a much greater effect on cGMP accumulation induced by sodium nitroprusside than did either agent when used alone. One interpretation of this result is that, in addition to the cGMP-specific PDE, the Ca^{2+}/calmodulin-stimulated PDE and/or the cGMP-stimulated PDE contribute to cGMP metabolism in the trachealis. Alternatively, the highest concentration of zaprinast used might not have been sufficient to maximally inhibit the activity of the cGMP-specific PDE. Consequently, the added effect of IBMX might have resulted solely from an incremental inhibition of this isozyme beyond that produced by zaprinast alone. The latter explanation is less likely than the former, in view of the fact that the highest concentration of zaprinast used (30 μM) was 300-fold above its K_i value (0.1 μM; Table 1) for inhibiting the cGMP-specific PDE (12). Regardless, it appears that cGMP hydrolysis in intact trachealis is not mediated by the same PDE isozymes that hydrolyze cAMP. The data from our experiments suggest that cGMP content is regulated by the cGMP-specific PDE and perhaps the Ca^{2+}/calmodulin-stimulated and/or the cGMP-stimulated PDEs, whereas cAMP content is regulated by the cGMP-inhibited and cAMP-specific PDEs.

Although both IBMX and the combination of IBMX and zaprinast increased sodium nitroprusside-stimulated cGMP accumulation to a much greater extent than did zaprinast alone, the maximum relaxant response to the nitrovasodilator was nearly identical under all three conditions. The most obvious explanation for this observation is that the substantial increase in cGMP accumulation produced by sodium nitroprusside in the presence of zaprinast is great enough to elicit maximal activation of the cGMP/cGMP-dependent protein kinase cascade. Consequently, any further elevation of cGMP content would be futile. Alternatively, this apparent discrepancy could be explained by the existence of subcellular compartments for guanylate cyclase, cGMP, PDE isozymes, and cGMP-dependent protein kinase. For example, elevations of cGMP content in a cellular pool other than that regulated by the cGMP-specific PDE might not result in an activation of cGMP-dependent protein kinase. A final possibility to consider is that cGMP does not mediate the relaxant response of canine trachealis to sodium nitroprusside and, as a corollary, PDE inhibitors potentiate sodium nitroprusside-induced relaxation by a mechanism(s) that is not related to an elevation of cGMP content.

The apparent specificity of the various PDE isozymes for cAMP or cGMP in the intact canine trachealis is difficult to explain solely on the basis of their kinetic characteristics. For example, the cGMP-specific PDE regulates cGMP content in the intact tissue, whereas the cGMP-inhibited PDE does not, even though the K_m values of these two isozymes for cGMP are virtually identical (12). Perhaps more surprising is the apparent failure of the Ca^{2+}/calmodulin-stimulated PDE to hydrolyze cAMP in the intact tissue. This conclusion is based upon the inability of the combination of IBMX, SK&F 94120, and Ro 20-1724 to produce a greater potentiation of the responses to isoproterenol than the combination of SK&F 94120 and Ro 20-1724. Thus, even though the Ca^{2+}/calmodulin-stimulated PDE has a low K_m for cAMP (1 μM) and accounts for a substantial portion of the total cAMP-hydrolyzing capacity of trachealis homogenates (12, 14), this enzyme does not appear to regulate cAMP content in the intact trachealis. The apparent cyclic nucleotide selectivity of PDE isozymes in the intact tissue could be accounted for by various factors, ranging from the presence of unique functional compartments for cyclases and PDEs to the assignment of erroneous *in vitro* kinetic constants to the various PDE isozymes. Obviously, further studies are required to address this issue.

A few caveats concerning the interpretation of the data presented in this study need to be considered. First, the isozyme-selective inhibitors used may have potentiated the mechanical and/or biochemical responses to relaxants by mechanisms in addition to direct inhibition of PDE. In fact, Harris and co-workers (25) have suggested that Ro 20-1724 and other compounds of this class may relax the guinea pig trachea by a mechanism that does not involve PDE inhibition. Another concern is the possibility that the agents used in this study potentiated the responses to isoproterenol not by inhibiting PDE but by acting as adenosine A_1 receptor antagonists. Because adenosine acts through A_1 receptors to inhibit adenylate cyclase activity, antagonism of endogenously released adenosine by IBMX, a known A_1 receptor antagonist, or other PDE inhibitors could enhance isoproterenol-induced cAMP accumulation and, consequently, relaxation. This possibility seems unlikely, in view of the fact that adenosine failed to inhibit isoproterenol-stimulated cAMP accumulation in the canine trachealis, implying that functional A_1-adenosine receptors are not present in this tissue. Another point of caution concerns our inability to measure precisely the intracellular concentrations of the various PDE inhibitors. Consequently, concentrations of inhibitors within the biophase of the catalytic site of an individual PDE isozyme could differ significantly from their concentrations in the extracellular medium. Finally, when cAMP or cGMP content is determined in a tissue containing a heterogeneous cell population, there is always a chance that a portion of the cyclic nucleotide accumulation being measured occurs in cells other than those of interest. This potential complication is unlikely to be an important factor in the present study, inasmuch as an overwhelming proportion of the total cell population of the canine trachealis is composed of smooth muscle cells (33).

The purpose of this study was to describe the roles of various PDE isozymes in regulating cyclic nucleotide content in canine trachealis. It was not our intent to define the role of cyclic nucleotides in regulating airway smooth muscle tone. Nonetheless, the data presented in this paper provide strong circumstantial evidence supporting the proposed role of cAMP and cGMP as second messengers mediating airway smooth muscle relaxation. Although absolute levels of cAMP and cGMP varied somewhat among different studies, there was a marked and

consistent correlation between the ability of the various isozyme-selective PDE inhibitors to potentiate drug-induced cyclic nucleotide accumulation and their ability to potentiate drug-induced relaxation. That is, on every occasion that PDE inhibitors increased cAMP or cGMP accumulation in response to isoproterenol or sodium nitroprusside, there was a parallel increase in drug-induced relaxation.

In conclusion, the results of this study suggest that cAMP hydrolysis in intact airway smooth muscle is mediated by PDE isozymes that are different from those that mediate cGMP hydrolysis. Moreover, the functional importance of various isozymes may differ, depending on whether cyclic nucleotides are being synthesized at a basal rate or under hormone-stimulated conditions. Finally, the results of this study not only demonstrate the utility of isozyme-selective PDE inhibitors as pharmacological probes but may also help identify molecular targets for a new class of bronchodilators.

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