Role of Low K_m Cyclic AMP Phosphodiesterase Inhibition in Tracheal Relaxation and Bronchodilation in the Guinea Pig

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ABSTRACT

This study evaluated the relationship between inhibition of the rolipram-sensitive and the CI-930-sensitive low K_m cyclic AMP-specific phosphodiesterase (PDE) isozymes (PDE III_{RO} and PDE III_C, respectively) and bronchomotor tone in the guinea pig. Rolipram and CI-930 exhibited biphasic concentration-response relationships for relaxation of carbachol-, histamine- and leukotriene D_4-contracted trachea. However, each agent produced a monophasic (sigmoidal) concentration-response curve when tested in the presence of a fixed concentration (3 μM) of the other. The same relationships were observed for inhibition of tracheal peak III PDE isolated via diethylaminoethyl-cellulose chromatography. Whereas CI-930 was approximately equipotent inhibiting PDE III_C and relaxing rolipram-pretreated trachea, rolipram was substantially more potent (EC_{50} = 0.02 μM) in relaxing CI-930-pretreated trachea than in inhibiting CI-930-pretreated PDE III (PDE III_{RO}, IC_{50} = 2.6 μM). Among a series of PDE inhibitors, there was a highly significant correlation (r = 0.89, P

< .01) between PDE III_C inhibition (i.e., PDE III in the presence of rolipram) and rolipram-pretreated tracheal relaxation, but not between PDE III_{RO} inhibition and CI-930-pretreated tracheal relaxation (r = 0.23). Nine of the PDE inhibitors used in this study have been reported to displace rolipram from a high-affinity binding site in rat brain. A highly significant correlation between relaxation of CI-930-pretreated trachea and displacement of rolipram binding by these agents was observed (r = 0.97, P < .0001). Significant correlations were also observed between in vivo bronchodilation (inhibition of histamine-induced bronchoconstriction) and PDE III_C inhibition (r = 0.88, P < .01), rolipram-displacing potency (r = 0.88, P < .001) and relaxation of CI-930-pretreated trachea (r = 0.94, P < .0005), but not PDE III_{RO} inhibition (r = 0.21). These data suggest that in the guinea pig PDE III_C inhibition produces bronchodilation whereas rolipram-induced bronchodilation is associated with a high-affinity binding site, which may or may not be the PDE III_{RO} isozyme.

There is evidence that suggests that cGMP and cAMP play an important role in the regulation of tracheal tone. The *beta* adrenergic receptor agonist, isoproterenol, induces the relaxation of trachea by stimulating the formation of cAMP *via* activation of adenylate cyclase (for review see Chu *et al.*, 1984a). Agents that inhibit cyclic nucleotide PDE, for example the methylxanthines theophylline and IBMX, have also been proposed to exert at least part of their tracheal relaxant activity and bronchodilator activity by increasing the intracellular levels of cAMP (Chu *et al.*, 1984b). Indeed, previous studies have shown a highly significant correlation between inhibition of canine tracheal cAMP-specific PDE and canine tracheal relaxation by seven methylxanthine derivatives (Polson *et al.*, 1982), and a significant correlation between relaxation of guinea pig trachea and inhibition of a crude cAMP PDE preparation by a number of chemically unrelated agents (Newman *et al.*, 1978).

Mammalian tissue contains multiple molecular forms of cyclic nucleotide PDE (for review see Wells and Hardman,

1977; Weishaar *et al.*, 1986; Beavo, 1988). These isozymes of PDE have been identified by a variety of techniques and are characterized by their affinity and specificity for substrate (cAMP and cGMP), differing maximum velocities, intracellular regulation by second messengers and susceptibility to inhibition by selective pharmacological agents (Weishaar *et al.*, 1986; Beavo, 1988; Silver *et al.*, 1988). At least two PDE isozymes which selectively hydrolyze cAMP with a high affinity have been identified. Although these low K_m cAMP-specific (also termed peak III) PDE isozymes from trachea, aorta and cardiac muscle elute from DEAE-cellulose columns at similar sodium acetate concentrations, there is evidence which suggests the presence of different PDE isozymes within these fractions (Silver *et al.*, 1988). In aortic smooth muscle, the concentration-response relationship to inhibition by some selective peak III inhibitors (e.g., milrinone and CI-930) are sigmoidal and monophasic, whereas with tracheal peak III PDE, the concentration-inhibition curves of these same agents and of rolipram, another selective low K_m cAMP PDE inhibitor, are much

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ABBREVIATIONS: cGMP, cyclic GMP; cAMP, cyclic AMP; PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; DEAE, diethylaminoethyl; LT, leukotriene; DTT, dithiothreitol.

shallower and appear to be biphasic. These observations suggest the possibility that two pharmacologically distinct PDE isozymes exist within the peak III PDE fraction of the canine tracheal smooth muscle (Cieslinski et al., 1988). The peak III PDE of trachea may contain both the CI-930-sensitive PDE (termed cardiotonic-sensitive or PDE III$_C$) and the rolipram-sensitive PDE (termed PDE III$_{RO}$).

Accordingly, the major focus of the present studies was to elucidate the roles of inhibition of the peak III PDE isozymes in causing relaxation of guinea pig airway smooth muscle and bronchodilation. A number of studies on the effect of selected agents on the different PDE isozymes have been reported in the literature (Weishaar et al., 1985; Silver et al., 1988) and many of these agents have been employed in the following experiments. Experiments were designed to determine: 1) the effect of selective and nonselective PDE inhibitors on relaxation of isolated trachea contracted by carbachol, histamine or LTD$_4$; 2) the tracheal relaxant effect and PDE inhibitory effect induced by rolipram, CI-930 and other PDE inhibitors in the presence of a selective inhibitor of one of the peak III PDE isozymes (either CI-930 or rolipram); and 3) the ability of these agents to inhibit histamine-induced bronchoconstriction in the guinea pig in vivo.

Methods

Intact guinea pig tracheal smooth muscle studies. Male and female Hartley guinea pigs weighing from 550 to 800 g were obtained from Buckberg Farms (Liberty, NY) and housed separately by sex in wire mesh cages. Guinea pigs were anesthetized with pentobarbital (50 mg/kg i.p.) and exsanguinated. Each trachea was excised rapidly and placed into an oxygenated modified Krebs' solution at room temperature of the following concentrations (in millimolar): NaCl, 137; KCl, 2.7; CaCl$_2$, 1.8; MgCl$_2$, 0.93; NaH$_2$PO$_4$, 0.35; NaHCO$_3$, 11.9; dextrose, 5.6; and Ca disodium EDTA, 0.026; pH = 7.3 to 7.4. Each trachea was cut longitudinally on the ventral side, the rings opened up into strips and suspended in 10-ml tissue baths under a 2-g load in the modified Krebs' solution at 37°C. After a 45-min equilibration period, tissues were tested for their contractile response by addition of 0.3 μM carbachol. This procedure was repeated 2 more times after washing. Tissues were then equilibrated for an additional 45 min. Sustained contractions were induced by addition of 0.3 μM carbachol, 0.3 μM LTD$_4$ or 10 μM histamine to the tissue baths. In the pretreatment studies, 3 μM CI-930 or 3 μM rolipram was added to the tissue baths 10 min before the addition of contractile agent. Isometric forces (grams) was recorded continuously on a Grass model 7 polygraph.

Relaxation is expressed as the mean ± S.E. Percentage of relaxation is defined as the percentage of the maximal relaxation induced by 300 μM papaverine. EC$_{50}$ values were calculated, where appropriate, by the probit method (Tallarida and Murray, 1986) and expressed as geometric means. If 50% relaxation was not reached at the highest concentration tested, EC$_{50}$ values were not calculated and results are expressed as percentage of relaxation at the highest concentration tested. EC$_{50}$ values were compared by an analysis of variance followed by a Student-Neuman Keul's Test (Tallarida and Murray, 1986). Significance was assigned as P < .05. In an attempt to quantifiy the complex concentration-response curve of rolipram and CI-930, the percentage of relaxation vs. log concentration-response curve was considered to be made up of two or three segments relating response to concentration for low, middle and high concentrations. Within each tissue, slopes were estimated for each of these segments. If the response curves were biphasic, the slopes of the middle segments would be expected to be different than that of either the high or the low segments. The slopes were compared by means of a two-way analysis of variance with the terms for tissue and slope, and Duncan's multiple range test to make comparisons among the different segments.

Preparation of PDE isozymes. Slight modifications of the methods of Weishaar et al. (1986) and Silver et al. (1988) were used to separate the isozymes of PDE. Tracheas were obtained from naive, pentobarbital-anesthetized guinea pigs (50 mg/kg i.p.) and placed immediately in ice-cold saline. Approximately 60 to 70 tracheas per preparation were used. In preliminary experiments, inhibition (by rolipram and CI-930) of PDE III extracted from trachealis muscle was similar to that of PDE III extracted from the remainder of the trachea (IC$_{50}$ values within the S.E.). Therefore, the whole trachea was used as the source of enzyme in subsequent experiments.

The tissues were minced with fine scissors and homogenized immediately in 10 volumes of extraction buffer that contained 10 mM Tris acetate, pH 7.5, 2 mM MgCl$_2$, 1 mM DTT and 2000 U/l of aprotinin. This and subsequent procedures were performed at 0–4°C. Tissue was homogenized with a Brinkman PT-20 polytron (3–6 bursts at medium setting; 20 sec/burst). The homogenate was then sonicated (20 sec; 6× at 80% of maximum) in a Heat Systems Ultrasonics sonicator and was centrifuged at 48,000 × g for 30 min. The resultant supernatant fraction was applied to a DEAE cellulose column (30 × 1.6 cm; 30 ml of bed volume) that had been equilibrated with 35 or 70 mM sodium acetate/ 1 mM DTT (pH = 6.5).

After application of the sample, the column was washed with 2 to 3 bed volumes of 35 or 70 mM sodium acetate/1 mM DTT (pH = 6.5). PDE isozymes were eluted with a continuous 70 to 1000 mM sodium-acetate gradient (containing 1 mM DTT, pH = 6.5, total volume = 400 ml). Fractions (4–6 ml) were collected and assayed for cyclic nucleotide hydrolysis using 1 μM cGMP or cAMP (fig. 1). Appropriate fractions, corresponding to PDE peak I and peak III, were pooled separately for each preparation and dialyzed against 70 mM sodium acetate/0.5 mM DTT (pH = 6.5) for approximately 20 hr. After dialysis, PDE fractions were concentrated to 14% of original volume with an Amicon ultrafiltration cell system (model 8050) containing a YM 10 membrane under 25 psi of nitrogen. The concentrated fractions were diluted with ethylene glycol to 50% and stored at −20°C. No significant changes in hydrolysis or sensitivity to inhibitors were noted for up to 2 months.

PDE activity was measured in reaction mixtures containing 40 mM Tris (pH = 8.0), 5 mM MgCl$_2$ and 1 mM DTT. For assessing PDE inhibition, the concentration of substrate was 0.2 μM of either [^{3}H] cAMP or [^{3}H]cGMP. None of the vehicles significantly affected PDE activity. Each assay was performed in triplicate. The concentration of inhibitor which produced 50% inhibition of cyclic nucleotide hydrolysis (IC$_{50}$) and 95% confidence intervals were calculated from concentration-response curves as described by Tallarida and Murray (1986). For cotreatment studies, a fixed concentration (3 μM) of either CI-930 or rolipram was added to each reaction mixture. Concentration-response curves (done in duplicate) from at least two separate enzyme preparations were generated for each agent.

In vivo **bronchodilation.** Male Hartley guinea pigs (360–500 g, Buckberg Farms) were anesthetized with pentobarbital, 40 mg/kg i.p. The trachea and both external jugular veins of each animal were cannulated and the guinea pig ventilated mechanically. A side arm of the tracheal cannula was connected to a pressure transducer (Statham B23AC) for the recording of airway pressure on a Grass model 7 polygraph.

After an equilibration period, histamine was infused until a steady-state bronchoconstriction was achieved (9–14 μg/kg/min i.v., to produce an airway pressure increase of 20–30 mm Hg). The test substance or vehicle was then administered i.v. in ascending doses (four to six doses per animal) at 10-min intervals. Ten minutes after the final dose was administered, the histamine infusion was terminated and airway pressure was allowed to attain a constant level (postinfusion pressure). Histamine-induced bronchoconstriction was defined as the difference between the airway pressure at steady-state bronchoconstriction and the post-infusion pressure; the bronchodilation activity of each dose of a compound was calculated as the percentage of reduction of histamine-induced bronchoconstriction, with each animal serving as its own control.

Materials. Isoproterenol, carbachol, theophylline, IBMX, hista-

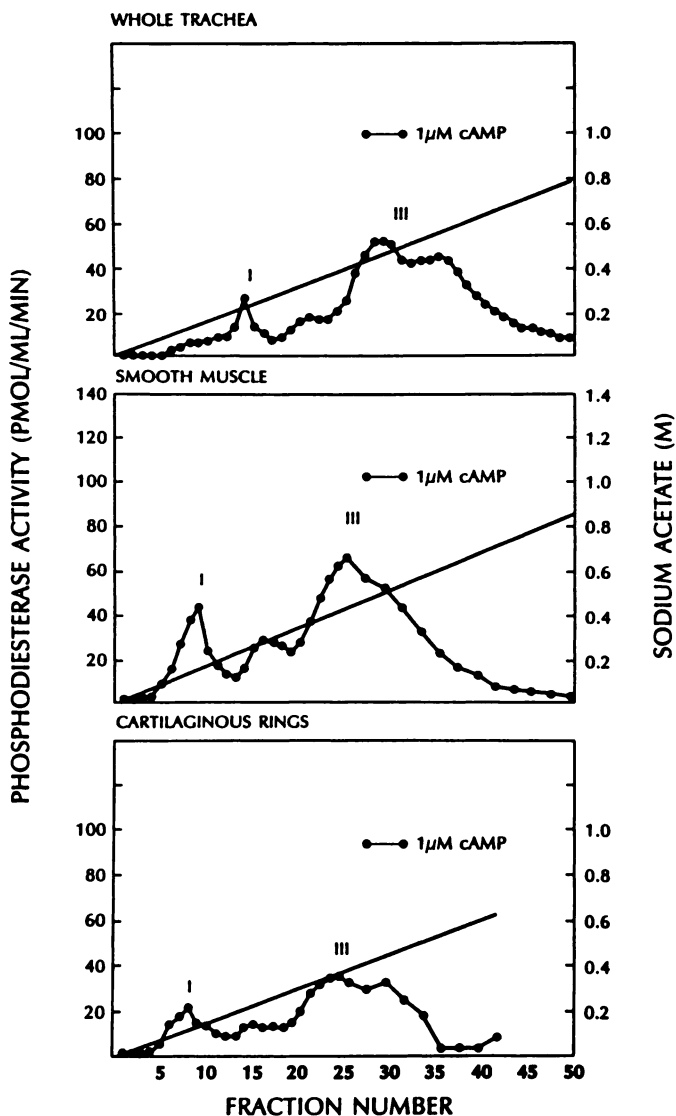## WHOLE TRACHEA

SMOOTH MUSCLE

CARTILAGINOUS RINGS

Fig. 1. Representative DEAE-cellulose chromatograms of cAMP phosphodiesterase activity from guinea pig whole trachea (top panel), tracheal smooth muscle (middle panel) and cartilaginous tracheal rings (bottom panel). Fractions were eluted with sodium-acetate (solid line) and peaks I and II were collected and assayed as described in the text. Experiments revealed similar pharmacological sensitivity to CI-930 and rolipram in peak III from all preparations.

mine diphosphate and papaverine were obtained from Sigma Chemical Co (St. Louis, MO). Rolipram, milrinone and CI-930 were synthesized at Sterling Research Group (Rensselaer, NY). Nitraquazone was a gift from Troponwerke (Cologne, FRG); Ro 20-1724 was a gift from Hoffmann-La Roche (Nutley, NJ); imazodan was a gift from Warner Lambert/Parke Davis (Ann Arbor, MI); piroximone was a gift from Merrell Dow (Cincinnati, OH); anagrelide was a gift from Bristol-Myers (East Wallingford, CT); proquazone was a gift from Sandoz (East Hanover, NJ); ICI 63,197 was a gift from ICI (Wilmington, DE). LTD$_4$ was purchased from Biomol Inc. (Plymouth Meeting, PA). Compounds that were not water soluble were dissolved in dimethylsulfoxide or ethanol and diluted in double-distilled H$_2$O. The effect of all vehicles was tested and was shown to be negligible.

Results

Inhibition of guinea pig tracheal PDE isozymes. Theophylline, papaverine, rolipram and CI-930 were tested for

TABLE 1

Inhibition of DEAE-cellulose Peaks I and III PDE from guinea pig trachea

Peaks I and III as separated by DEAE-cellulose column chromatography.

Agent	IC$_{50}$ (95% Confidence Intervals)	
	Peak I	Peak III
	μM	
Rolipram	226	59
	(190–269)	(47–75)
Papaverine	6.0	1.0
	(5–7)	(0.9–1.1)
Theophylline	280	131
	(252–308)	(118–146)
CI-930	54	1.0*
	(48–62)	(0.3–4.2)

* EC$_{50}$ values are highly variable from preparation to preparation due to shallowness of concentration-response curves and differences in the ratio of PDE III$_c$ to PDE III$_{RO}$ composition from different preparations.
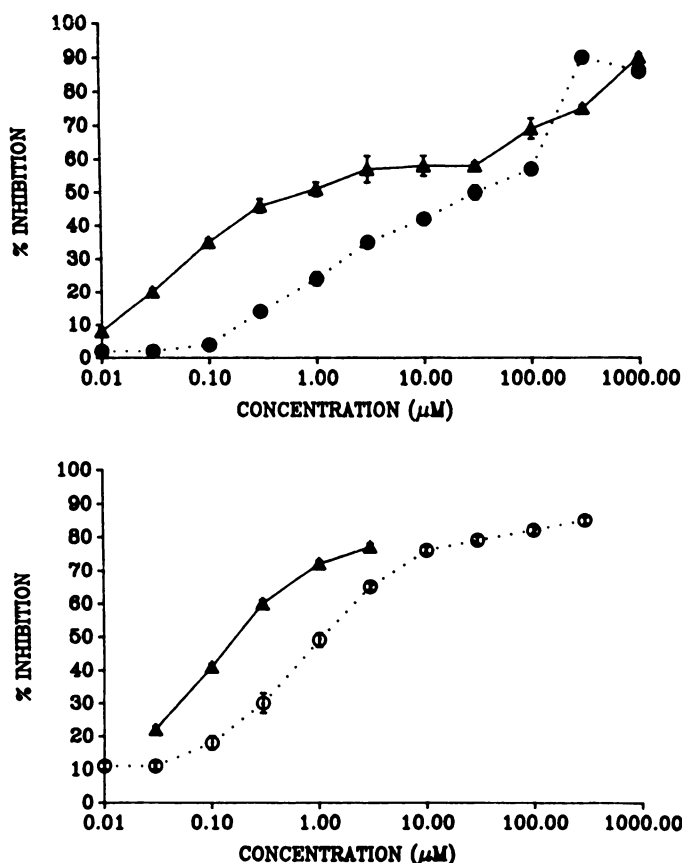

Fig. 2. Effect of rolipram and/or CI-930 on the hydrolysis of cAMP by peak III PDE from guinea pig trachea. In the top panel PDE III isozymes were treated with increasing concentrations of either rolipram (●) or CI-930 (▲). In the bottom panel PDE III isozymes were cotreated with a fixed concentration of CI-930 (3 μM) and increasing concentrations of rolipram (○) or a fixed concentration of rolipram (3 μM) and an increasing concentration of CI-930 (△). The concentration of cAMP used for the PDE assays was 0.2 μM. Each point is the mean and S.E. of at least two determinations from at least two different preparations.

inhibition of cGMP or cAMP hydrolysis by PDE peaks I and III, respectively, isolated from guinea pig trachea. Theophylline was relatively nonselective in its ability to inhibit PDE I or PDE III, whereas papaverine, CI-930 and rolipram were selective against PDE III (table 1).

Biphasic inhibition of PDE III from guinea pig trachea was

TABLE 2

Effect of selected PDE inhibitors in isolated Peak III PDE from guinea pig trachea

ND, not determined.

Agent	IC_{50} (95% Confidence Intervals)		
	Alone	+Rolipram*	+CI930*
	μM		
Papaverine	1.0	0.7	2.5
	(0.9–1.1)	(0.67–0.8)	(1.5–4.2)
IBMX	4.4	4.2	17
	(3.6–5.2)	(3.9–4.5)	(12–24)
Theophylline	131	455	220
	(118–146)	(126–167)	(178–272)
Rolipram	59	ND	2.6
	(47–75)		(1.7–4.1)
Proquazone	11	103	2.2
	(9–14)	(78–137)	(1.7–2.9)
Nitraquazone	51	ND	17
	(44–59)		(12–25)
ICI 63,197	71	140	12
	(44–114)	(127–154)	(7–23)
Ro 20-1724	47	146	13
	(34–63)	(134–160)	(7–23)
Milrinone	2.4	1.8	17
	(1.7–3.3)	(1.6–2.1)	(11–27)
CI-930	1.1	0.46	ND
	(0.3–4.7)	(0.2–0.9)	
Imazodan (CI-914)	38	6.2	499
	(21–68)	(1.7–23.1)	(338–736)
Piroximone	69	21	448
	(49–95)	(15–28)	(412–486)
Anagrelide	0.3[b]	0.04	ND
		(0.03–0.05)	

* Concentration of CI-930 and rolipram coincubation was 3 μM.

[b] Compound produced a maximum inhibition of approximately 50% from 0.3 to 300 μM.

observed with CI-930 or rolipram (fig. 2). These data suggested that guinea pig trachea contains two isozymes of PDE III: a rolipram-sensitive PDE (PDE III_{RO}) and a cardiotonic-sensitive PDE (PDE III_C). Pretreatment of PDE III with either CI-930 (3 μM) or rolipram (3 μM) reduced markedly the IC_{50} value obtained with other PDE inhibitors (fig. 2; table 2). Similar results were obtained with pretreatment of PDE III with 100 μM rolipram (data not shown), a concentration of the agent that fully inhibits PDE III_{RO}.

Intact trachea studies. Cumulatively increasing concentrations of carbachol (0.01–10 μM), LTD$_4$ (0.001–0.3 μM) or histamine (1–300 μM) produced concentration-dependent increases in contractile force of isolated guinea pig trachea. In subsequent tracheal relaxation studies, concentrations of carbachol, histamine and LTD$_4$ were chosen that produced approximately equal increases in active force (0.3, 30 and 0.3 μM, respectively). The force development (mean ± S.E.) in response to these agents was not different: carbachol (2.0 ± 0.2 g); histamine (1.6 ± 0.14 g); and LTD$_4$ (1.5 ± 0.16 g).

Theophylline (10–1000 μM) and papaverine (1–100 μM) produced concentration-dependent relaxation of contracted trachea (fig. 3). The concentration-relaxation relationship appeared to follow a sigmoidal curve, with nearly equal relaxant activity against all three contracting agents. Rolipram and CI-930 (fig. 4) relaxed isolated trachea in a biphasic manner as demonstrated by the differences in slopes between the low and middle concentrations and the high concentrations. Both agents were more potent against histamine- and LTD$_4$-induced contractions than against contractions induced by carbachol. In the case of rolipram, the first phase of relaxation occurred
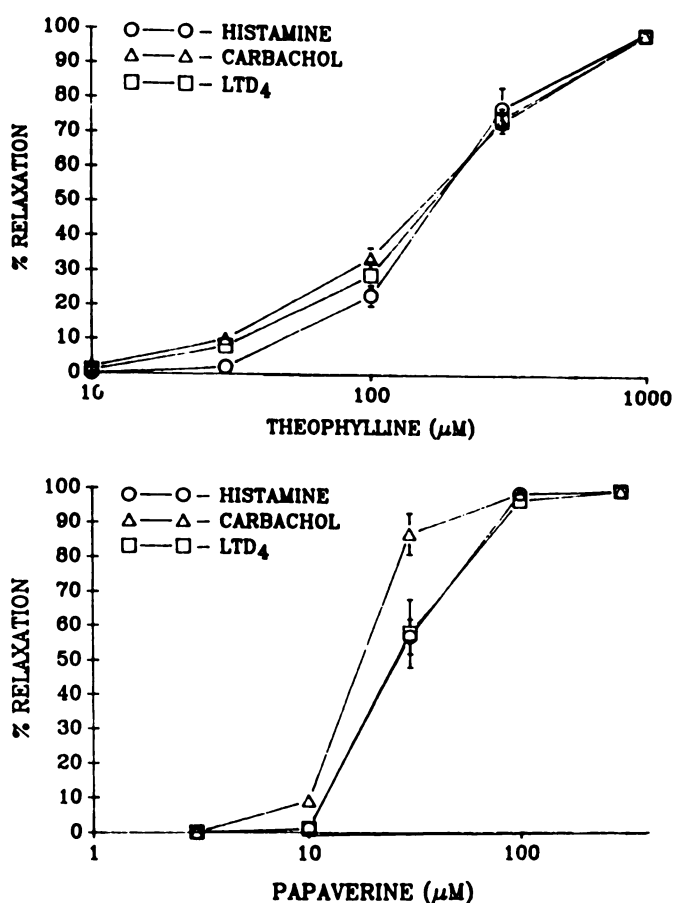

Fig. 3. Effect of cumulatively increasing concentrations of theophylline (top panel) or papaverine (bottom panel) on guinea pig tracheal strips precontracted with either carbachol (0.3 μM), histamine (10 μM) or leukotriene D$_4$ (0.3 μM). The isometric force (mean ± S.E.) developed was: carbachol, 2.0 ± 0.2 g; histamine, 1.6 ± 0.14 g; and LTD$_4$, 1.5 ± 0.16 g. Each point is the mean and S.E. of five to six tracheal strips.

between 0.01 to 1.0 μM, whereas the second phase occurred between 10 to 300 μM. For CI-930, the biphasic nature of the concentration-response curve was less pronounced than that obtained with rolipram. The first phase of the concentration-response curve occurred below 3 μM, whereas the second phase occurred between 30 and 300 μM. Isoproterenol (0.3–30 nM) also produced sigmoidal concentration-dependent tracheal relaxation (data not shown). This *beta* adrenergic agonist was more potent in relaxing hisatmine- and LTD$_4$-induced contractions in trachea relative to carbachol-induced contraction.

The biphasic nature of the rolipram and CI-930 concentration-relaxation relationships suggested that there may be two mechanisms involved in causing relaxation of guinea pig trachea. Pretreatment with one of the agents may inhibit one mechanism and allow for assessment of the effects of the remaining mechanism without interference. Concentrations (3 μM) of both rolipram and CI-930 were chosen that were between the first and second phase (plateau region) of the concentration-relaxation curve. Trachea was treated with either vehicle, CI-930 or rolipram, contracted with 0.3 μM carbachol and a cumulative concentration-relaxation curve was generated to either rolipram or CI-930. The results of these studies are shown in figure 5. As with inhibition of PDE isozymes, pretreatment with CI-930 or rolipram converted the biphasic relaxation curve to monophasic curves. Pretreatment with 3 μM
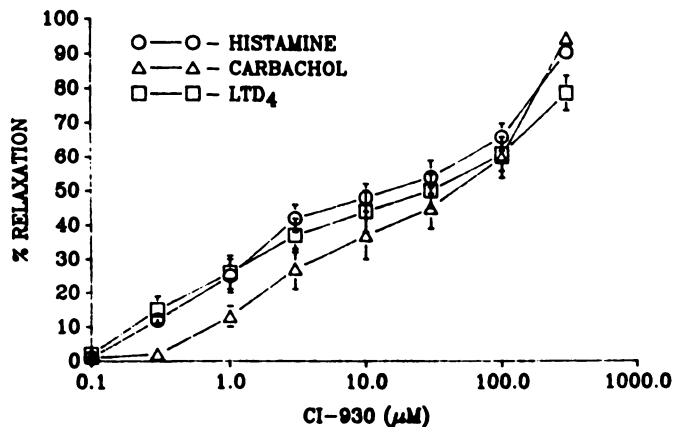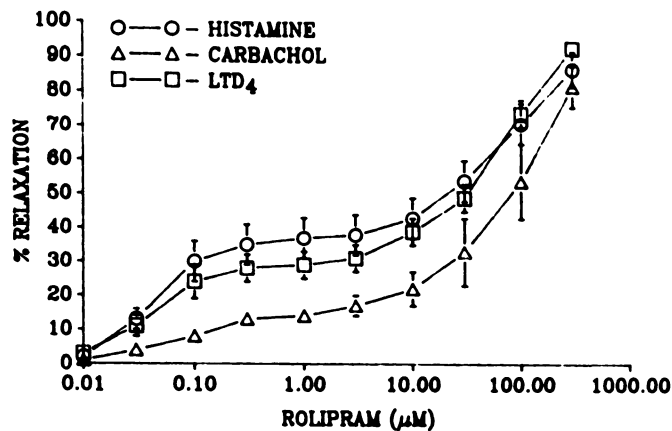
Fig. 4. Effect of cumulatively increasing concentrations of rolipram (top panel) or CI-930 (bottom panel) on guinea pig cut tracheal strips precontracted with either carbachol (0.3 μM), histamine (10 μM) or LTD$_4$ (0.3 μM). The isometric force (mean ± S.E.) developed was: carbachol, 2.0 ± 0.2 g; histamine, 1.6 ± 0.14 g; and LTD$_4$, 1.5 ± 0.16 g. Each point is the mean and S.E. of five to six tracheal strips.
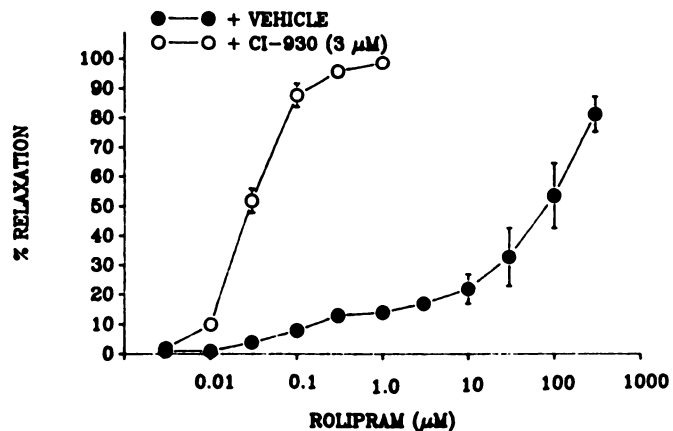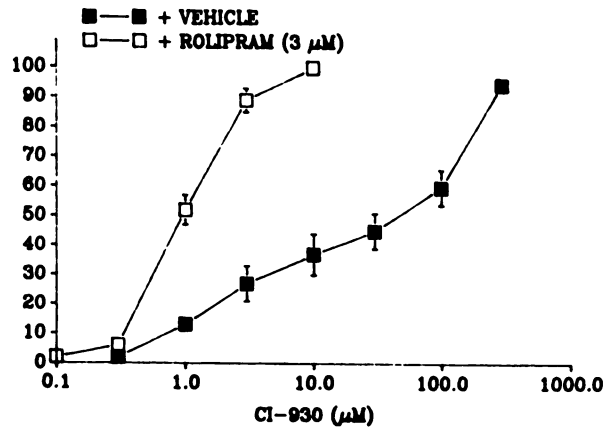
rolipram did not significantly alter the concentration-response relationship to subsequent relaxation induced by rolipram.

Bronchodilation by PDE inhibitors *in vivo*. The ED$_{50}$ values for inhibition of histamine-induced bronchoconstriction are shown in table 3. The most potent compounds were rolipram, ICI 63,197, nitraquazone and Ro 20-1724, with ED$_{50}$ values of 0.8 to 21 μg/kg. The least efficacious agent was proquazone, which produced only 40% inhibition at the highest dose tested (1 mg/kg).

Relationship between PDE isozyme inhibition and tracheal relaxation (*in vitro*) or bronchodilation (*in vivo*). Selected agents that have been described as PDE isozyme inhibitors were tested for their ability to relax CI-930- and/or rolipram-pretreated, carbachol-contracted trachea (table 4) and to inhibit guinea pig tracheal PDE III in the presence and absence of CI-930 or rolipram (table 2). Correlation coefficients for a group of PDE inhibitors were then determined for the relationship between isozyme inhibition and tracheal relaxation. A significant correlation between tracheal relaxation (in the presence of rolipram) and PDE III$_C$ inhibition (also in the presence of rolipram) was observed (R = 0.89, P < .01, n = 8; fig. 6). There was no significant correlation between tracheal relaxation (in the presence of CI-930) and inhibition of PDE III$_{RO}$ (r = 0.23, n = 9, fig. 6).

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Fig. 5. Effect of rolipram (3 μM) pretreatment on CI-930-induced relaxation (top panel) or CI-930 (3 μM) pretreatment on rolipram-induced relaxation (bottom panel) of carbachol-contracted guinea pig tracheal strips. Pretreatments were 5 min before administration of carbachol. When contractile force reached a maximal steady-state level, cumulatively increasing concentrations of CI-930 or rolipram were added to the tissue bath. Each point is the mean and S.E. of 9 to 12 tracheal strips.

TABLE 3
Bronchodilation *in vivo*

Anesthetized guinea pigs were infused i.v. with histamine. Each animal then received four to six ascending i.v. doses of a single test substance. ED$_{50}$ values were calculated from percentage of inhibition of histamine-induced bronchoconstriction in four to seven animals.

Compound	ED$_{50}$ (95% Confidence Intervals)
	mg/kg i.v.
Rolipram	0.0008 (0.0004–0.001)
ICI 63,197	0.005 (0.003–0.008)
Nitraquazone	0.008 (0.005–0.021)
Ro 20-1724	0.021 (.014–0.047)
Anagrelide	0.066 (0.24–0.18)
IBMX	0.11 (0.09–0.16)
CI-930	0.09 (0.041–0.17)
Imazodan (CI-914)	0.27 (0.095–0.75)
Papaverine	0.73 (0.19–2.7)
Theophylline	0.70 (0.44–1.2)
Enprofylline	1.16 (0.86–1.55)
Proquazone	40% @ 1 mg/kg

PDE inhibitors to inhibit histamine-induced bronchoconstriction in anesthetized guinea pigs (table 3) and to relax CI-930-pretreated, carbachol-contracted trachea (table 4) or to inhibit guinea pig tracheal PDE III isozymes (table 2) were evaluated. Comparable to the *in vitro* results, a significant correlation

TABLE 4

Effect of PDE inhibitors on relaxing carbachol-contracted guinea pig trachea in the presence and absence of CI-930 or rolipram

IA, inactive/failed to produce 50% relaxation at highest concentration tested; ND, not determined.

Agent	EC$_{50}$ (95% Confidence Intervals)[a]		
	Alone	+Rolipram[b]	+CI 930[b]
	μM		
IBMX	7.1	8.1	6.8
	(5.5–9.4)	(4.3–11.6)	(3.7–9.6)
Papaverine	30	6	9
	(12–52)	(4–9)	(5–13)
Theophylline	180	77	57
	(96–311)	(47–112)	(22–94)
Rolipram	100	ND	0.02
	(36–277)		(0.01–0.04)
Proquazone	IA	ND	20
			(10–43)
Nitraquazone	IA	ND	0.09
			(0.04–0.19)
ICI 63,197	46	ND	0.40
	(27–81)		(0.2–0.7)
RO 20-1724	30	ND	0.34
	(18–66)		(0.2–0.6)
Imazodan	39	2.5	ND
	(15–88)	(1.1–4.6)	
CI-930	80	1.0	ND
	(55–112)	(0.7–1.7)	
Milrinone	30	3.6	70
	(21–41)	(2.6–4.9)	(45–108)
Anagrelide	10	0.56	ND
	(7–17)	(0.31–0.88)	
Piroximone	33	74.4	ND
	(18–56)	(49–121)	

[a] EC$_{50}$ is defined as the concentration of PDE inhibitor that produces 50% relaxation of a maximally relaxed carbachol-contracted trachea by 300 μM papaverine. *n* = 4–9 tracheal preparations.

[b] Concentration of both CI-930 or rolipram pretreatment (15 min before addition of 0.3 μM carbachol) was 3 μM.

between bronchodilation and PDE III$_C$ inhibition was observed (*r* = 0.88, P < .01, *n* = 7; fig. 7). In contrast, no significant correlation was observed between bronchodilation and inhibition of PDE III$_{RO}$ (*r* = 0.21, *n* = 9; fig. 7). A highly significant correlation between relaxation of CI-930-pretreated trachea and bronchodilation was observed (*r* = 0.94, P < .0005, *n* = 8), whereas a significant correlation between relaxation of rolipram-ptretreated trachea and bronchodilation was also demonstrated (*r* = 0.80, P < .05, *n* = 7).

Other potential mechanisms of action of rolipram. Studies were also performed to explore other mechanisms of action possibly responsible for rolipram-induced tracheal relaxation. Rolipram-induced relaxation of guinea pig trachea, in the presence of CI-930 (3 μM), was not inhibited by the pretreatment with the following standard pharmacological agents: nadolol (1 μM), a *beta* adrenergic antagonist; tetrodotoxin (1 μM), a Na$^+$ channel inhibitor; apamin (1 μM) a bee venom polypeptide that has been shown to inhibit the release of the transmitter(s) involved in the nonadrenergic, noncholingeric inhibitory nervous system (MacKenzie and Burnstock, 1980); 8-phenyl theophylline (10 μM), an adenosine antagonist; and α-chymotrypsin (1 μM), a peptide shown to inhibit exogenously added vasoactive intestinal peptide (McKenzie and Burnstock, 1980; A. L. Harris, unpublished observations).

Discussion

These results have shown that addition of rolipram, a selective inhibitor of PDE III$_{RO}$, or CI-930, a selective inhibitor of
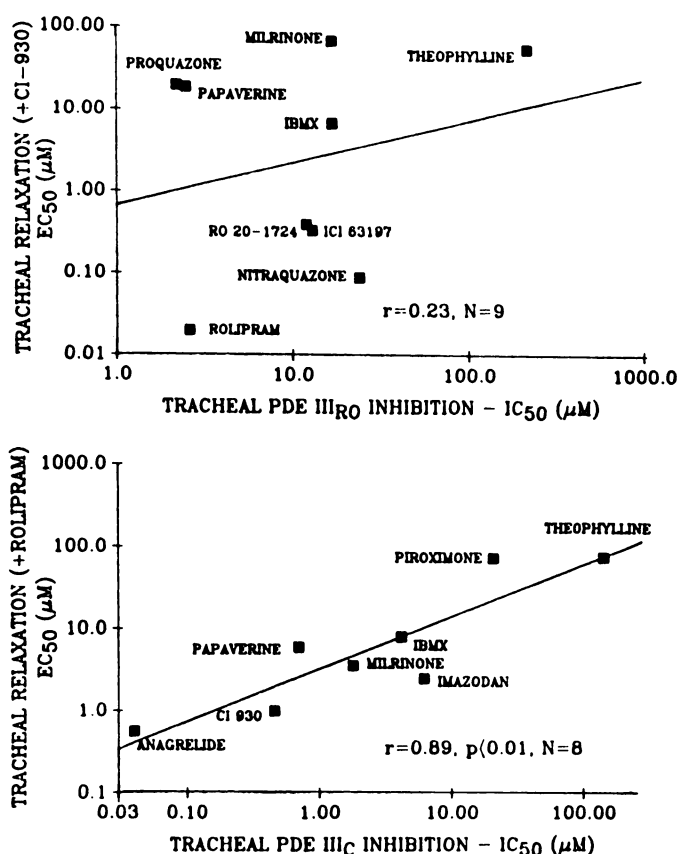

Fig. 6. *In vitro* tracheal relaxation as a function of PDE III$_{RO}$ (top panel) or PDE III$_C$ (bottom panel) inhibition in carbachol-contracted, CI-930-pretreated (3 μM; top panel) or rolipram-pretreated (3 μM; bottom panel) guinea pig trachea. Data for trachea are expressed as EC$_{50}$ values (micromolar; table 4) which are the concentrations of relaxant that produced 50% relaxation of carbachol-induced contraction; data for PDE inhibition are expressed as IC$_{50}$ values (micromolar; table 2) which are the concentrations of agent that produced 50% inhibition of cAMP hydrolysis by PDE III in the presence of 3 μM CI-930 (PDE III$_{RO}$) or 3 μM rolipram (PDE III$_C$). Each point is the mean of at least three determinations. Regression analysis for relaxation as a function of PDE III$_{RO}$ inhibition revealed a lack of a significant correlation (*r* = 0.23, P = .58, *n* = 9), whereas regression analysis for relaxation as a function of PDE III$_C$ inhibition revealed a significant correlation (*r* = 0.89, P < .01, *n* = 8).

PDE III$_C$, to histamine-, LTD$_4$- or carbachol-contracted guinea pig trachea produces biphasic concentration-relaxation curves. Pretreatment of trachea with a fixed concentration (3 μM) of either rolipram or CI-930 converted the concentration-relaxation curve of each agent into a monophasic, sigmoidal curve. Similar combination studies with peak III PDE from guinea pig trachea produced similar results. These results suggest that both PDE III$_{RO}$ and III$_C$ are involved in the regulation of intracellular cAMP in guinea pig trachea and that when one isozyme is inhibited, only partial relaxation of the trachea occurs. Also supportive of this hypothesis is the observation that theophylline and papaverine, nonselective inhibitors of both PDE III isozymes, produce monophasic, sigmoidal concentration-relaxation curves with isolated guinea pig trachea.

In the presence of rolipram, CI-930 had similar potencies in relaxing isolated trachea (EC$_{50}$ = 1 μM) and in inhibiting PDE III$_C$ (IC$_{50}$ = 0.3 μM). When other PDE inhibitors were evaluated in the presence of the same fixed concentration of rolipram, a highly significant correlation was established between PDE III$_C$ inhibition and relaxation of trachea (fig. 6, *r* = 0.89, P <
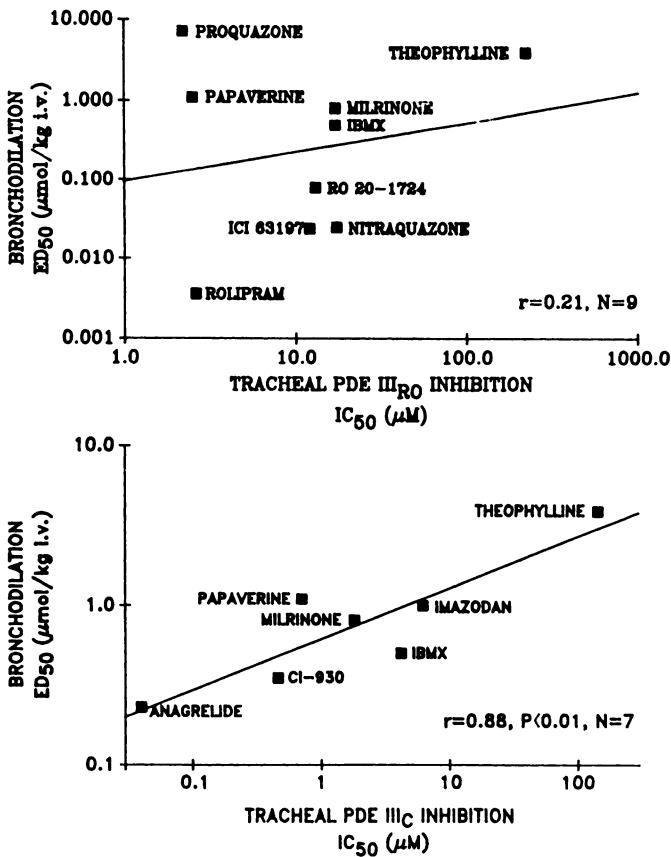
Fig. 7. Guinea pig bronchodilation (*in vivo*) as a function of PDE III$_{RO}$ (top panel) or PDE III$_C$ (bottom panel) inhibition. Bronchodilation data are expressed as ED$_{50}$ values (dose of agent that reduces histamine-induced bronchoconstriction by 50%; table 3) in anesthetized guinea pigs (n = 4–7). Data for PDE inhibition are expressed as IC$_{50}$ values (micromolar; table 2) which are the concentrations of agent that produced 50% inhibition of cAMP hydrolysis by PDE III in the presence of 3 μM CI-930 (PDE III$_{RO}$) or 3 μM rolipram (PDE III$_C$). Regression analysis for bronchodilation as a function of PDE III$_{RO}$ inhibition revealed a lack of a significant correlation (r = 0.21, n = 9), whereas regression analysis for bronchodilation as a function of PDE III$_C$ inhibition was significant (r = 0.88, P < .01, n = 7).

Fig. 8. Tracheal relaxation as a function of rolipram binding displacement (top panel; data obtained from Russo *et al.*, 1987) in carbachol-contracted, CI-930-pretreated (3 μM) guinea pig trachea and bronchodilation as a function of rolipram binding displacement (bottom panel). Tracheal relaxation data are expressed as EC$_{50}$ values (micromolar; table 4) which are the concentrations of relaxant that produced 50% relaxation of carbachol-induced contraction; bronchodilation data are expressed as ED$_{50}$ values (dose of agent that reduces histamine-induced bronchoconstriction by 50%; table 3) in anesthetized guinea pigs (n = 4–7); rolipram binding displacement data are expressed as IC$_{50}$ values (micromolar) which are the concentrations of agent that produced 50% inhibition of rat brain homogenate rolipram binding (Russo *et al.*, 1987). Each point is the mean of at least three determinations. Both tracheal relaxation as a function of rolipram binding displacement (r = 0.97, P = .0001, n = 9) and bronchodilation as a function of rolipram binding displacement (r = 0.88; P < .005, n = 8) are highly significant.

.01, n = 8). In the presence of CI-930, rolipram's EC$_{50}$ value for relaxation of trachea (0.02 μM) was significantly less than that required for PDE III$_{RO}$ isozyme inhibition (2.6 μM). When selected PDE inhibitors were analyzed in the presence of CI-930, no correlation was observed between PDE III$_{RO}$ inhibition and *in vitro* tracheal relaxation (r = 0.21, n = 9). This lack of correlation was most obvious with two structurally similar compounds, proquazone and nitraquazone. Both had similar inhibitory potency against PDE III$_{RO}$; however, nitraquazone was a substantially more potent relaxant of CI-930-pretreated trachea (EC$_{50}$ = 0.09 μM) than was proquazone (EC$_{50}$ = 70 μM). These results may be interpreted to suggest that the relationship between PDE III$_{RO}$ inhibition and its translation into smooth muscle relaxation may not be equivalent, such that much less inhibition of the PDE isozyme (5–15% at 20 nM rolipram) is needed to induce smooth muscle relaxation (50% at 20 nM). Alternatively, rolipram and selected PDE III inhibitors may bind to and inhibit a PDE isozyme at low concentrations with a reduction in total PDE activity that is too low to be detected. Another possibility is that these agents may be concentrated within specific compartments in the cell, thereby causing changes in force at much lower extracellular concen-
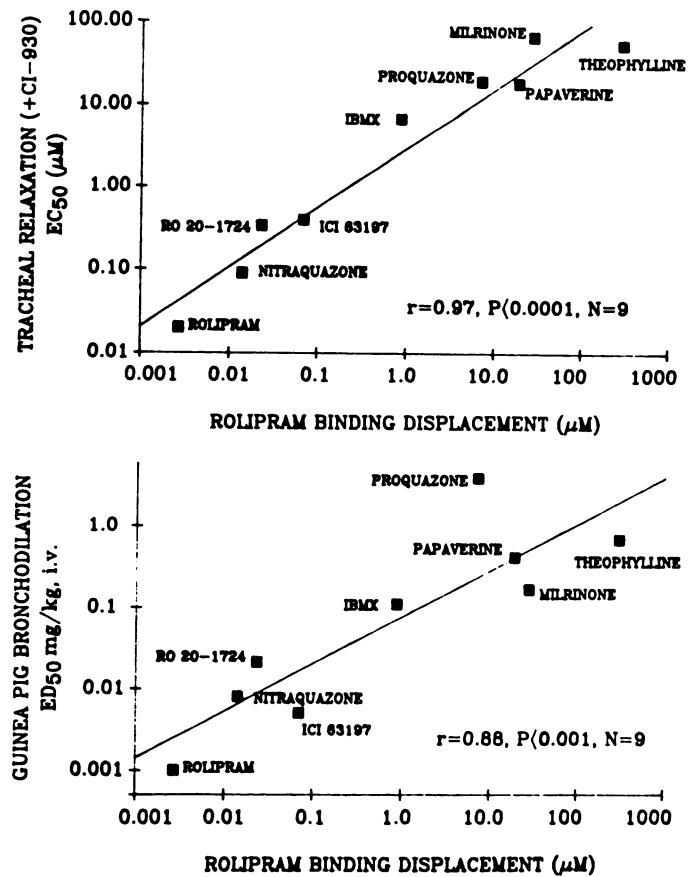
trations than that required to inhibit the isolated isozyme. Previous studies have shown that Ro 20-1724, a PDE III$_{RO}$ inhibitor that also potently displaces rolipram binding, can potentiate isoproterenol-induced bronchorelaxation, indicating functional PDE isozyme inhibition by this agent (Torphy *et al.*, 1988). Thus, these data suggest there may be both high- and low-affinity rolipram binding sites on a single PDE isozyme, or a separate high-affinity and low-affinity rolipram-sensitive PDE isozyme in the peak III PDE preparation.

These data would also be consistent with the hypothesis that rolipram possesses another activity, PDE inhibition, with potency in the nanomolar range. This activity appears to be related to a high-affinity rolipram binding site described in homogenized fractions of rat brain (Schneider *et al.*, 1986; Russo *et al.*, 1987). A highly significant correlation was observed in the present study between the ability of nine selected PDE inhibitors to relax CI-930-pretreated trachea and for these agents to displace rolipram binding in rat brain homogenates

(fig. 8; $r = 0.97$, P < .0001, $n = 9$). It is possible that this strong correlation between rolipram binding displacement and tracheal relaxation is due to inhibition of a low K_m cAMP PDE with nanomolar affinity for rolipram that is masked in the DEAE peak III fraction. A previous study by Schneider (1982) suggests that the rolipram binding site activity is associated with a soluble PDE fraction isolated from rat brain. Alternatively, another possible explanation is that rolipram induces smooth muscle relaxation by an undefined mechanism not exclusively related to PDE inhibition. However, data in the present study show that this putative mechanism(s) does not involve *beta* adrenergic or adenosine stimulating activity, neuronal (Na^+) conduction or activation of the nonadrenergic, noncholinergic inhibitory system.

In summary, the results of this study suggest the existence of two pharmacologically distinct isozymes of PDE contained in the peak III PDE DEAE-cellulose fraction of guinea pig trachea. One of these isozymes (PDE III_C) appears to play a role in relaxation of guinea pig trachea. A positive correlation was observed for inhibition of PDE III_C and relaxation of trachea; no significant correlation was apparent for inhibition of PDE III_{RO} and relaxation. A positive correlation was also observed between *in vitro* relaxation of guinea pig trachea or *in vivo* bronchodilation and previously published values for inhibition of rolipram binding. An exact cause-and-effect relationship between the rolipram binding and modulation of bronchomotor tone, however, has not been established. Future studies should focus on purification of the rolipram-sensitive PDE to better understand the relationships between high-affinity rolipram binding, PDE isozyme inhibition and bronchodilation, and examine if the low K_m cAMP PDE isozymes play an important role in modulating bronchomotor tone in airway smooth muscle of other species.

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