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CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ISOENZYMES IN GUINEA-PIG TRACHEAL MUSCLE AND BRONCHORELAXATION BY ALKYLXANTHINES

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Abstract—In this study the phosphodiesterase (PDE) isoenzymes in guinea-pig trachealis smooth muscle were separated by DEAE-Sepharose anion exchange chromatography, identified, and characterized. Furthermore the effect of theophylline and 1-*n*-butyl-3-*n*-propylxanthine (BPX) on the isolated PDE isoenzymes and on their tracheal relaxant effect were investigated and compared with the nonxanthine PDE inhibitors amrinone and Ro 20-1724. We identified five distinct isoenzymes in guinea-pig tracheal muscle; calcium/calmodulin-stimulated cyclic AMP PDE (PDE I), cyclic GMP-stimulated cyclic AMP PDE (PDE II), cyclic GMP-inhibited and amrinone-sensitive cyclic AMP PDE (PDE III), cyclic AMP-specific and Ro 20-1724-sensitive PDE (PDE IV), and cyclic GMP-specific PDE (PDE V). BPX strongly inhibited the PDE IV isoenzyme with high selectivity, while the inhibitory effect of theophylline was weak. The PDE IV inhibitors BPX and Ro 20-1724 synergistically increased the relaxant effect of the β_2-adrenoceptor agonist salbutamol in carbachol-contracted trachea much more strongly than theophylline. In contrast, amrinone, a PDE III inhibitor, hardly influenced the relaxant effect of salbutamol, suggesting that the PDE IV isoenzyme is functionally associated with β_2-adrenoceptors in guinea-pig trachea and that inhibition of this enzyme potentiates the ability of sulbutamol to increase the intracellular cyclic AMP content. These results indicate that the PDE IV isoenzyme plays a significant role in alkylxanthine-mediated relaxation of guinea-pig trachea.

Key words: PDE inhibitor; 1-*n*-butyl-3-*n*-propylxanthine; PDE IV; β_2-adrenoceptor; salbutamol; synergism

Seven families of PDE‡ isoenzymes have been reported [1]. These enzyme families differ in their substrate specificity, affinity for cyclic purines, and regulatory properties, with a variable tissue distribution (see review by Beavo [2]). Among them the PDE IV isoenzyme is of significant functional importance in tracheal smooth muscle in mammals [3–5]. In the guinea-pig a possible relationship between PDE IV inhibition and bronchorelaxation has been also suggested using selective PDE isoenzyme inhibitors [6, 7], but there are few reports of identification of the PDE isoenzymes in guinea-pig tracheal muscle.

Theophylline is one of the most effective and frequently used drugs for the treatment of asthma, but its extrapulmonary reactions impair its usefulness [8, 9]. The mechanism underlying the airway reactivity of xanthines such as theophylline have been proposed to be PDE inhibition, adenosine antagonism, or others [6, 9] and have not been completely clarified. We have studied structure–activity relationships to obtain selective bronchodilators from xanthine derivatives [10–14], and have established, using guinea-pigs, that the tracheal relaxant effect of xanthine derivatives is closely related to their inhibitory activity against PDE IV but not with their affinity for adenosine receptors [15]. In those studies, we found that substitutions of long alkyl chain at the N1 position of the xanthine nucleus increased the selectivity for bronchorelaxation vs heart stimulation.

The purpose of this study was to identify and characterize PDE isoenzymes present in guinea-pig tracheal smooth muscle, to assess the inhibitory effects of theophylline and BPX, a novel airways selective relaxant, on these PDE isoenzymes and to confirm the functional involvement of PDE IV inhibition in tracheal relaxation evoked by these alkylxanthines and other known nonxanthine PDE inhibitors.

MATERIALS AND METHODS

This study was done using tracheas from guinea-pigs (male, Hartley strain, 300–500 g, Nippon SLC, Hamamatsu, Japan) killed by cervical dislocation followed by exsanguination. Tissues were rapidly removed and placed in the relevant buffer.

Separation and characterization of PDE isoenzymes. The method reported by Reeves *et al.* [16] was used to separate the PDE isoenzymes. Tracheal muscle was homogenized in a buffer (composition

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‡ Abbreviations: PDE, phosphodiesterase; BPX, 1-*n*-butyl-3-*n*-propylxanthine; DEAE, diethylaminoethylether.

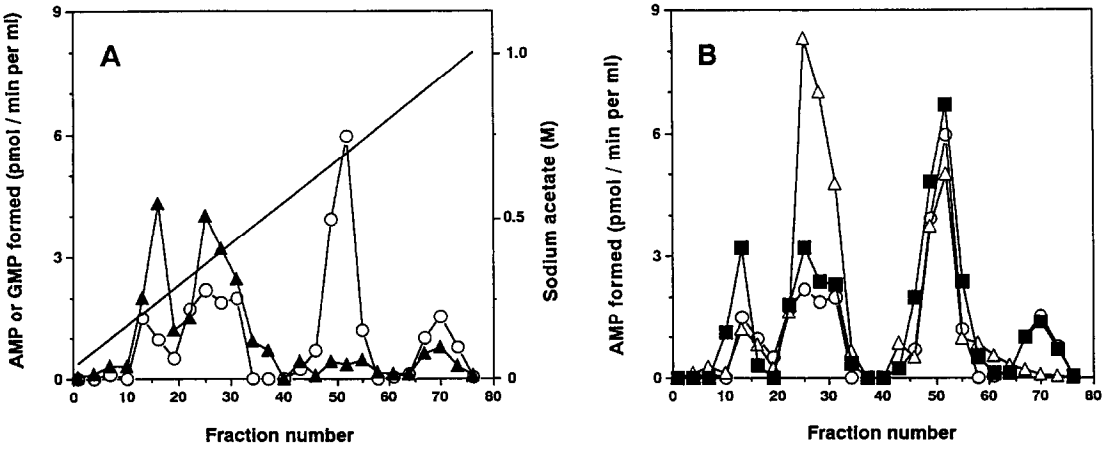

Fig. 1. A typical elution profile of guinea-pig tracheal smooth muscle PDE activity from the DEAE-Sepharose anion exchange column. The 10,000 *g* supernatants of tracheal muscle homogenates were put on the column and eluted with a linear sodium acetate gradient. (A) PDE activity measured in the presence of 1 μM [^{3}H]cyclic AMP (○) or 1 μM [^{3}H]cyclic GMP (▲). (B) [^{3}H]cyclic AMP (1 μM) hydrolysis in the absence (○) or presence of calcium/calmodulin (■) or 1 μM cold cyclic GMP (△).

in mM: Bistris 20, 2-mercaptoethanol 5, benzamidine 2, EDTA 2, phenylmethylsulfonyl fluoride 0.05, sodium acetate 50; pH 6.5). The homogenate was then centrifuged at 10,000 *g* for 15 min, and the supernatant was applied to a column (3 × 0.5 cm) of DEAE-Sepharose CL-6B pre-equilibrated with the buffer. The flow rate was 0.4 mL/min throughout the chromatography. The column was washed with the buffer and PDE activities were eluted with a linear gradient of 0.05–1.0 M sodium acetate in buffer. The eluate was collected in 0.5-mL fractions and assayed for cyclic AMP and cyclic GMP PDE activity by a two-step assay method described by Thompson and Appleman [17]. PDE activity was measured in a reaction mixture containing 40 mM Tris, 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol (pH 8.0) and either cyclic AMP (1 μM) or cyclic GMP (1 μM). Calmodulin-stimulated cyclic nucleotide hydrolysis and cyclic GMP-inhibited cyclic AMP hydrolysis were assessed by the addition of 50 U/mL calmodulin plus 3 mM $CaCl_2$ and 1 μM cold cyclic GMP, respectively.

For calculations of K_m and V_{max}, the concentrations of cyclic AMP and cyclic GMP were varied. Kinetic data were obtained from Lineweaver–Burk plots.

Protein was measured using a Bio-Rad assay kit, based on the method by Bradford [18].

Inhibition of PDE activity. The concentration of substrate (cyclic AMP or cyclic GMP) was fixed at 1 μM for all experiments. Each assay was performed in duplicate and the concentration of inhibitor that produced 50% inhibition of hydrolysis (IC_{50}) was calculated by a nonlinear least squares method program, MULTI, written by Yamaoka *et al.* [19]. All assays were repeated at least three times.

Tracheal muscle relaxation. Relaxation of isolated tracheal ring chains was measured through an isometric transducer. Tracheal ring chains were

Table 1. Effects of theophylline, BPX and selective PDE inhibitors on PDE activities in guinea-pig trachea

Inhibitor	IC_{50} (μM) PDE I*	PDE II†	PDE III‡	PDE IV‡	PDE V§
Theophylline	>300‖	>300	>300	114 ± 11	>300
BPX	44 ± 1	64 ± 16	102 ± 5	0.23 ± 0.03	45 ± 1
Ro 20-1724	>300	>300	>300	0.22 ± 0.04	>300
Amrinone	>300	>300	8.2 ± 2.1	>300	>300

* Cyclic AMP (1 μM) as substrate in the presence of $CaCl_2$ (3 mM) and calmodulin (50 U/mL).

† Cyclic AMP (1 μM) as substrate in the presence of cyclic GMP (1 μM).

‡ Cyclic AMP (1 μM) as substrate.

§ Cyclic GMP (1 μM) as substrate.

‖ 50% inhibition was not achieved at 300 μM, the maximum concentration soluble in the assay mixture.

Values are expressed as the means ± SE of three experiments.

Table 2. Kinetic characteristics of guinea-pig tracheal PDE isoenzymes

Isoenzyme	K_m (μM) Cyclic AMP	K_m (μM) Cyclic GMP	V_{max} (nmol/min per mg protein) Cyclic AMP	V_{max} (nmol/min per mg protein) Cyclic GMP
I*	64	8.9	1.9	2.0
II†	138	26	15	4.9
III	1.5	ND‡	3.0	ND
IV	8.8	ND	5.0	ND
V	ND	5.4	ND	0.7

* In the presence of $CaCl_2$ (3 mM) and calmodulin (50 U/mL).
† In the presence of cyclic GMP (1 μM).
‡ Not determined, because of low activity with substrate concentration at 1 μM.
All values are means of two to three measurements.

placed in a 10-mL thermostatically controlled organ bath (37°) containing Krebs–Henseleit solution (pH 7.4), gassed with 95% O_2–5% CO_2. Preparations were treated with PDE inhibitor for 15 min and then contracted with carbachol at a concentration producing 50% of maximum contraction in each preparation. When the response had plateaued, salbutamol was added cumulatively.

Statistics. Values are given as means ± SE of three to five measurements. The regression lines were calculated with the program for the nonlinear least-squares method (MULTI) [19]. Statistical significance was measured with Student's *t*-test.

Materials. BPX was synthesized in our laboratory [13]. Theophylline, amrinone, cyclic AMP, cyclic GMP, calmodulin, carbachol and salbutamol were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Ro 20-1724 was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). DEAE-Sepharose CL-6B was obtained from Pharmacia (Uppsala, Sweden). [^{3}H]Cyclic AMP (sp. act. 1.05 TBq/mmol) and [^{3}H]cyclic GMP (sp. act. 0.37 TBq/mmol) were from New England Nuclear (Boston, MA, U.S.A.). BPX, amrinone, and Ro 20-1724 were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide used in each assay was below 0.25% at which the solvent did not influence assays. All other agents were prepared in the respective assay buffer.

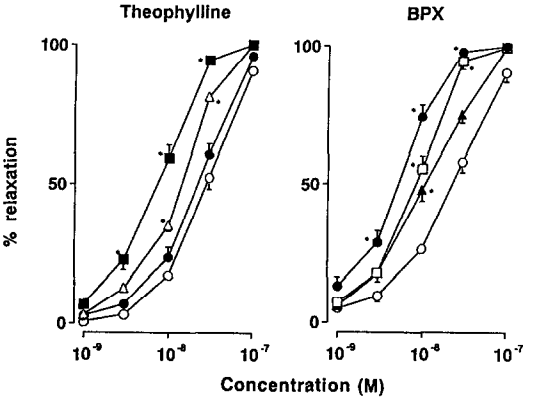

Fig. 2. Effects of theophylline and BPX on the relaxation by salbutamol of the carbachol-contracted guinea-pig trachea. The trachea ring chain preparations were treated without (○) or with a PDE inhibitor at 1 μM (▲), 3 μM (□), 10 μM (●), 30 μM (△) or 100 μM (■) for 15 min before administration of the 50% contractile concentration of carbachol. Values are the means ± SE from three to five experiments. *Significant difference from the salbutamol alone control, $P < 0.05$.

RESULTS

A representative chromatogram of PDEs separated from guinea-pig tracheal muscle is shown in Fig. 1. When assayed with 1 μM cyclic AMP as substrate, four peaks of PDE activity were resolved by the column. The first peak eluted by low sodium acetate concentrations was markedly activated by calcium/calmodulin, indicating this peak contains PDE I. When assayed with 1 μM cyclic GMP, two sharp peaks appeared. These cyclic GMP-hydrolytic activities were barely influenced by calcium/calmodulin (data not shown). Therefore, the faster eluted cyclic GMP-hydrolytic enzyme might be defined as PDE V, although it could not be clearly separated from the PDE I fractions. Another peak of cyclic GMP-hydrolytic activity was eluted in the same fractions as the second peak of cyclic AMP-hydrolytic activity. This activity was significantly increased by the addition of cyclic GMP and is PDE II. The major peak which hydrolysed cyclic AMP but not cyclic GMP was not affected by calcium/calmodulin or cyclic GMP, but was selectively inhibited by a PDE IV inhibitor, Ro 20-1724 [16] (Table 1). This enzyme was thus identified as PDE IV. The fifth enzyme was eluted by high sodium acetate, had low cyclic AMP hydrolytic activity and was inhibited by cyclic GMP and a selective PDE III inhibitor, amrinone [20] (Table 1), indicating it to be the PDE III isoenzyme.

The kinetic characteristics of PDE isoenzymes isolated from guinea-pig tracheal muscle are presented in Table 2. PDE I had a low affinity for cyclic AMP. PDE II hydrolysed both cyclic nucleotides with low affinity and high activity. PDE III had high affinity for cyclic AMP but low activity. PDE IV showed relatively high affinity and activity for cyclic AMP. PDE V showed high affinity but

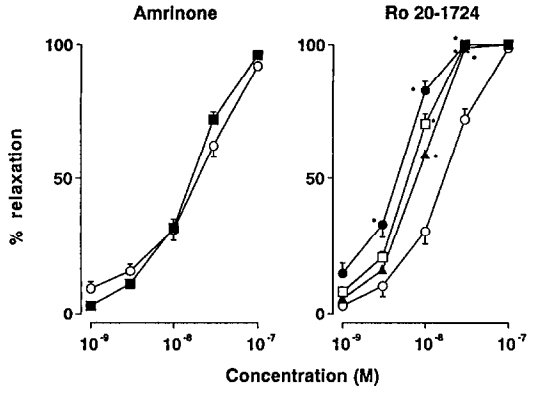

Fig. 3. Effects of the selective PDE inhibitors amrinone and Ro 20-1724 on the relaxation by salbutamol of the carbachol-contracted guinea-pig trachea. Symbols and note are similar to the footnote in Fig. 2. *Significant difference from the salbutamol alone control, $P < 0.05$.

Table 3. Combined effects of PDE inhibitors on the relaxant effect of salbutamol on carbachol-contracted guinea-pig trachea

Inhibitor	IC_{50} (nM)	Potentiation rate†
Salbutamol alone	20.8 ± 7.9	—
+theophylline (10 μM)	17.0 ± 3.0	1.2
(100 μM)	6.8 ± 1.2*	3.1
+BPX (10 μM)	5.1 ± 1.5*	4.1
+Ro 20-1724 (10 μM)	5.8 ± 0.4*	3.6
+amrinone (100 μM)	14.9 ± 1.1	1.4

The IC_{50} was the concentration of salbutamol producing 50% relaxation with or without pretreatment with a PDE inhibitor (concentrations used were indicated in parenthesis). Data are the means ± SE from three to five experiments.

* Significant difference from the salbutamol alone control, $P < 0.05$.

† IC_{50} from salbutamol alone was divided by IC_{50} from combined experiment with the inhibitors.

low activity for cyclic GMP. Because of the small amounts of enzymes and low activity, further kinetic analyses were not done; e.g. kinetic parameters of cyclic AMP for PDE V and cyclic GMP for PDE III and IV. The 50% inhibitory activity of theophylline, BPX, amrinone, and Ro 20-1724 on the isoenzymes isolated is indicated in Table 1. Amrinone and Ro 20-1724 selectively inhibited cyclic AMP-hydrolytic activities of PDE III and IV, respectively, as described elsewhere [16, 20]. Theophylline weakly inhibited PDE IV activity. BPX had a strong inhibitory effect on the PDE IV isoenzyme and was highly selective for this isoenzyme.

The effects of PDE inhibitors on β_2-adrenoceptor-mediated relaxation of guinea-pig trachea are shown in Figs 2 and 3. Alkylxanthines significantly shifted the concentration–relaxation curve of salbutamol to the left in a concentration-dependent manner. Theophylline increased the potency of the β_2-agonist by about 3-fold at 100 μM, and BPX produced a 4-fold shift at 10 μM. Although a PDE III inhibitor, amrinone, hardly affected the relaxant effect of salbutamol even at 100 μM, a PDE IV inhibitor, Ro 20-1724, concentration-dependently potentiated the relaxation, with a similar magnitude of BPX (Table 3).

DISCUSSION

This study shows that guinea-pig tracheal smooth muscle contains five PDE isoenzymes which have affinities for cyclic AMP and cyclic GMP similar to the isoenzymes from human tracheal muscle [5]. This is the first paper to identify PDE isoenzymes I to V in guinea-pig tracheal muscle. Tanaka *et al.* [21] have isolated cyclic GMP PDE from guinea-pig trachea and reported a relationship between tracheal relaxation and PDE inhibition by 3-alkylxanthines. Recently, we have found that extending the alkyl chain length at the N3-position of the xanthine nucleus increases not only the pharmacological activities of the xanthines, such as tracheal relaxation and heart stimulation, but also inhibitory activities on PDE I, III, and IV isoenzymes [15]. We also reported that increasing the length of the alkyl chain at the N1-position increased the selectivity of xanthines for the PDE IV from the brain [15], which lead to the development of BPX, a potent and selective bronchodilator [13]. In this study we found that BPX inhibited the PDE IV isoenzyme more effectively than other isoenzymes from the guinea-pig tracheal smooth muscle and suggest a functional role of PDE IV inhibition in alkylxanthine-induced bronchorelaxation in guinea-pigs.

Harris *et al.* [22] suggested that both PDE III and IV are involved in the regulation of intracellular cyclic AMP and the contractility of guinea-pig trachea using several nonselective and selective PDE inhibitors. It is known that airway smooth muscle from guinea-pigs and man has predominantly β_2-adrenoceptors [23–25]. Tomkinson *et al.* [7] suggested that the β_2-adrenoceptor is functionally related to PDE IV but not PDE III in bovine and guinea-pig trachea. This study also shows that theophylline and PDE IV selective inhibitors Ro 20-1724 and BPX but not amrinone synergistically increased the tracheal relaxant effect of the β_2-adrenoceptor agonist salbutamol. These data support the suggestion of a functional association of PDE IV with β_2-adrenoceptors in guinea-pig trachea and suggest that the inhibition of the PDE IV isoenzyme potentiates β_2-adrenoceptor-mediated relaxation through an increase of intracellular cyclic AMP. On the other hand, inhibitors of PDE III and V cause tracheal relaxation by themselves, and possibilities of involvement of these isoenzymes or complex contributions of PDE III and PDE IV to the tracheal relaxation remain uncertain [3, 5, 26].

In conclusion, this study identified five PDE isoenzymes in guinea-pig tracheal muscle and indicates that PDE IV inhibition plays a major role in tracheal relaxation evoked by alkylxanthines.

REFERENCES

1. Michaeli T, Bloom TJ, Martins T, Loughney K, Ferguson K, Riggs M, Rodgers L, Beavo JA and Wigler M, Isolation and characterization of a previously undetected human cAMP phosphodiesterase by complementation of cAMP phosphodiesterase-deficient *Saccharomyces cerevisiae*. *J Biol Chem* **268**: 12925–12932, 1993.
2. Beavo JA, Multiple isozymes of cyclic nucleotide phosphodiesterase. In: *Advances in Second Messenger and Phosphoprotein Research* (Eds. Greengard P and Robison GA), pp. 1–38. Raven Press, New York, 1988.
3. Shahid M, van Amsterdam RGM, de Boer J, Ten Berge RE, Nicholson CD and Zaagsma J, The presence of five cyclic nucleotide phosphodiesterase isoenzyme activities in bovine tracheal smooth muscle and the functional effects of selective inhibitors. *Br J Pharmacol* **104**: 471–477, 1991.
4. Cortijo J, Bou J, Beleta J, Cardelus I, Llenas J, Morcillo E and Gristwood RW, Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with human bronchus. *Br J Pharmacol* **108**: 562–568, 1993.
5. Torphy TJ, Undem BJ, Cieslinski LB, Luttmann MA, Reeves ML and Hay DWP, Identification, characterization and functional role of phosphodiesterase isozymes in human airway smooth muscle. *J Pharmacol Exp Ther* **265**: 1213–1223, 1993.
6. Howell RE, Multiple mechanisms of xanthine actions on airway reactivity. *J Pharmacol Exp Ther* **255**: 1008–1014, 1993.
7. Tomkinson A, Karlsson JA and Raeburn D, Comparison of the effects of selective inhibitors of phosphodiesterase types III and IV in airway smooth muscle with differing β-adrenoceptor subtypes. *Br J Pharmacol* **108**: 57–61, 1993.
8. Piafsky KM and Ogilvie RI, Dosage of theophylline in bronchial asthma. *New Engl J Med* **292**: 1218–1222, 1975.
9. Persson CGA, Andersson KE and Kjellin G, Effects of enprofylline and theophylline may show the role of adenosine. *Life Sci* **38**: 1057–1072, 1986.
10. Takagi K, Hasegawa T, Kuzuya T, Ogawa K, Watanabe T, Satake T, Miyamoto K, Wakusawa S and Koshiura R, Structure–activity relationship in 3*N*-alkyl-xanthine derivatives. *Jpn J Pharmacol* **46**: 373–378, 1988.
11. Miyamoto K, Takagi K, Sakai R, Wakusawa S, Koshiura R, Nadai M, Apichartpichean R and Hasegawa T, Correlation between hydrophobicity of *N*-alkylxanthine derivatives and their biological activities on guinea-pig isolated tracheal smooth muscle. *J Pharm Pharmacol* **41**: 844–847, 1989.
12. Miyamoto K, Sakai R, Yamamoto Y, Konno K, Sanae F, Hasegawa T and Takagi K, Selective bronchodilators from 1-(5′-oxohexyl)xanthines. *J Pharm Pharmacol* **44**: 888–892, 1992.
13. Sakai R, Konno K, Yamamoto Y, Sanae F, Takagi K, Hasegawa T, Iwasaki N, Kakiuchi M, Kato H and Miyamoto K, Effects of alkyl substitutions of xanthine skeleton on bronchodilation. *J Med Chem* **35**: 4039–4044, 1992.
14. Miyamoto K, Yamamoto Y, Kurita M, Sakai R, Konno K, Sanae F, Ohshima T, Takagi K, Hasegawa T, Iwasaki N, Kakiuchi M and Kato H, Bronchodilator activity of xanthine derivatives substituted with functional groups at the 1- or 7-position. *J Med Chem* **36**: 1380–1386, 1993.
15. Miyamoto K, Kurita M, Ohmae S, Sakai R, Sanae F and Takagi T, Selective tracheal relaxation and phosphodiesterase-IV inhibition by xanthine derivatives. *Eur J Pharmacol* **267**: 317–322.
16. Reeves ML, Leigh BK and England PJ, The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. *Biochem J* **241**: 535–541, 1987.
17. Thompson WJ and Appleman MM, Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry* **10**: 311–316, 1971.
18. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
19. Yamaoka K, Tanigawara Y, Nakagawa T and Uno T, A pharmacokinetic analysis program (MULTI) for microcomputer. *J Pharmacobio-Dyn* **4**: 879–885, 1981.
20. Silver PJ, Hamel LT, Perrone MH, Bently RG, Bushover CR and Evans DB, Differential pharmacologic sensitivity of cyclic nucleotide phosphodiesterase isozymes isolated from cardiac muscle, arterial and airway smooth muscle. *Eur J Pharmacol* **150**: 85–94, 1988.
21. Tanaka H, Ogawa K, Takagi K, Satake T and Hidaka H, Inhibition of cyclic GMP phosphodiesterase by xanthine derivatives relaxes guinea-pig trachealis smooth muscle. *Clin Exp Pharmacol Physiol* **18**: 163–168, 1991.
22. Harris AL, Connell MJ, Ferguson EW, Wallace AM, Gordon RJ, Pagani ED and Silver PJ, Role of low K_m cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea pig. *J Pharmacol Exp Ther* **251**: 199–206, 1989.
23. O'Donnell SR, An examination of some β-adrenoceptor stimulant for selectivity using the isolated trachea and atria of the guinea-pig. *Eur J Pharmacol* **19**: 371–379, 1972.
24. Carswell H and Nahorski SR, β-Adrenoceptor heterogeneity in guinea-pig airways: comparison of functional and receptor labelling studies. *Br J Pharmacol* **79**: 965–971, 1983.
25. Zaagsma J, van der Heijden PJCM, van der Schaar MWG and Bank CMC, Comparison of functional β-adrenoceptor heterogeneity in central and peripheral airway smooth muscle of guinea-pig and man. *J Recept Res* **3**: 89–106, 1983.
26. Howell RE, Sickels BD and Woeppel SL, Pulmonary antiallergic and bronchodilator effects of isozyme-selective phosphodiesterase inhibitors in guinea pigs. *J Pharmacol Exp Ther* **264**: 609–615, 1993.