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Effects on tracheal smooth muscle of adenosine and methylxanthines, and their interaction

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The relaxant effects of xanthine and 15 different methylxanthines were studied in a guinea-pig isolated tracheal ring preparation. Substitution in the 3- and 1-positions of the xanthine molecule were found to be of greatest importance for improving tracheal relaxant potency. The unsubstituted xanthine and all 9-methylated xanthines were weakly active. Adenosine occasionally contracted (slightly) the tracheae but a concentration-dependent relaxation was always recorded. Endogenous adenosine did not seem to contribute to the tracheal tone. Xanthines methylated in the 1-position consistently antagonized the relaxant effect of adenosine and produced graded rightward shifts of the concentration-response line to adenosine (competitive antagonism). Xanthines with an unsubstituted 1-position or with comethylation in the 9-position were generally without antagonism. The results support our view that bronchodilating xanthine derivatives lacking universal adenosine antagonism can be produced.

Little is known about the biological role of adenosine in the lung. It seems that opposite actions can be produced both on smooth muscle tension and on release of histamine from various cells (Fredholm et al 1979; Marquardt et al 1978; Marone et al 1979). Theophylline at low and therapeutic concentrations antagonizes adenosine's bronchorelaxant effects but potent bronchodilator alkylxanthines have been produced that seem to lack antagonism at important adenosine receptor sites (Persson & Kjellin 1981; Persson et al 1981; Lunell et al 1982; Persson & Erjefält 1982).

The xanthine molecule can be substituted at several positions to give derivatives that qualitatively and quantitatively differ from theophylline (Bock 1920; Armitage et al 1961; Goodsell et al 1971; Bergstrand et al 1978; Persson 1980; Persson & Kjellin 1981 and unpublished observations). To examine which positions are most important for bronchodilation and antagonism of adenosine-induced relaxation, respectively, xanthine and a variety of methylxanthines have been evaluated in a tracheal preparation. Some results have been preliminarily communicated (Karlsson & Persson 1981b; Persson et al 1982).

MATERIALS AND METHODS

Guinea-pigs of either sex, 200–400 g, were killed by a blow on the head and the trachea rapidly dissected. Open tracheal rings were prepared (Karlsson & Persson 1981a) and suspended under a resting tension of 0·6 g in 50 ml organ baths with heated (37 °C) Krebs solution (mM: NaCl 118·0, KCl 4·6, $CaCl_2$ 2·5, $MgSO_4$ 1·15, $NaHCO_3$ 24·9, KH_2PO_4 1·15, glucose 5·5, pH = 7·4) and gassed with 5% CO_2 in oxygen. The preparations were left to equilibrate for about 1 h during which a stable spontaneous tension between 0·6 and 2·0 g was attained.

Isometric tension changes were measured with Grass strain-gauge transducers (FT03) and recorded on a Grass polygraph model 5. The drugs were added cumulatively by injection to the organ baths until no further effect was obtained.

Relaxant dose-response (D/R) curves were evaluated to xanthine and the methylxanthines in tracheal preparations contracted by carbachol $5{\cdot}5 \times 10^{-7}$ mol litre^{-1}. Intrinsic tone preparations were used in all experiments involving studies of adenosine.

D/R-curves to adenosine were evaluated in the absence and presence of different concentrations of the xanthines. Initially, in each preparation, adenosine control-curves (in the absence of xanthines) were evaluated at least twice to obtain stable tissue sensitivity to adenosine. After this protocol adenosine-induced relaxations were reproducible since EC50 values from two to four D/R-curves to adenosine, obtained in each preparation during the day, generally deviated less than 10% from the mean value in that preparation.

To study adenosine-antagonism, different concentrations of the xanthines were added to the bath

* Correspondence.

before D/R-curves to adenosine were evaluated. The adenosine EC50 value obtained after pretreatment for 20 min did not differ from that obtained after 5-min contact so the xanthines were added 5 to 10 min before adenosine.

Dipyridamole at 2 μmol litre^{-1}, when added 15 min before adenosine, produced an approximately 50-fold leftward shift of the adenosine-curve. This concentration which did not affect baseline tension or D/R-curves to the xanthines, was always present when adenosine-effects were evaluated.

Mean ± s.e.m. values were calculated for each concentration of the drugs. EC50 values (the concentrations of drug producing 50% of its own maximal response under the chosen experimental condition) are mean ± s.e.m. results from each individual D/R curve. Adenosine EC50 values in the presence of xanthines were compared with mean adenosine control values from the same preparation, only. Thus, adenosine-antagonistic potency-values of each compound are the mean of concentration ratios from individual preparations. Statistical differences, based on EC50 values, were calculated by use of Student's *t*-test for unpaired observations.

Adenosine, adenosine-5′-triphosphate, carbachol, 1,3,7-trimethylxanthine (caffeine) and dipyridamole were dissolved in 0·9% NaCl (saline). Other compounds were dissolved in 1 equiv. of 0·5 mol litre^{-1} NaOH and then diluted in saline. Drugs used were: adenosine (Sigma), adenosine-5′-triphosphate (ATP, Sigma), adenosine deaminase, EC 3.5.4.4 Type III, (Sigma), carbachol chloride (Ph. Nord.), dipyridamole (Persantin, Boehringer, Ingelheim), theophylline, anhydrous (Knoll), xanthine (Merck), 3,7-dimethylxanthine (theobromine, Ph.Nord.), 1,3,7-trimethyl-xanthine (caffeine, Ph.Nord), 1-methylxanthine, 7-methylxanthine, 8-methylxanthine, 9-methylxanthine, 1,7-dimethylxanthine, 1,9-dimethylxanthine, 3,9-dimethylxanthine and 1,3,9-trimethylxanthine (Fluka), 3-methylxanthine, 3,8-dimethylxanthine and 1,3,8-trimethylxanthine were formed by the classical Traube route (Traube 1900). 1,8-dimethylxanthine was prepared according to Cook et al (1949). Tetrodotoxin (Sankyo).

RESULTS

Tracheal relaxation by xanthine and various methylxanthines

Open guinea-pig tracheal rings, spontaneously contracted (0·6 to 2·0 g) or contracted by 5·5 · 10^{-7} mol litre^{-1} carbachol (corresponding to 70% of a maximum carbachol-contraction, Karlsson & Persson 1981a) were relaxed in a concentration-related way by the methylxanthines.

When repeatedly exposed to high concentrations of methylxanthines, the rings gradually lost their ability to regain a spontaneous tone after rinsing. Therefore, the statistical evaluation of relative relaxant potencies was made with carbachol-contracted rings. The rank order and degree of relaxation among the methylxanthines in a preliminary study were the same, whether evaluated at basal tone or at the chosen carbachol concentration.

Mean EC50 values for tracheal relaxation by the methylxanthines are given in Table 1. However, xanthine and compounds methylated in the 9-position produced maximum relaxations (in concentrations close to saturation) less than 50% of a maximum theophylline relaxation.

Table 1. Tracheal relaxation and adenosine-antagonism by methylxanthines. EC50-values were obtained in carbachol-contracted (5·5 · 10^{-7} mol litre^{-1}) tracheal rings. pA_2 values and slopes are derived from Schild plots for antagonism of adenosine-induced relaxation of intrinsic tone preparations.

Position(s) of methyl group(s)	EC50±s.e.m. mmol litre^{-1}	pA_2	Slope	95% confid. limits
– -Xanthine	**	NA		
1–	1·81 ± 0·07*	4·40	1·09	0·81–1·37
3–	1·24 ± 0·11	NA		
7–	2·12 ± 0·25	NA		
8–	3·41 ± 0·21	NA		
9–	**	NA		
1·3-Theophylline	0·32 ± 0·01	4·92	1·18	0·77–1·60
1·7	0·90 ± 0·19	4·63	1·26	0·77–1·81
1·8	0·84 ± 0·02	4·95	1·01	0·51–1·51
1·9	**	NA		
3·7-Theobromine	0·83 ± 0·08	NA		
3·8-	0·60 ± 0·05	NA		
3·9-	**	NA		
1.3.7-Caffeine	0·43 ± 0·04	4·42	0·84	0·35–1·34
1.3.8-	0·30 ± 0·03	4·95	0·88	0·48–1·28
1.3.9-	**	NA		

* Each value is the mean of 4 preparations.
** Less than 50% of a maximum theophylline induced relaxation was produced.
NA – No antagonism, see text.

Among monosubstituted xanthines, methylsubstitution in the 3-position gave more active compounds than substitution in 1-, 7- or 8-position. 9-Methylxanthine was almost inactive. The disubstituted methylxanthines exhibited the following rank order of potency: 1,3>3,8>3,7>1,8>1,7. The 1,9 and 3,9-dimethylxanthine were almost inactive. The potencies among trisubstituted compounds ranked: 1,3,8>1,3,7; 1,3,9 being almost inactive (Table 1).

Substitution in the 8-position in di- and trisubstituted xanthines produced, in contrast to monosubstituted xanthines, more active compounds than substitution in the 7-position.

Consistent with this, 1,3,8-trimethylxanthine was the most active compound, closely followed by 1,3-dimethylxanthine. Mean EC50 values for theophylline in intrinsic tone (60 ± 11 μmol litre^{-1}; n = 4) and in carbachol-contracted (320 ± 10 μmol litre^{-1}; n = 4) preparations agree with previously reported values (Karlsson & Persson 1981a).

Effects of adenosine

The first evaluation (dipyridamole absent) of adenosine occasionally yielded a slight contraction followed by a concentration-related relaxation. The contraction, when it occurred, did not show repeatability. Whether this slight contraction was affected by tetrodotoxin (TTX 3 μmol litre^{-1}) was examined in four tracheae. Another four preparations were used as controls and in two an initial contraction to adenosine was recorded. Two out of the four TTX-treated tracheae also responded with an initial contraction to adenosine.

Isolated tracheal rings with spontaneous tone or contracted by carbachol were consistently relaxed by adenosine in a dose-related way when dipyridamole, was present. The maximum relaxation by adenosine in intrinsic tone or in carbachol-contracted rings was 56·7 and 17·4%, respectively, of a maximum theophylline relaxation. The corresponding mean adenosine EC50 values were 9·8 ± 0·7 μmol litre^{-1} (n = 47) and 12·1 ± 3·0 μmol litre^{-1} (n = 4). D/R curves to adenosine in intrinsic tone preparations are shown in Fig. 1 (solid lines).

To study the possibility that an endogenous tone to adenosine contributed to the contractile state of the tracheal preparation, adenosine deaminase (0·1 u ml^{-1}) was added to the spontaneously contracted preparations. No change in tone was produced. The deaminase completely abolished the relaxation to exogenously added adenosine (100 μmol litre^{-1}) but reduced only slightly the sensitivity of the preparation to the relaxant effects of ATP.

Adenosine-antagonism by xanthine derivatives

The effect of adenosine on spontaneous tone, was evaluated in the presence of different concentrations of xanthine and of methylxanthines. The drugs were used in concentrations producing up to 50% relaxation (of their own maximum) of the intrinsic tone tracheae. Additional graded relaxant responses to adenosine could always be obtained, and from results with non-blocking xanthines it was obvious that a decrease in tone (by the xanthines) itself did not influence the adenosine evaluations.

Only xanthines methylated in the 1-position consistently shifted the D/R curve to adenosine to the right and these produced concentration-related shifts, without any decrease in maximum adenosine

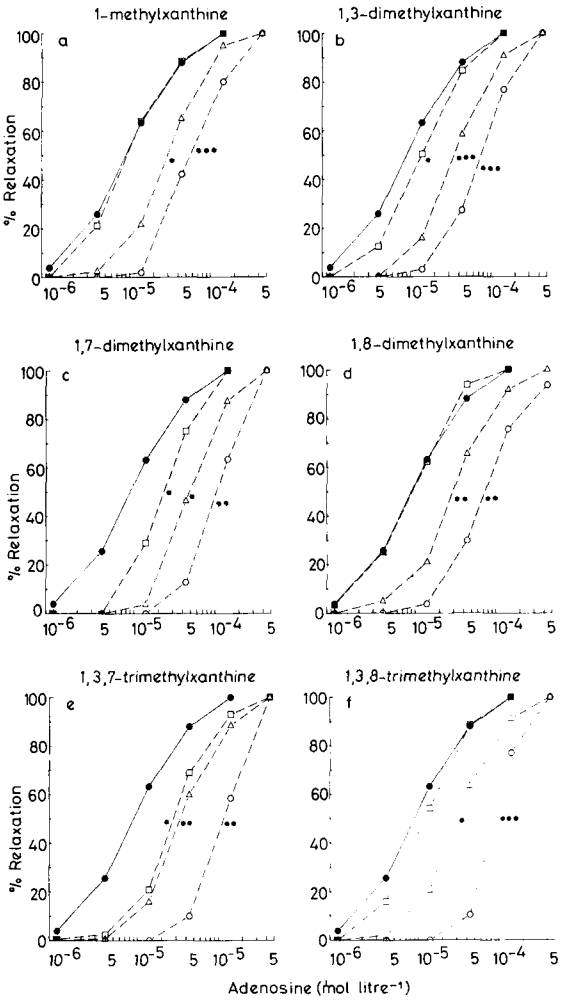


FIG. 1. Antagonism by methylxanthines of adenosine-induced relaxation of tracheal rings. Dose-response (D/R) lines to adenosine in the absence of methylxanthines shown in a–f (solid lines, ●) are mean results from 47 preparations. D/R lines to adenosine in the presence of different concentrations of methylxanthines, shown in a–f (broken line, open symbols), are mean results from 4 to 10 preparations. Adenosine effects were always studied in the presence of 2 μmol litre^{-1} dipyridamole. Statistical differences (Student's *t*-test) between mean EC50 values for adenosine in the absence and presence of methylxanthine are denoted by: $P < 0{\cdot}05$, *; $P < 0{\cdot}01$, **; $P < 0{\cdot}001$, ***. Doses in μmol litre^{-1}: a: 1-methylxanthine; 15 (□), 50 (△), 150 (○); b: 1,3-dimethylxanthine, 10 (□), 30 (△), 60 (○); c: 1,7-dimethylxanthine 15 (□), 50 (△), 150 (○); d: 1,8-dimethylxanthine, 15 (□), 50 (△), 150 (○); e: 1,3,7-trimethylxanthine, 30 (□), 90 (△), 300 (○); f: 1,3,8-trimethylxanthine, 8(□), 23 (△), 69 (○).

relaxation (Fig. 1). This antagonism was not much affected by methylation in the 3-,7- and 8-positions or combinations thereof (Table 1). However, xanthine and 9-methylated xanthines were always inactive (Fig. 2). Schild plots of the active compounds indicated that the antagonism was competitive, the slopes not differing significantly from 1 ($P > 0{\cdot}05$, Table 1) (see Arunlakshana & Schild 1959). The low theobromine (3,7-dimethylxanthine) concentration (100 μmol litre^{-1}) produced a slight, but significant, increase in the adenosine EC50 value ($P < 0{\cdot}01$), whereas the higher concentration (150 μmol litre^{-1}) was inactive in this respect. 3,8-Dimethylxanthine (100 μmol litre^{-1}) also showed a weak, but significant, ($P < 0{\cdot}05$) antagonism (Fig. 2).

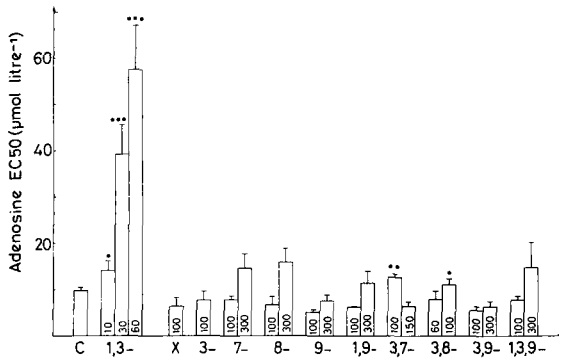


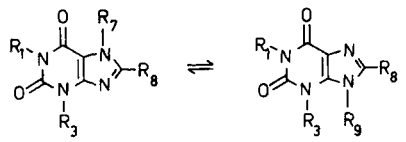
FIG. 2. Adenosine EC50 values in tracheal preparations in the absence or presence of 'non-blocking' xanthines. For comparison the values obtained with theophylline are included. Positions of methyl groups in the tested compounds are shown below concentration bars; xanthine is indicated by X. Concentrations of compounds (μmol litre^{-1}) are shown within bars. Adenosine control (C) EC50 value (± s.e.m.) is the mean of 47 preparations. Each methylxanthine concentration was studied in 3 to 10 preparations. Adenosine EC50 values in the presence of the xanthines were compared statistically (Student's *t*-test) only with mean control values from the same preparations. Significant differences are denoted by $P < 0{\cdot}05$, *; $P < 0{\cdot}01$, **.

The presence of adenosine (10 μmol litre^{-1}) in the bath attenuated the theophylline-induced relaxation. The EC50 value of theophylline was increased about twice (n = 4). Addition of adenosine to markedly methylxanthine-relaxed preparations often produced a transient, slight contraction which did not impede evaluation of adenosine antagonism.

DISCUSSION

The bronchorelaxant effect of theophylline is considered to be the major therapeutic action and xanthines are effective relaxants irrespective of which mediator has contracted the bronchi (unpublished work by Karlsson, Persson and Sonmark). The degree of mediator-induced contraction will determine both the potency and efficacy of theophylline as a tracheal relaxant in vitro (Karlsson & Persson 1981a). A high sensitivity was desirable in the present study because many xanthines are poorly soluble in relation to their potency. Thus intrinsic tone tracheae or a low concentration of contractile agent were employed.

Xanthine itself and 9-methylsubstituted xanthine derivatives produced only weak relaxations. Derivatives with substitutions at other positions of xanthine were effective relaxants. In summary, the 3-position, then the 1-position, was the most important for bronchodilator potency. Excepting the monosubstituted compounds, substitution in the 8-position yielded more potent xanthines than substitution in the 7-position. In line with these observations 1,3,8-trimethylxanthine was the most potent bronchorelaxant of the methylxanthines. The activity disappears with substitution in the 9-position. With substitution in the 9-position the double bond is moved to 7-8 from the otherwise preferred location. of 8-9 (see chemical structure).



Chemical structure of the two tautomeric forms of xanthine ($R_1 = R_3 = R_7 = R_8 = R_9 = H$). The different compounds were obtained by substituting with methyl groups at the positions denoted by R.

The structure-activity findings are largely in agreement with results with xanthine derivatives substituted with groups larger than methyl (Kjellin & Persson 1980; Persson et al 1981; and unpublished observations).

In agreement with the report by Farmer & Farrar (1976), the effect of adenosine on intrinsic tone tracheae in the present study was characterized as relaxation. The preparations moderately contracted by carbachol behaved similarly. On the other hand, a slight adenosine-induced contraction has been reported to occur occasionally in tracheal preparations, although the consistent response was relaxation (Fredholm et al 1979; Persson et al 1982; Coleman 1980). In the present study, contraction was infrequent and experiments with tetrodotoxin suggested it to be a smooth muscle effect.

The degree of relaxation by adenosine was much dependent on the degree of contraction of the

muscle. In the carbachol-contracted preparations the induced relaxations reached only 20% of the maximum methylxanthine-induced effect (compared with 60% in intrinsic tone preparations).

Low and therapeutic concentrations of theophylline produce a surmountable antagonism at adenosine receptors (Rall 1980). This mechanism has been suggested to reflect its bronchodilator effect (Marquardt et al 1978; Fredholm et al 1979; Welton & Simko 1980). In guinea-pig tracheae Fredholm et al (1979) observed that adenosine produced a rightward shift of the D/R curve to theophylline. Such an interaction was shown in the present study and could be expected because in a preparation relaxed by adenosine, theophylline would produce dual opposing actions: a contraction through antagonism of adenosine and a relaxation through direct smooth muscle effects. The findings by Fredholm et al (1979), therefore, may not be interpreted as a mechanism explaining the bronchodilator effects of xanthines. Nor is it likely that adenosine contributes to the muscle tone of the isolated tracheae because addition of an adenosine catabolizing enzyme (deaminase) or blocking the cellular uptake of adenosine (dipyridamole, cf. Coleman 1976) was not associated with any change in tension. It seems that significant tracheal relaxant effects of xanthines are produced by mechanisms unrelated to an interaction with adenosine.

It can be argued that adenosine receptors mediating relaxation of bronchial smooth muscle should not be blocked, because at the concentrations reported to occur for instance in the hypoxic lung (Mentzer et al 1975), adenosine may be active as a relaxant autacoid (cf. Persson et al 1981). It is thus of both theoretical and practical interest that a potent bronchodilator xanthine (3-propylxanthine, enprofylline) has been shown to be practically devoid of antagonist activity against inhibitory effects of adenosine in smooth muscle (trachea) and nervous tissue (myenteric plexus) (Persson et al 1981, 1982). The present findings, in agreement with results obtained with enprofylline, indicate those positions of the xanthine molecule that are critical for retaining and loosing, respectively, an adenosine antagonist activity in airway smooth muscle. The general finding was that the various 1-methylxanthines were potent adenosine antagonists. However, as with the bronchodilator effect, adenosine antagonist activity was lost by substitution in the 9-position. The abolition of antagonist (and relaxant) activity by alkylating the 9-position is remarkable considering that this is the position where the purine ring is substituted in adenosine. There was no consistent result suggesting that substitution at any other position could compensate for a lack of methyl in the 1-position (cf. Fig. 2). Among the active 1-methylxanthines, there was only about a 3-fold potency difference, the most potent being 1,3,8-tri- and 1,8-dimethylxanthine and the least, 1-methylxanthine. The results suggest that in addition to methyl in the 1-position, substitutions in 3- and/or 8-positions may be favourable for adenosine antagonist potency.

The slopes of the linear Schild plot regressions for all the active antagonists were not different from 1, indicating competitive antagonism at the adenosine receptors. Despite the relatively large spread in values this result is at apparent variance with the work of Coleman (1980) who reported significant adenosine antagonism by caffeine and theophylline in guinea-pig tracheal smooth muscle but their slope values were significantly less than 1. Confirming the results by Coleman (1976) it was observed that dipyridamole 2 μmol litre^{-1} produced a 50-fold leftward shift in the dose response curve to adenosine. This high sensitivity may be a necessary prerequisite to show the antagonism by xanthines. In the absence of dipyridamole there seems to be no antagonism by theophylline and caffeine of the tracheal relaxant effects of adenosine (Jones et al 1980; unpublished work).

Little work has been published on structure-activity of adenosine antagonists compared with the extensive studies of adenosine receptor stimulants (cf. Baer & Drummond 1979). Scholtholt et al (1972) studied inhibitory effects of a fixed dose of different xanthine derivatives on the coronary vasodilator effect of dipyridamole. Accepting that the effect of dipyridamole reflected adenosine receptor stimulant actions, this qualitative study was the first to hint at the importance of substitution at the 1-position for adenosine blocking activity with xanthines. In apparent contrast to later work (Green & Stanberry 1977; Karlsson & Persson 1981b), Huang et al (1972) reported that 1-methylxanthine did not antagonize neuronal effects (increase in cAMP) of adenosine. When the present study was under way (cf. Karlsson & Persson 1981b) a report by Bruns (1981) appeared, showing many features of structure-activity relationships for adenosine antagonism by xanthine derivatives. Bruns, who studied adenosine antagonism by measuring the effect on fibroblast cyclic AMP concentrations, also indicated the importance of the 1-position, but among the joint 1-methylxanthines studied, the order of antagonist

potencies differed much from our findings in the trachea. We have also observed that different blocking 1-methylated xanthines are not similarly active in tracheal smooth muscle and in a myenteric plexus preparation (Karlsson & Persson 1981b, and unpublished work) which suggests that blocking xanthines may not be equally active at all adenosine receptors.

In conclusion, we have found that antagonism of the effect of adenosine on the guinea-pig trachea is unrelated to the tracheal relaxant action of xanthine derivatives. The potential value of a xanthine lacking in adenosine antagonism is illustrated by enprofylline which shares with the 1H-methylxanthines only a low ability to antagonize the inhibitory effects of adenosine in tracheal smooth muscle and enteric nervous tissue (Persson et al 1981). This 'non-blocking', 3-propylxanthine derivative is a potent bronchodilator in animals and man but seems to be devoid of theophylline-like diuretic and c.n.s.-stimulant behavioural effects (Persson & Kjellin 1981; Persson et al 1981; Persson & Erjefält 1982; Lunell et al 1982).

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