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# Mechanism of xanthine-induced relaxation of guinea-pig isolated trachealis muscle

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**1** Four 3-alkylxanthines (3-methylxanthine, 3-n-propylxanthine (enprofylline), 3-n-butylxanthine and 3-iso-butylxanthine) and four 1-methyl-3-alkylxanthines (1-methyl-3-methylxanthine (theophylline), 1-methyl-3-n-propylxanthine, 1-methyl-3-n-butylxanthine and 1-methyl-3-iso-butylxanthine (IBMX)), were compared in terms of cyclic AMP phosphodiesterase (PDE) inhibition and trachealis muscle relaxation. The relationship between xanthine structure and cyclic AMP PDE inhibition was also studied.

**2** Xanthine induced relaxation of guinea-pig isolated trachealis muscle was measured against spontaneous tone.

**3** The four 1-methyl-3-alkylxanthines were each significantly more potent than the corresponding 3-alkylxanthines in relaxing the isolated trachealis muscle. The 1-methyl-3-alkylxanthines were similarly more potent than the corresponding 3-alkyl derivatives in inhibiting low $K_m$ cyclic AMP PDE. There was a strong positive correlation between low $K_m$ cyclic AMP PDE inhibition and the tracheal smooth muscle relaxation evoked by the xanthine derivatives.

**4** Since methylation of the 1-position of each 3-alkylxanthine increased the potency of the derivative in inhibiting low $K_m$ cyclic AMP PDE and in relaxing trachealis muscle and since a strong positive correlation was observed between the relaxant $EC_{50}$ and the $K_i$ value of each xanthine derivative, it is suggested that low $K_m$ cyclic AMP PDE inhibition by xanthines plays an important role in their tracheal relaxant effect.

## Introduction

Xanthines such as theophylline are effective bronchodilators and various studies have been performed to elucidate their mechanism of action. Katsuki & Murad (1977) and Polson *et al.* (1982) studied the cyclic nucleotide phosphodiesterase (PDE) inhibitory effect of xanthine agents. Persson *et al.* (1982) and Fredholm & Persson (1982) examined the adenosine-antagonizing effect of xanthine agents. Peach (1972) studied the use of xanthine agents to control intracellular calcium. Farmer & Chick (1967) and Stubbs *et al.* (1984) observed the effect of xanthine agents on the release of catecholamines from the adrenal medulla. Horrobin *et al.* (1977) studied inhibition induced by xanthine of prostaglandin synthesis in vascular smooth muscle.

However, the pharmacological actions of xanthine agents have not been fully clarified by any of these studies for the following three reasons: (1) In the first three studies, the concentration of the xanthine inhibiting PDE or antagonizing adenosine did not correlate with the concentration effective in causing relaxation. (2) Xanthine agents are strong smooth muscle relaxants even where the maintenance of muscle tone is independent of prostaglandin synthesis. (3) Whether or not xanthine agents directly influence the release of catecholamines from the adrenal medulla remains uncertain. It has recently been reported that bronchodilators such as agonists at $\beta$-adrenoceptors, xanthine agents and dibutyryl cyclic AMP induce hyperpolarization of the cell membranes in guinea-pig isolated trachealis muscle with a muscle relaxant effect (Honda *et al.*, 1986). $\beta$-Adrenoceptor stimulants and xanthine agents tested in combination acted to relax the isolated trachealis muscle in guinea-pigs (Persson & Gustafsson, 1986). Also recently, a significant relationship was found between PDE inhibition and trachealis muscle relaxation although there was no obvious correlation between the increase in intracellular cyclic nucleotides and trachealis muscle relaxation (Bryson & Rodger, 1987).

Therefore, the cyclic nucleotide PDE system is once again receiving attention. We compared the cyclic AMP PDE inhibitory effect of various xanthine derivatives with the myorelaxant effect of these derivatives on tracheal preparations obtained from

**Table 1** $K_i$ values for cyclic AMP phosphodiesterase and $-\log EC_{50}$ values for the relaxant effects of various xanthine derivatives on guinea-pig isolated trachealis muscle ($n = 6$)

| *Xanthine derivative* | $K_i$ *value* (μM) *Low* $K_m$ cyclic AMP 0.1 ~ 0.2 (μM) | $-log\ EC_{50}$ (M) *Spontaneous tone* |
|---|---|---|
| 3-Alkylxanthine | | |
| 3-Methylxanthine | 122 ± 2 | 3.85 ± 0.05 |
| 3-n-Propylxanthine (Enprofylline) | 42 ± 1 | 5.14 ± 0.25 |
| 3-n-Butylxanthine | 32 ± 2 | 5.08 ± 0.11 |
| 3-iso-Butylxanthine | 37 ± 2 | 4.92 ± 0.10 |
| 1-Methyl-3-alkylxanthine | | |
| Theophylline | 56 ± 2* | 4.55 ± 0.13* |
| 1-Methyl-3-n-propylxanthine | 1.9 ± 0.2* | 6.01 ± 0.05* |
| 1-Methyl-3-n-butylxanthine | 1.2 ± 0.3* | 5.87 ± 0.11* |
| IBMX | 5.0 ± 0.5* | 6.05 ± 0.14* |

Low $K_m$ cyclic AMP PDE: $K_m = 0.61 \pm 0.04\ \mu M$, $V_{max} = 49.5 \pm 5\ pmol\ mg^{-1}\ min^{-1}$.
$K_i$ values were determined by the method of Dixon (1953), and the $EC_{50}$ values were calculated from log · probit plots of the individual relaxation responses.
* Significantly different ($P < 0.01$) from each 3-alkylxanthine corresponding to 1-methyl-3-alkylxanthine.
All data are presented as mean ± s.e.mean.

guinea-pigs, in order to clarify how the trachealis muscle relaxant effect of xanthine agents correlates with PDE inhibition. We also investigated the changes in tracheal relaxant potency and PDE inhibitory potency which resulted from methylation of the 1-position of 3-alkylxanthines.

## Methods

### *Tissue preparation and trachealis muscle relaxation*

Male Hartley guinea-pigs weighing 200–400 g were stunned and bled, and the trachea was removed. One tracheal ring was cut from the pharyngeal end of each trachea. The ring was opened by cutting through the cartilaginous region diametrically opposite the trachealis muscle, and the ends of the cartilaginous region were ligated. The preparation was placed in an organ bath of 1 ml volume. Tension was measured with an isometric transducer (Minebea Co., Ltd, UL 10GR), and tension changes were recorded with a pen recorder (Matsushita Communication Industrial Co., Ltd, VP-6621A). Using the method of Baba *et al.* (1985), the preparation was perfused at a constant flow of 1.5 ml $min^{-1}$ at 37°C in Krebs solution of the following composition (mM): NaCl 137, $KHCO_3$ 5.9, $CaCl_2$ 2.4, $MgCl_2$ 1.2, and glucose 11.8. Isoprenaline ($2 \times 10^{-5}$ M) was added to produce complete relaxation, then tension of about 0.5 g was applied to the preparation. The preparation was again perfused at 37°C in Krebs solution and spontaneous tone was allowed to develop. After the tension had stabilized, the preparation was then perfused for 20 min in the presence of a xanthine derivative at various concentrations. Relaxation occurring on exposure of the tissue to a $Ca^{2+}$-free medium containing 0.01 mM EGTA was defined as 100%, and the relaxation caused by each xanthine was calculated in terms of this standard.

### *Cyclic AMP phosphodiesterase assay*

Cyclic AMP PDE activity was measured by a method similar to that devised by Thompson & Appleman (1971). Trachealis muscle was homogenized in Tris HCl buffer (40 mM Tris HCl, 10 mM $MgCl_2$ and 4 mM 2-mercaptoethanol). The homogenate was cooled and centrifuged at 10,000 *g* in a refrigerated centrifuge. The supernatant fluid was collected. A mixture consisting of 50 μl of the test xanthine solution or vehicle solution, 25 μl of cyclic AMP ($1 \times 10^{-7}$–$4 \times 10^{-5}$ M) and 25 μl of [$^3$H]-cyclic AMP (1 mCi $ml^{-1}$) was placed in a glass test tube incubated at 30°C for 5 min; 100 μl of the supernatant solution from the tissue homogenate was added and the mixture was incubated at 30°C for 10 min. The reaction mixture was placed in boiling water for 1 min to stop the reaction. After the mixture had been cooled with water, 50 μl of *Crotalus atrox* snake venom solution was added and the mixture was incubated at 30°C for 10 min. Following that, an ion exchange resin (Dowex 1-X2) was added, and the mixture was allowed to stand for 15 min. The mixture was centrifuged at 3,000 r.p.m. for 5 min in a bench centrifuge. The supernatant solution was collected. The radioactivity in 0.5 ml of the supernatant was measured with a liquid scintillation counter. The $K_m$ value and the $V_{max}$ value were obtained from Lineweaver-Burk plots. Since diphasic

graphs are observed for guinea-pig trachealis muscle, a high $K_m$ enzyme value and a low $K_m$ enzyme value can be obtained. The $K_i$ value was obtained by the method of Dixon (1953).

Drugs used were isoprenaline (Sigma), 3-methylxanthine (Sigma), theophylline (Sigma), 1-methyl-3-iso-butylxanthine (IBMX, Sigma), 3-n-propylxanthine, 3-n-butylxanthine, 3-iso-butylxanthine, 1-methyl-3-n-propylxanthine and 1-methyl-3-n-butylxanthine (synthesized in our laboratories), EGTA (Sigma), cyclic AMP (Yamasa), [$^3$H]-cyclic AMP (Yamasa), Tris HCl (Sigma), 2-mercaptoethanol (Sigma), Dowex 1-X2 (Dow Chemical), *Crotalus atrox* snake venom (Sigma).

*Statistical analysis*

All the results are expressed as the mean ± s.e.mean, and the *t* test without correspondence was applied. *P* values less than or equal to 0.05 were considered to indicate a significant difference between means.

## Results

*Relaxation by xanthine derivatives*

Table 1 shows the $-\log EC_{50}$ values for the trachealis muscle in the condition of spontaneous tone while Figure 1 shows the concentration-response curves for 1-methyl-3-alkylxanthines and the corresponding 3-alkylxanthines for trachealis in spontaneous tone. These results reveal that relaxant potency of xanthine derivatives is increased both when a 3-methyl substituent is replaced by a longer 3-alkyl chain and when a 3-alkyl derivative is additionally methylated in the 1-position.

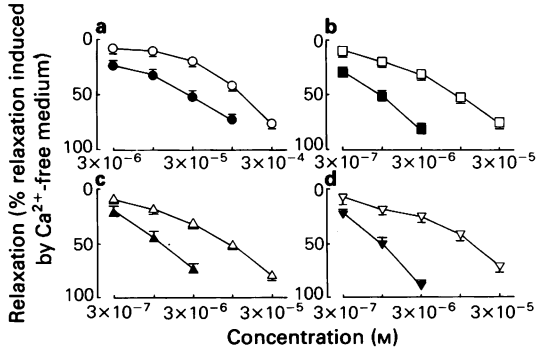


**Figure 1** Log concentration-relaxation curves of the 3-alkyl xanthines and the 1-methyl-3-alkyl xanthines: (a) (○) 3-methylxanthine and (●) theophylline; (b) (□) 3-n-propylxanthine (enprofylline) and (■) 1-methyl-3-n-propylxanthine; (c) (△) 3-n-butylxanthine and (▲) 1-methyl-3-n-butylxanthine; (d) (▽) 3-iso-butylxanthine and (▼) 1-methyl-3-iso-butylxanthine. Each curve was derived from experiments on tracheal smooth muscle specimens from six guinea-pigs. Data points represent means with s.e.mean shown by vertical bars.

*Cyclic AMP phosphodiesterase activity*

For guinea-pig tracheal smooth muscle, low (high affinity) and high (low affinity) $K_m$ cyclic AMP PDE activities were observed. Examination of the $K_i$ values of individual xanthine derivatives revealed that for the low $K_m$ enzyme, inhibitory potency of the xanthines is increased both when a 3-methyl substituent is replaced by a longer 3-alkyl chain and when a 3-alkyl derivative is additionally methylated in the 1-position (Table 1).

## Discussion

Studies by Persson *et al.* (1982) revealed that alkyl-substitution in the 1-position of the xanthine nucleus yielded a xanthine with adenosine antagonist activity. This observation stimulated active discussion on the relationship between the bronchodilator action and adenosine-antagonizing action of xanthines. However, theophylline and related substituted methylxanthines such as IBMX, 8-phenyltheophylline, caffeine, and theobromine are effective inhibitors of adenosine at $A_1$- and $A_2$-purinoceptors in concentrations between 20 and 100 times lower than those required to inhibit phosphodiesterase (Fredholm & Persson, 1982). Furthermore, enprofylline, which has been shown to be almost entirely free of antagonism of the functional and biochemical effects of adenosine, has a greater bronchodilator potency than some xanthines alkylated in the 1-position (Lunell *et al.*, 1982., Persson, 1983). We have therefore come to believe that the adenosine-antagonizing action of xanthine does not represent the main mechanism of its bronchodilator effect.

A number of studies have also been conducted on the mechanism by which the PDE inhibitory action of xanthine and the related increase in intracellular cyclic AMP concentration may lead to the relaxation of trachealis muscle. Doubt has been cast on the operation of this mechanism by observation of a difference between the effective blood concentration of theophylline and its PDE inhibitory activity in the isolated airway (Polson *et al.*, 1978; Bergstrand, 1980). Furthermore, drugs such as papaverine and dipyridamole, which are strong PDE inhibitors, are not effective as bronchodilators *in vivo* (Ruffin & Newhouse, 1981).

However, in consideration of a recent finding that there was a supra-additive interaction between terbutaline and both theophylline and enprofylline, which are PDE inhibitors (Persson & Gustafsson,

1986), and also of a recent report that PDE inhibitors such as papaverine, IBMX, SKF 94120, etc., showed a strong relaxant effect on guinea-pig isolated trachealis muscle (Bryson & Rodger, 1987), a strong correlation seems to be suggested between the PDE inhibitory action and the bronchodilator action of xanthine. Furthermore, a significant correlation has been observed between the *in vitro* PDE inhibitory activity and the bronchodilator potency of xanthine (Newman *et al.*, 1978; Polson *et al.*, 1982).

We conducted experiments with 2 main objectives: one was to look for a possible structure-activity relationship by comparing the 3-alkylxanthine derivatives with their corresponding 1-methyl-3-alkylxanthine analogues in terms of their cyclic AMP PDE inhibitory action and their relaxant effect on guinea-pig isolated trachealis. The 1-methyl-3-alkyl derivatives were more potent in inhibiting high affinity cyclic AMP PDE than their 3-alkyl analogues and similar potency differences were obtained for relaxation of trachealis muscle. Furthermore, in both groups of analogues, an increase in the size of the 3-alkyl substituent from methyl to n-butyl increased potency as regards both enzyme inhibition and tracheal relaxation. A structure-activity relationship thus became obvious; that is, 1-methylation and a longer 3-alkyl chain not only elevate high affinity cyclic AMP PDE inhibitory potency, but also increase potency for relaxation of trachealis muscle. These findings usefully supplement the structure-activity relationship for the adenosine-antagonizing action of xanthines reported by Persson *et al.* (1982). There are reports that alkylation of the 1- and 3-position increases tracheal relaxant potency and that, within limits, an increase in the size of the 3-alkyl analogues also increases bronchodilator potency (Parker *et al.*, 1956; Persson *et al.* 1985; Takagi *et al.*, 1988). For tracheal smooth muscle, cyclic AMP PDE activity is of 2 types, i.e., low $K_m$ (high-affinity) and high $K_m$ (low-affinity). The tissue concentration of cyclic AMP is normally no greater than 1 $\mu$M. Therefore the high affinity enzyme of $K_m$; $0.61 \pm 0.04$ $\mu$M is considered to be the isozyme of greatest physiological importance.

For xanthine-induced inhibition of the low $K_m$ enzyme, we found a clear, strong correlation with the relaxant effect on trachealis muscle ($r = 0.916$, $P < 0.01$) (Figure 2). This was the other main objective of our experiments. Polson *et al.* (1982) examined cyclic AMP PDE activity in canine trachea and detected high-affinity cyclic AMP PDE activity (peak V), the $K_m$ of which was $0.63 \pm 0.09$ $\mu$M. They reported that, for 6 different methylxanthines (1-methylxanthine, 3-methylxanthine, 7-methylxanthine, caffeine, theophylline and IBMX) a strong correlation existed between xanthine-induced inhibition of high-affinity cyclic AMP PDE and of isolated canine trachealis muscle contracted by means of methacholine. Despite the different animal species used, the results of the present study are therefore consistent with those of earlier studies.

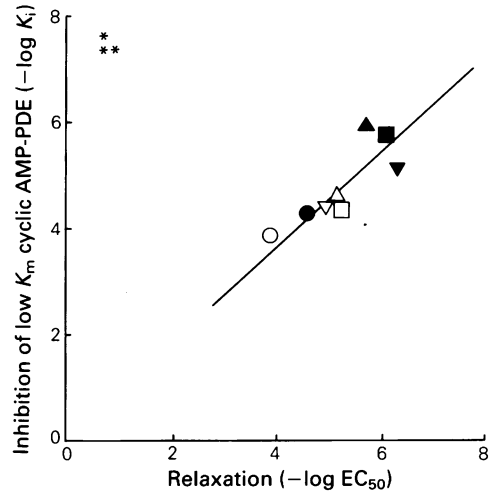


**Figure 2** Correlation between inhibition of low $K_m$ cyclic AMP phosphodiesterase and relaxation of guinea-pig isolated trachealis muscle. Data from Table 1: (○) 3-methylxanthine; (●) theophylline; (□) 3-n-propylxanthine (enprofylline); (■)1-methyl-3-n-propylxanthine; (△) 3-n-butylxanthine; (▲)1-methyl-3-n-butylxanthine; (▽) 3-iso-butylxanthine; (▼)1-methyl-3-iso-butylxanthine (IBMX). * $r = 0.916$, ** $P < 0.01$.

On the basis of all the above results, it can be concluded that in the guinea-pig the low $K_m$ cyclic AMP PDE inhibitory activity of xanthine derivatives is closely related to their tracheal relaxant activities *in vitro*.

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