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The relaxant and spasmogenic effects of some xanthine derivatives acting on guinea-pig isolated trachealis muscle

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1 Caffeine (10 mM)-induced relaxation of guinea-pig isolated trachealis was attenuated and converted to a small spasmogenic response on cooling to 22°C. The relaxant response was restored on rewarming to 37°C and was abolished by indomethacin (2.8 μM). Cooling to 22°C in the presence of indomethacin revealed spasmogenic responses to caffeine which were abolished on rewarming to 37°C.

2 Trachealis treated with indomethacin (2.8 μM) was repeatedly dosed with acetylcholine (ACh, 10 μM). Caffeine (1 or 10 mM), added as each ACh-induced spasm reached equilibrium, transiently augmented but then suppressed the spasm. On cooling from 37°C to 12°C, the increment in spasm evoked by caffeine increased relative to the spasm evoked by ACh.

3 Trachealis treated with indomethacin (2.8 μM) was repeatedly dosed with caffeine (10 mM). At 37°C caffeine had little effect but it caused spasm when the tissue was cooled to 32°C. Spasm amplitude increased as cooling progressed to 12°C. Similar results were obtained with caffeine (1 mM).

4 At 37°C, caffeine, enprofylline, 1,3,7,9-tetramethylxanthinium (TMX), theobromine, theophylline, xanthine and forskolin each caused concentration-dependent suppression of tracheal tone. Among the xanthine derivatives the rank order of potency was enprofylline > theophylline > caffeine > theobromine > xanthine > TMX.

5 In trachealis treated with indomethacin (2.8 μM) and maintained at 12°C, the xanthines each caused concentration-dependent spasm. The rank order of potency was theobromine $\geqslant$ theophylline $\geqslant$ caffeine $\geqslant$ enprofylline > xanthine > TMX. Forskolin was devoid of spasmogenic activity.

6 Trachealis treated with indomethacin (2.8 μM) and maintained at 12°C was repeatedly dosed with either caffeine (10 mM) or potassium chloride (KCl, 40 mM). Caffeine-induced spasm was attenuated in a Ca^{2+}-free medium containing EGTA (2 mM), modestly at first but subsequently more profoundly. KCl did not evoke spasm at 12°C but at 37°C the KCl-induced spasm was virtually abolished at its first trial in the Ca^{2+}-free, EGTA-containing medium.

7 It is concluded that caffeine, other alkylated xanthines and xanthine itself share a spasmogenic action in guinea-pig isolated trachealis which is best observed when the tissue is treated with indomethacin (2.8 μM) and maintained at 12°C. The spasmogenic action represents the release of Ca^{2+} from intracellular sites of sequestration and may not depend on the intracellular accumulation of cyclic AMP. The rank order of spasmogenic potency of the xanthine derivatives differs markedly from their rank order of potency in suppressing the spontaneous tone of the trachealis observed at 37°C. Since, at 12°C, TMX is spasmogenic at concentrations identical to those causing relaxation at 37°C, it is likely that TMX penetrates the cell. The relaxant effects of TMX do not, therefore, indicate that methylxanthine-induced relaxation is mediated by a receptor located on the external surface of the cell.

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Introduction

It has been recognised for many years that methylxanthines such as caffeine and theophylline can cause relaxation of airways smooth muscle *in vitro* and it is commonly assumed that this action stems from the ability of the methylxanthines to inhibit the activity of intracellular cyclic AMP-phosphodiesterase. However, the ability of a quaternized xanthine derivative (1,3,7,9-tetramethylxanthinium; TMX) to evoke relaxation of isolated airways smooth muscle led Persson (1985a,b) to suggest that the methylxanthines might evoke relaxation by activating a receptor site located on the external surface of the cell membrane.

Caffeine is a methylxanthine which is currently enjoying widespread (e.g. Klockner & Isenburg, 1985; Meisheri *et al.*, 1985; Benham & Bolton, 1986) use as a pharmacological agent which acts to release Ca^{2+} from its intracellular storage sites within smooth muscle. By liberating Ca^{2+} from internal stores, caffeine can activate the intracellular contractile machinery. Hence caffeine can cause spasm of many types of smooth muscle including that of the airways (Ito & Itoh, 1984a,b).

In the present experiments we have examined the relaxant and spasmogenic effects of a variety of xanthine derivatives in guinea-pig isolated trachealis. The experiments were designed to check the validity of the suggestion that the relaxant effects of methylxanthines are mediated by a receptor located on the external surface of the cell (Persson, 1985a,b) and to shed further light on the spasmogenic activity of caffeine. In the latter respect it was our intention to examine the mechanism of the spasmogenic action of caffeine, whether spasmogenic activity was shared by other xanthine derivatives and, if so, whether the spasmogenic action was observed at concentrations similar to those producing relaxation. Results of some of the present experiments have been communicated to the British Pharmacological Society (Boyle *et al.*, 1988).

Methods

Guinea-pigs (350–700 g) of either sex were killed by stunning and bleeding. Tracheae were excised, cleaned of adhering adipose and connective tissue and opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis. Small segments of trachea were set up for the isometric recording of tension changes as previously described (Foster *et al.*, 1983).

(A) *The tracheal response to caffeine (10 mM): influence of tissue tone and temperature*

Segments of trachea were set up in Krebs solution maintained at 37°C and were dosed with caffeine (10 mM) at intervals throughout the experiment. Each dose of caffeine was allowed 15 min tissue contact before washout. The tissue was washed several times over a 30 min period before administering the next dose.

(B) *The tracheal response to caffeine (1–10 mM) as observed in the presence of indomethacin and acetylcholine: the influence of temperature*

Tracheal segments were set up at 37°C and allowed to equilibrate for 1 h. Indomethacin (2.8 μM) was then added to the Krebs solution and tissue tone was allowed to dissipate (30–40 min). ACh (10 μM) was administered and, when the resulting spasm was approaching equilibrium, caffeine (10 mM) was added. When tissue tone subsequently returned to the pre-ACh value both the caffeine and the ACh were washed from the tissue. Bath temperature was lowered in 5C° steps to 7°C. At each temperature step the tissue was exposed to ACh and then caffeine as described above.

(C) *The spasmogenic response to caffeine: influence of temperature*

Tracheal segments were set up in indomethacin-containing Krebs solution as described in (B) and then dosed repeatedly with caffeine (1 or 10 mM) essentially as described in (A) above. The initial caffeine dose was administered at a temperature of 37°C but thereafter the tissue was cooled progressively in 5C° steps to a temperature of 7°C. A single caffeine dose was administered at each temperature step.

(D) *Concentration-response relationships for the relaxant actions of the xanthines and forskolin*

These experiments were carried out at 37°C. The ability of caffeine, enprofylline, TMX, theobromine, theophylline and xanthine to suppress the spontaneous tone of the trachealis muscle was examined by the construction of sequential log concentration-response curves. Starting at a value close to the threshold for activity, the concentration of each xanthine was raised in twofold steps (15 min contact) until a maximal response was obtained. Between successive doses of the xanthine the tissue was washed at 10 min intervals until tone was restored.

The concentration-response relationship for each xanthine was examined on a fresh tissue. However, each tissue was ultimately dosed with caffeine 4 mM so that the maximal response of all the xanthines could be related to that of caffeine. The concentration-relaxation relationship (at 37°C) for forskolin was studied in a fashion similar to that described for the xanthines.

In a separate series of experiments the relaxant action of forskolin was studied in trachealis treated with indomethacin (2.8 μM) and maintained at 12°C. Tissues were dosed repeatedly with ACh (1 mM). Forskolin was added as the spasm induced by each ACh dose reached equilibrium and was allowed 15 min tissue contact.

(E) *Concentration-response relationships for the spasmogenic actions of the xanthines and forskolin*

These experiments were carried out in Krebs solution containing 2.8 μM indomethacin and maintained at 12°C. Following an equilibration period of 1 h the spasmogenic actions of caffeine, enprofylline, TMX, theobromine, theophylline and xanthine were examined by the construction of sequential log concentration-response curves, essentially as described in (D) above. Forskolin was tested for spasmogenic activity in a fashion similar to that described for the xanthines.

(F) *Effects of a Ca^{2+}-free, EGTA-containing medium on spasmogenic responses to caffeine and KCl*

Segments of trachea were set up in Krebs solution containing indomethacin (2.8 μM) and maintained at 12° or 37°C. A series of four doses of caffeine (10 mM) or KCl (40 mM) was administered. Each dose of spasmogen was allowed 15 min tissue contact. The tissue was then washed several times over a 30 min period before administering the next dose. Following the initial dose, test tisses were exposed to Ca^{2+}-free Krebs solution containing indomethacin (2.8 μM) and EGTA (2 mM). The doses of caffeine or KCl were then repeated three times. Time-matched control tissues were treated similarly but were not exposed to the Ca^{2+}-free EGTA-containing medium.

Drugs and solutions statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. The following substances were used: acetylcholine chloride (Sigma), caffeine (Sigma), enprofylline (AB Draco/Astra), ethyleneglycol-*bis* (β-amino-ethylether)-NN'-tetra-acetic acid (EGTA, Sigma), forskolin (Sigma), indomethacin (Sigma), potassium chloride (Hopkins & Williams), theobromine (Sigma), theophylline (Sigma) and xanthine (Sigma). 1,3,7,9-Tetramethylxanthinium methyl sulphate was synthesized in the laboratories of Mundipharma AG, Basel, Switzerland. Its identity and purity (98% + 2% water) were checked by elemental analysis, nuclear magnetic resonance spectroscopy, and thin layer chromatography. Stock solutions of ACh, indomethacin and forskolin were prepared in absolute ethanol, those of other drugs in twice-distilled water. The Krebs solution used in all the experiments had the following composition (mM): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1. In some experiments the Krebs solution contained indomethacin (2.8 μM). This concentration of indomethacin profoundly inhibits the synthesis of prostglandins by guinea-pig lung tissue (Vane, 1971) and thereby suppresses tracheal tone (Farmer *et al.*, 1974). The significance of differences between means was assessed by use of either a one-tailed or a two-tailed unpaired *t* test.

Results

(A) *The tracheal response to 10 mM caffeine: influence of tissue tone and temperature*

These initial experiments were designed to reveal the experimental circumstances under which caffeine might cause spasm rather than relaxation of the trachealis. At 37°C the first 10 mM dose of caffeine caused relaxation equivalent to full suppression of the spontaneous tone of the tracheal segments. Tone was restored by washing. When bath temperature was reduced to 22°C some loss (50–80%) of spontaneous tone occurred. Caffeine again evoked full relaxation. Thereafter washing no longer led to tone restoration and two subsequent doses of caffeine each evoked small spasmogenic responses (Figure 1).

Following several washes and restoration of the bath temperature to 37°C, tissue tone rose towards its initial value. Caffeine again caused full relaxation. Following the caffeine washout, addition of indomethacin (2.8 μM) to the bath fluid caused loss of tissue tone which became complete after 30–40 min. Caffeine then caused no observable response. On lowering bath temperature to 22°C, 3 successive doses of caffeine each evoked small spasmogenic responses. The spasmogenic response to caffeine was abolished when the bath temperature was restored to 37°C.

(B) *The tracheal response to caffeine (1 and 10 mM) as observed in the presence of indomethacin and acetylcholine: the influence of temperature*

In these experiments ACh (10 μM) was used to

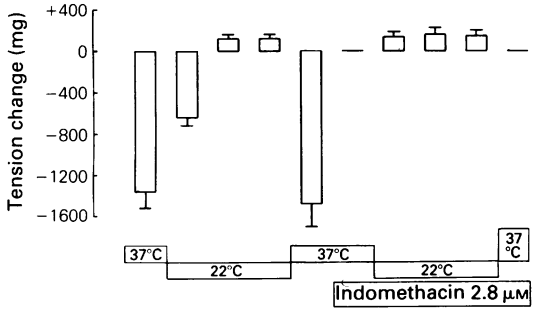

Figure 1 The influence of temperature and tissue tone on the response of guinea-pig isolated trachealis to 10 mM caffeine. The abscissa scale indicates consecutive (left to right) dosage with 10 mM caffeine. The ordinate scale indicates the tension change (+ = spasm, − = relaxation) in mg. Horizontal bars indicate the bath temperature and the presence of indomethacin (2.8 μM) in the bath fluid. Column height represents the mean of values from at least 6 tissues and vertical bars the s.e.mean.

restore tone in tissues in which spontaneous tone had been suppressed by indomethacin (2.8 μM). The principal objective of the experiments was to determine whether tone loss was a prerequisite for unmasking the spasmogenic action of caffeine.

ACh-induced spasm of trachealis treated with indomethacin reached equilibrium after approximately 3min. The addition of 10 mM caffeine at this time evoked a biphasic response (Figure 2). Initially caffeine caused a small increase in the ACh-induced spasm but this was followed by a secondary and complete suppression of the ACh-induced spasm. On cooling the tissue progressively to 12°C, it was observed that the size of the caffeine-induced tension rise increased relative to the ACh-induced spasm. As the tissue was cooled below 27°C this effect was accentuated by a progressive decrease in the amplitude of the ACh-induced spasm. The secondary suppression of ACh-induced spasm remained evident even at 12°C. Similar results were obtained when 1 mM caffeine was tested against the ACh standard.

(C) *The spasmogenic response to caffeine: influence of temperature*

Results obtained in (A) and (B) above clearly demonstrated that, compared with suppression of tone, cooling was a more important factor in unmasking the spasmogenic activity of caffeine. Accordingly, experiments were performed under conditions of zero tone to establish the optimal temperature for expression of the spasmogenic action of caffeine.

In the presence of indomethacin (2.8 μM), and at

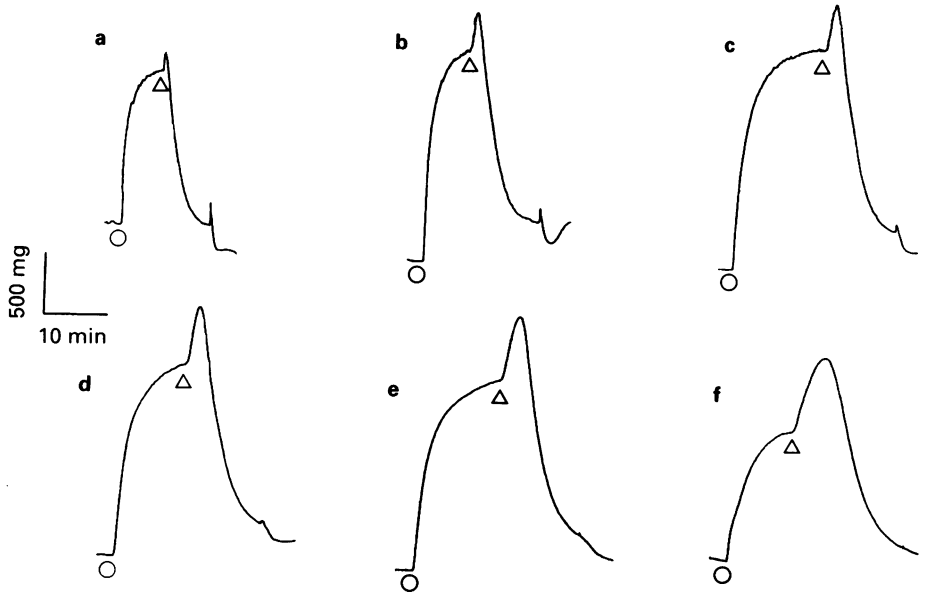

Figure 2 Guinea-pig trachealis treated with indomethacin (2.8 μM): the influence of cooling on the interaction between caffeine and acetylcholine (ACh). The recordings illustrate the mechanical activity of a single preparation of trachea at (a) 37, (b) 32, (c) 27, (d) 22, (e) 17 and (f) 12°C. (○) = application of 10 μM ACh; (△) = application of 10 mM caffeine. Indomethacin (2.8 μM) was present throughout. Note the initial promotion and subsequent suppression by caffeine of the ACh-induced spasm. Note also that cooling increases the size of the caffeine-induced spasm relative to that produced by ACh.

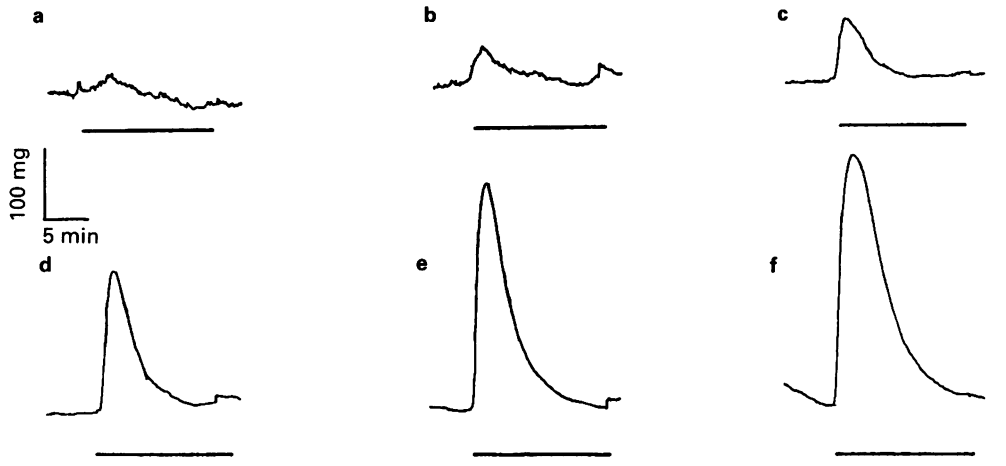

Figure 3 Guinea-pig isolated trachealis treated with indomethacin (2.8 μM): recordings illustrating the influence of temperature on the spasmogenic response evoked by dosing with caffeine (10 mM). The recordings illustrate the mechanical activity of a single preparation of trachea at (a) 37, (b) 32, (c) 27, (d) 22, (e) 17 and (f) 12°C. The horizontal bar under each tracing represents the application of 10 mM caffeine for 15 min. Indomethacin (2.8 μM) was present throughout. Note that cooling to 12°C increases the size of the spasm evoked by caffeine.

37°C, 10 mM caffeine evoked little observable response. At 32°C 10 mM caffeine evoked slight spasm. This peaked within the 15 min contact time and thereafter began to decline. At the end of the period of drug contact the developed tension was 10% or less of its peak value. As the tissue was cooled progressively to 12°C, the size of caffeine-induced spasm became greater (Figures 3 and 4). However, as the tissue was cooled, there was an increase in the rate at which the caffeine-induced tension developed and declined after reaching its peak value. The residual tension at the end of the drug contact time therefore altered very little with cooling. When bath temperature was reduced to 7°C, the spasm evoked by 10 mM caffeine was smaller than that observed at 12°C and the rate of decay of peak tension was also reduced.

The effects of cooling were similar in experiments where 1 mM caffeine was used. However, 1 mM caffeine did not evoke spasm until the tissue was cooled to 22°C and the size of the spasm continued to increase as temperature was reduced to 7°C (Figure 4). Again, peak tension was achieved within the 15 min contact time and thereafter tension began to decline. However, at all temperatures, the rate of tension decay was smaller than that observed for 10 mM caffeine.

(D) *Concentration-response relationships for the relaxant actions of xanthines and forskolin*

Log concentration-relaxation curves of xanthine derivatives were constructed in order to determine

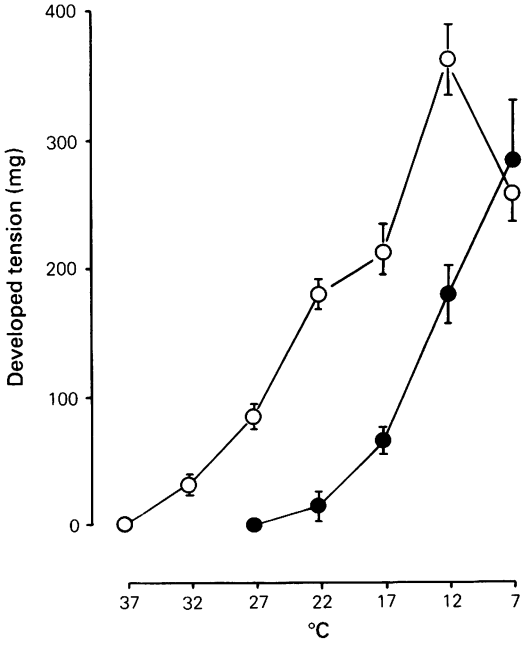

Figure 4 Guinea-pig isolated trachealis treated with indomethacin (2.8 μM): the influence of temperature on the spasmogenic response evoked by dosing with caffeine 1 (●) or 10 (○) mM. The abscissa scale indicates bath temperature on an arithmetic scale. The ordinate scale indicates tension developed in mg. Data represent the means of determinations from at least six tissues; s.e.mean shown by vertical bars.

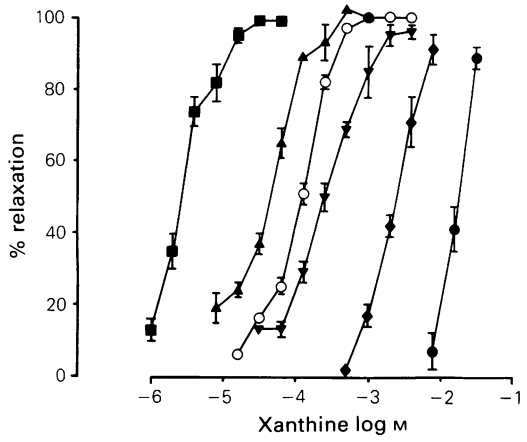

Figure 5 The relaxant effects of some xanthine derivatives tested against the spontaneous tone of guinea-pig isolated trachealis maintained in Krebs solution at 37°C. The abscissa scale represents the molar concentration of the xanthine derivative on a log scale. The ordinate scale represents relaxation expressed as a percentage of the relaxation induced by caffeine (10 mM). The illustrated log concentration-effect curves are for enprofylline (■), theophylline (▲), caffeine (○), theobromine (▼), xanthine (◆) and 1,3,7,9-tetramethylxanthinium (●). Data indicate means of values from at least 6 tissues; s.e.mean shown by vertical bars.

their relative order of relaxant potency. At 37°C, caffeine, enprofylline, TMX, theobromine, theophylline and xanthine each evoked concentration-dependent suppression of the spontaneous tone of the trachealis. The xanthines shared the same maximal effect (full suppression of spontaneous tone) and the slopes of their log concentration-effect curves were similar (Figure 5). pD_2 values for the relaxant actions of the xanthines are presented in Table 1 from which it is clear that the relative order of potency was enprofylline > theophylline > caffeine > theobromine > xanthine > TMX.

Forskolin (0.1 nM–1 μM) also produced concentration-dependent suppression of the spontaneous tone of the trachealis. The maximal effect of forskolin was equivalent to that of caffeine (Figure 6) and the pD_2 value for the relaxant action of forskolin was 7.60 ± 0.05 (mean ± s.e.mean; $n = 11$).

In tissues treated with indomethacin (2.8 μM) and maintained at 12°C, forskolin (10–100 μM) was able partially to suppress the spasm evoked by ACh (1 mM). Expressed as a percentage of the relaxation evoked by caffeine (10 μM), forskolin 10 and 100 μM evoked relaxation of 13 ± 3% and 31 ± 4% (mean ± s.e., $n = 7$) respectively.

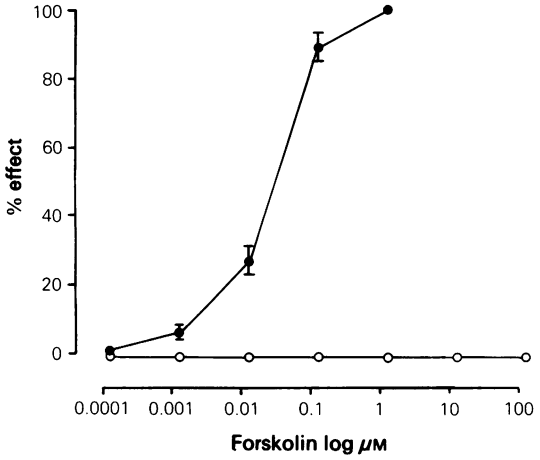

Figure 6 Effects of forskolin in guinea-pig isolated trachealis maintained in Krebs solution at 37°C (●) or maintained at 12°C in Krebs solution containing 2.8 μM indomethacin (○). The abscissa scale represents the concentration (μM) of forskolin on a log scale. The ordinate scale represents the relaxation (●) or the spasm (○) each expressed as a percentage respectively of the relaxation or spasm evoked by caffeine (10 mM). Data indicate means of values from at least 6 tissues; s.e.mean shown by vertical bars.

Table 1 pD_2 values for some xanthines acting as relaxants or spasmogenic agents in guinea-pig trachealis

	Suppression of spontaneous tracheal tone at 37°C	*Spasm evoked in presence of (2.8 μM) indomethacin at 12°C*
Caffeine	3.91 ± 0.03 (6)	3.30 ± 0.05 (6)
Enprofylline	5.59 ± 0.02 (6)	3.20 ± 0.05 (12)
Theobromine	3.59 ± 0.05 (6)	3.51 ± 0.03 (15)
Theophylline	4.36 ± 0.03 (8)	3.42 ± 0.06 (6)
Xanthine	2.64 ± 0.05 (6)	2.33 ± 0.03 (6)
TMX	1.75 ± 0.03 (6)	<2.0 (6)

Values represent mean ± s.e.mean. Figures in parentheses = no. of observations.
TMX = 1,3,7,9-tetramethylxanthinium.

(E) *Concentration-response relationships in tests of the spasmogenic properties of xanthines and forskolin*

These experiments were performed to determine whether the spasmogenic activity of caffeine was shared by other xanthine derivatives and to compare the relative order of spasmogenic potency of the xanthines with their relative order of relaxant potency.

Caffeine had spasmogenic effects in tissue treated with indomethacin (2.8 μM) and maintained at 12°C. The threshold concentration was 125 μM. Over the concentration range 125 μM–1 mM, the concentration-dependent spasm induced by caffeine developed slowly and reached a peak value towards the end of the period of drug contact (15 min). In a concentration > 1 mM, the caffeine-induced spasm developed more rapidly, reached a peak well within 15 min and thereafter began to decline. Log concentration-peak tension curves for caffeine and other xanthines are presented in Figure 7.

Enprofylline, theobromine and theophylline each had spasmogenic actions similar to that of caffeine. The maximal effects of enprofylline and theophylline were identical to that of caffeine, but the maximal effect of theobromine was slightly smaller than that of caffeine. The linear portions of the log concentration-effect curves of these four xanthines were virtually superimposable (Figure 7).

Xanthine, too, was spasmogenic though its spasmogenic threshold concentration was considerably higher than that of caffeine. The log concentration-effect curve for xanthine shown in Figure 7 suggests that its maximal effect was approximately 50% of that of caffeine. However, at the highest concentrations (8–16 mM) of xanthine used in these experiments the drug did not fully dissolve in the Krebs solution and the log concentration-effect relationship may have been distorted for that reason.

TMX also had spasmogenic activity with a threshold concentration of 8 mM. The limited amount of TMX at our disposal prevented us from exploring the full log concentration-effect relationship but it was clearly less potent than the other xanthines tested (Figure 7). pD_2 values for the spasmogenic actions of the xanthines are presented in Table 1 from which it is clear that the relative order of potency was theobromine ≈ theophylline ≈ caffeine ≈ enprofylline > xanthine > TMX.

Forskolin was devoid of spasmogenic activity over the concentration range 0.1 nM–100 μM (Figure 6).

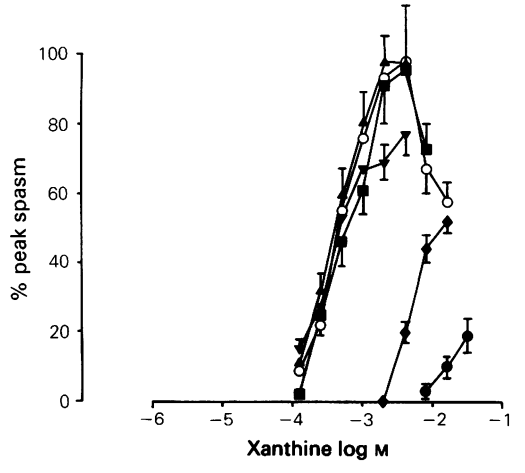

Figure 7 The spasmogenic effects of some xanthine derivatives as observed in guinea-pig isolated trachealis maintained at 12°C in Krebs solution containing 2.8 μM indomethacin. The abscissa scale represents the molar concentration of the xanthine derivative on a log scale. The ordinate scale represents peak spasm expressed as a percentage of the peak spasm evoked by caffeine (10 mM). The illustrated log concentration-effect curves are for enprofylline (■), theophylline (▲), caffeine (○), theobromine (▼), xanthine (◆) and 1,3,7,9-tetramethylxanthinium (●). Data indicate means of values from at least 6 tissues; s.e.mean shown by vertical bars.

(F) *Effects of a Ca^{2+}-free, EGTA-containing medium on spasmogenic responses to caffeine and KCl*

These experiments were performed to assess the dependency of caffeine-induced spasm on extracellular Ca^{2+}. In test tissues, the first dose of caffeine applied after tissue exposure to Ca^{2+}-free Krebs solution containing 2 mM EGTA yielded spasm which was approximately 78% of the initial value. Subsequent spasmogenic responses were less than 11% of the initial value (Table 2).

In time-matched control tissues the first two spasmogenic responses to caffeine were of equal magnitude. Thereafter responses to caffeine became progressively smaller. However, this decline in the size of the caffeine-induced spasm was less prominent than that observed in the test tissues (Table 2).

KCl (40 mM) failed to evoke spasm from trachealis treated with indomethacin (2.8 μM) and maintained at 12°C. However, KCl evoked spasm when the bath temperature was raised to 37°C. In time-matched control tissues the spasmogenic response to successive doses of KCl remained constant in amplitude. When test tissues were exposed to Ca^{2+}-free Krebs solution containing 2 mM EGTA, the KCl-induced spasm was virtually abolished even for the first KCl dose applied in the Ca^{2+}-free medium (Table 2).

Table 2 The effects of Ca^{2+}-free EGTA-containing Krebs solution on spasmogenic responses of guinea-pig isolated trachealis to caffeine or KCl

	Caffeine (10 mM) 12°C		*KCl* (40 mM) 37°C	
Dose no.	*Test tissues*	*Time-matched controls*	*Test tissues*	*Time-matched controls*
1	100	100	100	100
2	77.5 ± 5.9	100.7 ± 1.49	2.5 ± 2.1	99.4 ± 2.9
3	10.7 ± 1.2	82.1 ± 6.5	0	96.8 ± 3.5
4	9.7 ± 1.1	58.0 ± 5.4	0	103.3 ± 3.6

For each group of tissues the response to the initial spasmogen dose was defined as 100% and responses to subsequent doses are expressed as % of the response to the initial dose. Experiments with caffeine were conducted at 12°C, those with KCl at 37°C. Indomethacin (2.8 μM) was present throughout all experiments. For test tissues only, responses to the second and subsequent spasmogen doses were obtained in Ca^{2+}-free Krebs solution containing 2 mM EGTA. Data indicate means of responses from 6 tissues ± s.e.mean.

Discussion

Relaxant versus spasmogenic activity of the methylxanthines: establishment of optimal conditions for observing spasmogenic activity

Ito & Itoh (1984a,b) were able to demonstrate spasmogenic effects of caffeine in feline isolated trachealis, a tissue which lacks intrinsic tone. Guinea-pig isolated trachealis exhibits spontaneous tone and it was reasoned at the outset of the present study that the well-recognised (e.g. Persson, 1985a,b; Allen *et al.*, 1986; Honda *et al.*, 1986) ability of the methylxanthines to suppress this tone would frustrate attempts to demonstrate their spasmogenic activity. Accordingly, preliminary experiments were performed with caffeine in order to establish optimal conditions for observing the spasmogenic activity of the methylxanthines.

Cooling guinea-pig trachealis from 37°C to 22°C converted caffeine (10 mM)-induced relaxant responses to small spasmogenic responses (Figure 1). However, since cooling also caused a fall in tissue tone, the observed change from relaxant to spasmogenic responses could have been a consequence either of the cooling or of the decrease in tone. In tissue rewarmed to 37°C, tone suppression with indomethacin abolished relaxant responses to caffeine but did not reveal spasmogenic activity. Spasmogenic responses to caffeine only appeared when the indomethacin-treated tissue was cooled to 22°C (Figure 1). This strongly suggested that cooling was more important than a decrease in tissue tone for revealing the spasmogenic action of caffeine.

This finding was re-inforced by the results of the experiments where caffeine was added during ACh-induced spasm of indomethacin-treated tissue. The ability of caffeine at 37°C transiently to augment the ACh-induced spasm suggests that the spasmogenic activity of caffeine can be manifest at physiological temperatures and under circumstances where the tissue is generating tension. However, in these same experiments, cooling greatly improved the expression of the spasmogenic action of caffeine (Figure 2).

When indomethacin-treated tissues were subjected to repeated caffeine (10 mM) challenges and progressively cooled, the amplitude of the caffeine-induced spasm increased as the temperature was lowered to 12°C. Thereafter further cooling led to a decrease in spasm amplitude (Figure 4). These findings led us to believe that when guinea-pig trachealis is treated with indomethacin (2.8 μM) and cooled to 12°C the biochemical processes which underlie the relaxant effects of caffeine (or the mechanical expression of those processes) are suppressed relative to those which underlie its spasmogenic effects. Accordingly, under such conditions the spasmogenic action of caffeine is seen to its best advantage. The spasmogenic actions of caffeine and other methylxanthines examined in the present work were therefore studied in indomethacin (2.8 μM)-treated tissue maintained at 12°C.

Mechanism of the spasmogenic actions of the methylxanthines

The present experiments have yielded two observations which suggest that the mechanism underlying the spasmogenic effects (as seen at 12°C in tissue treated with indomethacin 2.8 μM) of the methylxanthines differs from that underlying their relaxant effects when tested against the spontaneous tone of the trachea at 37°C. Firstly, the rank order of potency of the xanthine derivatives in eliciting spasm differs from their rank order of potency in evoking relaxation. Secondly, forskolin is an adenylate

cyclase activator that suppresses the spontaneous tone of guinea-pig trachealis at concentrations (10^{-8}–10^{-6} M) causing an increase in the tissue content of cyclic AMP (Tsukawaki *et al.*, 1987). In the present study, forskolin was able to mimic the relaxant effects of the methylxanthines but it failed to mimic their spasmogenic activity.

In trachealis treated with indomethacin (2.8 μM) and maintained at 12°C, forskolin was able partially to suppress spasm induced by ACh. This suggests that, even at 12°C, forskolin is capable of stimulating adenylate cyclase and hence the production of cyclic AMP. Accordingly, the failure of forskolin to mimic the spasmogenic effects of the methylxanthines at 12°C suggests that accumulation of cyclic AMP is not important for the expression of their spasmogenic activity.

Caffeine retains the ability to evoke spasm of airways smooth muscle when the tissue is exposed to a Ca^{2+}-free, EGTA-containing medium (Ito & Itoh, 1984a; present study). Furthermore, caffeine retains spasmogenic activity when tested on saponin-permeabilised smooth muscle (Meisheri *et al.*, 1985). Observations of this kind lend support to the widely-held (e.g. Ito & Itoh, 1984a,b) belief that, in smooth muscle, the spasmogenic activity of caffeine represents its direct action in releasing Ca^{2+} from intracellular sites of sequestration.

In the present study the spasmogenic activity of caffeine was observed to be shared by enprofylline, TMX, theophylline, theobromine and xanthine. The observed rank order of potency of these substances in evoking spasm is probably not a true index of their affinity for a receptor site at the level of the intracellular Ca^{2+} store, for the relative potency measurements of these compounds are almost certainly influenced by their different abilities to cross the plasmalemma. Nevertheless it is clear that promotion of Ca^{2+} release from the intracellular stores is an action shared by various methylxanthines and, since xanthine itself is active, alkylation of the xanthine nucleus is not crucial for expression of the action.

The site at which methylxanthines exert their relaxant effects in airways smooth muscle

The long-held belief that methylxanthines evoke relaxation of airways smooth muscle by crossing the cell membrane and inhibiting intracellular phosphodiesterase has recently been challenged. Persson (1985a,b) observed that the quaternized xanthine derivative, TMX, retained the ability to relax airways smooth muscle *in vitro*. Since quaternized xanthines should not cross the plasmalemma Persson (1985a,b) argued that the relaxant activity of the methylxanthines might be mediated at a receptor located on the external surface of the cell.

The present study has confirmed that TMX (8–32 mM) has relaxant activity when tested against the spontaneous tone of guinea-pig trachea as observed at 37°C. However, when identical concentrations of TMX were tested in trachealis treated with indomethacin (2.8 μM) and maintained at 12°C, spasmogenic effects were observed. As discussed above, these spasmogenic effects represent the direct effect of TMX on the intracellular Ca^{2+} store. Accordingly it seems likely that, at concentrations of 8–32 mM, TMX crosses the cell membrane.

The argument (Persson, 1985a,b) that the relaxant effects of TMX indicate an extracellular site for the relaxant action of methylxanthines therefore becomes very questionable. Indeed, the ability of TMX to produce relaxation, but only at concentrations that are spasmogenic, prompts the counter-argument – that the relaxant effects of the methylxanthines are mediated at an intracellular site.

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