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Effects of leukotriene D_4 on the mechanical and electrical properties of guinea-pig isolated trachealis

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1 The effects of leukotriene D_4 (LTD_4) on mechanical and electrical activity were examined in guinea-pig isolated trachealis muscle and compared with two other bronchoconstrictors, methacholine and potassium chloride (KCl).

2 LTD_4 elicited concentration-dependent increases in tension in trachealis muscle which were slower in time course than responses induced by either methacholine or KCl. The maximum response to LTD_4 was approximately 85% of the methacholine maximum.

3 At a concentration close to the EC_{50} for tension changes, LTD_4 had no significant effect on either transmembrane potential or slow wave activity recorded in single trachealis cells.

4 At a concentration close to the EC_{90} for tension changes, LTD_4 caused significant membrane depolarization, transiently reduced the amplitude and increased the frequency of slow wave discharge and ultimately abolished slow wave discharge. LTD_4-induced depolarization was less marked, and developed more slowly, than that evoked by either methacholine or KCl.

5 These results show that LTD_4 can elicit substantial increases in tension without altering transmembrane potential and are consistent with the view that LTD_4 initiates contraction mainly through potential-independent mechanisms. However, at high concentrations the depolarization evoked by LTD_4 allows the possibility that potential-dependent mechanisms may contribute to the spasm.

Introduction

Since the discovery that 'slow reacting substance of anaphylaxis' (SRS-A) comprises three leukotriene derivatives of arachidonic acid (leukotriene C_4 (LTC_4), LTD_4 and LTE_4) there has been considerable interest in the bronchoconstrictor action of these agents (Dahlen *et al.*, 1980; 1983; Krell *et al.*, 1981; Jones *et al.*, 1982) and their possible role as mediators in bronchial asthma. LTD_4 elicits bronchoconstriction which is not associated with an increased uptake of extracellular calcium ions (Raeburn & Rodger, 1984) and is only partly antagonized by Ca^{2+}-channel blockers (Jones *et al.*, 1982; Advenier *et al.*, 1983; Weichman *et al.*, 1983). It is, however, blocked completely by a Ca^{2+} antagonist, TMB-8 (Weichman *et al.*, 1983) which is thought to act intracellularly (Malagodi & Chiou, 1974). This has led to the suggestion that LTD_4 initiates contraction mainly through the release of intracellularly stored Ca^{2+} ions (Raeburn & Rodger, 1984).

It is believed that agonists induce contraction in smooth muscle in at least three different ways: (a) depolarization of the cell membrane which promotes opening of voltage-operated Ca^{2+}-channels (VOCs), causing movement of Ca^{2+} from the extracellular compartment into the cytoplasm; (b) activation of receptor-operated Ca^{2+}-channels (ROCs), which also results in Ca^{2+} influx and (c) release of Ca^{2+} into the cytoplasm from intracellular storage sites (see Bolton & Large, 1986). Some bronchoconstrictor agents such as KCl act through mechanism (a) and the contraction is thus entirely dependent on changes in membrane potential (Coburn & Yamaguchi, 1977). The action of other agents such as acetylcholine (ACh) is thought to involve a combination of all three mechanisms (Farley & Miles, 1977; Bolton & Large, 1986). Furthermore the relative contribution to contraction of the different mechanisms may vary according to the

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concentration of agonist (Farley & Miles, 1978). As stated above it is thought that LTD_4 acts mainly through release of intracellular Ca^{2+} but the effects of LTD_4 on the electrical properties of airway smooth muscle have not been studied. Hence it is not known whether there is a potential-dependent component to its action. We decided, therefore, to examine the spasm induced by LTD_4 in guinea-pig trachealis muscle using mechanical and electrophysiological recording techniques. The effects of KCl and methacholine were also studied for comparison. A preliminary account of these results has been presented to the British Pharmacological Society (McCaig & Rodger, 1986).

Methods

Animals

Guinea-pigs (male Dunkin-Hartley, 350–450 g body weight) were killed by a blow to the head and the trachea was rapidly excised. Ring segments of trachea, obtained by sectioning between adjacent cartilage bands, were removed from the mid-cervical portion of the trachea for tension studies and adjacent segments containing 4–6 cartilage rings were taken for electrophysiological studies.

Tension studies

Tracheal segments were set up in an organ bath at 37°C containing Krebs solution (composition, mM): Na^+127, K^+5.9, Ca^{2+}2.5, Mg^{2+}1.2, Cl^-121, $H_2PO_4^-$1.2, SO_4^{2-}1.2, HCO_3^-25, glucose 11) and gassed with a mixture of 95% O_2:5%CO_2. Flurbiprofen, 10^{-6} M, was present throughout all experiments in order to inhibit both the spontaneous and agonist-induced generation of cyclo-oxygenase products (Rome & Lands, 1975) e.g. prostaglandin E_2 (PGE_2), PGI_2 and thromboxane A_2, known to be produced during tissue contraction (Orehek *et al.*, 1973; Weichman *et al.*, 1982). Tension was recorded with an isometric tension transducer coupled to a pen recorder (both Devices). An initial resting tension of 1 g was imposed and the tissue was left to equilibrate for 60 min. Cumulative concentration-effect curves were constructed according to the method of Van Rossum (1963). Thus, each agonist was added to the tissue bath at the peak effect achieved by the preceding concentration. For methacholine and LTD_4 the concentrations were increased step-wise in 10 fold increments; for KCl increments were 2 fold. At the end of each experiment a single concentration of methacholine (1×10^{-4} M) was administered in order to gauge the maximum contractile response of the tissue. Results are expressed as a percentage of this methacholine-induced maximum response. In some experiments the concentration-effect curves were constructed before and after treatment of the tissues with atropine (1×10^{-7} M). In these experiments the control concentration-effect curve was constructed first and then the tissues were washed repeatedly until pre-drug tension levels were obtained. The tissue was then incubated for 60 min with atropine, after which the drug under test was added in a cumulative fashion until a maximum response was obtained.

Electrophysiological studies

Segments of trachea were opened by means of a longitudinal cut made diametrically opposite the trachealis muscle and were pinned to the base of the recording chamber (volume 10 ml) through which Krebs solution flowed at a rate of 5 ml min^{-1}. The temperature was maintained at 37°C and the bathing solution was equilibrated in the reservoir with 95% O_2:5%CO_2 (dead space of tubing <2 ml). In preliminary experiments the effects of flurbiprofen on resting electrical properties of trachealis cells were examined. Thereafter, flurbiprofen, 10^{-6} M, was present in the superfusing solution throughout each experiment. A small segment of the mucosal layer was removed carefully with watchmakers forceps to facilitate microelectrode penetration of the smooth muscle cells. After a 40 min equilibration period single trachealis cells were impaled with glass microelectrodes (resistance 60–80 MΩ, filled with 0.5 M KCl). Voltage signals were fed through a unity-gain amplifier (World Precision Instruments) displayed on an oscilloscope (Tektronix) and recorded continuously on a pen recorder (Gould).

Transmembrane potential and slow wave activity (when present) were recorded for 20–30 s in each of a sample of 10–15 cells. A further impalement was then made and maintained, where possible, during exposure to LTD_4, KCl or methacholine. Once the response had stabilized the electrode was withdrawn and a second group of 10–15 cells was impaled, each for 20–30 s. A further impalement was made and maintained during washout of the drug. In a number of cases the microelectrode was dislodged from the cell during exposure to an agonist, particularly at the higher concentrations of KCl and methacholine (which induced rapid, powerful contractions of the trachea). After washout of the agonist the preparation was left for 30 min and then a second concentration of the agonist was applied in the manner described above.

Drugs

The following drugs were used: atropine sulphate (Sigma); flurbiprofen (Boots); KCl (Sigma); and

methacholine chloride (Sigma). LTD_4 was a gift from Miles Laboratories. In tension studies drugs were added directly to the bath in a volume of 10 μl. In electrophysiological studies drugs were added to the reservoir of Krebs solution. All concentrations are expressed as the molar concentration of salt in the bathing solution.

Statistics

Mean responses were compared by a two-tailed, unpaired t test. Values of $P < 0.05$ were regarded as significant.

Results

Tension studies

In the presence of flurbiprofen the isolated tracheal preparations failed to generate any spontaneous tone. In initial experiments in which preparations were set up in Krebs solution without flurbiprofen, spontaneous tone developed over a 30 min period (increasing from the initial applied resting tension of 1 g to approximately 2 g). Addition of flurbiprofen at this time induced relaxation, re-establishing resting levels (1 g) within 30 min. Flurbiprofen was without effect on the concentration-effect curves obtained with either KCl or methacholine. In contrast, responses to LTD_4 were significantly augmented, in accordance with data published previously (Raeburn & Rodger, 1984).

In the presence of flurbiprofen, LTD_4 evoked concentration-dependent increases in tension in tracheal segments (Figure 1). Responses were relatively slow to develop (15–20 min) and reached a maximum equivalent to 83 ± 5% (mean ± s.e.mean, $n = 5$) of the methacholine maximum for these tissues. The pD_2 for LTD_4 was 8.3 ± 0.3 ($n = 5$). After washout tension was gradually restored to baseline over a period of 45 min.

In the presence of flurbiprofen, methacholine and KCl also induced concentration-dependent increases in tension (Figure 1). Responses were rapid and readily reversible on washout of the agonist. It is clear from Figure 1 that LTD_4 is almost two orders of magnitude more potent than methacholine, although the maximum response is smaller. Responses to LTD_4 and KCl were unaffected by pretreatment of the tissues with atropine (1×10^{-7} M). For LTD_4 the mean pD_2 values before and after atropine were 8.2 ± 0.1 and 8.15 ± 0.09, respectively; for KCl the mean pD_2 values were 1.83 ± 0.05 and 1.85 ± 0.1. In contrast, the concentration-effect curves for methacholine were shifted significantly to the right in the presence of atropine. The mean pD_2 values in the absence and presence of atropine were 5.97 ± 0.21 and 3.8 ± 0.18 ($n = 6$), respectively. This represents in excess of a 200 fold shift to the right of the methacholine concentration-effect curve.

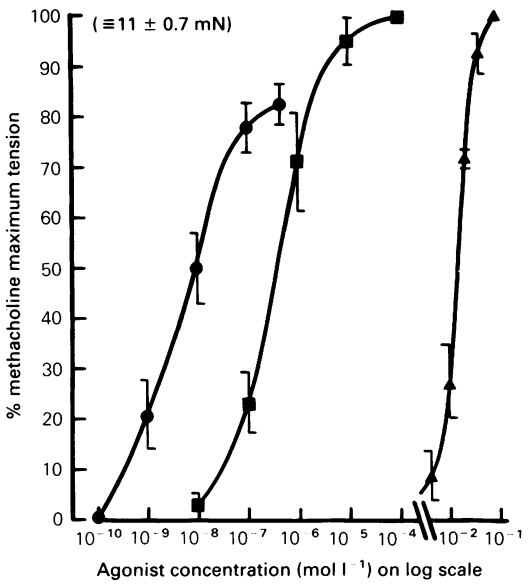

Figure 1 Cumulative concentration-effect curves for leukotriene D_4 (LTD_4, ●), methacholine (■) and potassium chloride (KCl, ▲) on guinea-pig isolated trachealis, constructed in the presence of flurbiprofen, 10^{-6} M. The increase in tension is expressed as percentage of the methacholine maximum (ordinate scale). Values are mean of 3–5 observations, vertical bars show s.e.mean.

Electrophysiological studies

The effects of the three bronchoconstrictor drugs on the electrical activity of the guinea-pig trachealis were examined in the presence of flurbiprofen. Initially, therefore, it was important to establish whether flurbiprofen itself affected resting electrical properties. As shown in Table 1, flurbiprofen, 10^{-6} M, had no significant effect on transmembrane potential, or on slow wave amplitude or frequency of discharge.

The effects of LTD_4 on electrical activity were examined at two concentrations, 5×10^{-9} M and 10^{-7} M, which were close to the EC_{50} and EC_{90}, respectively, for the drug, as assessed in tension studies. LTD_4, 5×10^{-9} M, had no significant effect on transmembrane potential or slow wave characteristics as shown in Table 1. LTD_4, 10^{-7} M, did however cause significant depolarization (Table 1 and Figure 2a). The mean depolarization recorded

Table 1 Effects of flurbiprofen (Flur) and of LTD_4, methacholine (MCh) and KCl on transmembrane potential (Em) and electrical slow wave characteristics in single guinea-pig trachealis cells

			Slow wave	
	Drug concentration (M)	*Em* (mV)	*Amplitude* (mV)	*Frequency* (Hz)
	Control[a]	−42.5 ± 1.0 (38)	4.8 ± 0.6 (36)	0.74 ± 0.24 (16)
Flur	10^{-6}	−41.8 ± 0.7 (54)	5.5 ± 0.7 (49)	0.66 ± 0.25 (39)
	Control[b]	−40.0 ± 0.7 (102)	5.3 ± 0.4 (96)	0.69 ± 0.02 (55)
LTD_4	5×10^{-9}	−38.1 ± 0.5 (42)	5.4 ± 0.7 (26)	0.77 ± 0.04 (21)
	10^{-7}	−33.0 ± 0.5*** (132)	2.7 ± 0.3*** (56)	0.96 ± 0.02*** (41)
	Control[b]	−39.8 ± 0.7 (39)	5.9 ± 1.0 (15)	0.77 ± 0.05 (13)
MCh	5×10^{-7}	−32.6 ± 0.5*** (24)	4.4 ± 1.9 (9)	1.06 ± 0.10** (8)
	10^{-5}	−28.5 ± 0.9*** (45)	2.3 ± 0.9 (3)	0.87 ± 0.18 (3)
	Control[b]	−41.7 ± 0.8 (26)	6.7 ± 1.1 (26)	0.64 ± 0.02 (26)
	10^{-2}	−35.5 ± 1.4** (19)	8.4 ± 0.7 (19)	0.68 ± 0.03 (19)
KCl	2×10^{-2}	−24.1 ± 1.0*** (24)	4.1 ± 1.1 (18)	0.84 ± 0.06*** (18)
	4×10^{-2}	−21.7 ± 0.7*** (32)	2.1 ± 0.6* (7)	0.94 ± 0.07*** (7)

Results include observations made in separate groups of cells sampled before and after exposure to spasmogen and in cells in which the microelectrode impalement was maintained during exposure to spasmogen. The first set of control observations (Control[a]) were made in drug-free Krebs solution. Subsequent control observations (Control[b]) were made in Krebs solution containing flurbiprofen, 10^{-6} M, prior to the addition of spasmogen. Measurements were made after drug contact times of 10–15 min (LTD_4), 5–12 min (KCl and MCh) and 30–60 min (Flur). Results for slow wave activity exclude cells which were quiescent in the resting state and cells in which slow wave activity was abolished by the spasomgen.

Figures in parentheses indicate the number of cells impaled.

Significant difference from control values at * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

by sampling groups of cells before and during application of LTD_4 (10^{-7} M) was 7.2 mV and this was consistent with the depolarization recorded during continuous impalement of single cells (7.0 ± 0.7 mV, $n = 4$, see Figure 2). The depolarization, as gauged by taking the mean depolarization per preparation, was also very similar (7.8 ± 1.2 mV). The depolarization began 3 min after the start of perfusion with the LTD_4-containing Krebs solution and was maximal after 10 ± 1.5 min ($n = 4$). At this concentration (10^{-7} M) LTD_4 also affected slow wave activity (Table 1 and Figure 2a). Initially there was a reduction in amplitude and an increase in frequency of discharge, followed by a period when slow waves ceased or were barely detectable (time from exposure to drug to cessation 9 ± 1 min; $n = 5$). On washout there was a gradual repolarization of cells over approximately 20 min and slow wave activity recommenced after 13 ± 3 min ($n = 3$) in drug-free Krebs solution.

The effects of KCl were examined at three concentrations, 1, 2 and 4×10^{-2} M. The EC_{50} obtained in the tension studies was between 1 and 2×10^{-2} M and 4×10^{-2} M is close to the EC_{90}. KCl evoked a concentration-dependent depolarization (Table 1 and Figure 2b). At 1×10^{-2} M KCl tended to increase the amplitude of slow waves without affecting frequency whereas at higher concentrations slow wave amplitude was reduced and frequency increased (Figure 2b). Slow waves were abolished at either 2 or 4×10^{-2} M KCl. The effects developed more rapidly than those of LTD_4, depolarization beginning after 1–2 min and reaching a peak at 3–5 min, with slow waves ceasing between 2 and 4 min. On washout repolarization took approximately 5 min, and slow waves resumed in 3 min.

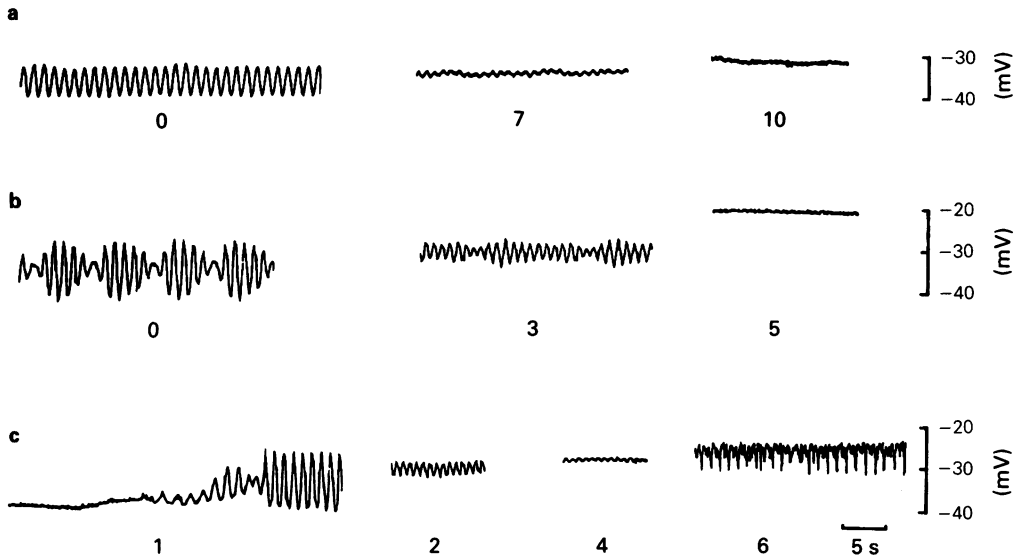

Figure 2 The electrical activity recorded intracellularly in single cells from three guinea-pig isolated trachealis preparations during exposure to (a) leukotriene D_4, 10^{-7} M, (b) KCl, 2×10^{-2} M and (c) methacholine, 10^{-5} M. Segments of records are from a single cell for each drug. Numbers below the traces refer to the time in min from the addition of the agonist to the reservoir of Krebs solution.

Methacholine also elicited concentration-dependent depolarization of the trachealis (Table 1 and Figure 2c). Methacholine at a concentration of 5×10^{-7} M, which was close to the EC_{50} in tension studies, caused significant depolarization with no change in slow wave amplitude but an increase in frequency. At the higher concentration of 10^{-5} M (close to EC_{90}) methacholine evoked further depolarization, with a reduction in amplitude and increase in frequency of slow waves which rapidly gave way to cessation of discharge. In one quiescent preparation, slow wave activity was initiated on exposure to methacholine. Slow wave discharge gradually became smaller in amplitude and faster as the cell depolarized, then ceased (Figure 2c). Like those of KCl, the effects of methacholine were rapid in onset, depolarization beginning at 1–2 min and peaking after 3–4 min. At the height of the depolarization, a few minutes after slow waves ceased, a complex series of oscillations in potential with rapid hyperpolarizations was seen in a number of cells (Figure 2c). Such activity was reported previously during exposure of guinea-pig trachealis to ACh (Ahmed *et al.*, 1984). This persisted until washout of the methacholine and, as the cell repolarized, gradually gave way to slow wave activity.

In order to allow some comparison among the three agonists used, transmembrane potential at the various concentrations of the drugs was plotted against the tension (as percentage of maximal tension induced by methacholine) (Figure 3). It is clear that LTD_4 evokes substantially less depolarization to produce a given increase in tension than does KCl. Thus LTD_4, 10^{-7} M, evoked an increase in tension of 79% of the methacholine maximum and depolarization of 7 mV, whilst KCl, 2×10^{-2} M, evoked a smaller increase in tension (70% of methacholine maximum) with a much greater degree of depolarization (17.6 mV). The results strongly suggest that LTD_4 also induces less depolarization for a given increase in tension than does methacholine. LTD_4, 10^{-7} M, for example, evokes a bigger increase in tension than methacholine, 5×10^{-7} M, although the two agonists at these concentrations ellicit a similar degree of depolarization (Table 1 and Figure 3). However, it remains to be shown whether the relatively small depolarization induced by LTD_4 is critical for contraction.

Discussion

These results confirm that LTD_4 elicits concentration-dependent contraction in guinea-pig trachealis, as reported previously (Raeburn & Rodger, 1984), the maximum response amounting to approximately 85% of the methacholine maximum. Responses are slower to develop than those induced

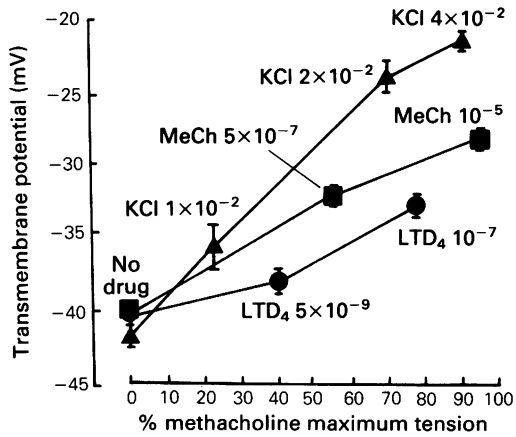

Figure 3 The relationship between transmembrane potential (ordinate scale) and tension (measured in separate experiments as the percentage of the methacholine maximum) (abscissa scale) in the guinea-pig isolated trachealis at various concentrations of the three agonists, Leukotriene D_4 (LTD_4) (●), methacholine (■) and KCl (▲). Transmembrane potential values are the mean of 19–132 observations, vertical bars show s.e. mean. For methacholine, 5×10^{-7} M, and LTD_4, 5×10^{-9} M, tension was estimated from Figure 1 by interpolation in the relevant concentration-effect curves. For all other concentrations of the 3 agonists, tension was directly assessed.

by methacholine or KCl and are also slower to dissipate after washing. At a concentration just below its EC_{50}, LTD_4 had no significant effect on either transmembrane potential or slow wave activity, suggesting that the contraction may be mediated by potential-independent mechanisms. LTD_4, at a higher concentration, did elicit significant depolarization and produced a transient reduction in amplitude and an increase in frequency of slow wave discharge abolishing slow wave activity altogether in most preparations. Thus at higher concentrations a potential-dependent component to the contraction could exist.

The results obtained are consistent with the view that in concentrations up to its EC_{50}, the contractions induced by LTD_4 occur through potential-independent mechanisms. The role of potential-dependent effects, however, is harder to define. At a high concentration LTD_4 does depolarize the trachealis but the degree of depolarization is small compared to that of KCl. Responses to LTD_4 can be partly blocked by verapamil (Raeburn & Rodger, 1984), which is though to act by blocking VOCs, which would be consistent with the present results. Thus in the presence of verapamil depolarization would be unable to induce Ca^{2+} influx through VOCs and this component of the response would be lost. It has not been possible, however, to demonstrate an increase in the lanthanum-resistant Ca^{2+} content of trachealis during exposure to LTD_4, even at high concentrations (Raeburn & Rodger, 1984), suggesting either that Ca^{2+} influx is not involved in the response or that the magnitude of Ca^{2+} influx is beneath the level of detection using the 'lanthanum' technique. On the other hand, LTD_4, like the other agonists tested, transiently increased slow wave frequency and, since slow waves are Ca^{2+}-dependent (Small, 1982; McCaig, 1986), it seems likely that LTD_4 acts in some way to modulate Ca^{2+}-channel opening.

Such seemingly-conflicting results have also been obtained with ACh. Thus ACh-induced spasm of trachealis is blocked partially by verapamil (Farley & Miles, 1978) but does not seem to involve an increase in Ca^{2+} content of the trachealis muscle (Ahmed *et al.*, 1984). Again, it has been suggested that there is some Ca^{2+} influx but that the amount is too low to be detected by the lanthanum technique (Ahmed *et al.*, 1984). One way of reconciling the conflicting data would be to suggest that there is a small influx of Ca^{2+} through VOCs which then acts intracellularly to cause Ca^{2+}-induced Ca^{2+}-release from storage sites as has been proposed in feline airway smooth muscle (Ito & Itoh, 1984). If this were so, verapamil would block the influx of Ca^{2+} and block this component of the contraction. The intracellularly-acting Ca^{2+}-antagonist TMB-8 would be expected to block both this component and the direct effects of LTD_4 on intracellularly-stored Ca^{2+} thus causing complete antagonism of the LTD_4 response, as has been reported (Weichman *et al.*, 1983).

The complex oscillations in membrane potential with hyperpolarizing spikes seen at the higher concentration of methacholine are similar to those reported during exposure of guinea-pig trachealis to ACh (Ahmed *et al.*, 1984). Such activity was never seen during exposure to KCl, despite its greater capacity to induce depolarization, or to LTD_4 (present study), but was reported to occur on exposure to histamine (Ahmed *et al.*, 1984). Spontaneous transient outward currents have been demonstrated in dispersed single visceral and vascular smooth muscle cells (Benham & Bolton, 1986). It was suggested that these currents might be caused by activation of Ca^{2+}-dependent K^+-channels in the cell membrane brought about by cyclical changes in intracellular Ca^{2+} ion concentration, perhaps as a result of bursts of release and subsequent reuptake of Ca^{2+} ions from intracellular stores. It is likely that in whole tissues where the cells are electrically coupled, such currents would dissipate quickly without giving rise to discernible fluctuations in membrane potential. Hyperpolarizing spikes might

be produced, however, if changes in intracellular Ca^{2+} levels became synchronized in the presence of an agonist or if the agonist acted to reduce intercellular electrical coupling. Treatment of guinea-pig trachealis with K^+-channel blockers, such as tetraethylammonium or procaine, suppresses ACh- or histamine-induced 'noise', supporting the idea that this does involve K^+-channel activation (Small *et al.*, 1987; Boyle *et al.*, 1988). Interestingly, suppression of ACh-induced noise does not appear to affect the contractile response.

Flurbiprofen induced relaxation of the guinea-pig trachealis, supporting the view that endogenous production of prostaglandins is involved in the maintenance of resting tone in this tissue. Relaxation, however, was not associated with hyperpolarization or suppression of slow wave activity in single trachealis cells, indicating that tone and slow wave activity may be dissociated, and that slow waves are not dependent on prostaglandin production. This finding is in good agreement with that of Boyle *et al.* (1988) who found that the cyclo-oxygenase inhibitor, indomethacin, failed to suppress slow wave discharge in guinea-pig trachealis, but is at variance with the work of Honda & Tomita (1987) in which they report abolition of slow waves by indomethacin in the same tissue.

In conclusion, LTD_4 has a potent spasmogenic action in guinea-pig trachealis. The spasm evoked by concentrations of LTD_4 lying within the lower half of its concentration-effect curve is accompanied by little or no depolarization. The spasm evoked by higher concentrations of LTD_4 is accompanied by depolarization which may lead to suppression of slow wave activity. It is possible that low concentrations of LTD_4 may evoke contraction by a potential-independent mechanism. Contraction evoked by higher concentrations of LTD_4, however, could involve potential-dependent mechanisms.

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