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Lack of effect of leukotriene D_4 on Ca-uptake in airway smooth muscle

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1 The effects of verapamil on leukotriene D_4 (LTD_4)- and KCl-induced contractions and ^{45}Ca-uptake were examined in guinea-pig isolated tracheal smooth muscle.

2 Both LTD_4 (0.1 to 200 nmol l^{-1}) and KCl (8 to 125 mmol l^{-1}) produced concentration-dependent increases in tension in the tracheal preparations.

3 Verapamil (1 μmol l^{-1}) inhibited the tension responses induced by both LTD_4 and KCl.

4 LTD_4 failed to increase the lanthanum-resistant Ca content of tracheal smooth muscle at either low (EC_{25}; 3 nmol l^{-1}) or high (EC_{90}; 50 nmol l^{-1}) concentrations. Verapamil did not modify this result.

5 KCl (90 mmol l^{-1}) increased the lanthanum-resistant Ca content of the smooth muscle by approximately 60% over basal levels. This effect was completely inhibited by verapamil (1 μmol l^{-1}).

6 It is concluded that in this tissue, LTD_4 utilizes principally an intracellular source of Ca^{2+} to initiate contraction whereas KCl is dependent upon the uptake of Ca^{2+} from the extracellular compartment. It is suggested that the inhibitory effects of verapamil may reflect an intracellular mechanism of action directed against Ca^{2+} release initiated by LTD_4.

Introduction

Slow reacting substance of anaphylaxis (SRS-A) has long been considered to be an important chemical mediator in asthma (Orange & Austen, 1969). Recently, it has been shown that SRS-A consists of a mixture of three leukotrienes derived from arachidonic acid (leukotriene C_4 (LTC_4), LTD_4 and LTE_4) all of which possess potent, long-lasting bronchoconstrictor activity (Dahlen *et al.*, 1980; 1983; Krell *et al.*, 1981; Jones *et al.*, 1982). The contractile responses of airway smooth muscle to LTD_4 have been shown to be directly mediated and Ca^{2+}-dependent but only partially inhibited by high concentrations of the voltage-dependent Ca^{2+}-channel blocking drugs D600, nicardipine, nifedipine and verapamil (Jones *et al.*, 1982; Advenier *et al.*, 1983; Weichman *et al.*, 1983). In contrast, however, the calcium antagonist TMB-8, which is thought to possess an intracellular mechanism of action (Malagodi & Chiou, 1974), inhibited completely the responses to LTD_4 (Weichman *et al.*, 1983). These observations suggest that LTD_4 may utilize an intracellular source of Ca^{2+} to initiate contraction rather than depend upon the uptake of extracellular Ca^{2+}.

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We have investigated this possibility by comparing the effects of LTD_4, in the absence and presence of verapamil, on tension responses and Ca-uptake in airway smooth muscle preparations of the guinea-pig.

A preliminary account of these findings has been presented to the British Pharmacological Society (Raeburn & Rodger, 1984).

Methods

Male Dunkin-Hartley guinea-pigs were killed by stunning and bleeding. Tracheae were rapidly excised from the animals, dissected free of extraneous connective tissue and then prepared as follows.

(a) *Tension studies*

Spirally-cut preparations of tracheae were suspended in Krebs-Henseleit solution at 37°C and bubbled with a mixture containing 95% O_2 and 5% CO_2. An initial stretching tension of 20 mN was applied to the tissues which were left for 60 min to equilibrate, during which time the bathing medium was changed

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three times. Changes in tension were recorded using isometric force-displacement transducers (FTO3C; Grass Instruments, Quincy, Mass.) coupled to a Grass (model 7) curvilinear ink-writing polygraph. The composition of the Krebs-Henseleit solution used in these studies was as follows (in mmol l^{-1}); NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.7.

Following equilibration the tissues were treated with flurbiprofen (1 μmol l^{-1}) in order to inhibit the generation of cyclo-oxygenase products (Rome & Lands, 1975) e.g. prostaglandin E_2 (PGE_2), PGI_2 and thromboxane A_2 (TxA_2), known to be produced during tissue contraction induced by some agonists (Orehek *et al.*, 1973; Weichman *et al.*, 1982). Flurbiprofen remained in the bathing solution for the duration of the experiment. Sixty minutes after the addition of flurbiprofen a cumulative concentration-effect curve to either LTD_4 or KCl was constructed according to the method of Van Rossum (1963). Following washout and return to baseline tension the tissues were incubated for 30 min with verapamil (1 μmol l^{-1}), after which a second cumulative concentration-effect curve was constructed.

(b) *Ca-uptake studies*

The lanthanum-resistant calcium content of trachealis muscle was measured according to the original method described by Van Breemen *et al.* (1972) but incorporating the modifications introduced by Godfraind (1976) and Deth (1978). For a review of the lanthanum method the reader is directed to a recent article by Daniel *et al.* (1983).

Strips of trachealis muscle approximately 10 mm long and 2 mm wide, weighing between 10 and 20 mg were dissected free of cartilage under a dissection microscope and allowed to equilibrate in 3 ml Tris-buffered Krebs-Henseleit solution (Tris-KHS), bubbled with 100% O_2, at 37°C for 30–45 min. The composition of the Tris-KHS was (in mmol l^{-1}) NaCl 137, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 11.7 and Tris 2.5. In each experimental run one or two strips of trachealis muscle served as controls, the remainder acting as test strips. After equilibration test tissues were transferred to 1 ml of fresh Tris-KHS at 37°C containing ^{45}Ca, 0.5 μCi ml^{-1}. The tissues were allowed to take up ^{45}Ca from the 'loading' solution for 5 min (to allow the ^{45}Ca to equilibrate with the tissue's extracellular space) at which point either vehicle, KCl (90 mmol l^{-1}) or LTD_4 (3 or 50 nmol l^{-1}) was added to the loading solution and left in contact for a further 15 min. At the end of this period tissues were removed from these media and placed in 3 ml of 0.5°C Ca^{2+}-free Tris-KHS containing 50 mmol l^{-1} $LaCl_3$ and left for 60 min. Tissues were then removed, blotted on absorbent paper, weighed and left in 3 ml EDTA (5 mmol l^{-1}) solution overnight to extract the ^{45}Ca present. The following day the EDTA extracts and 100 μl aliquots of the loading and La^{3+}-containing solutions were prepared for scintillation counting to determine ^{45}Ca content of the tissues and media, using triton X-toluene scintillation fluid (BDH). The radioactivity present was measured on a Packard Tricarb 460 CD spectrometer.

Ca-uptake was calculated according to the formula:

$$\text{Ca-uptake (nmol g}^{-1}\text{ tissue)} = \frac{\text{c.p.m. in tissue}}{\text{tissue wet weight (g)}} \times \frac{\text{nmol Ca}^{2+}\text{ in 100 μl labelled medium}}{\text{c.p.m. in 100 μl labelled medium}}$$

where c.p.m. = activity of ^{45}Ca expressed as counts per min.

In the experiments in which flurbiprofen (1 μmol l^{-1}) and verapamil (1 μmol l^{-1}) were used they were present throughout the initial equilibration period and also during the 'loading' procedure.

(c) *Drugs and solutions*

The following drugs were used: flurbiprofen (Boots) lanthanum chloride (BDH), methacholine chloride (BDH) and verapamil hydrochloride (Abbott). Leukotriene D_4 was a gift from Dr J. Rokach, Merck-Frosst, Canada. It was stored as a stock solution (20 μmol l^{-1}) in distilled water at −80°C until the day of use. After thawing, dilutions of LTD_4 were made in distilled water and stored at 4°C until required. Fresh solutions of the deep frozen LTD_4 stock were made up daily.

^{45}Ca was supplied as an aqueous solution of $CaCl_2$ by the Radiochemical Centre, Amersham. The specific activity of the material supplied was 2.13 mCi 141 μg^{-1} Ca^{2+}.

(d) *Statistical analysis*

The significance for differences between mean values was assessed using a two-tailed, unpaired *t* test.

Results

(a) *Tension studies*

LTD_4 (0.1 to 100 nmol l^{-1}) and KCl (8 to 125 mmol l^{-1}) both produced concentration-related increases in tension of the tracheal preparations up to

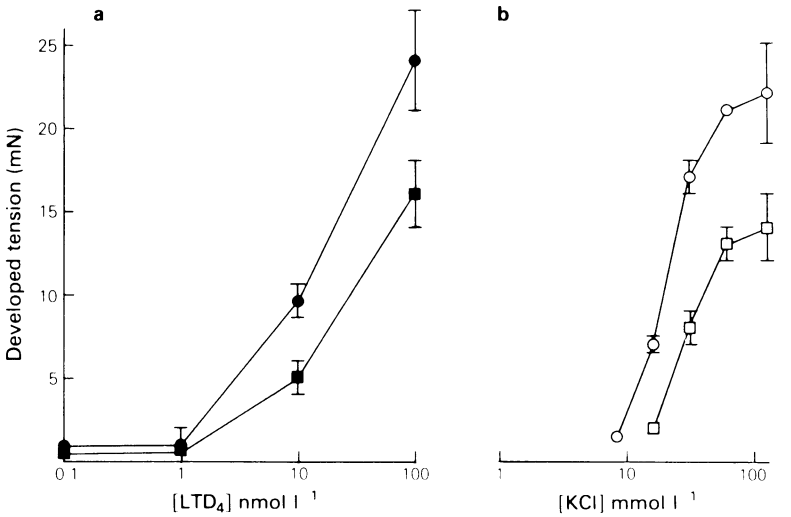

Figure 1 Effects of verapamil ($1\ \mu mol\ l^{-1}$) on (a) leukotriene D_4 (LTD_4)- and (b) KCl-induced contractions of guinea-pig isolated tracheal preparations. Each point represents the mean response (mN) of 4–6 preparations; s.e. mean shown by vertical lines. Flurbiprofen ($1\ \mu mol\ l^{-1}$) was present throughout the experiments. (a) LTD_4 before (●) and after (■) verapamil; (b) KCl before (○) and after (□) verapamil.

a maximum of 24 ± 3 mN for LTD_4 and 22 ± 3 mN for KCl. (Figure 1). It should be mentioned that the presence of flurbiprofen in the medium significantly augmented the maximum tension responses to LTD_4 whereas KCl maxima were little affected (data not shown). In each case the contractions were slow to develop taking approximately 15 min to achieve peak tension for each concentration of the drug used. KCl-induced contractions were readily reversed to control levels by washing (within 20 min) whereas LTD_4-induced contractions were only slowly reversible taking approximately 45 min to return to baseline tension levels with repeated washing. Repeat concentration-effect curves to both drugs were highly reproducible within the same tissue. Verapamil ($1\ \mu mol\ l^{-1}$) had no effect on baseline tension but inhibited the responses to both LTD_4 and KCl. The maximum response to KCl was depressed by approximately 36% (Figure 1b). It was not feasible to test concentrations of LTD_4 in excess of $0{\cdot}1\ \mu mol\ l^{-1}$ because of the limited supply of the eicosanoid. Thus no data are available regarding the effect of verapamil upon the maximum tension response induced by LTD_4. However, contractions elicited by the highest concentration of LTD_4 used ($0.1\ \mu mol\ l^{-1}$) were depressed by approximately 33% (Figure 1a) in the presence of verapamil.

(b) *Ca-uptake studies*

Initially these studies were performed in tracheal preparations that included some cartilage. Figure 2 illustrates, however, that the presence of such cartilage complicates studies involving Ca-uptake in that significantly greater amounts of Ca^{2+} are taken up by the tissue than can be attributed to the smooth muscle alone. Furthermore, in tissues that contained cartilage, KCl-depolarizing solutions failed to stimulate Ca-uptake; instead there occurred a significant reduction in Ca-uptake compared to basal (unstimulated) levels (data not shown). Consequently, all further studies on Ca-uptake were performed in cartilage-free preparations (see Methods). Those concentrations of LTD_4 that produced approximate-

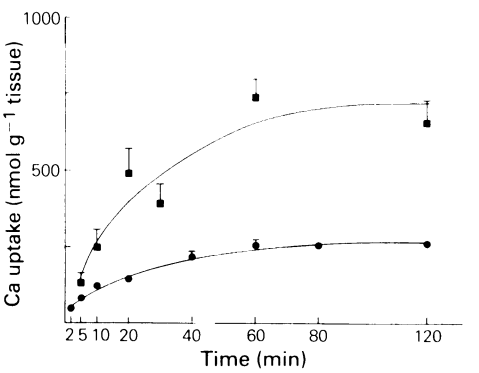

Figure 2 Time course of Ca uptake into guinea-pig isolated tracheal preparations. Tracheal smooth muscle alone (●, $n = 6-10$); tracheal smooth muscle with cartilage present (■, $n = 4$). Each point is plotted as the mean with the s.e.mean represented by the vertical lines.

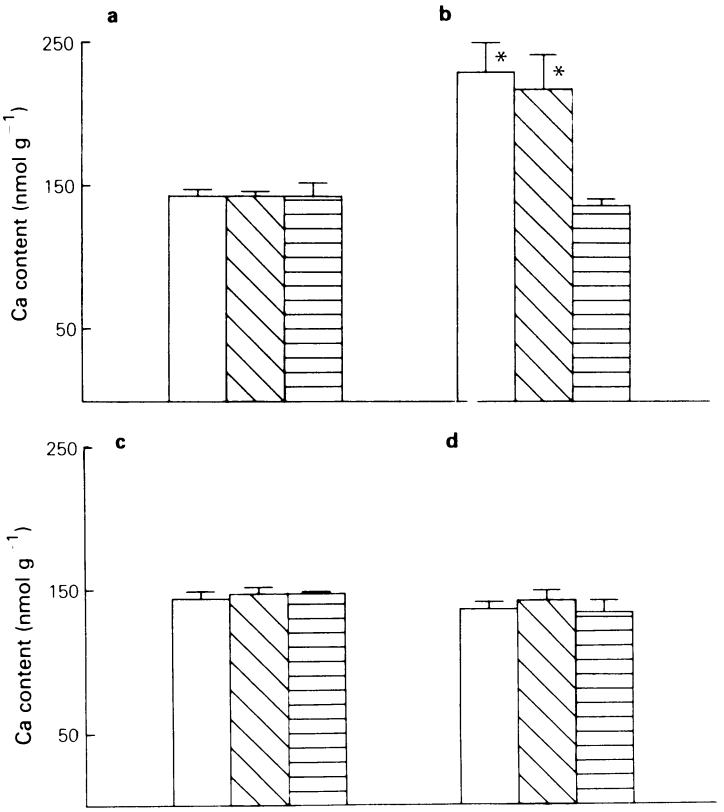

Figure 3 Lanthanum-resistant Ca content of guinea-pig tracheal smooth muscle (devoid of cartilage). (a) Basal i.e., unstimulated Ca-uptake and (b) KCl-stimulated uptake (90 mmol l^{-1}). (c) Leukotriene D_4 (LTD_4) at a low (3 nmol l^{-1}) and (d) at a high (50 nmol l^{-1}) concentration. Open columns, control; diagonally hatched columns, flurbiprofen present throughout (1 μmol l^{-1}); horizontally hatched columns, flurbiprofen (1 μmol l^{-1}) and verapamil (1 μmol l^{-1}) both present throughout (see Methods for full description). Each column represents the mean of 4–10 observations and vertical lines show s.e.mean. * Denotes significantly different from control ($P < 0.001$, unpaired t test).

ly 25% (EC_{25}; 3 nmol l^{-1}) and 90% (EC_{90}; 50 nmol l^{-1}) of the maximum contractile response to LTD_4 were selected for study and compared with effects produced by an EC_{90} of KCl (90 mmol l^{-1}). The results are summarized in Figure 3. LTD_4 failed to stimulate (or inhibit) Ca-uptake at either of the concentrations used. In contrast, and as one would expect, KCl significantly increased the lanthanum-resistant Ca-content by about 60% over basal levels. The presence of flurbiprofen in the medium did not affect these results.

Verapamil (1 μmol l^{-1}) was without effect on basal Ca-uptake nor did it modify the LTD_4 response. The KCl-stimulated Ca-uptake was, however, completely inhibited by verapamil.

Discussion

These results show that LTD_4 does not stimulate Ca-uptake into guinea-pig airway smooth muscle at concentrations which produce approximately 25% and 90% of its maximum contractile response. In contrast, but in accord with previously published work (Foster *et al.*, 1983), KCl increased the lanthanum-resistant Ca-content of the airway smooth muscle, an effect that was completely inhibited by verapamil. These data, therefore, are consistent with the hypothesis that in this tissue LTD_4 utilizes principally an intracellular source of Ca^{2+} to initiate contraction whereas KCl is dependent upon uptake of Ca^{2+} from the extracellular compartment.

Verapamil, however, reduced the contractile responses to *both* LTD_4 and KCl. Almost identical effects have recently been obtained by Weichman *et al.* (1983). In the case of KCl these findings can be interpreted as an inhibition by verapamil of Ca^{2+}-uptake via voltage-dependent Ca^{2+} channels opened as a consequence of KCl-induced membrane depolarization (Coburn, 1977; Bolton, 1979; Foster *et al.*, 1983; 1984). Such an explanation, however, is not tenable for LTD_4. Since we could detect no increase in Ca-uptake at either of the concentrations of LTD_4 used, the effect of verapamil on LTD_4-induced contractions cannot be attributed to an inhibition of Ca^{2+}- influx from the extracellular compartment. At present we are unable to advance a suitable explanation for the effects of verapamil on LTD_4-induced contractions other than to invoke an intracellular mechanism of action directed against Ca^{2+} release initiated by LTD_4.

The observation that cartilage present in the tracheal preparations could contribute to the total uptake of Ca by the tissue is in complete agreement with the recent findings of Foster *et al.* (1983). Also in accord with these authors' results is our observation that KCl stimulation reduced the lanthanum-resistant Ca content of tissue containing cartilage. In the present study, the values obtained for the lanthanum-resistant Ca content of the tissues are significantly lower than those observed by Foster *et al.* (1983). This is most probably attributable to differences in methodology, in particular the concentrations of $LaCl_3$ employed to displace extracellularly-bound ^{45}Ca and reduce ^{45}Ca loss from within the cells (50 mmol l^{-1} $LaCl_3$ in the present study, whereas 10 mmol l^{-1} was used by Foster *et al.* (1983)).

Recently attention has been focused on the potential use of calcium channel blocking drugs in the treatment of bronchospasm associated with asthma (McFadden, 1981; Triggle, 1983). However, with the possible exception of exercise-induced asthma (Barnes *et al.*, 1981; Cerrina *et al.*, 1981; Patel, 1981a,b) there is little convincing evidence, either *in vitro* or *in vivo*, that voltage-dependent Ca^{2+}-channel blocking drugs inhibit bronchoconstriction induced by a variety of different spasmogens (Patel, 1981c; Patel & Al-Shamma, 1982; Advenier *et al.*, 1983; Cerrina *et al.*, 1983; Drazen *et al.*, 1983; Russi *et al.*, 1983; Weiss & Mullick, 1983), at other than high concentrations. If the results described in this study in guinea-pig airways can be applied to man, they may provide a possible explanation for these findings. In the event that leukotrienes in human airways depend upon an intracellular rather than an extracellular source of Ca^{2+} to support contraction, then the absence of membrane translocation of Ca^{2+} via voltage-dependent channels would render the currently available Ca^{2+}-channel blocking drugs ineffective as antagonists of leukotriene-induced bronchospasm. Since the leukotrienes (SRS-A) are considered to be important chemical mediators of asthma (see Introduction for references) such Ca^{2+}-channel blocking drugs would therefore be of limited clinical value as bronchodilators. Clearly before such an explanation can be accepted further work using electrophysiological and ^{45}Ca-flux techniques is required especially in human airway smooth muscle treated with a wide variety of different bronchoconstrictor drugs.

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