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LEUKOTRIENE EFFECT IN AIRWAYS SMOOTH MUSCLE: CALCIUM DEPENDENCY AND VERAPAMIL INHIBITION

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ABSTRACT

The extracellular calcium $(Ca^{++})_E$ dependency of the synthetic leukotrienes (LT) C_4, D_4 and E_4 and inhibition of LTC_4 by verapamil was demonstrated on guinea pig trachealis *in vitro*. LTC_4 exhibited an intrinsic potency 170 X greater than histamine. Tension development by LTC_4, D_4 and E_4 was equipotent at 2.5 mM $(Ca^{++})_E$. Tension studies in 0.5 mM $(Ca^{++})_E$ indicate these three leukotrienes to be $(Ca^{++})_E$ dependent with a potency ranking of $LTC_4 > LTD_4$ and LTE_4 ($P < 0.05$ and $P < 0.001$ respectively). Inhibition of LTC_4 by verapamil yielded an IC_{50} of approximately 1.1×10^{-4}M with complete inhibition at 2.2×10^{-4}M; this antagonism was non-competitive. The specific dependency of verapamil inhibition of LTC_4 to $(Ca^{++})_E$ was demonstrated by reversal of verapamil antagonism with 5.0 mM $(Ca^{++})_E$. Synthetic leukotrienes C_4, D_4 and E_4 are dependent upon $(Ca^{++})_E$ for their myotropic activity, an action non-competitively inhibited by the calcium channel blocker verapamil.

INTRODUCTION

Recent studies have characterized the leukotrienes, a group of extremely potent biological substances derived from arachidonic acid via lypoxygenase pathways. These lipid substrates are distinct from prostaglandins and thromboxanes and are believed to be the active components of SRS-A (1). The leukotrienes (LT) may be released by immunological events

(viz. anaphylaxis) or be released by non-specific agents as calcium ionophores from mast cells, mononuclear macrophages and granulocytes (2,3). Following release, native leukotrienes have been shown to be highly active in smooth muscle including the airways of animal and human sources by evoking a uniquely slow but potent contraction. For example, available dose-response studies indicate that leukotrienes are approximately 100 times more potent than histamine in contracting guinea pig trachealis and 50 to 500-fold more active than histamine in ileal smooth muscle (4,5). The structural elucidation of these important metabolites of arachidonic acid, LTC_4, LTD_4 and LTE_4, permitted their subsequent chemical synthesis and pharmacologic evaluation. Thereafter the potent biological activity of these synthetic leukotrienes upon airways smooth muscle was demonstrated in several studies (6,7) to be qualitatively similar to those previously reported for impure extracts of SRS-A (8). As agonist-induced smooth muscle contraction is dependent upon calcium $(Ca^{++})_E$ for contraction, we examined the influence of extracellular Ca^{++} upon guinea pig isometric trachealis tension for the synthetic leukotrienes LTC_4, LTD_4 and LTE_4. In addition, as we have reported total inhibition of airways smooth muscle tension in experimental *in vitro* anaphylaxis by the calcium channel antagonist verapamil, the action and mechanism of this antagonist was evaluated against these synthetic leukotrienes (9).

METHODS AND MATERIALS

Adult male Hartley guinea pigs (Elm Hill Breeding Laboratory, Chelmsford, Mass., U.S.A.) weighing 450 to 600 g were sacrificed by stunning and exsanguination. The trachea was removed, placed in a Krebs-Henseleit (KH) buffer at 37° C, gassed with 95% O_2 and 5% CO_2 and cut into rings (10). For tension measurements the cartilage was cut, and one end was fastened with No. 50 cotton thread to a fixed clamp; the other end was fastened with a thin piece of platinum wire to a Grass FT03C force displacement transducer amplified by a Hewlett-Packard 8805B amplifier to record isometric tension changes, in milligrams, on a precalibrated Hewlett-Packard 7754A thermal tip polygraph; full scale was 5 g. The muscle was oriented parallel to the direction of the force displacement. The strips were suspended at 37° C under 2 g of initial tension in a 20 ml chamber (Harvard) containing 10 ml KH solution aerated with 95% O_2 and 5% CO_2. Optimal length-tension relationships for these experiments were determined by exposing the muscle to increasing tensions of 0.25, 0.5, 1.0, 2.0 and 3.0 g against histamine, 1.0 µg/ml; verification of the 2 g resting tension was conducted periodically. Daily variation in mean maximal tension to 1.0 µg/ml histamine in our preparation is $\leq$ 10%, with mean maximal values of 1200 $\pm$ 120 mg (SE). Resting tension was less than 10% of the maximum active tension generated. All tracheas were analyzed for force development, and those exhibiting baseline instability ($\pm$ 10%), inconsistent rates or erratic responses were discarded. All preparations were initially equilibrated for 60 minutes with the bath fluid changed three times. Before any new experiment the bath was flushed three times, and the muscles re-equilibrated for 10 minutes. PO_2, PCO_2 and pH were monitored in an 813 blood gas analyzer (Instrumentation Laboratories, Inc.); PCO_2 and PO_2 ranged from 33 to 44 mm Hg and 350 to 550 mm Hg respectively, with a mean pH of 7.42 $\pm$ 0.02.

Solutions and Drugs

The Krebs-Henseleit buffer was prepared as follows: NaCl, 118.1 mM; KCl, 4.7 mM; $NaHCO_3$, 24.8 mM; $CaCl_2$, 2.52 mM; $MgSO_4.7H_2O$, 2.4 mM; KH_2PO_4, 1.10 mM; glucose, 10 mM; in distilled, deionized water. Histamine dihydrochloride, acetylcholine (Sigma Pharmaceuticals) and verapamil HCl (gift of Knoll Pharmaceuticals) were prepared daily in distilled, deionized water to produce the desired final bath concentrations; volume additions to the 10 ml bath volume were 0.10 ml or less. Synthetic LTC_4, LTD_4 and LTE_4 (as free acid) were the generous gifts of Merck Frosst Laboratories, Montreal, Canada. These were solubilized in methanol (90%), freshly prepared daily, with final organ bath methanol concentrations less than 0.10%, this concentration having no significant effect on the trachealis preparation.

A fresh trachealis ring was employed for each individual drug study. Due to leukotriene supply limitations, cumulative dose-response was employed where feasible. Preliminary experiments indicated that unit dose LTC_4 1.6×10^{-7} M gave the same approximate maximal response as cumulative dosing. Hence we did not observe significant tachyphylaxis in guinea pig trachealis under these conditions, similar to the findings of Ghelani et al. in human bronchus (11). In addition the stability and potency of our synthetic leukotrienes were bio-assayed daily in our preparation; random mean tensions on control trachealis was always in the range of 1100 mg $\pm$ 150 mg (SE) for LTC_4 1.6×10^{-7} M.

Statistical Analysis

Data were analyzed for competitiveness by regression analysis for determination of slope and linearity. Slopes were compared by a Student's t test utilizing a pooled residual mean square with a P value < 0.05 for significance. Two population regression co-efficients were also analyzed by variance ratio tests (12). Student's t test was employed for difference of means ($\pm$ SE). All calculations were computed by a Hewlett-Packard Model 9810A Calculator and Hewlett-Packard statistical programs. Dose-response data were plotted arithmetically and double-reciprocal (Lineweaver-Burk) graphs were also constructed for determination of competitiveness. Estimates of median effective dose (EC_{50}) were determined visually.

RESULTS

1. Effect of leukotriene C_4 (LTC_4), histamine and acetylcholine on tension.

The response to LTC_4 in our guinea pig trachealis system was characterized by a slow and sustained increase in isometric tension compared to agonist histamine. For example, in an average experiment employing maximal concentrations, 1.6×10^{-5}M histamine induced a tension response within 30 seconds, reached a maximum in 5 - 10 minutes and then decayed to 1/2 maximum by 20 minutes (Figure 1). In contrast, LTC_4 (1.6×10^{-7}M) induced a slow increase in tension commencing in 60 seconds, attaining a

maximum tonic plateau in approximately 20 - 30 minutes with a decay to 1/2 maximum in 3 - 5 hours, even with multiple KH bath buffer exchanges. The threshold concentration for LTC_4 was $1.5 \times 10^{-9}M$.

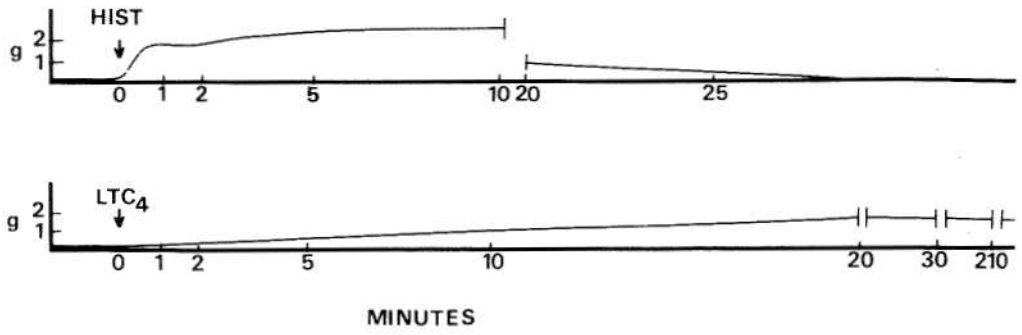

Figure 1. Isometric tension responses to histamine ($1.6 \times 10^{-5}M$) and LTC_4 ($1.6 \times 10^{-7}M$) in guinea pig trachealis muscle. Ordinate isometric tension in grams, abscissa time; temporal interruptions indicated on tension curves. Redrawn from original tracings.

Cumulative, time-independent, concentration-response curves for LTC_4, histamine and acetylcholine are shown in Figure 2. The mean maximal isometric tension to these three agonists was greater with histamine (2250 mg), while LTC_4 and acetylcholine exhibited equimaximal tensions approximately 1/2 that of histamine. The mean EC_{50} values derived from these plots are as follows: LTC_4 $3.2 \times 10^{-8}M$, histamine $5.5 \times 10^{-6}M$, acetylcholine $1.5 \times 10^{-6}M$. Hence on a molar basis, the intrinsic potency of LTC_4 is 170-fold greater than histamine and 46-fold greater than acetylcholine in trachealis smooth muscle.

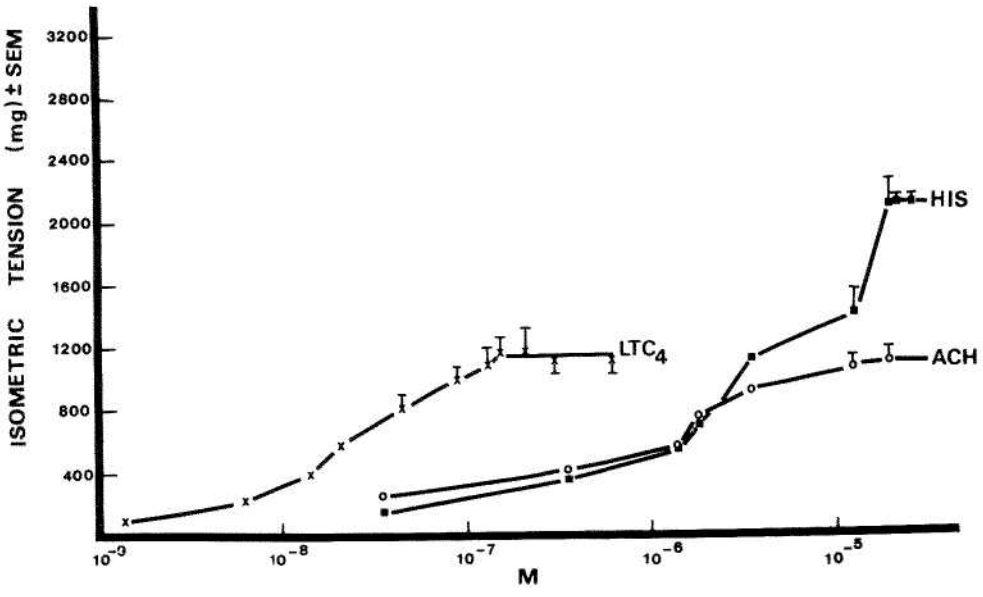

Figure 2. Cumulative dose-response of guinea pig trachealis muscle to LTC_4 (1.6×10^{-9} to 8.0×10^{-7}M, n = 8); histamine (5.5×10^{-8} to 3.8×10^{-5}M, n = 7); and acetylcholine (5.5×10^{-8} to 1.6×10^{-5}M, n = 7). (Note that unit dose LTC_4 at 1.6×10^{-7}M yields 1150 mg tension.) See text.

2. Dependency upon extracellular calcium.

The dependency of isometric trachealis tension to LTC_4 upon extracellular calcium $(Ca^{++})_E$ was documented over a range of 0 to 2.52 mM $(Ca^{++})_E$ (see Figure 3A). In 0 mM $(Ca^{++})_E$ no tension was elicited by LTC_4 up to 3.2×10^{-8}M. The relative potencies of the synthetic leukotrienes LTC_4, LTD_4 and LTE_4 were then compared at 3.2×10^{-8}M (the EC_{50} of LTC_4) at 0.5 mM and 2.52 mM $(Ca^{++})_E$. The results (Figure 3B) reveal two salient features. First, LTD_4 and LTE_4 are shown to be dependent upon $(Ca^{++})_E$ as was LTC_4. At 2.52 mM Ca^{++}, LTC_4 is statistically equipotent to LTD_4 and LTE_4 in tension development ($P > 0.5$, $P > 0.4$, respectively), although LTC_4 exhibits a tendency toward greater myoactivity. Second, at subphysiologic

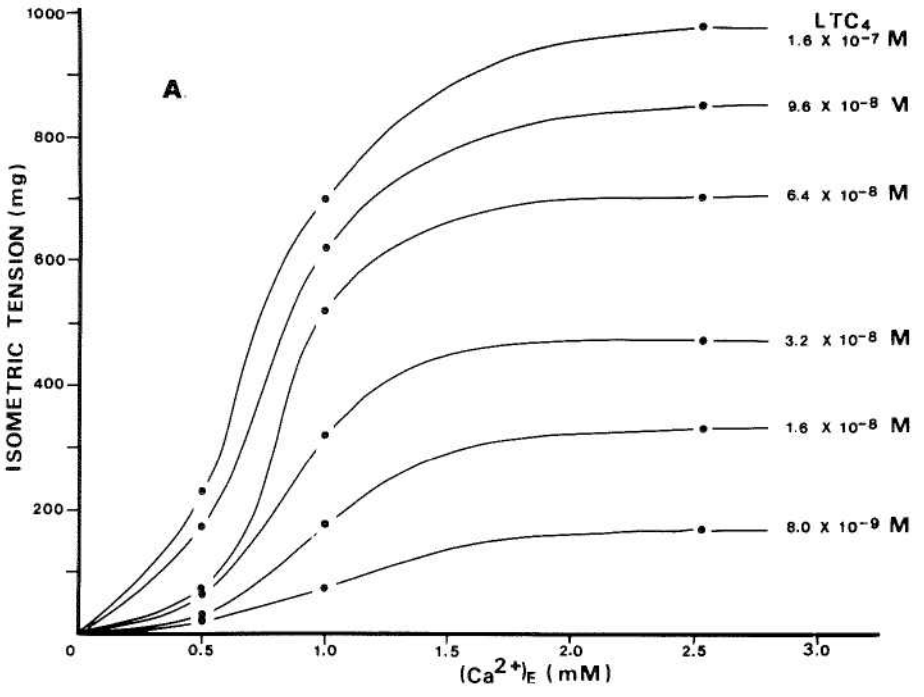

Figure 3A. Extracellular calcium $(Ca^{++})_E$ dependence of LTC_4 induced isometric tension of guinea pig trachealis muscle. Cumulative dosing was employed for each calcium concentration (mM). Each point represents mean of three experiments. Similar data exist at 0 mM $(Ca^{++})_E$; incubation time was one hour in Ca^{++}-free medium prior to LTC_4 dose. All data observations are at 20 minutes.

$(Ca^{++})_E$ 0.5 mM, all leukotrienes tested yield a reduced isometric tension with a potency order of LTC_4 > LTD_4 and LTE_4. Statistically this ranking was shown to be significantly different: LTC_4 vs. LTD_4, $P < 0.05$, and LTC_4 vs. LTE_4, $P < 0.001$ ($n = 4$ for all cited experiments). From the individual data of these observations we computed the percentage reduction in isometric tension in 2.52 mM vs. 0.5 mM Ca^{++} for each leukotriene tested. The mean percent $\pm$ SE reductions were as follows: $LTC_4 = 27.0 \pm 7.1\%$, $LTD_4 = 35.5 \pm 9.6\%$, $LTE_4 = 50.0 \pm 3.7\%$. Significance values were LTC_4 vs. LTD_4 $P = 0.5$, LTC_4 vs. LTE_4 $P < 0.02$, LTD_4 vs. LTE_4 $P = 0.2$.

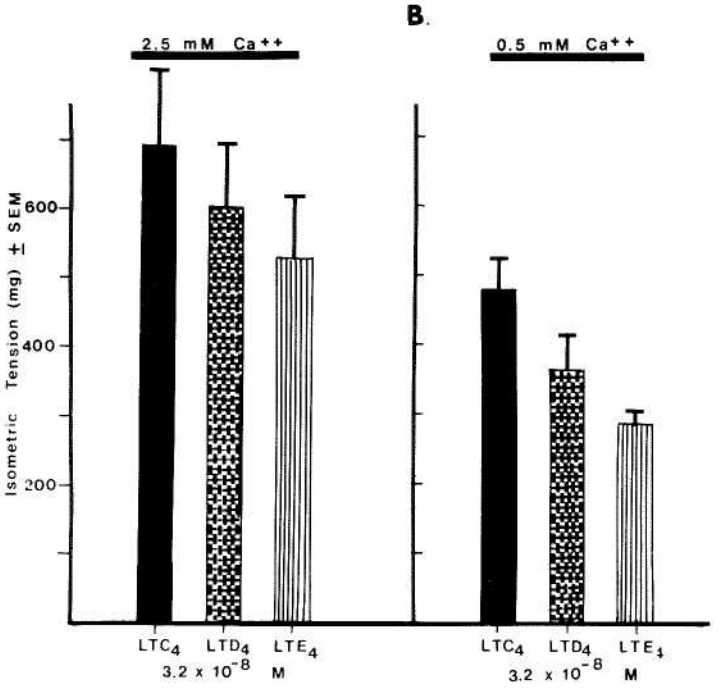

Figure 3B. Comparison of LTC_4, LTD_4, LTE_4 potency and calcium dependency at 2.5 and 0.5 mM $(Ca^{++})_E$, at an equimolar concentration of 3.2×10^{-8}M (the determined EC_{50} of LTC_4). $P < 0.05$ for LTC_4 at 2.5 mM vs. 0.5 mM Ca^{++}. N = 4 for each leukotriene. See text.

3. Inhibition by verapamil.

Because of its qualitatively greater intrinsic potency, LTC_4 was selected for detailed study. A concentration-dependent inhibition of LTC_4 myoactivity was observed following pretreatment of the trachealis preparation with verapamil. Complete inhibition occurred with 2.2×10^{-4}M verapamil (Figure 4). Depression of the absolute maximal tension was also confirmed by construction of a double-reciprocal plot. Additionally, initial slopes were determined from linear regression with a four point assay and compared: LTC_4 control mean slope 1.3 ± 0.8 (n = 8); LTC_4 plus verapamil 1.1×10^{-4}M mean slope 0.46 ± 0.10 (n = 5, $P < 0.01$ compared to LTC_4 control); LTC_4 plus verapamil 1.5×10^{-4}M mean slope 0.23 ± 0.11 (n = 5, $P < 0.001$ compared to LTC_4 control). Hence a reduction in maximal contractile tension with a non-parallel rightward displacement of the concentration-response curve was observed with verapamil.

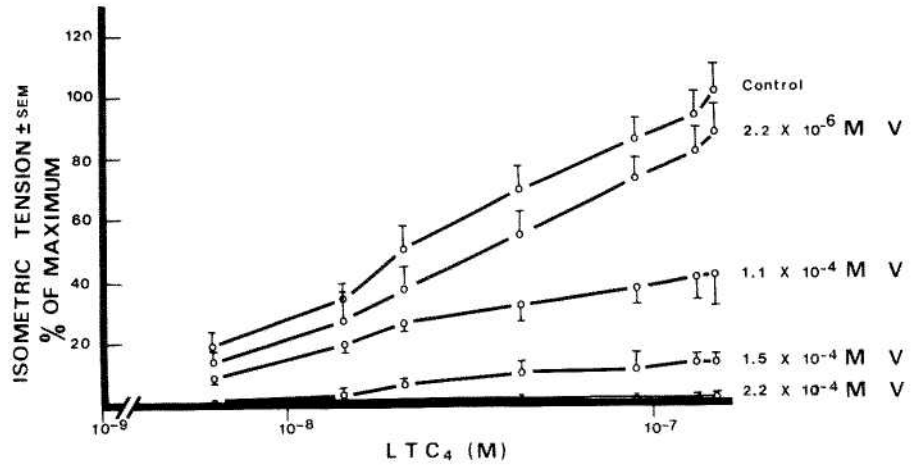

Figure 4. Inhibitory effect by verapamil pretreatment on isometric tension produced by LTC_4. Inhibition of LTC_4 was calculated from the point of maximal phasic tension at 20 - 25 minutes following agonist exposure by cumulative dose response. Each data point is 5 - 8 experiments. Complete inhibition occurred with 2.2×10^{-4}M verapamil, with an approximate 50% inhibitory concentration (IC_{50}) of 1.1×10^{-4}M. See text.

Finally, the specific dependency of verapamil antagonism to LTC_4 upon extracellular calcium was demonstrated by comparing the inhibitory action of verapamil first at 2.5 mM and then at 5.0 mM $(Ca^{++})_E$ (Figure 5). In this experiment, verapamil effect was compared in four separate groups of muscles: a) control LTC_4 cumulative dose-response in 2.5 mM $(Ca^{++})_E$, b) identical LTC_4 cumulative dose-response with 1.1×10^{-4}M verapamil in 2.5 mM $(Ca^{++})_E$, c) control LTC_4 cumulative dose-response in 5.0 mM $(Ca^{++})_E$, and d) identical LTC_4 cumulative dose-response with 1.1×10^{-4}M verapamil in 5.0 mM $(Ca^{++})_E$. The inhibition by verapamil at 2.5 mM Ca^{++} was reversed in 5.0 mM $(Ca^{++})_E$ as measured by analysis of maximal tension and slope of the dose-response curve. (See Figure 5 legend.) 5.0 mM $(Ca^{++})_E$ had no significant effect upon the control responses of LTC_4 as compared to 2.5 mM $(Ca^{++})_E$ ($P = 0.4$).

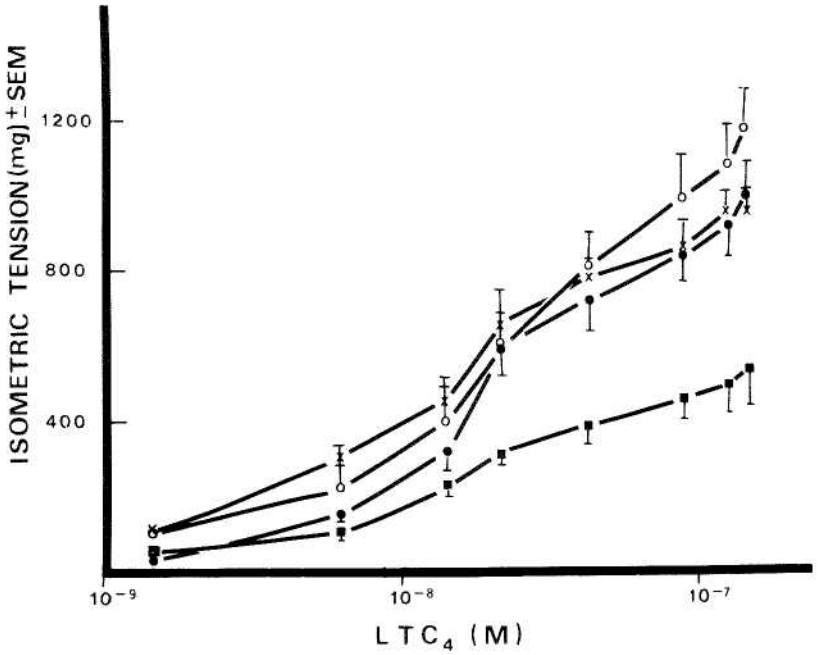

Figure 5. Reversal of verapamil inhibition of LTC_4 with 5.0 mM $(Ca^{++})_E$. 0 = cumulative dose - isometric tension response to LTC_4 at 2.5 mM $(Ca^{++})_E$, control muscles; X = cumulative dose - response of LTC_4 in 5.0 mM $(Ca^{++})_E$, control muscles; ■ = effect of verapamil (1.1×10^{-4}M) inhibition of LTC_4 response at 2.5 mM $(Ca^{++})_E$; ● = reversal of verapamil (1.1×10^{-4}M) inhibition at 5.0 mM $(Ca^{++})_E$. Mean tension at 1.6×10^{-7}M LTC_4 and 5.0 mM $(Ca^{++})_E$ with verapamil was not statistically different from control muscle tension at 2.5 mM or 5.0 mM $(Ca^{++})_E$ at this same LTC_4 concentration ($P = 0.3$). Each data point represents five experiments ± SE after 20 minutes of agonist incubation. Slope changes are visually apparent.

DISCUSSION

This study demonstrates an extracellular calcium $(Ca^{++})_E$ dependency of synthetic leukotrienes upon isometric tension in guinea pig trachealis smooth muscle and their subsequent inhibition by the calcium channel blocking agent verapamil. As in previous reports, we observed an LTC_4 isometric tracheal tension response which was significantly slower to develop (and to relax) and of greater duration compared with histamine (Figure 1), features of a slow-reacting substance (4,7). It has also

been reported that LTC_4 and LTD_4 contract small as well as large (central) airways while LTB_4 is largely active in peripheral airways in both human and guinea pig models (13). Interestingly, Morris et al. documented the release of LTB_4 in substantial concentrations from guinea pig lung during anaphylaxis (ANA), suggesting a role for leukotrienes in the bronchoconstriction associated with ANA (2).

The maximal histamine contracture *in vitro* was greater than any leukotriene evaluated, although such maximal concentrations are unlikely to exist under physiologic conditions. For example, the maximal response to histamine (1.6×10^{-5}M) was a mean force of 2250 mg while LTC_4 at the maximal concentration tested (1.6 to 2.2×10^{-7}M) yielded a mean maximal force of 1200 mg. However, the relative potency of LTC_4 estimated from EC_{50} values indicate a physiologic effect in trachealis approximately 170-fold greater than histamine. Similar potency data has been reported. For example, Sirois et al. in a superfusion system rated synthetic LTC_4 and LTD_4 70 X more active than histamine on guinea pig trachealis (7), and Holme et al. determined LTC_1 potency to be 1000 X on guinea pig trachealis and 100 X on ileum compared to histamine (6). These data are consistent with other results showing LTC isolated from mouse mastocytoma to be two orders of magnitude more potent than histamine (14). In the cited study of Sirois et al., LTA_4, LTB_4, LTC_4 and LTD_4 were determined to be over 100 X more potent than histamine in guinea pig lung parenchymal strips.

The dependency of myocontraction induced by synthesized leukotrienes upon the extracellular calcium concentration has not been reported before, but it is consistent with a similar influence of calcium upon other smooth muscle agonists as histamine, acetylcholine, $PGF_{2\alpha}$ and depolarizing potassium (15). In the present investigation, in 2.5 mM $(Ca^{++})_E$ while there was a potency trend in isometric tension of $LTC_4 > LTD_4 > LTE_4$, these leukotrienes were equipotent by statistical analysis. However, in 0.5 mM $(Ca^{++})_E$, this potency rank differential was statistically validated with $LTC_4 > LTD_4$ and LTE_4, albeit with a small magnitude difference. The possibility of a slightly greater dependence by LTE_4 than LTC_4 upon $(Ca^{++})_E$ is suggested by its relatively greater percentage decrement (50%) compared to LTC_4 ($P < 0.02$) in 0.5 mM Ca^{++}. In general, LTC_4 and LTD_4 exhibit equipotency in guinea pig trachea at physiologic calcium concentrations, whereas LTD_4 is 10 X more active in ileum (7). Intact animal data indicate LTD_4 more potent than LTC_4 when administered intravenously in unanesthetised guinea pigs (4). In human bronchial studies *in vitro*, Dahlén et al. observed approximate equipotency of LTC_4 and LTD_4 (16). While a recent evaluation in normal human subjects with aerosol drug delivery revealed a substantial potency of LTC_4 against histamine, comparison with LTD_4 was not provided (17). Differences in leukotriene responses in airways may vary for a variety of experimental, biological or pharmacological reasons; the reader is referred to the excellent discussion by Sirois et al. on this subject (7). Nevertheless, the physiological consequences of small *in vitro* differences in leukotriene potency may not be significant, as Samuelsson has detailed, notably since the composition of native SRS-A represents a mixture of LTC and LTD whose effect will depend upon the conditions for their generation, their relative concentrations and bioactivity, features which are yet undefined (18).

We have recently reported the inhibition of isometric tension following passive *in vitro* ANA in guinea pig trachealis by verapamil with a mean IC_{50} of $2 \times 10^{-4}M$ in a protection design experiment (9). We also observed the inhibitory action of verapamil upon receptor-initiated contractures (histamine) and depolarization-initiated contraction with 80 mM KCl (9). Concurrently, the influence of $(Ca^{++})_E$ in ANA including mast cell mediator release and smooth muscle force attributable to SRS-A has been detailed (19,20). Because of these observations and the $(Ca^{++})_E$ dependency of the synthetic leukotrienes observed in the present study, we evaluated the inhibitory action of the calcium channel blocker verapamil against LTC_4. A concentration-related inhibition of LTC_4 by verapamil was observed with an IC_{50} of $\sim 1.1 \times 10^{-4}M$ and with 100% inhibition at $2.2 \times 10^{-4}M$ verapamil (Figure 4). These inhibitory concentrations of verapamil are, interestingly, of the same order of magnitude for both the Schultz-Dale ANA reaction and for synthetic leukotrienes in guinea pig trachealis (9). This inhibition, characterized by both a depression of the maximum response and a non-parallel rightward shift of the concentration-response curve, is traditionally interpreted as non-competitive inhibition (21). Double-reciprocal analysis (data not shown) also confirmed this form of antagonism of verapamil to LTC_4.

The interaction of verapamil with calcium has been shown in rabbit aorta, guinea pig gastric strips and other models to be a reversible antagonism, defined as a reversion of inhibition when the extracellular calcium concentration is increased (22). We confirmed the specific dependency of verapamil inhibition in airways smooth muscle by similarly increasing the $(Ca^{++})_E$ from 2.5 to 5.0 mM thereby overcoming the verapamil inhibition of LTC_4 (Figure 5). From these observations, we conclude the nature of verapamil inhibition of LTC_4 myocontraction is via an influx antagonism, an effect not specific for the LTC_4 receptor site. At the observed molar inhibitory concentration of $10^{-4}M$ verapamil, it is possible that verapamil may not be highly specific in inhibiting slow calcium channels. Current interpretations of the potency of verapamil inhibition in smooth muscle has been generally based upon observations in non-airway smooth muscle models (e.g. vascular tissue). However, data does exist for an IC_{50} verapamil concentration in this molar range for non-lung tissue (23). In fact, Goodman has recently stressed that currently available calcium channel blockers must be employed in airways smooth muscle at considerably greater concentrations than those used with vascular smooth muscle (24). In addition the binding of labelled calcium antagonists in cardiac muscle is of higher affinity (371 $fmol^{-1}$ of protein) compared to an intermediate activity of lung tissue (106 $fmol^{-1}$) (25). Hence at present it is clear that some controversy remains concerning this issue. While selective antagonism of leukotriene myocontraction by FPL 55712 has been reported (26), Krell et al. have recently expressed caution in interpreting the mechanisms of action of this inhibitor (27). No studies to date have linked calcium dynamics or calcium inhibitors with leukotriene activity in airways smooth muscle. However, in guinea pig ileal longitudinal smooth muscle, Findlay et al. have shown that partially purified SRS-A and purified LTD_4 could be inhibited by D-600 by approximately 40% and 60% respectively (28). While it is suggested that verapamil or nifedipine may be effective inhibitors of human asthma, available studies do not provide direct evidence for specific leukotriene antagonism.

Nevertheless our *in vitro* data supports the hypothesis that extracellular calcium highly influences tracheal smooth muscle activity to the leukotriene components of SRS-A, which are intimately involved in the pathophysiology of allergic disorders. Furthermore it is presumed that calcium channel antagonists may affect not only the penetration of extracellular calcium through the cell membrane, but also the movement of calcium in the cytosol (29). Hence inhibition by presumably "non-specific" calcium channel blockers as verapamil may be effective not only against leukotrienes, but also against other agonists which exhibit a qualitatively similar dependence upon calcium activation of various cellular sources for their smooth muscle contractile activity. We have previously indicated that non-specific calcium inhibition by local anesthetics, e.g. lidocaine, may serve as a model for influencing calcium in smooth muscle airways activity, although the sites of action of local anesthetics may be other than via calcium channels (10). The use of calcium antagonists of whatever drug class permits not only the clarification of basic mechanisms, but also illustrates the potential therapeutic application to human airway reactive diseases such as asthma.

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