Bioconversion of C-6 Sulfidopeptide Leukotrienes by the Responding Guinea Pig Ileum Determines the Time Course of its Contraction

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ABSTRACT The naturally occurring sulfidopeptide leukotrienes, leukotriene (LT) C_4 (LTC_4) [5(*S*)-hydroxy-6(*R*)-S-glutathionyl-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid] and its cysteinylglycine (LTD_4) and cysteinyl (LTE_4) analogs, which are derived by peptide cleavage, differ in the concentrations required to elicit comparable contractions of the guinea pig ileum, with respective potencies of 1.2:5:1. The effect of the ongoing bioconversion of LTC_4 and LTD_4 on the contractile response of the guinea pig ileum to each was determined by recording the pattern of the contraction and quantitating the initial agonist and its metabolic products. The contraction was elicited by radiolabeled agonist, and its conversion products were sampled at defined intervals and resolved by their retention times on reverse-phase high performance liquid chromatography. After a latent period of 60 s, LTC_4 initiated a linear response, followed by a slower, progressive response to a maximum level that was maintained without relaxation. The metabolic conversion of LTC_4 was <5% during the linear phase of contraction and complete inhibition of bioconversion of LTC_4 to LTD_4 by the presence of serine-borate complex did not alter the pattern of the spasmogenic response. As the maximum response in the presence of serine-borate complex was three-quarters of that obtained without the inhibitor of bioconversion, the predominant response was to LTC_4 itself. The spasmogenic response of the ileum to LTD_4 was immediate, linear to a maximum level, and immediately followed by a marked relaxation. That the failure of LTD_4 to sustain a contraction was due to its immediate, rapid, and quantitative conversion to the less potent LTE_4 was established by pharmacologically inhibiting and anatomically deleting the converting activity. In the presence of L-cysteine the conversion of LTD_4 to LTE_4 was largely inhibited and the maximum contractile response was well maintained. After anatomic removal of the mucosa that contained the LTD_4 dipeptidase activity, the longitudinal smooth muscle preparation gave a maximal response to LTD_4 that was fully maintained. Thus, bioconversion is not a prerequisite for the spasmogenic activity of LTC_4 and accounts for the transient response of the ileum to LTD_4.

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INTRODUCTION

The C-6 sulfidopeptide leukotrienes (LT)[1] LTC_4, LTD_4, and LTE_4 together constitute the biological activity ascribed to slow reacting substance of anaphylaxis (1–5). Their biosynthesis is initiated by the oxidative metabolism of arachidonic acid by 5-lipoxygenase to yield 5-hydroperoxyeicosatetraenoic acid, which is converted enzymatically to LTA_4, 5-6-oxido-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid (6). LTC_4, 5(*S*)-hydroxy-6(*R*)-S-glutathionyl-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid is formed via conjugation of

[1] *Abbreviations used in this paper*: LT, leukotriene; RPHPLC, reverse-phase high performance liquid chromatography.

LTA_4 with glutathione by a glutathionyl-S-transferase (7). Glutamic acid and glycine are sequentially cleaved from the glutathionyl domain at the C-6 to yield LTD_4, 5(*S*)-hydroxy-6(*R*)-S-cysteinyl-glycine-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid; and LTE_4, 5(*S*)-hydroxy - 6(*R*) - S - cysteinyl - 7,9 - *trans*,11,14 - *cis* - eicosatetraenoic acid, respectively (2–5). Formation of the eicosatetraenoic acid metabolite, LTD_4, and the eicosatrienoic acid metabolite, LTD_3, by cleavage of glutamic acid from LTC_4 and LTC_3, respectively, has been demonstrated with partially purified γ-glutamyl transpeptidase (8, 9), and more recently with a homogeneous preparation of this enzyme (10). Conversion of LTD_4 to LTE_4 by cleavage of the carboxy terminal glycine has been separately achieved with highly purified rat kidney dipeptidase and aminopeptidase (10). LTC_4 is also converted to LTD_4 and to LTE_4 in vitro by a number of cell types and tissue extracts (11–16) and these bioconversions are suppressed by agents capable of inhibiting γ-glutamyl transpeptidase and dipeptidases, respectively (9, 12, 17).

In vitro studies of the amounts of agonist that are equally potent in eliciting spasmogenic activity have yielded molar ratios of $LTC_4/LTD_4/LTE_4$ for guinea pig ileum of 4.2:1:5, for guinea pig parenchymal lung strips of 100:1:30 (18), and of LTC_4/LTD_4 for human bronchial tissues of ~1:1 (19, 20). The molar ratio of $LTC_4/LTD_4/LTE_4$ for amounts that are equally potent in decreasing human and guinea pig myocardial contractility are 25:1:265 and 10:1:2,000, respectively (21). Thus, in in vitro studies, LTD_4 is generally the most potent and LTE_4 the least potent, but there is as much as a 2-log variation in the molar ratios of equal potencies with different tissues.

Possible explanations for these differences in relative potencies for the sulfidopeptide leukotrienes could relate to both receptor-determined and non–receptor-determined muscle functions. Receptor-determined differences could result from a separate receptor for each sulfidopeptide leukotriene; a single receptor with different affinities for each; or a single receptor that recognizes only LTD_4 and LTE_4, so that LTC_4 must be converted to LTD_4 to elicit a biological response. Substantially different rates of bioconversion and/or inactivation for each sulfidopeptide leukotriene by the responding tissue(s) would represent a second major variable. Functional inactivation of LTC_4 occurs during the respiratory burst of stimulated human polymorphonuclear leukocytes and is mediated by the action of hypochlorous acid to generate chiral LTC_4 sulfoxides and 6-*t*-LTB_4 diastereoisomers (22). However, there is no information on the metabolic conversion or inactivation of the sulfidopeptide leukotrienes during an elicited spasmogenic response or on the effect of such metabolism on the apparent spasmogenic potencies of these sulfidopeptide leukotrienes. To evaluate the effects of bioconversion and inactivation, a model was developed in which the metabolic processing of radiolabeled LTC_4 and LTD_4 was assessed quantitatively during the period when the nonvascular smooth muscle contractile response was initiated and maintained. These studies demonstrate that differences in rates of bioconversion represent one important variable in determining the intensity and time course of the elicited contractile response. In addition, the enzyme converting LTD_4 to LTE_4 is shown to reside in the mucosa rather than in the muscle layer of the guinea pig ileum.

METHODS

Materials. L-cysteine, L-serine, atropine sulfate, histamine diphosphate (Sigma Chemical Co., St. Louis, MO), and high performance liquid chromatography-grade methanol (Burdick & Jackson Laboratories, Muskegon, WI) were purchased from the manufacturers. LTC_4, LTD_4, and LTE_4 were prepared as previously described (5, 23, 24) and stored in 10 mM phosphate buffer (pH 6.8)/ethanol, 4:1 (vol/vol) at −70°C under argon until use. $[^3H]LTC_4$ (25 Ci/mmol) and $[^3H]LTD_4$ (25 Ci/mmol) were prepared as described (25) and provided by New England Nuclear (Boston, MA).

Assay of biological activity. A 4- to 6-cm segment of distal guinea pig ileum or ileal longitudinal muscle strip was placed in an organ bath at 37°C for recording isotonic contractions in response to the sulfidopeptide leukotrienes (26). Longitudinal muscle strips were prepared from the distal 10-cm segment of the ileum (27) by mechanically removing the outer layer of the longitudinal muscle.

Measurement of $[^3H]LTC_4$ and $[^3H]LTD_4$ bioconversion. 5 ng of $[^3H]LTC_4$ (1×10^5 cpm) or 3.6 ng of $[^3H]LTD_4$ (1×10^5 cpm) were added to an organ bath containing the smooth muscle preparation. At defined time intervals, 200-μl samples of the suspension medium were removed and added to 300 μl of methanol at −20°C. 25 μl of 0.5 N acetic acid were added, and the samples were centrifuged at 8,000 *g* for 2 min in a model B microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). Each of the supernatants was mixed with 0.5–1.0 μg of leukotriene standards and reverse-phase high performance liquid chromatography (RPHPLC) was performed with a C_{18} column and an isocratic solvent of methanol/water/acetic acid (65:34.9:0.1, vol/vol) pH 5.6, at a flow rate of 1 ml/min (2). Absorbance at 280 nm (A_{280}) was continuously monitored with an on-line Hitachi spectrophotometer. 1-ml fractions were collected and assessed for radioactivity after addition of Hydrofluor (National Diagnostics, Inc., Somerville, NJ). In selected experiments, the functional and biochemical responses to a radiolabeled leukotriene were determined a second time on the same ileal tissue after it had been thoroughly washed and preincubated for 10 min with 10 mM of either L-cysteine or serine-borate complex. The radiolabeled leukotrienes associated with the smooth muscle preparation were analyzed both after extensive washing and after extraction of the tissue. Washing was carried out with 2 ml of RPHPLC buffer (65% methanol, 34.9% water, and 0.1% acetic acid) at 37°C for 30 min and then overnight at −20°C followed by pooling of the wash buffers. The tissue was then homogenized in 80% ethanol by using a TRI-R STIR-R homogenizer (TRI-R Instruments, Inc., Rockville Centre, NY) at room temperature

for 5 min. The ethanol extract was cleared by centrifugation and the sediment washed with 10 ml of 80% ethanol. The particulate debris was again sedimented and the ethanolic solutions combined, dried in a rotary evaporator, and dissolved in the RPHPLC buffer. The ethanol-extracted debris was solubilized in 80% ethanol and one-third was counted in 10 ml of Aquasol (New England Nuclear). The pooled, wash-buffers and the ethanol extract were each analyzed and quantitated for their content of radiolabeled leukotrienes by RPHPLC and scintillation counting.

RESULTS

Bioconversion of LTC_4 and LTD_4 by responding guinea pig terminal ileum. In each of four experiments with 5 ng of $[^3H]LTC_4$ (1×10^5 cpm), the contractile response was delayed 60 s in onset. The contraction was then linear to 2 min, progressed to a plateau at 30 min, and maintained this maximum for the remainder of the 50-min observation period (Fig. 1). Minimal conversion, <5%, of $[^3H]LTC_4$ to $[^3H]LTD_4$ and $[^3H]LTE_4$ was apparent at 5 min. By 30 min, the time of maximum contraction, 24% of the $[^3H]LTC_4$ had been converted. At 50 min, with the maximal contraction still being maintained, conversion of $[^3H]LTC_4$ was 35%, with 8% appearing as LTD_4 and 27% as LTE_4. LTE_4 was detectable at 10 min and increased linearly up to 50 min at a rate of 8 fmol/cm segment per min. The recoveries of radiolabeled leukotrienes from the organ bath diffusate at each time point for the four experiments averaged 70±5% (mean±SD), and 72±10% of this activity was recovered from RPHPLC; no products other than LTC_4, LTD_4, and LTE_4 were identified. In a separate experiment, the C-6 sulfidopeptide leukotrienes that were in the diffusate and associated with the tissue were analyzed 12 min after introduction of the radiolabeled LTC_4. The ratio of $LTC_4/LTD_4/LTE_4$ in the diffusate that contained 52% of the recovered counts was 6.0:0.7:3.3; the leukotrienes that were associated with the tissue and releasable by washing were present in a similar ratio and represented 0.1% of the counts recovered. The ethanol extract of the tissue and the residual tissue debris contained no measurable counts.

In four experiments with 3.6 ng of $[^3H]LTD_4$ (1×10^5 cpm), the contractile response was virtually immediate and progressed linearly to a maximum amplitude within 40 s (Fig. 2). The maximum was not maintained and the response declined almost linearly from 1 to 6 min and then progressively until 11 min, at which point the response plateaued at ~20% of maximum. The $[^3H]LTD_4$ declined in a linear fashion with time, with the conversion to $[^3H]LTE_4$ reaching 82% by 25 min. The recoveries of radiolabeled leukotrienes from the organ bath diffusate at each time point for the four experiments averaged 90±7%, and 76±10% of this activity was recovered after RPHPLC; no products other than LTD_4 and LTE_4 were detected.

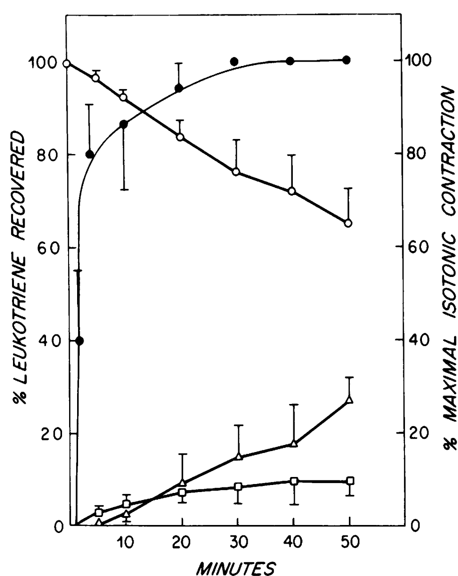

FIGURE 1 Time course of contractile response to 5 ng of $[^3H]LTC_4$ from guinea pig ileum, expressed as percentage of maximal response (● —— ●), and of the metabolism of $[^3H]LTC_4$ (○) to $[^3H]LTD_4$ (□) and $[^3H]LTE_4$ (△), expressed as percentage of leukotriene recovered. Data represent the mean±SD of four experiments in which the maximal contractions were 0.75, 0.05, 7.5, and 0.3 V.

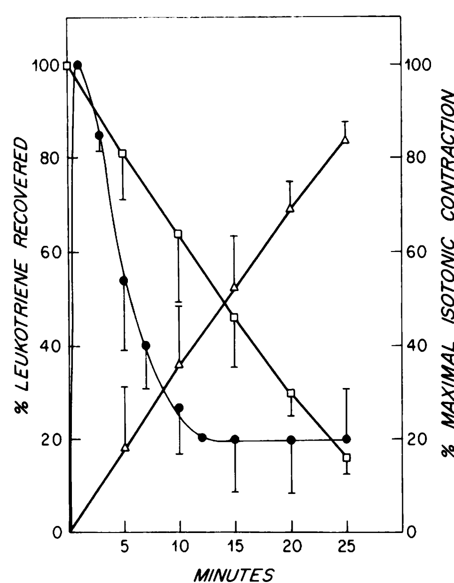

FIGURE 2 Time course of contractile response to 3.6 ng of $[^3H]LTD_4$ from guinea pig ileum, expressed as percentage of maximal response (● —— ●), and of the metabolism of $[^3H]LTD_4$ (□) to $[^3H]LTE_4$ (△), expressed as percentage of leukotriene recovered. Data represent the mean±SD of four experiments in which the maximal contractions were 1.2, 0.9, 0.6, and 1.0 V.

Inhibition of bioconversion of LTC_4 to LTD_4 by L-serine-borate complex and of LTD_4 to LTE_4 by L-cysteine. Because L-serine-borate complex and L-cysteine both contract the muscle preparation, a 15-min period was allowed after addition of either agent during which the muscle tension returned to base line. In two similar experiments with 5 ng of [^{3}H]LTC_4, the time course of response without and with L-serine-borate complex included a 1-min lag time, a linear brisk contraction from 1 to 2 min, a slower progressive contraction to a maximum, and a plateau with a slight decline out to 40 min (Fig. 3). In the presence of L-serine-borate complex, the rate of contraction was lower and the maximum achieved was less than in its absence. Without the serine-borate complex, 20 and 30% of the [^{3}H]LTC_4 in the two experiments was converted to [^{3}H]LTD_4 and [^{3}H]LTE_4 at 40 min, whereas in the presence of inhibitor there was no apparent conversion in either experiment. The overall recoveries without and with serine-borate complex averaged 54 and 50% for the two experiments. In a separate experiment conducted in the presence of 1 mM serine-borate complex, C-6 sulfidopeptide leukotrienes that were in the diffusate and associated with the tissue were separately analyzed 5 min after introduction of the radiolabeled LTC_4. The diffusate contained 53.8% of the recovered counts, all of which were LTC_4. The wash-buffer and the ethanolic extract of the tissue each contained <0.3% of the recovered counts and >90% of these counts eluted as LTC_4; the residual tissue debris contained no measurable counts.

After the contractile effect of the serine-borate complex had subsided, the presence of this inhibitor (10 mM) did not alter the contractile response of the smooth muscle to 50 ng of histamine or to 2 ng of LTD_4.

The rates of the immediate linear contraction and the maximum amplitude evoked by 3.6 ng of [^{3}H]LTD_4 were not altered by the presence of 10 mM L-cysteine in three experiments. In the presence of L-cysteine, the maximum contraction was maintained for 4 min and declined to a plateau at two-thirds of the maximal response, whereas without cysteine the contraction was not maintained and declined rapidly to a plateau of one-sixth maximum. In the absence of the L-cysteine, the [^{3}H]LTD_4 was linearly and quantitatively converted to [^{3}H]LTE_4, whereas in the presence of L-cysteine, [^{3}H]LTD_4 metabolism was <20%. The capacity of L-cysteine to minimize metabolism of [^{3}H]LTD_4, to prolong the maximum response, and to augment the plateau response as shown for one experiment (Fig. 4), was similar in the two additional experiments. The overall recoveries without and with cysteine averaged 65 and 56.5%, respectively, for the three experiments.

Bioconversion of LTC_4 and LTD_4 by guinea pig ileum longitudinal muscle strips. The contractile response of a longitudinal muscle strip to 5 ng of [^{3}H]LTC_4 included a 1-min delay, a linear contraction from 1 to 2 min, progression to a plateau at 25 min, and maintenance of this maximum contraction for the duration of observation to 45 min (Fig. 5). There was

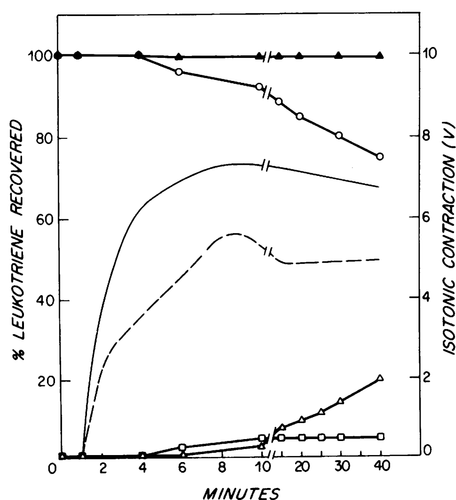

FIGURE 3 Time course of contractile response to 5 ng of [^{3}H]LTC_4 from guinea pig ileum (in volts) in the presence (— —) and absence (——) of 10 mM serine-borate complex and of the metabolism of [^{3}H]LTC_4 in the presence of serine-borate complex (▲) and in its absence (○), which allowed conversion to [^{3}H]LTD_4 (□) and [^{3}H]LTE_4 (△).

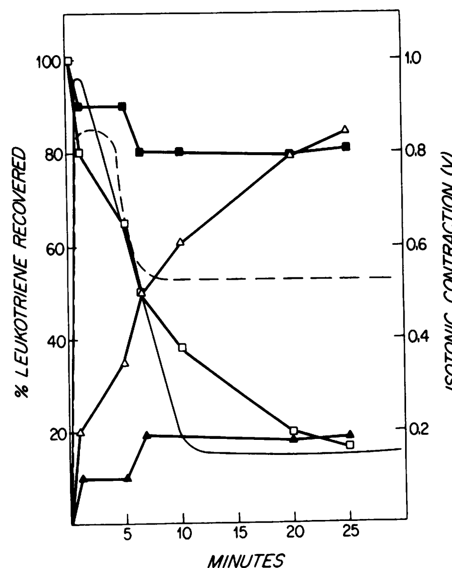

FIGURE 4 Time course of contractile response to 3.6 ng of [^{3}H]LTD_4 from guinea pig ileum (in volts) in the presence (— —) and absence (——) of 10 mM L-cysteine and of the metabolism of [^{3}H]LTD_4 (□) to [^{3}H]LTE_4 (△) in the presence (filled) and absence (open) of L-cysteine.

a gradual decline in [^{3}H]LTC$_4$ and at 40 min, 15% had been converted to [^{3}H]LTD$_4$ without further processing to [^{3}H]LTE$_4$. In the second experiment, the spasmogenic response was similar in form, and 20% of [^{3}H]LTC$_4$ was metabolized to [^{3}H]LTD$_4$ at 50 min. The recoveries of radiolabeled leukotriene from the diffusate at each time point averaged 75% for the two experiments and 72% of this activity was recovered after RPHPLC; no products other than LTC$_4$ and LTD$_4$ were detected.

In four experiments with 3.6 ng of [^{3}H]LTD$_4$ (1×10^5 cpm), each contractile response of the longitudinal muscle strips was immediate and linear in progression to 2 min, followed by a progressively slower contraction to maximum at 20 min with persistence to 50 min (Fig. 6). Bioconversion of [^{3}H]LTD$_4$ was minimal at 30 min, reflecting the lack of the converting enzyme in the mucosa-free muscle preparations. The recoveries of radiolabeled leukotriene from the diffusates at each time point averaged 82.5% for the four experiments and 86% of this activity was recovered after RPHPLC; no products other than LTD$_4$ and LTE$_4$ were detected.

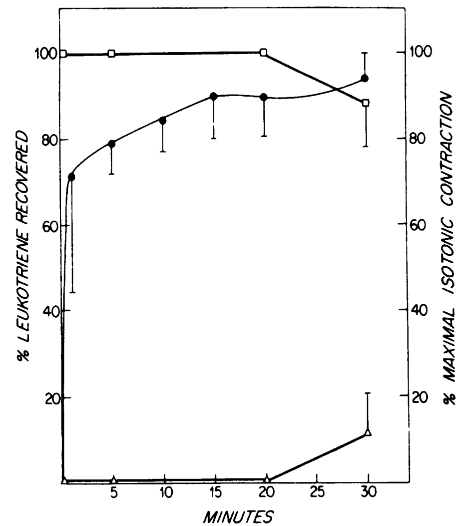

FIGURE 6 Time course of contractile response to 3.6 ng of [^{3}H]LTD$_4$ from longitudinal muscle strips of guinea pig ileum, expressed as percentage of maximal response (● —— ●), and of the metabolism of [^{3}H]LTD$_4$ (□) to [^{3}H]LTE$_4$ (Δ), expressed as percentage of leukotriene recovered. Data represent the mean±SD of three experiments in which the maximal contractions were 0.97, 0.5, and 2.5 V.

DISCUSSION

In order to characterize the effects of the associated bioconversion of LTC$_4$ and LTD$_4$ during the spasmogenic response of guinea pig ileum elicited by each agonist, the contraction and the conversion products in the diffusate were recorded simultaneously over time. The contraction was initiated with radiolabeled C-6 sulfidopeptide leukotriene, and at fixed intervals the labeled leukotrienes in the diffusate were resolved by their respective retention times on RPHPLC. The recoveries of radiolabeled products from the organ bath diffusate were 70 and 90% for [^{3}H]LTC$_4$ and [^{3}H]LTD$_4$ inputs, respectively, and 72 and 76% of these activities were recovered after RPHPLC (Figs. 1 and 2). The losses in the diffusate are attributed to binding by the glass, and those occurring during chromatography are comparable with other studies with unlabeled and labeled leukotrienes (16, 22). No products other than the sulfidopeptide leukotrienes were identified. That the bioconversions were not epiphenomena but were related to the muscle response was established by attenuating the metabolism of the C-6 sulfidopeptide leukotrienes with enzyme inhibitors and with anatomic deletion of the LTD$_4$ dipeptidase activity from the responding muscle preparation. With these interventions it was possible to distinguish the time course of the onset of the intrinsic activity of LTC$_4$ from that of LTD$_4$ and to define the effect of metabolism on the persistence of the spasmogenic effect of each agonist.

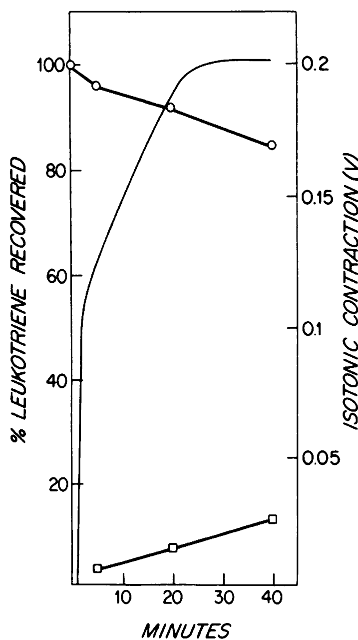

FIGURE 5 Time course of contractile response to 5 ng [^{3}H]LTC$_4$ of a longitudinal muscle strip of guinea pig ileum, expressed in volts (——), and of the metabolism of [^{3}H]LTC$_4$ (O) to [^{3}H]LTD$_4$ (□).

The response of the guinea pig ileum to [^{3}H]LTC$_4$ included a 60-s lag phase, a linear contraction to about two-thirds maximum over 60 s, and a slow further contraction to maximum, which was then maintained

(Fig. 1). As the metabolic conversion was <5% at 5 min, and was only 35% (8% LTD_4 and 27% LTE_4) at 50 min, the pattern of the response was attributed to LTC_4 rather than to its conversion products. The introduction of serine-borate complex (28) completely prevented the bioconversion of LTC_4 and reduced the maximum response to three-quarters of that observed without the inhibitor (Fig. 3). Thus, on the guinea pig ileum, LTC_4 has an inherent spasmogenic activity that is predominantly independent of its bioconversion to LTD_4.

The spasmogenic response of the guinea pig ileum to LTD_4 was immediate and progressed to a maximum at 60 s, which was maintained only briefly (Fig. 2). A period of rapid relaxation was then followed by a plateau at ~20% of the maximal contractile response. As the plot of the metabolic conversion of LTD_4 to LTE_4 was linear and passed through the origin, the conversion rate of 46.6 fmol/cm segment per min accounts for the transient nature of the maximal response to LTD_4. The apparent lower rate of conversion of LTD_4 to LTE_4, as compared with the rate of muscle relaxation (Fig. 2), most likely reflects the lag between tissue events and their presentation in the surrounding diffusate. Nonetheless, because of an equilibrium between association and dissociation of agonists, the contractile response observed does reflect the bioconversion and distribution of the agonists available for reassociation. This interpretation is supported by the finding of a more sustained contraction when the conversion of LTD_4 to LTE_4 was almost completely inhibited by the introduction of L-cysteine to the organ bath (Fig. 4). With anatomical deletion of the LTD_4 dipeptidase activity by removing the mucosa, the longitudinal smooth muscle preparation gave a contractile response to LTD_4 that maintained its maximum (Fig. 6).

The spasmogenic response of the guinea pig ileum and that of the longitudinal smooth muscle preparation to LTC_4 are delayed in onset by 1 min and are much more persistent than those occurring in response to LTD_4. The different latency for initiation of the contractile response distinguishes the inherent activities of these leukotrienes and is not, as previously postulated, due to the requirement for the bioconversion of LTC_4 to an active principle LTD_4 (29, 30), because the latent period for initiation of contraction was not altered by inhibition of this conversion. Further, the persistence of the LTC_4 contraction at its maximum, as compared with the transient maximal response to LTD_4, is due to resistance of the LTC_4 to conversion to LTD_4, which is in turn rapidly metabolized to LTE_4. The conversion rate of LTC_4 to LTD_4 by a single longitudinal smooth muscle preparation is ~5 fmol/cm segment per min (Fig. 5) and compares favorably with an average value of 8 fmol/cm segment per min for the two-step conversion of LTC_4 to LTE_4 by the intact ileum, because the conversion of LTC_4 to LTD_4 is the rate-limiting step (Fig. 1). In contrast, the conversion of LTD_4 to LTE_4 by the dipeptidase activity in the ileal preparation bearing mucosa was 46.6 fmol/cm segment per min (Fig. 2). The marked difference of the persistence of the contractile response to LTD_4 in the absence of the mucosal LTD_4 dipeptidase activity dramatically demonstrates that maintenance of a maximal response is determined by the continual integrity of the agonist and is not due to down-regulation by the muscle itself.

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