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An alternative pathway for metabolism of leukotriene D_4: effects on contractions to cysteinyl-leukotrienes in the guinea-pig trachea

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1 Contractions of guinea-pig tracheal preparations to cysteinyl-leukotrienes (LTC_4, LTD_4 and LTE_4) were characterized in organ baths, and cysteinyl-leukotriene metabolism was studied using radiolabelled agonists and RP-HPLC separation.

2 In the presence of S-hexyl GSH (100 μM) the metabolism of [^{3}H]-LTC_4 into [^{3}H]-LTD_4 was inhibited and the LTC_4-induced contractions were resistant to $CysLT_1$ receptor antagonism but inhibited by the dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 (0.3–3 μM) with a pA_2-value of 6.8 ± 0.2.

3 In the presence of L-cysteine (5 mM), the metabolism of [^{3}H]-LTD_4 into [^{3}H]-LTE_4 was inhibited and the LTD_4-induced contractions were inhibited by the $CysLT_1$ receptor antagonist ICI 198,615 (1–10 nM) with a pA_2-value of 9.3 ± 0.2. However, at higher concentrations of ICI 198,615 (30–300 nM) a residual contraction to LTD_4 was unmasked, and this response was inhibited by BAY u9773 (1–3 μM).

4 In the presence of the combination of S-hexyl GSH with L-cysteine, the LTD_4-induced contractions displayed the characteristics of the LTC_4 contractile responses, i.e. resistant to $CysLT_1$ receptor antagonism, increased maximal contractions and slower time-course. This qualitative change of the LTD_4-induced contraction was also observed in the presence of S-decyl GSH (100 μM), GSH (10 mM) and GSSG (10 mM).

5 S-hexyl GSH, S-decyl GSH, GSH and GSSG all stimulated a formation of [^{3}H]-LTC_4 from [^{3}H]-LTD_4.

6 In conclusion, GSH and GSH-related compounds changed the pharmacology of the LTD_4-induced contractions by stimulating the conversion of LTD_4 into LTC_4. Moreover, the results indicate that, in addition to the metabolism of LTC_4 into LTD_4 and LTE_4, also the formation of LTC_4 from LTD_4 may regulate cysteinyl-leukotriene function.

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Abbreviations: γ-GT, γ-glutamyl transpeptidase; GSH, reduced glutathione; GSSG, oxidized glutathione; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4

Introduction

Drugs that inhibit the actions of cysteinyl-leukotrienes (LTC_4, LTD_4 and LTE_4) have been established as a new treatment of asthma (Drazen *et al*., 1999). The current class of clinically introduced leukotriene receptor antagonists was developed mainly on the basis of functional studies of smooth muscle preparations *in vitro*, with the guinea-pig trachea and human bronchus being particularly predictive of the therapeutic effects of anti-leukotrienes in human subjects (Dahlén, 2000). The receptor mediating the major component of asthmatic bronchoconstriction caused by cysteinyl-leukotrienes is referred to as $CysLT_1$ (Dahlén, 2000), and the molecular characterization of this receptor was recently reported (Lynch *et al*., 1999; Sarau *et al*., 1999).

In isolated human pulmonary veins, the contractile effects of cysteinyl-leukotrienes cannot be blocked by $CysLT_1$ receptor antagonists (Labat *et al*., 1992). The term $CysLT_2$ has been introduced for the receptor that is resistant to $CysLT_1$ receptor antagonists but inhibitable by the leukotriene analogue BAY u9773, and a CysLT receptor with these characteristics was recently cloned (Heise *et al*., 2000; Takasaki *et al*., 2000; Nothacker *et al*., 2000). There are however findings from functional studies that indicate the presence of additional receptors for cysteinyl-leukotrienes. For example, a functional CysLT receptor displaying different antagonist properties was recently described in human and porcine pulmonary arteries (Bäck *et al*., 2000a, b).

One caveat when studying CysLT receptors is the metabolic conversion of LTC_4 into LTD_4 and LTE_4 in biological systems. For example, in the initial studies utilizing guinea-pig trachea, the $CysLT_1$ receptor antagonist FPL 55712 inhibited the contractions to all cysteinyl-leukotrienes (Krell *et al*., 1981; Jones *et al*., 1983). However, LTC_4 is metabolized by a γ-glutamyl transpeptidase (γ-GT) that removes the γ-glutamyl group from the peptide chain of

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LTC_4, thus yielding LTD_4 (Örning & Hammarström, 1980). When this metabolism is inhibited by either L-serine borate or glutathione, the contractions to LTC_4 are potentiated and no longer inhibited by $CysLT_1$ receptor antagonists (Snyder *et al*., 1984; Snyder & Krell, 1984; Weichman & Tucker, 1985; Hand & Schwalm, 1987), but by the dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 (Tudhope *et al*., 1994). These observations have resulted in the hypothesis that in the guinea-pig trachea, LTD_4 and LTE_4 activate a $CysLT_1$ receptor, which is pharmacologically similar to the $CysLT_1$ receptor mediating contraction of human bronchi (Buckner *et al*., 1990). On the other hand, LTC_4 activates a $CysLT_2$ receptor with similar antagonist properties as the $CysLT_2$ receptor in human pulmonary veins (Labat *et al*., 1992). However, this hypothesis may be too simplistic and one of the aims of this study was to assess the possibility of different subtypes of receptors for LTD_4 in the guinea-pig trachea. Although this has previously been proposed by other investigators (Krell *et al*., 1983; Hand *et al*., 1989), no studies have been performed in order to characterize such LTD_4 receptor subtypes. LTD_4 is metabolized by a dipeptidase that cleaves off a glycyl residue of the peptide chain, yielding LTE_4 (Hammarström, 1981), and the contractions to LTD_4 in the guinea-pig trachea have been shown to be potentiated when the metabolism into LTE_4 is inhibited by L-cysteine (Snyder *et al*., 1984).

In order to assess the function and agonist properties of each individual cysteinyl-leukotriene, interventions must thus be attempted at the level of these metabolizing enzymes. However, the literature covering the use of various inhibitors of cysteinyl-leukotriene metabolism in both functional and binding studies is heterogeneous, making comparisons between published studies difficult. The present study was therefore initiated to obtain an overall assessment of how different inhibitors of cysteinyl-leukotriene conversion affect metabolism and contractile responses to cysteinyl-leukotrienes. For the reason of available reference data, the guinea-pig trachea was selected for this investigation. However, metabolism of cysteinyl-leukotrienes takes place in a number of different tissues, including the human lung (Kumlin & Dahlén, 1990), suggesting that findings in the guinea-pig trachea may be applicable also to other systems. During this study it was interestingly discovered that S-hexyl GSH, suggested to be an inhibitor of the human leukotriene selective γ-GT (Nicholson, personal communication), profoundly changed the pharmacology of LTD_4. These findings may help to interpret some previously published conflicting results (Tomioka *et al*., 1991; Wikström Jonsson, 1997; Ravasi *et al*., 2000) and may facilitate the design of future studies of CysLT receptors.

Methods

Tissue preparation

Male Dunkin Hartley guinea-pigs (from SVA, Uppsala, Sweden), weighing 300–450 g, were asphyxiated and bled. The trachea was removed and immediately put into ice-cold Tyrode's solution (composition, mM): NaCl 149.2; KCl 2.7; $NaHCO_3$ 11.9; $CaCl_2$ 1.8 $MgCl_2$ 0.5; NaH_2PO_4 0.4 and glucose 5.5. The trachea was cleaned of surrounding tissue and cut into a spiral at an angle of 45°.

Contractions

One spirally cut trachea was divided into four equal preparations that were set up in 5 ml organ baths containing Tyrode's solution and gassed with 6.5% CO_2 in O_2 at 37°C. Initial experiments showed no difference in reactivity to histamine and LTD_4 between distal or proximal portions of the trachea. The preparations were set up with a cotton thread to EMKA IT1-25 or Grass FT-03 force-displacement transducers connected to EMKA or Grass 7D amplifiers, respectively. Changes in isometric tension were measured with an EMKA IOX data acquisition system. The preparations were placed under an initial tension of 15 mN and tension was adjusted during the experiments in order to keep a constant resting tension of 10 mN. After a 90 min equilibration period, with washes every 10 min, the experimental protocol started with the administration of a single dose of histamine (3 μM) in order to test tissue viability. After wash and resting tension readjustment a cumulative concentration-response curve for histamine was established by the addition of increasing concentrations (0.3–30 μM). After a further period of 60 min with washes every 10 min and readjustment of resting tension, the preparations were incubated for 30 min in the absence or presence of the following compounds: L-serine borate (45 mM; Weichman & Tucker, 1985), reduced glutathione (GSH, 10 mM; Hand & Schwalm, 1987), oxidized glutathione (GSSG, 10 mM), acivicin (1 mM; Snyder *et al*., 1991), S-hexyl GSH (100 μM; Nicholson, personal communication; Bäck *et al*., 1996; Wikström Jonsson, 1997), S-decyl GSH (100 μM), glutamic acid (1 mM) and/or L-cysteine (5 mM; Snyder *et al*., 1984; Bäck *et al*., 1996). In experiments with CysLT receptor antagonists, these were administered 15 min before the addition of cysteinyl-leukotrienes. At the end of the treatment periods, cumulative concentration-response curves for LTC_4, LTD_4 or LTE_4 (0.1 nM–10 μM) were established. When the cysteinyl-leukotriene contraction reached a plateau at the end of the cumulative dosing, a maximal contraction was determined by simultaneous addition of histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM). The contractions to histamine, acetylcholine and KCl were 14 ± 1.2 mN ($n = 37$) for controls, which was not significantly different when compared with each of the different treatment groups (data not shown).

Measurements of cysteinyl-leukotriene metabolism

In each experiment two spirally cut guinea-pig tracheas (see above) were cut into eight parts each. Four parts, representing a mix of proximal and distal parts from the two tracheas (mean weight: 72 ± 1.8 mg, $n = 117$) were put into a test-tube containing either 0.5 ml phosphate buffered saline (PBS buffer, control) or 0.5 ml PBS buffer containing inhibitors of cysteinyl-leukotriene metabolism. After 30 min incubation at 37°C, [^{3}H]-LTC_4, [^{3}H]-LTD_4 or [^{3}H]-LTE_4 (3.7 kBq, 1 pmol) was added to the tubes. The final concentration of each radiolabelled cysteinyl-leukotriene was 2 nM, which in preliminary experiments was shown to be a suitable concentration in order to obtain a reproducible

metabolism of [^{3}H]-LTC_4. The reaction was stopped after 30 min (Snyder *et al*., 1984) by the addition of 0.5 ml ethanol. In each experiment one tube containing only 0.5 ml PBS buffer (no trachea) was incubated with [^{3}H]-cysteinyl-leukotrienes in order to exclude any non-enzymatic degradation. The supernatants were injected onto a RP-HPLC column (Nucleosil C_{18}) and eluted with methanol/water/acetic acid (72/28/0.1; pH adjusted to 4.2 with NH_4OH) as mobile phase at a flow rate of 0.8 ml min^{-1} as previously described (Kumlin & Dahlén, 1990). The retention times for the individual cysteinyl-leukotrienes were determined with authentic standards and the distribution of radioactivity in 0.8 ml fractions was determined by liquid scintillation counting. The total amount of radioactivity detected as cysteinyl-leukotrienes corresponded to the radioactivity obtained in the single peak of the unmetabolized cysteinyl-leukotriene in the PBS control (no trachea).

Drugs

Acivicin, L-serine, boric acid, reduced glutathione (GSH), oxidized glutathione (GSSG), S-hexyl GSH, S-decyl GSH, L-cysteine, histamine and acetylcholine were obtained from Sigma (St. Louis, MO, U.S.A.). PBS buffer (composition: 0.14 M NaCl, 0.003 M KCl, 0.01 M phosphate buffer pH 7.4) was from Medicago (Uppsala, Sweden). LTC_4, LTD_4, LTE_4 and BAY u9773 were purchased from Cascade Biochem Ltd (Reading, U.K.) or Cayman Chemical (Ann Arbor, MI, U.S.A.) and concentrations and purity were checked by u.v.-spectrometry and RP-HPLC. [14,15,19,20-^{3}H]-LTC_4, [14,15,19,20-^{3}H]-LTD_4 and [14,15,19,20-^{3}H]-LTE_4 (0.37 MBq ml^{-1}) were purchased from NEN (Boston, MA, U.S.A.) and purity was confirmed with RP-HPLC. ICI 198,615 (Snyder *et al*., 1987), MK 571 (Jones *et al*., 1989) and SKF 104,353 (Hay *et al*., 1987) were kindly provided by AstraZeneca (Alderley, U.K.), Merck Frosst (Montreal, Canada) and Smith Kline Beecham (Swedeland, PA, U.S.A.), respectively.

L-serine borate was prepared from equimolar concentrations of L-serine and boric acid dissolved in water and buffered at pH 7.4 with 10 M NaOH. GSH and GSSG were dissolved in either Tyrode's (for contraction experiments) or PBS buffer (for incubation experiments) and buffered at pH 7.4 with 10 M NaOH. S-hexyl GSH and S-decyl GSH were dissolved in Tyrode's/PBS buffer with 10% ethanol and 10% Tris buffer (1 M). ICI 198,615 and SKF 104,353 were dissolved in dimethyldisulfoxide and subsequently diluted in Tyrode's solution. Solutions of cysteinyl-leukotrienes and BAY u9773 were obtained by diluting stock solutions of 1–8 mM into 20% ethanol in water. All other drugs were dissolved in either Tyrode's or PBS buffer.

Data analysis

All data are expressed as means ± s.e.mean. Contractions are expressed as per cent of the final contraction to histamine, acetylcholine and KCl. The time-course of contractions was calculated as time from addition to 50% ($T_{50\%}$) of the peak response to 3 nM of LTC_4 or LTD_4. Results of the incubations with [^{3}H]-cysteinyl-leukotrienes are expressed as per cent of the total radioactivity detected in the three cysteinyl-leukotriene peaks.

The maximal contraction (E_{max}) is expressed as the contraction induced by the highest concentration of each agonist, and the half-maximum effective concentrations (EC_{50} values) were calculated by linear regression from each individual experiment. The pD_2 value was calculated as the negative log of the EC_{50} value. In experiments with CysLT receptor antagonists, a dose ratio (DR) of EC_{50} values in the presence and absence of antagonist was calculated for each experiment relative to the paired control. The apparent dissociation constant (pK_B-value) was calculated for each antagonist concentration as the negative log of the following equation: $[B]/(DR-1)$, where [B] is the concentration of the antagonist and DR the dose-ratio. The pA_2 value was determined as the mean of all pK_B-values according to MacKay (1978). In addition, in order to confirm linearity, schild plot analysis was performed on the means of the DRs (Arunlakshana & Schild, 1959).

Statistical evaluation was performed using a either a Student's *t*-test (pairwise comparisons) or a one way analysis of variances (ANOVA) test followed by Dunnett's test (multiple comparisons). A *P*-value of less than 0.05 was considered significant.

Results

LTC_4 contractions and metabolism

LTC_4 contracted the guinea-pig tracheal preparations (Figure 1A, Table 1) and [^{3}H]-LTC_4 was metabolized into [^{3}H]-LTD_4 and [^{3}H]-LTE_4 (Figure 1B). In the presence of L-cysteine (5 mM) in combination with either L-serine borate (45 mM) or S-hexyl GSH (100 μM), the LTC_4 contractions were significantly potentiated and the metabolism of [^{3}H]-LTC_4 was inhibited (Figure 1A,B). These results were not different from results obtained with either S-hexyl GSH or L-serine borate alone (Table 1). In addition, the LTC_4-induced contractions were potentiated and the metabolism of [^{3}H]-LTC_4 into [^{3}H]-LTD_4 and [^{3}H]-LTE_4 was inhibited by GSH (10 mM) and acivicin (1 mM), but significantly less than for either L-serine borate (45 mM) or S-hexyl GSH (100 μM; Table 1).

In the presence of S-hexyl GSH and L-cysteine the contractions to LTC_4 were resistant to the $CysLT_1$ receptor antagonist ICI 198,615 (300 nM, Figure 1C, Table 2) but inhibited in a competitive manner by the combined $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 (0.3–3 μM), with a pA_2 value determined to 6.8 ± 0.2 (Figure 1D inset, Table 2). The results with CysLT receptor antagonists on LTC_4-induced contractions were not significantly different between preparation treated with L-cysteine in combination with either L-serine borate or S-hexyl GSH (Table 2). In addition, S-hexyl GSH did not alter either the E_{max} or the time-course of the LTC_4-induced contractions compared with L-serine borate (Table 2).

LTD_4 contractions and metabolism

LTD_4 contracted the guinea-pig tracheal preparations ($pD_2 = 7.5 \pm 0.2$, $n = 11$; Figure 2A) and the metabolism of [^{3}H]-LTD_4 into [^{3}H]-LTE_4 was inhibited by L-cysteine (5 mM, Figure 2B). L-cysteine (5 mM) significantly potentiated the LTD_4-induced contractions (Figure 2A) and the pD_2-value

(8.7 ± 0.2) was not significantly different from that of LTC_4 in the presence of S-hexyl GSH (Table 1). However, the maximal contraction (E_{max}) to LTD_4 in the presence of L-cysteine (Figure 2A) was significantly lower than the E_{max} for

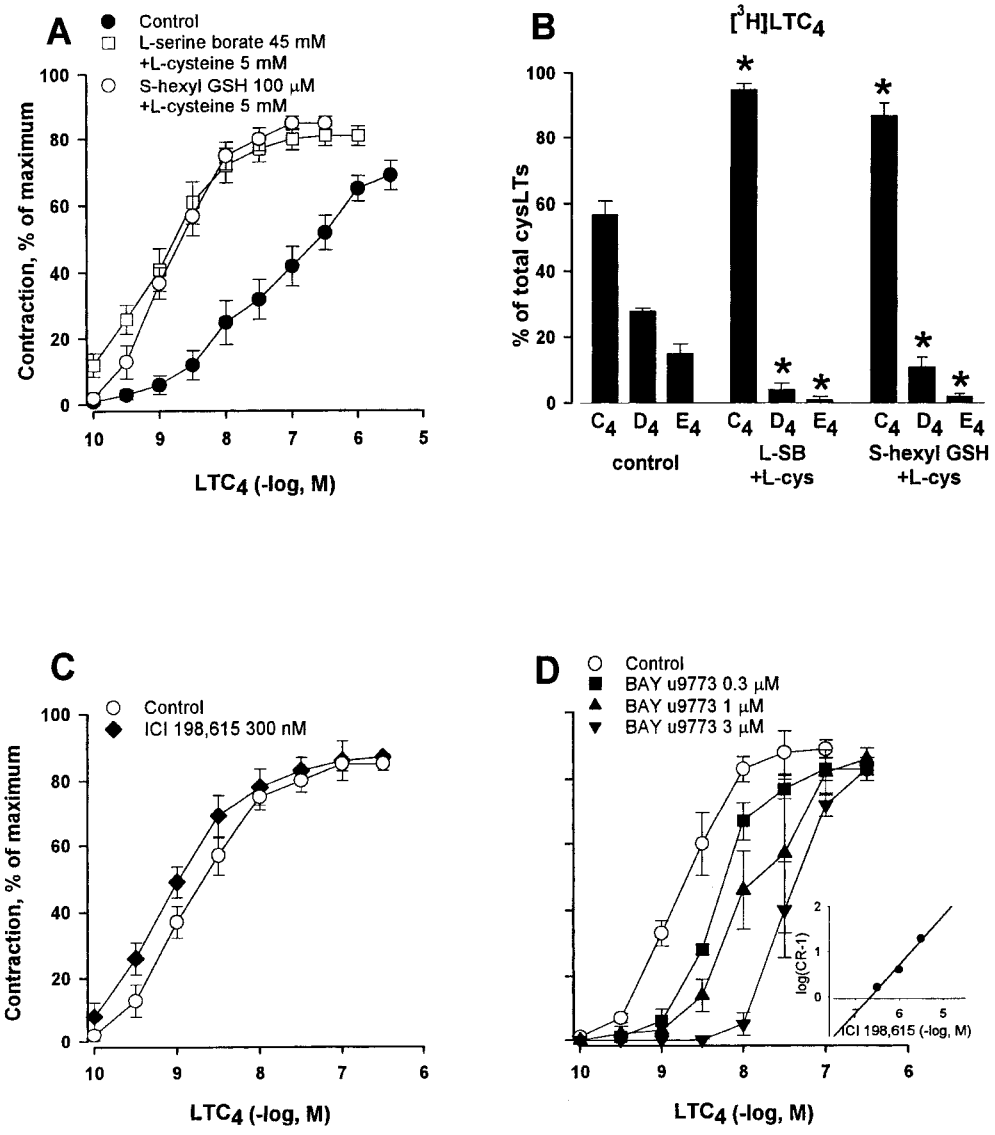

Figure 1 Concentration-effect curves for leukotriene C_4 (LTC_4) in guinea-pig tracheal spiral preparations (A, $n=7-12$) and metabolism of radiolabelled leukotriene C_4 ([^{3}H]-LTC_4, B, $n=4-7$) in the absence and presence of L-cysteine (5 mM, L-cys) in combination with either L-serine borate (45 mM, L-SB) or S-hexyl GSH (100 μM). In (C, $n=4$) and (D, $n=4-10$) preparations were treated with S-hexyl GSH (100 μM) and L-cysteine (5 mM) in the absence and presence of either the $CysLT_1$ receptor antagonist ICI 198,615 (C) or the $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 (D). Contractions are presented as per cent of a maximal contraction to histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM) and metabolism as per cent of total radioactivity. Vertical lines represent s.e.mean and (*) indicates a significant difference ($P<0.05$) compared with control.

Table 1 The contractions to leukotriene C_4 (LTC_4) and the metabolism of [^{3}H]-LTC_4 in the guinea-pig trachea in the absence and presence of inhibitors of metabolism

	Contractions			*Metabolism* (% of total cysLTs)			
	n	pD_2	E_{max} (%)	*n*	[^{3}H]-LTC_4	[^{3}H]-LTD_4	[^{3}H]-LTE_4
Control	12	7.3±0.20	69±4	7	57±4	28±1	15±3
S-hexyl GSH	5	8.9±0.17*	85±3*	4	92±3*	4±2*	3±1*
L-serine borate	6	8.8±0.12*	80±4*	4	96±2*	3±2*	1±1*
GSH	8	9.0±0.09*	87±4*	4	82±5*	17±5*†	1±1*
Acivicin + L-cys	5	8.6±0.26*	83±4*	4	76±4*†	21±4†	3±1*

*Indicates a significant difference ($P<0.05$) compared with control, and † indicates $P<0.05$ compared with preparations treated with S-hexyl GSH.

Table 2 The contractions to leukotrine C_4 (LTC_4) in the guinea-pig trachea in the presence of the combination of L-cysteine with either S-hexyl GSH or L-serine borate as inhibitors of LTC_4 metabolism

	n	pD_2	E_{max} (%)	pK_B
	S-hexylGSH (100 μM) + *L-cysteine* (5 mM)			
Control ($T_{50\%}$:403±42 s)	7	8.9±0.14	88±1	
ICI 198,615 300 nM	4	9.0±0.1	86±6	
BAY u9773 0.3 μM	5	8.2±0.12*	83±3	6.7±0.34
BAY u9773 1 μM	5	7.7±0.25*	86±3	6.9±0.36
BAY u9773 3 μM	6	7.4±0.15*	84±2	6.9±0.15
	L-serine borate (45 mM) + *L-cysteine* (5 mM)			
Control ($T_{50\%}$:396±27 s)	5	8.7±0.15	80±4	
ICI 198,615 300 nM	4	8.7±0.05	81±4	
MK 571 3 nM	4	8.8±0.24	86±3	
SKF 104,353 30 nM	4	8.9±0.14	82±5	
BAY u9773 3 μM	4	6.9±0.31*	77±3	7.0±0.32

The time-course of the control contractions is expressed as time (in seconds) from addition to 50% of the peak response to 3 nM of LTC_4 ($T_{50\%}$). All data are expressed as means±s.e.mean. *Indicates a significant difference ($P<0.05$) compared with control.

LTC_4 in the presence of L-cysteine + S-hexyl GSH (Figure 1A). The $CysLT_1$ receptor antagonist ICI 198,615 inhibited the LTD_4-induced contractions in a competitive manner at lower concentrations of antagonist (1–10 nM, Figure 2C). The pK_B values calculated at these antagonist concentrations were not significantly different from each other, and a pA_2 value of 9.3±0.2 was determined (Figure 2C inset, Table 3). However, higher concentrations of ICI 198,615 (30–300 nM) did not further displace the concentration response curves and in addition, at 100 and 300 nM of ICI 198,615 the E_{max} was slightly but significantly depressed compared with controls (Figure 2C, Table 3). The dual $CysLT_1/CysLT_2$ receptor antagonist BAY u9773 (1–3 μM) inhibited the residual contraction to LTD_4 in the presence of ICI 198,615 (300 nM) in an apparently competitive manner (Figure 2D).

Effect of γ-glutamyl-containing compounds on CysLT receptor antagonism and LTD_4 metabolism

Addition of S-hexyl-GSH (100 μM), but not of L-serine borate (45 mM) significantly enhanced the LTD_4-induced contractions compared with L-cysteine alone, without altering the pD_2 value (Figure 3A, Table 3). In the presence of the combination of L-cysteine with S-hexyl GSH, the E_{max} for LTD_4 was not significantly different from the E_{max} for LTC_4 (Tables 2 and 3). Also the time-course of the LTD_4-induced contractions was significantly prolonged by S-hexyl GSH, and again not significantly different from that of LTC_4 (Tables 2 and 3). In contrast to L-serine borate, incubation of guinea-pig trachea with [^{3}H]-LTD_4 in the presence of S-hexyl GSH (100 μM), yielded a peak of radioactivity corresponding to [^{3}H]-LTC_4 (Figure 3B). Interestingly, the contractions to LTD_4 were not inhibited by ICI 198,615 (Figure 3C), in the presence of S-hexyl GSH and L-cysteine but competitively inhibited by BAY u9773 with a pA_2 value determined to 6.9±0.1 (Figure 3D inset, Table 3). The $CysLT_1$ receptor antagonists (MK 571, 3 nM and SKF 104,353, 30 nM) inhibited the LTD_4-induced contractions in the presence of L-cysteine, but not in the presence of the combination of L-cysteine with S-hexyl GSH (Table 3). In fact, the findings with the CysLT receptor antagonists against LTD_4-induced contractions in the presence of S-hexyl GSH and L-cysteine were identical to those observed against LTC_4, namely that the contractions to this agonist were resistant to the $CysLT_1$ receptor antagonists ICI 198,615 (300 nM), MK 571 (3 nM) and SKF 104,353 (30 nM) but inhibited by the dual $CysLT_1/CysLT_2$ receptor antagonist BAY u9773 (Table 2).

Likewise, after incubation of guinea-pig trachea with [^{3}H]-LTD_4 in the presence of L-cysteine in combination with one of the γ-gluatmyl donors GSH (10 mM), GSSG (10 mM) or S-decyl-GSH (100 μM), a peak of [^{3}H]-LTC_4 was detected (Figure 4A–C). In addition, after pre-treatment with these compounds, ICI 198,615 (300 nM) did not inhibit the LTD_4-induced contractions. In contrast, in the presence of the combination of glutamic acid with L-cysteine, which did not stimulate metabolism of [^{3}H]-LTD_4 into [^{3}H]-LTC_4, ICI 198,615 (300 nM) inhibited the LTD_4-induced contractions (Figure 4D). These results also show that in the presence of L-cysteine and the γ-glutamyl-donors, the antagonist sensitivity, time-course of the contractions as well as the maximal contractions (E_{max}) to LTD_4 were all significantly different from LTD_4-induced contractions in the presence of L-cysteine alone, but not significantly different from LTC_4-induced contractions (Tables 2 and 3).

LTE_4 contractions and metabolism

The contractions to LTE_4 were inhibited by ICI 198,615 (1 nM; Figure 5A) and the pK_B value obtained in controls was 9.4±0.2 ($n=5$) which was not significantly different from that obtained in the presence of the combination of S-hexyl-GSH with L-cysteine (9.8±0.2, $n=4$, Figure 5B). In addition, the pK_B values were not significantly different from those obtained for LTD_4 (in the presence of L-cysteine) at the same antagonist concentration (Figure 2, Table 3). In contrast to the LTD_4-induced contractions, the contractions to LTE_4 were abolished by 300 nM of ICI 198,615 (Figure 5A,B). [^{3}H]-LTE_4 was not metabolized, either in controls or in the presence of the combination of S-hexyl GSH with L-cysteine (Figure 5C).

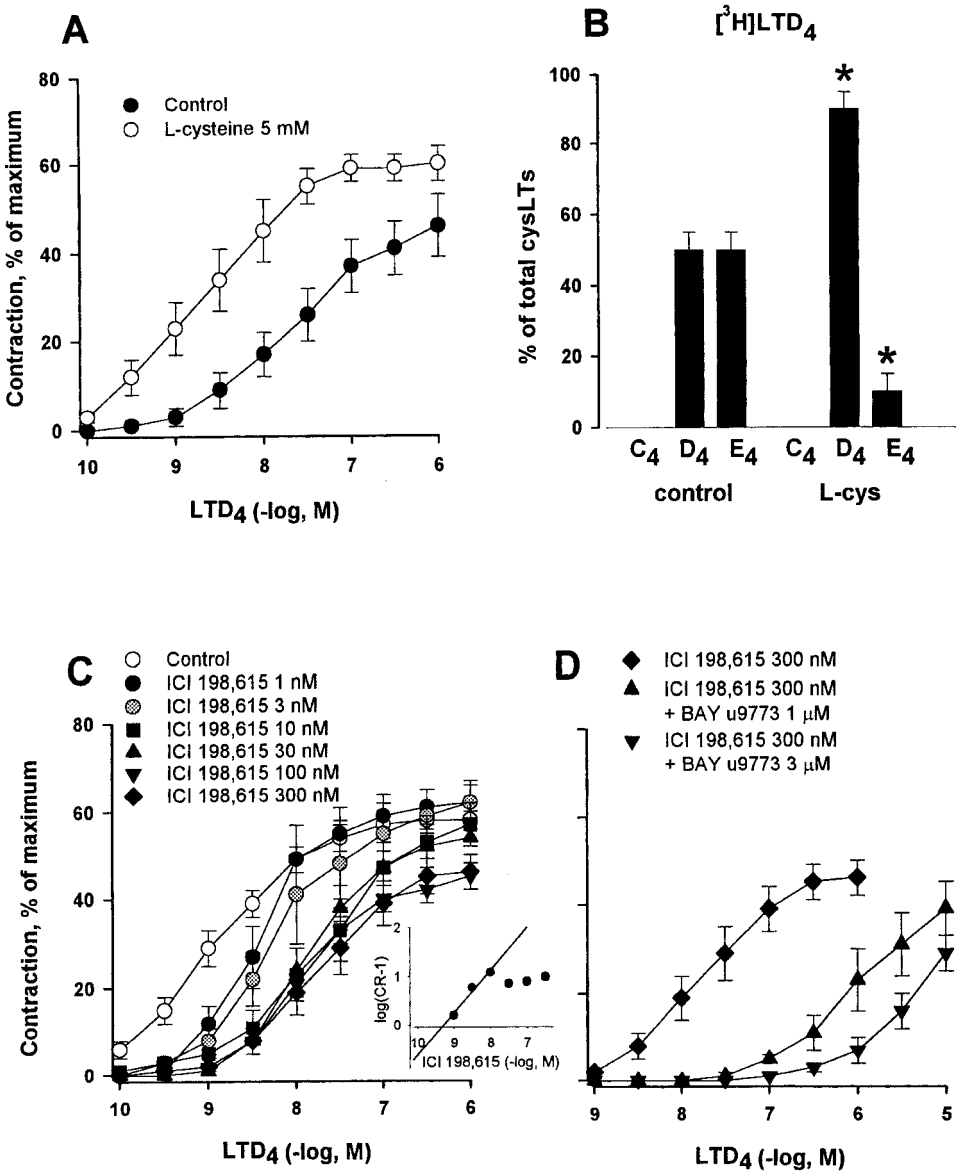

Figure 2 Concentration-effect curves for leukotriene D_4 (LTD_4) in guinea-pig tracheal spiral preparations (A, $n=6-8$) and metabolism of radiolabelled leukotriene D_4 ([^{3}H]-LTD_4, B, $n=5$). In (C, $n=5-14$) and (D, $n=4$) preparations were treated with L-cysteine (5 mM) in the absence and presence of the $CysLT_1$ receptor antagonist ICI 198,615 (C) or the combination of ICI 198,615 (300 nM) with the $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 (D). Contractions are presented as per cent of a maximal contraction to histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM) and metabolism as per cent of total radioactivity. Vertical lines represent s.e.mean and (*) indicates a significant difference ($P<0.05$) compared with control.

Discussion

In untreated guinea-pig tracheal preparations, LTC_4, D_4 and E_4 induced similar contractions. However, when their metabolism was inhibited, the contractile responses to the individual cysteinyl-leukotrienes exhibited a specific profile in terms of potency, maximal contractions, time-course of contractions and sensitivity to CysLT receptor antagonists, suggesting the possibility of correlating biochemical and functional results.

When metabolism of [^{3}H]-LTC_4 was inhibited by S-hexyl GSH and L-cysteine, the contractions to LTC_4 were resistant to $CysLT_1$ receptor antagonism but competitively inhibited by the dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 with a pA_2 value of 6.8, supporting the hypothesis that, in the guinea-pig trachea, LTC_4 activates $CysLT_2$ receptors (Tudhope *et al*., 1994). In addition, the inhibition of the LTC_4-induced contractions by BAY u9773 was not different whether L-serine borate or S-hexyl GSH was used as an inhibitor. Since unspecific effects have been documented for L-serine borate (Gardiner *et al*., 1990), S-hexyl GSH may represent a useful alternative as an inhibitor of LTC_4 metabolism.

In the presence of L-cysteine, the LTD_4-induced contractions were inhibited by the $CysLT_1$ receptor antagonist ICI 198,615. Lower concentrations of ICI 198,615 (1–10 nM)

Table 3 The contractions to leukotrine D_4 (LTD_4) in the guinea-pig trachea in the presence of either L-cysteine or the combination of L-cysteine with γ-glutamyl-containing compounds or glutamic acid

	n	pD_2	E_{max} (%)	pK_B
		L-cysteine (5 μM)		
Control ($T_{50\%}$:202±16 s)	17	8.7±0.17	54±2	
ICI 198,615 1 nM	6	8.3±0.15*	62±4	9.3±0.24
ICI 198,615 3 nM	5	7.9±0.27*	64±5	9.5±0.37
ICI 198,615 10 nM	8	7.6±0.17*	62±4	9.1±0.25
ICI 198,615 30 nM	6	7.9±0.11*	54±5	8.3±0.13
ICI 198,615 100 nM	7	8.0±0.12*	45±3*	
ICI 198,615 300 nM	11	7.7±0.15*	48±4*	
MK 571 3 nM	4	7.6±0.16*	54±5	9.2±0.31
SKF 104,353 30 nM	4	6.9±0.05*	50±4	8.0±0.36
		S-hexylGSH (100 μM)+*L-cysteine* (5 mM)		
Control ($T_{50\%}$:433±16 s†)	28	8.8±0.07	86±1†	
ICI 198,615 10 nM	5	8.7±0.09	90±1	
ICI 198,615 30 nM	5	8.6±0.14	84±2	
ICI 198,615 100 nM	7	8.8±0.27	85±2	
ICI 198,615 300 nM	5	8.9±0.11	84±2	
MK571 3 nM	3	9.0±0.11	84±2	
SKF 104,353 30 nM	3	8.9±0.24	82±5	
BAY u9773 0.3 μM	7	8.1±0.08*	90±3	6.8±0.36
BAY u9773 1 μM	10	7.9±0.10*	85±2	6.8±0.14
BAY u9773 3 μM	4	7.2±0.03*	86±2	7.4±0.19
BAY u9773 10 μM	4	6.4±0.16*	82±2	7.2±0.21
		GSH (10 mM)+*L-cysteine* (5 mM)		
Control ($T_{50\%}$:349±31 s†)	7	9.2±0.16	81±4†	
ICI 198,615 300 nM	7	9.1±0.19	84±4	
		GSSG (10 mM)+*L-cysteine* (5 mM)		
Control ($T_{50\%}$:415±30 s†)	4	9.3±0.17	81±7†	
ICI 198,615 300 nM	4	9.1±0.24	83±5	
		S-decyl GSH (100 μM)+*L-cysteine* (5 mM)		
Control ($T_{50\%}$:371±25 s†)	4	8.8±0.22	88±3†	
ICI 198,615 300 nM	4	8.8±0.26	85±6	
		Glutamic acid (1 mM)+*L-cysteine* (5 mM)		
Control ($T_{50\%}$:158±73 s)	5	8.7±0.32	56±4	
ICI 198,615 300 nM	5	7.4±0.12*	47±9*	

The time-course of the control contractions is expressed as time (in seconds) from addition to 50% of the peak response to 3 nM of LTD_4 ($T_{50\%}$). All data are expressed as mean±s.e.mean. *Indicates a significant difference ($P<0.05$) compared with control, and † indicates $P<0.05$ compared with preparations treated with L-cysteine.

competitively inhibited the LTD_4-induced contractions with a pA_2 value of 9.3, while at higher concentrations (30–100 nM) no further shift of the LTD_4 concentration response curve was observed. The residual contraction to LTD_4 in the presence of ICI 198,615 (300 nM) was inhibited by BAY u9773, suggesting that LTD_4 activated both $CysLT_1$ and $CysLT_2$ receptors. Previous studies have discussed the possibility of two different receptors for LTD_4 in the guinea-pig trachea, and that ICI 198,615 and other $CysLT_1$ receptor antagonists preferentially interact with the high affinity receptor (Krell *et al*., 1983; Hand *et al*., 1989; Jones *et al*., 1989). The present report extends these findings and indicates that the $CysLT_1$ receptor may represent the high affinity receptor for LTD_4 and $CysLT_2$ the low affinity receptor for LTD_4 in the guinea-pig trachea.

Interestingly, after pre-treatment with the combination of S-hexyl GSH and L-cysteine, the contractions observed after LTD_4 administration were not inhibited by ICI 198,615 but competitively inhibited by the dual $CysLT_1$ and $CysLT_2$ receptor antagonist BAY u9773 with a pA_2 value of 6.8, indicating that the high affinity $CysLT_1$ component was no longer present. The resistance of the contractions to ICI 198,615 and inhibition by BAY u9773, observed after LTD_4 administration in the presence of S-hexyl GSH, were identical to results obtained with LTC_4 (present study, Tudhope *et al*., 1994). In addition, the slower time-course and increased maximal contractions observed for LTD_4 in the presence of S-hexyl GSH were also similar to LTC_4. Taken together these findings indicate that, in the presence of S-hexyl GSH, the contractions observed after LTD_4 administration exhibited similar characteristics to the LTC_4-induced $CysLT_2$ contractile responses.

This change of the LTD_4-induced $CysLT_1$ response, which mimicked the LTC_4-induced $CysLT_2$ response, was not due to a change of the $CysLT_1$ receptor since the antagonism of LTE_4 at the $CysLT_1$ receptor was not affected by the combination of S-hexyl GSH with L-cysteine. This latter finding also indicates that the resistance of the LTD_4 response to ICI 198,615 was not due to an alteration of the antagonist, which is further supported by the results with

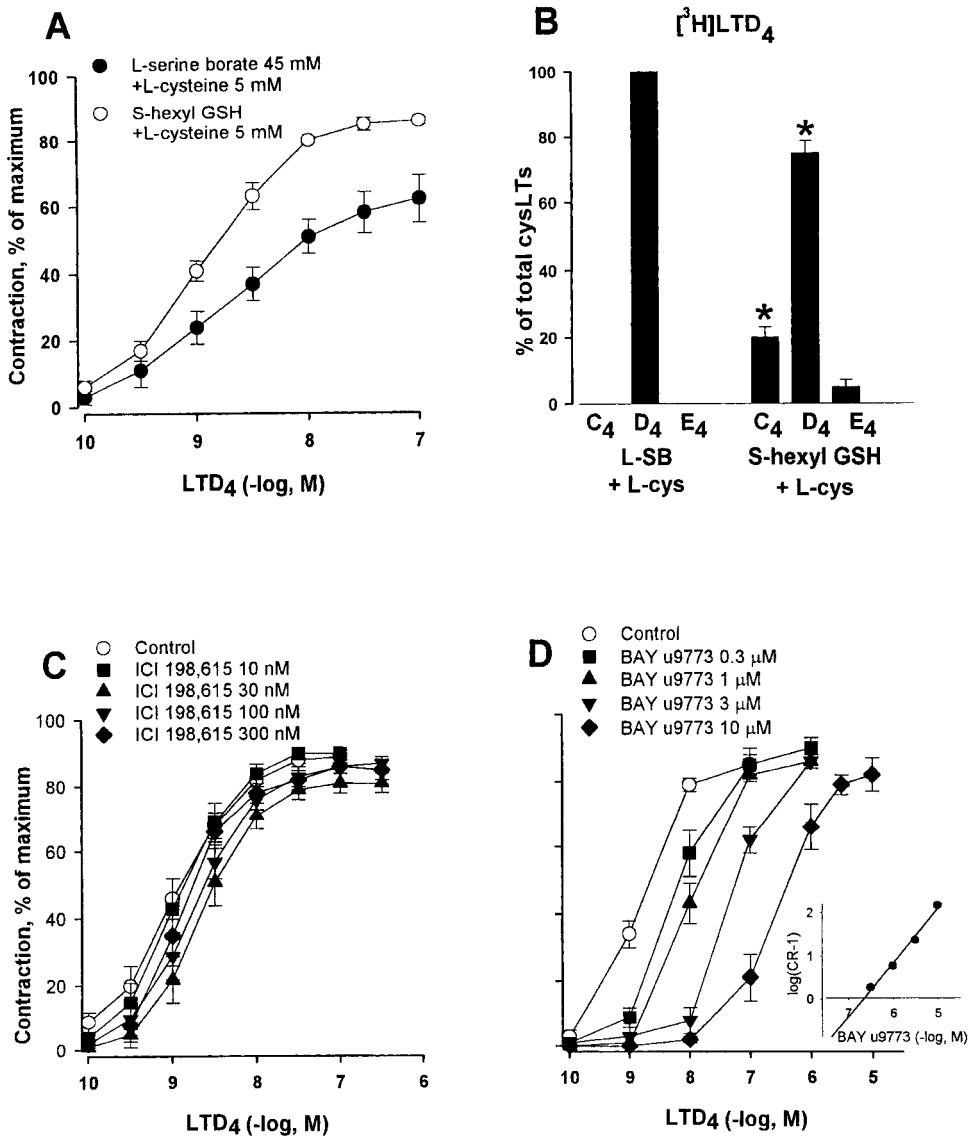

Figure 3 Concentration-effect curves for leukotriene D_4 (LTD_4) in guinea-pig tracheal spiral preparations (A, $n=4-10$) and metabolism of radiolabelled leukotriene D_4 ([^{3}H]-LTD_4, B, $n=4-7$). In (C, $n=5-11$) and (D, $n=4-14$) preparations were treated with S-hexyl GSH (100 μM) and L-cysteine (5 mM) in the absence and presence of either the $CysLT_1$ receptor antagonist ICI 198.615 or the $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 (D). Contractions are presented as per cent of a maximal contraction to histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM) and metabolism as per cent of total radioactivity. Vertical lines represent s.e.mean and (*) indicates a significant difference ($P<0.05$) compared with control.

MK 571 and SKF 104,353, namely that also these two structurally unrelated $CysLT_1$ receptor antagonists inhibited LTD_4-induced contractions in the absence but not in the presence of S-hexyl GSH.

Moreover, the biochemical experiments established that S-hexyl GSH stimulated the formation of [^{3}H]-LTC_4 from [^{3}H]-LTD_4 in the guinea-pig trachea and that [^{3}H]-LTC_4 was formed from [^{3}H]-LTD_4 also after treatment of the guinea-pig trachea with GSH, GSSG and S-decyl GSH. Formation of LTC_4 from LTD_4 has not previously been described in airway or smooth muscle preparations but, in studies using purified γ-GT (Anderson *et al.*, 1982) and guinea-pig liver and kidney homogenates (Hammarström, 1981) as well as perfused rat liver (Wettstein *et al.*, 1989), similar biochemical findings have been reported. S-hexyl GSH, S-decyl GSH, GSH and GSSG all contain γ-glutamyl groups that may be transferred to an acceptor, in this case LTD_4, which will yield LTC_4 (Hammarström, 1981).

Taken together, the results of the present report indicate that the enhanced maximal contraction, slower time-course and resistance to the $CysLT_1$ receptor antagonist ICI 198,615 observed for LTD_4 in the presence of the γ-glutamyl-containing compounds, were due to formation of LTC_4 acting on $CysLT_2$ receptors. However, although the biochemical and functional findings coincide, there was not an exact correlation between the results. It is probable that the biochemically detected conversion represents an underestimate of the actual proportion of [^{3}H]-LTC_4 formed from [^{3}H]-LTD_4 in the tissue, since the analyses were performed on the supernatants. This is similar to previous reports

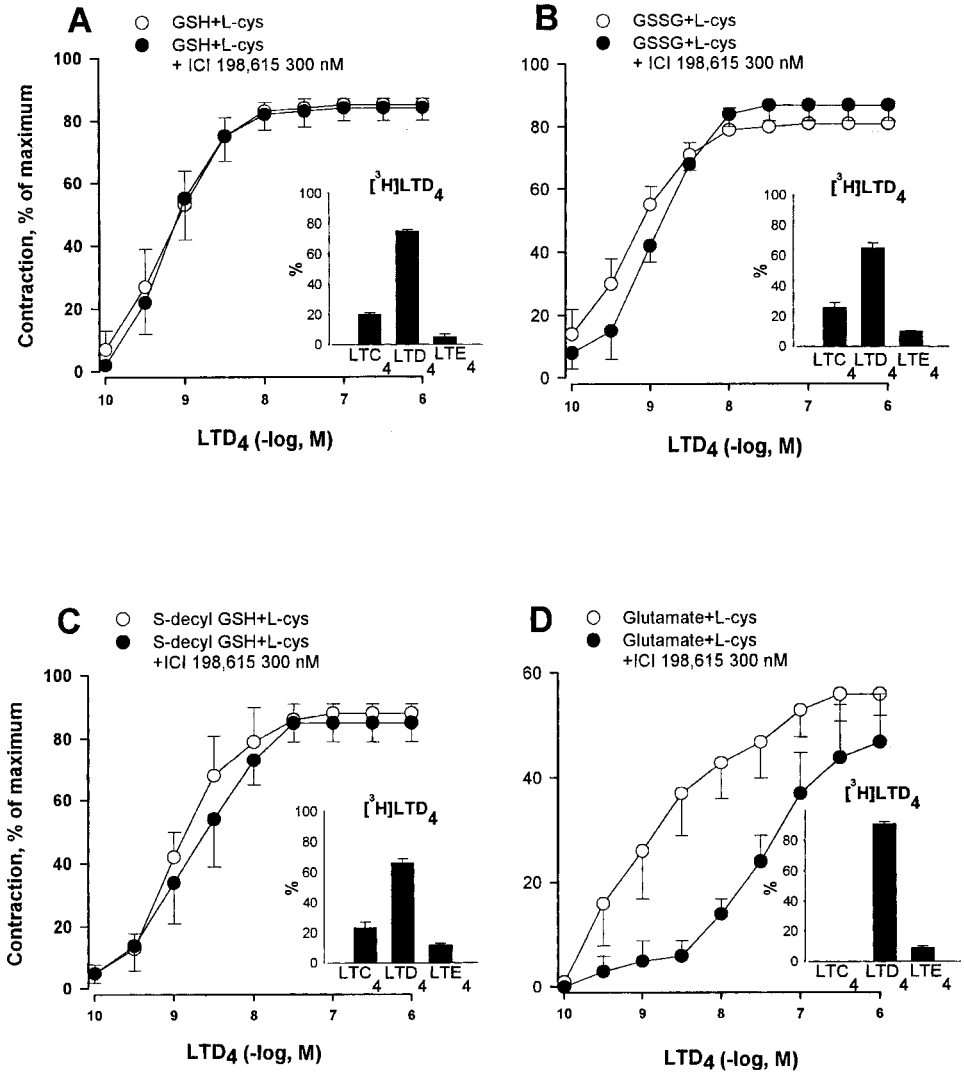

Figure 4 Concentration-effect curves for leukotriene D_4 (LTD_4) and the metabolism of radiolabelled leukotriene D_4 ([^{3}H]-LTD_4) in the guinea-pig trachea in the presence of the combination of L-cysteine (5 mM, L-cys) with (A) reduced glutathione (10 mM, GSH), (B) oxidized glutathione (10 mM, GSSG), (C) S-decyl GSH (100 μM) or (D) glutamic acid (1 mM). The contractions were studied in the absence and presence of the $CysLT_1$ receptor antagonist ICI 198,615 (300 nM). The contractions ($n=4-7$) are presented as per cent of a maximal contraction to histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM) and metabolism ($n=4$) as per cent of total radioactivity (shown as insets). Vertical lines represent s.e.mean.

evaluating the pharmacological effects of biochemically measured LTC_4 metabolism in the guinea-pig trachea (Snyder *et al*., 1984; Weichman & Tucker, 1985) and suggests that there is a slow diffusion of the converted leukotrienes from the tissue back into the buffer. The correlation between formation of [^{3}H]-LTC_4 from [^{3}H]-LTD_4 and change of the contractile response to LTD_4 is further supported by the results with glutamic acid, which did not stimulate [^{3}H]-LTC_4-formation from [^{3}H]-LTD_4. Accordingly, the contractions observed after LTD_4 administration were not altered by glutamic acid.

The stimulation of the formation of LTC_4 from LTD_4 by GSH and S-conjugated GSH may complicate studies of cysteinyl-leukotrienes and CysLT receptors, since these compounds often are used as inhibitors of LTC_4 metabolism in functional studies (Hand & Schwalm, 1987; Hand *et al*., 1989; Bäck *et al*., 1996). For example, the contradictory results of previous reports on the effects of $CysLT_1$ receptor antagonists on LTD_4-induced contractions in ovine airways (Tomioka *et al*., 1991; Wikström Jonsson, 1997) may relate to the use of S-hexyl GSH in the latter study. In addition, S-decyl GSH is commonly used as an inhibitor of non-receptor binding in agonist binding studies using radioactive cysteinyl-leukotrienes (Norman *et al*., 1987; Takasaki *et al*., 2000). Ravasi *et al*. (2000) recently reported that there was a difference between LTC_4 and LTD_4 binding to human lung membranes, whereas in the presence of S-decyl GSH, the profile of antagonism of LTD_4 binding was identical to that for LTC_4. In view of the results in the present study, it is probable that the alteration by S-decyl GSH observed by those investigators (Ravasi *et al*., 2000) was due to formation of LTC_4 from LTD_4 and in the presence of S-decyl GSH, only LTC_4 binding was studied regardless of which agonist was added. In addition, the results of that study (Ravasi *et*

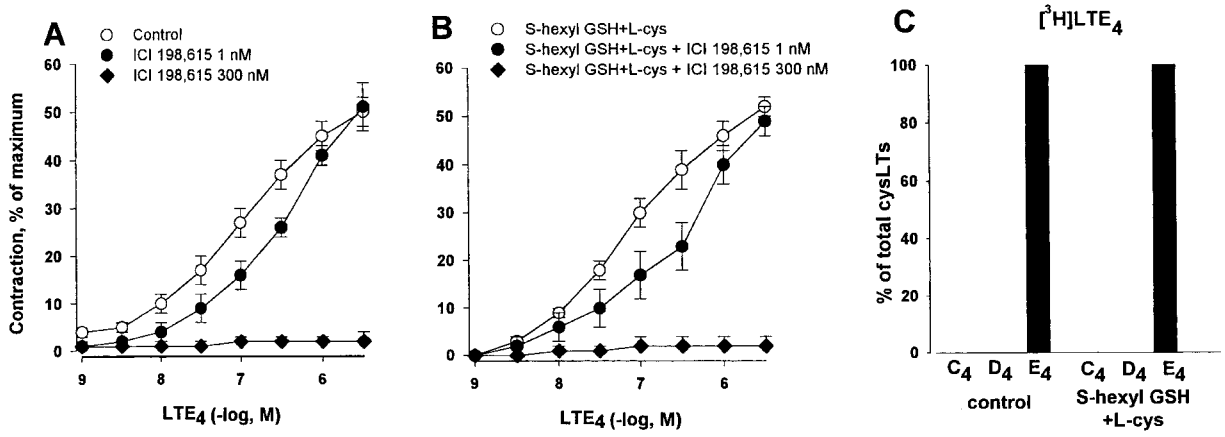

Figure 5 Concentration-effect curves for leukotriene E_4 (LTE_4) in guinea-pig tracheal spiral preparations (A and B) and the lack of metabolism of radiolabelled leukotriene E_4 ([^{3}H]LTE_4, C, $n=4$). The contractions were studied in the absence (A, $n=5$) and presence (B, $n=4$) of the combination of S-hexyl GSH (100 μM) with L-cysteine (5 mM, L-cys). Preparations were pretreated with the $CysLT_1$ receptor antagonist ICI 198,615 (1 and 300 nM). The contractions are presented as per cent of a maximal contraction to histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM) and metabolism as per cent of total radioactivity. Vertical lines represent s.e.mean.

al., 2000) indicate that the conversion of LTD_4 into LTC_4, described in the present report, takes place and may affect cysteinyl-leukotriene function also in the human lung.

In summary, LTC_4 activated a $CysLT_2$ receptor in the guinea-pig trachea, whereas LTD_4 activated both $CysLT_1$ and $CysLT_2$ receptors. In contrast, the LTE_4-induced contractions were abolished by a high concentration of ICI 198,615, suggesting that LTE_4 solely interacts with the $CysLT_1$ receptor in the guinea-pig trachea. In addition, the functional response to LTD_4 was changed into an LTC_4 response in the presence of γ-glutamyl-donors that induced [^{3}H]-LTC_4 formation from [^{3}H]-LTD_4. Together these results indicate that in addition to the metabolism of LTC_4 into LTD_4 and LTE_4, also the formation of LTC_4 from LTD_4 affects functional responses to cysteinyl-leukotrienes and should be considered in pharmacological studies of these inflammatory mediators. Moreover, this alternative metabolic pathway of LTD_4 may represent an endogenous mechanism for control of responses to cysteinyl-leukotrienes, both by delaying their catabolism and by changing what CysLT receptor is activated.

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