

## Specific receptors for leukotriene C4 on a smooth muscle cell line.

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*J Clin Invest.* 1983;72(4):1516-1519. <https://doi.org/10.1172/JCI111109>.

### Research Article

Specific receptors for leukotriene C4 (LTC<sub>4</sub>) have been identified on an intact smooth muscle cell line, DDT1 MF-2 cells derived from the Syrian hamster vas deferens. Specific [<sup>3</sup>H]LTC<sub>4</sub> binding at a fixed input at 4 degrees C was rapid, reached a plateau at 86% of total binding at 60 min, and was reversible upon addition of excess homoligand. With incremental inputs of radioligand and a constant cell number, specific [<sup>3</sup>H]LTC<sub>4</sub> binding reached a plateau indicative of saturable binding sites. LIGAND analysis of the Scatchard plot demonstrated a single high affinity binding site with a dissociation constant (K<sub>d</sub>) of 5 nM. With incremental inputs of unlabeled LTC<sub>4</sub>, LIGAND analysis of the Scatchard plot demonstrated a single high affinity site with a K<sub>d</sub> of 4.4 nM and in some experiments an additional low affinity site with a K<sub>d</sub> of 634 nM. The myotonically active structural analogues of LTC<sub>4</sub>, 5(R),6(S)-LTC<sub>4</sub>, 11-trans-LTC<sub>4</sub>, and C1-monoamide-LTC<sub>4</sub>, competed effectively with radiolabeled LTC<sub>4</sub> such that the relative K<sub>d</sub> values of these heteroligands were within one log of that of the homoligand. In contrast, the other native sulfidopeptide leukotrienes, leukotriene D<sub>4</sub> and leukotriene E<sub>4</sub>, exhibited relative K<sub>d</sub> values that were 2-3 logs less than that of LTC<sub>4</sub>. Thus, the high affinity receptor on the DDT1 smooth muscle cell line is specific for a single constituent, LTC<sub>4</sub>, of slow reacting [...]

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# Specific Receptors for Leukotriene C<sub>4</sub> on a Smooth Muscle Cell Line

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**ABSTRACT** Specific receptors for leukotriene C<sub>4</sub> (LTC<sub>4</sub>) have been identified on an intact smooth muscle cell line, DDT<sub>1</sub> MF-2 cells derived from the Syrian hamster vas deferens. Specific [<sup>3</sup>H]LTC<sub>4</sub> binding at a fixed input at 4°C was rapid, reached a plateau at 86% of total binding at 60 min, and was reversible upon addition of excess homoligand. With incremental inputs of radioligand and a constant cell number, specific [<sup>3</sup>H]LTC<sub>4</sub> binding reached a plateau indicative of saturable binding sites. LIGAND analysis of the Scatchard plot demonstrated a single high affinity binding site with a dissociation constant (*K<sub>d</sub>*) of 5 nM. With incremental inputs of unlabeled LTC<sub>4</sub>, LIGAND analysis of the Scatchard plot demonstrated a single high affinity site with a *K<sub>d</sub>* of 4.4 nM and in some experiments an additional low affinity site with a *K<sub>d</sub>* of 634 nM. The myotonically active structural analogues of LTC<sub>4</sub>, 5(R),6(S)-LTC<sub>4</sub>, 11-*trans*-LTC<sub>4</sub>, and C<sub>1</sub>-monoamide-LTC<sub>4</sub>, competed effectively with radiolabeled LTC<sub>4</sub> such that the relative *K<sub>d</sub>* values of these heteroligands were within one log of that of the homoligand. In contrast, the other native sulfidopeptide leukotrienes, leukotriene D<sub>4</sub> and leukotriene E<sub>4</sub>, exhibited relative *K<sub>d</sub>* values that were 2–3 logs less than that of LTC<sub>4</sub>. Thus, the high affinity receptor on the DDT<sub>1</sub> smooth muscle cell line is specific for a single

constituent, LTC<sub>4</sub>, of slow reacting substance of anaphylaxis.

## INTRODUCTION

The C-6 sulfidopeptide leukotriene, 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTC<sub>4</sub>),<sup>1</sup> a product of the oxidation of arachidonic acid by the 5-lipoxygenase pathway, undergoes proteolytic cleavage to 5(S)-hydroxy-6(R)-S-cysteinyl-glycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTD<sub>4</sub>) and 5(S)-hydroxy-6(R)-S-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTE<sub>4</sub>); and these leukotrienes together constitute the biological activity ascribed to slow-reacting substance of anaphylaxis (1–4). The structural determinants of LTC<sub>4</sub> and LTD<sub>4</sub> function include the presence of a hydrophobic omega region (5), and the geometrical relationships between the eicosanoid carboxyl and the appendages at C-5 and C-6, suggesting the existence of true receptors for this class of agonist (6). In the present study, the saturable and reversible binding of [<sup>3</sup>H]LTC<sub>4</sub> to intact smooth muscle cells from the hamster vas deferens DDT<sub>1</sub> cell line (7) provides evidence for a receptor for LTC<sub>4</sub>; and competition analyses indicate that it is distinct from the putative receptors for LTD<sub>4</sub> and LTE<sub>4</sub>.

Dr. Krilis is a recipient of an Applied Health Science Fellowship from the Medical Research Council of Australia. Address reprint requests to Dr. Austen, 604 Seeley G. Mudd Building, 250 Longwood Ave., Boston, MA 02115.

Received for publication 13 June 1983 and in revised form 19 July 1983.

<sup>1</sup> Abbreviations used in this paper: LTC<sub>4</sub>, 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTD<sub>4</sub>, 5(S)-hydroxy-6(R)-S-cysteinyl-glycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTE<sub>4</sub>, 5(S)-hydroxy-6(R)-S-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid.

## METHODS

**Materials.** LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub> and the structural leukotriene analogues of LTC<sub>4</sub>—5(R),6(S)-LTC<sub>4</sub>, C<sub>1</sub>-monoamide-LTC<sub>4</sub>, deamino-LTC<sub>4</sub>, and 11-*trans*-LTC<sub>4</sub>—were prepared as described (4, 6, 8, 9) and stored under argon in 0.05 M phosphate buffer, pH 6.8, containing 20% ethanol at -70°C. [14,15-<sup>3</sup>H]LTC<sub>4</sub> (40 Ci/mmol, lot 1796-297, and 42.8 Ci/mmol, lot 1917-088) was supplied by New England Nuclear, Boston, MA. FPL 55712 was supplied by Fisons, Ltd., Loughborough, United Kingdom.

**Receptor-binding assay.** The hamster vas deferens DDT<sub>1</sub> MF-2 cells were provided by Dr. J. S. Norris (University of Arkansas, Little Rock, AR) and were grown in monolayer cultures in 250-ml culture flasks in Dulbecco's modified Eagle's medium containing 4.5 g of glucose/liter, 1% penicillin-streptomycin, and 10% fetal calf serum (7). Monolayer cultures of DDT<sub>1</sub> MF-2 cells were harvested with a rubber policeman; washed twice with Tris-saline, pH 7.4, containing 140 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 11.2 mM D-glucose, and 10 mM Tris-hydroxy methylaminomethane; and sedimented at 500 g for 10 min at room temperature and resuspended in Tris-HCl, pH 7.3. Cell number was determined with a hemocytometer.

Smooth muscle cells ( $0.5\text{--}1 \times 10^6$ ) in 0.5 ml Tris-HCl, pH 7.3, containing 1 mM serine-borate complex and 1 mM CaCl<sub>2</sub>, were combined in 3.5-ml polypropylene tubes with varying concentrations (0.4–13.7 nM) of [<sup>3</sup>H]LTC<sub>4</sub> for saturation experiments and with fixed amounts of [<sup>3</sup>H]LTC<sub>4</sub> ranging from 0.4 to 1.5 nM and varying amounts of cold ligands at 4°C for competition experiments. Incubations were routinely continued for 60 min at 4°C and the contents of each tube were filtered under vacuum through separate glass fiber filters (Boehringer Mannheim Biochemicals, Indianapolis, IN) on a Hoefer filtration manifold (Hoefer Scientific Instruments, San Francisco, CA). Each filter was washed five times with 2-ml portions of cold Tris-HCl buffer, pH 7.3, containing 1 mM serine-borate complex, and then placed in a scintillation vial containing 1 ml of ethanol/water, 4:1 (vol/vol) solution overnight at 25°C. 5 ml of Aquasol (New England Nuclear) was added, and the mixtures were incubated for an additional 4–6 h at 25°C before radioactivity was determined in a scintillation counter (Mark II, Tracor Analytic, Inc., Elk Grove, IL). Total and nonspecific binding of [<sup>3</sup>H]LTC<sub>4</sub> were determined as the mean of triplicate assays carried out in the absence and in the presence of 3.2 μM unlabeled LTC<sub>4</sub>. Specific binding was calculated as the difference between total and nonspecific binding at each concentration of [<sup>3</sup>H]LTC<sub>4</sub>. Binding data were analyzed by the LIGAND computer program (10).

## RESULTS

**Binding of [<sup>3</sup>H]LTC<sub>4</sub> to the DDT<sub>1</sub> smooth muscle cells.** The total and specific binding of [<sup>3</sup>H]LTC<sub>4</sub> to  $0.3 \times 10^6$  DDT<sub>1</sub> smooth muscle cells at 4°C increased rapidly over the first 10 min of interaction, progressed slowly to equilibrium at 60 min, and remained stable for up to 120 min. In three experiments, specific binding accounted for  $86 \pm 8.0\%$  (mean  $\pm$  SD) of the total binding at 60 min (Fig. 1 a). Nonspecific binding was maximal at the earliest time point assessed and thereafter was independent of time.

Dissociation of the specifically bound [<sup>3</sup>H]LTC<sub>4</sub> was

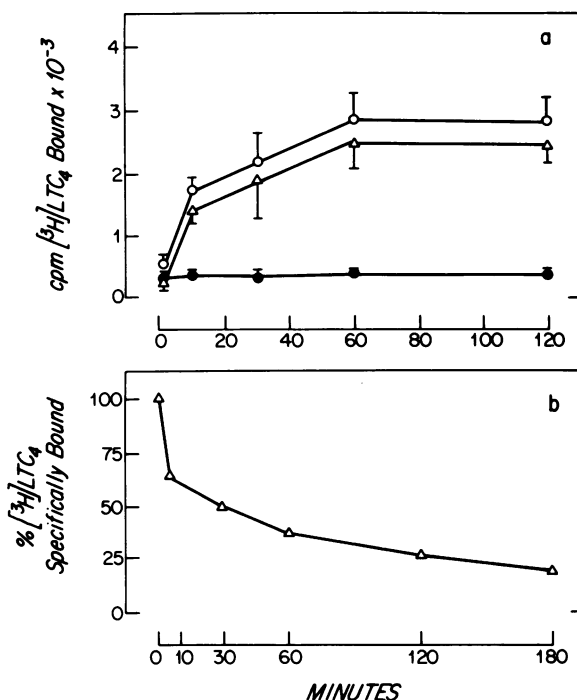


FIGURE 1 (a) Time course of total binding (O), nonspecific binding (●), and specific binding (Δ) of [<sup>3</sup>H]LTC<sub>4</sub> to DDT<sub>1</sub> cells. Each point represents the mean  $\pm$  SD of three different experiments, each performed in triplicate and using 1.2–1.5 nM [<sup>3</sup>H]LTC<sub>4</sub>. (b) Reversibility of specific [<sup>3</sup>H]LTC<sub>4</sub> binding to the DDT<sub>1</sub> cells after a 60-min incubation at 4°C, by addition of an excess of unlabeled LTC<sub>4</sub> (3.2 μM) and continued incubation at 4°C. The points represent the mean binding of two experiments, each performed in duplicate, expressed as a percent of specific residual binding.

assessed in two experiments by the addition of 3.2 μM unlabeled LTC<sub>4</sub> to replicate tubes containing  $0.5 \times 10^6$  smooth muscle cells that had previously been incubated with 1.5 and 1.2 nM [<sup>3</sup>H]LTC<sub>4</sub> for 60 min at 4°C; the quantity of [<sup>3</sup>H]LTC<sub>4</sub> remaining specifically bound was evaluated at subsequent time intervals up to 180 min (Fig. 1 b). Dissociation of [<sup>3</sup>H]LTC<sub>4</sub> occurred with an initial rapid fall at 5 min followed by a slower dissociation to 78% reversibility at 180 min.

Incubation of increasing numbers of the smooth muscle cells from  $0.25$  to  $3.5 \times 10^6$  and a constant concentration of radioligand (1.5 nM) revealed that specific [<sup>3</sup>H]LTC<sub>4</sub> binding was incremental over the entire range of cell numbers and was linear for up to  $1 \times 10^6$ .

With increasing concentrations of radioligand and  $10^6$  smooth muscle cells, specific binding of [<sup>3</sup>H]LTC<sub>4</sub> increased to 0.24 pmol/ $10^6$  cells at concentrations of 6.5 nM [<sup>3</sup>H]LTC<sub>4</sub> or higher, indicating saturability of specific receptors (Fig. 2). Nonspecific binding increased progressively up to the maximum input as-

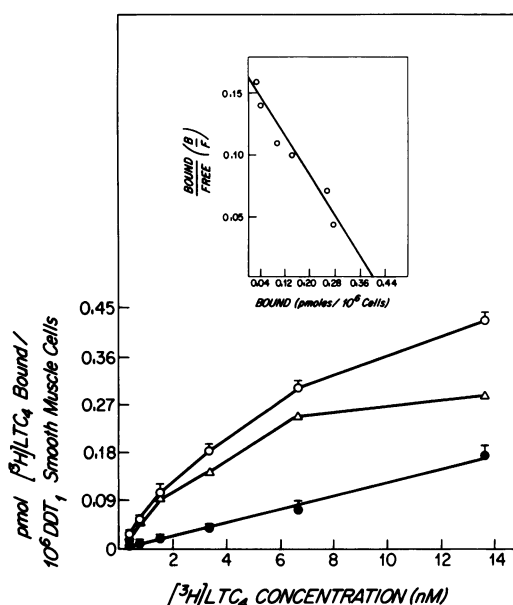


FIGURE 2 Total binding (O), nonspecific binding (●), and specific binding (Δ) of [<sup>3</sup>H]LTC<sub>4</sub> as a function of incremental inputs of [<sup>3</sup>H]LTC<sub>4</sub>. Each point represents the mean±SD of triplicate determinations. (Inset) Scatchard analysis of binding of [<sup>3</sup>H]LTC<sub>4</sub> to DDT<sub>1</sub> smooth muscle cells analyzed with LIGAND (10).

essed. A Scatchard plot of the data analyzed by the LIGAND computer program (10), described one class of binding sites with a dissociation constant ( $K_d$ ) of 5 nM and specific binding of 0.4 pmol/10<sup>6</sup> cells (Fig. 2, inset). In a comparable experiment, one class of binding sites was described with a  $K_d$  of 5.1 nM and a specific binding of 0.28 pmol/10<sup>6</sup> cells.

To evaluate the specificity of the [<sup>3</sup>H]LTC<sub>4</sub> binding sites, sulfidopeptide leukotrienes, structural analogues of LTC<sub>4</sub>, and FPL 55712 were assessed for the capacity to inhibit the specific binding of [<sup>3</sup>H]LTC<sub>4</sub>. LTC<sub>4</sub> at concentrations from 32 pM to 3.2 μM reduced [<sup>3</sup>H]LTC<sub>4</sub> binding in a dose-dependent manner, giving dissociation constants of 4.4±6.2 nM (mean±SD,  $n = 7$ ) for a high affinity site and a specific binding of 0.59±0.5 pmol/10<sup>6</sup> cells. In four competition studies, a low affinity site with a  $K_d$  of 634±871 nM (mean±SD) and a specific binding of 5.15±1.54 pmol/10<sup>6</sup> cells (mean±SD) was also recognized. The abilities of 5(R),6(S)-LTC<sub>4</sub> (relative  $K_d = 11$  nM), 11-*trans*-LTC<sub>4</sub> (relative  $K_d = 10.9$  nM), and C<sub>1</sub>-monoamide-LTC<sub>4</sub> (relative  $K_d = 12.5$  nM) to inhibit the binding of [<sup>3</sup>H]LTC<sub>4</sub> were approximately equal and were two- to threefold less potent than LTC<sub>4</sub> (Fig. 3). LTD<sub>4</sub> (relative  $K_d = 2.0$  μM), LTE<sub>4</sub> (relative  $K_d > 10$  μM), deamino-LTC<sub>4</sub> (relative  $K_d > 10$  μM) and FPL 55712 (relative  $K_d = 7.65$  μM) were minimally active.

## DISCUSSION

The characteristics of [<sup>3</sup>H]LTC<sub>4</sub> binding to intact DDT<sub>1</sub> smooth muscle cells satisfy the criteria for a true receptor (11). Specific [<sup>3</sup>H]LTC<sub>4</sub> binding to intact DDT<sub>1</sub> smooth muscle cells at 4°C was rapid over the first 10 min of incubation, reached a plateau at 60 min for 86% of the total binding (Fig. 1 a), and was reversible by the homologous unlabeled ligand to ~80% of the specifically bound radioligand (Fig. 1 b). Specific binding of a fixed input of [<sup>3</sup>H]LTC<sub>4</sub> was a linear function of DDT<sub>1</sub> smooth muscle cell numbers and accounted for 90±3% of total binding at each cell num-

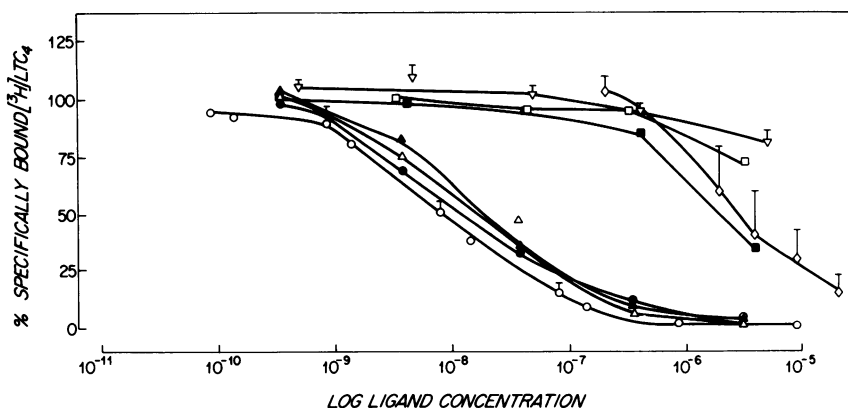


FIGURE 3 Percent inhibition of specific [<sup>3</sup>H]LTC<sub>4</sub> binding by incremental concentrations of LTC<sub>4</sub> (O), 5(R),6(S)-LTC<sub>4</sub> (●), 11-*trans*-LTC<sub>4</sub> (Δ), C<sub>1</sub>-monoamide LTC<sub>4</sub> (▲), deamino-LTC<sub>4</sub> (□), LTD<sub>4</sub> (■), LTE<sub>4</sub> (▽), and FPL 55712 (◇). Data are presented as the mean at each point ±SD when there were three or more separate experiments.

ber, as would be expected under equilibrium conditions. For a fixed number of DDT<sub>1</sub> cells and incremental concentrations of radioligand, the number of binding sites was saturable and indicated a single binding site with a  $K_d$  of 5 nM (Fig. 2) and 5.1 nM in two experiments. Analysis of the competition between a single concentration of [<sup>3</sup>H]LTC<sub>4</sub> and incremental concentrations of unlabeled LTC<sub>4</sub> with a fixed number of DDT<sub>1</sub> cells (Fig. 3) revealed a high affinity binding site in seven experiments with a  $K_d$  of  $4.4 \pm 6.2$  nM. A low affinity receptor with a  $K_d$  of 634 nM was also detected in four analyses. The inconsistent ability to demonstrate a low affinity site may reflect dissociation of radioligand from this site during the filtration washing procedure (12).

The [<sup>3</sup>H]LTC<sub>4</sub> binding sites on the DDT<sub>1</sub> smooth muscle cells are not considered to represent  $\gamma$ -glutamyl transpeptidase because binding was performed in the presence of 1 mM serine-borate complex, a transition state inhibitor of  $\gamma$ -glutamyl transpeptidase (13). In addition, the  $K_d$  values of the binding sites for [<sup>3</sup>H]LTC<sub>4</sub> are 4.4 and 634 nM, as compared with a Michaelis constant ( $K_m$ ) of  $\sim 5.6$   $\mu$ M for kidney  $\gamma$ -glutamyl transpeptidase (14).

Myotonically active analogues of LTC<sub>4</sub>, 11-*trans*-LTC<sub>4</sub> and C<sub>1</sub>-monoamide-LTC<sub>4</sub>, which are, respectively, 71 (4) and 40% (15) as potent as LTC<sub>4</sub> on the guinea pig ileum, displaced specifically bound [<sup>3</sup>H]LTC<sub>4</sub> from the DDT<sub>1</sub> smooth muscle cells with relative  $K_d$  values of 34 and 40% of that of LTC<sub>4</sub>. In contrast, deamino-LTC<sub>4</sub>, which has 1.7% of the LTC<sub>4</sub> agonist activity (Lewis, R. A., S. Krilis, J. M. Drazen, E. J. Corey, and K. F. Austen, unpublished observations), has a  $K_d$  value of <1% of LTC<sub>4</sub> (Fig. 3).

The other myotonically active natural leukotrienes displaced specifically bound [<sup>3</sup>H]LTC<sub>4</sub> weakly with the relative  $K_d$  being 2.0  $\mu$ M for LTD<sub>4</sub> and >10  $\mu$ M for LTE<sub>4</sub>. The putative leukotriene receptor antagonist, FPL 55712, demonstrated dose-dependent inhibition of binding of [<sup>3</sup>H]LTC<sub>4</sub> to the DDT<sub>1</sub> smooth muscle cell line with a relative  $K_d$  of only 7.65  $\mu$ M, indicating that it is not a potent, selective receptor antagonist for LTC<sub>4</sub> in this cell line. Thus, the receptor on this non-vascular smooth muscle cell line is highly specific for LTC<sub>4</sub> and cannot be considered to be a class receptor for the sulfidopeptide leukotrienes.

## ACKNOWLEDGMENTS

This work was supported by grants AI-00399, AI-07722, AI-10356, AI-20081, AM-05577, and RR-05669 from the National Institutes of Health, and a grant from the National Science Foundation.

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