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Characterization of the cloned guinea pig leukotriene B_4 receptor: comparison to its human orthologue

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Abstract

A cDNA clone coding for the guinea pig leukotriene B_4 (BLT) receptor has been isolated from a lung cDNA library. The guinea pig BLT receptor has an open reading frame corresponding to 348 amino acids and shares 73% and 70% identity with human and mouse BLT receptors, respectively. Scatchard analysis of membranes prepared from guinea pig and human BLT receptor-transfected human embryonic kidney (HEK) 293 EBNA (Epstein–Bar Virus Nuclear Antigen) cells showed that both receptors displayed high affinity for leukotriene B_4 (K_d value of $\sim$0.4 nM) and were expressed at high levels (B_{max} values ranging from 9 to 12 pmol/mg protein). The rank order of potency for leukotrienes and related analogs in competition for [^{3}H]leukotriene B_4 specific binding at the recombinant guinea pig BLT receptor is leukotriene B_4 > 20-OH-leukotriene B_4 > 12(*R*)-HETE ((5*Z*,8*Z*,10*E*,12(*R*)14*Z*)-12-hydroxyeicosatetraen-1-oic acid) > 12(*S*)-HETE ((5*Z*,8*Z*,10*E*,12(*S*)14*Z*)-12-Hydroxyeicosatetraen-1-oic acid) > 20-COOH-leukotriene B_4 > U75302 (6-(6-(3-hydroxy-1*E*,5*Z*-undecadienyl)-2-pyridinyl)-1,5-hexanediol) $\gg$ leukotriene C_4 = leukotriene D_4 = leukotriene E_4. For the human receptor the rank order of 12(*S*)-HETE, 20-COOH-leukotriene B_4 and U75302 was reversed. *Xenopus* melanophore and HEK aequorin-based reporter gene assays were used to demonstrate that the guinea pig and human BLT receptors can couple to both the cAMP inhibitory and intracellular Ca^{2+} mobilization signaling pathways. However, in the case of the aequorin-expressing HEK cells (designated AEQ17-293) transfected with either the guinea pig or human BLT receptor, expression of $G_{\alpha 16}$ was required to achieve a robust Ca^{2+} driven response. Leukotriene B_4 was a potent agonist in functional assays of both the guinea pig and human BLT receptors. U-75302 a leukotriene B_4 analogue which possesses both agonistic and antagonistic properties behaved as a full agonist of the guinea pig and human BLT receptors in AEQ17-293 cells and not as an antagonist. The recombinant guinea pig BLT receptor will permit the comparison of the intrinsic potencies of leukotriene B_4 receptor antagonists used in guinea pig in vivo models of allergic and inflammatory disorders.

Keywords: Leukotriene B_4; (Guinea pig); BLT receptor; HEK 293; Melanophore; Aequorin

1. Introduction

Leukotriene B_4, the first leukotriene to be discovered (Borgeat and Samuelsson, 1979), is formed from leukotriene A_4 which is the precursor to all of the bioactive leukotrienes including the cysteinyl leukotrienes, leukotriene C_4, leukotriene D_4 and leukotriene E_4. Made primarily in cells of the myeloid lineage, leukotriene B_4 is a potent leukocyte chemoattractant and chemokinetic agent in vitro (Ford-Hutchinson et al., 1980) and can cause the movement of leukocytes from the blood into the extravascular space in vivo (Dahlen et al., 1981). Thus, leukotriene B_4 plays an important role in the host inflammatory response. Recently, leukotriene B_4 has also been shown to be an activating ligand for the nuclear receptor, peroxisome proliferator-activated receptor (PPAR) α (Devchand et al., 1996), through which the duration of an inflammatory response and clearance of leukotriene B_4 in the liver is proposed to be controlled.

The inflammatory effects of leukotriene B_4 are mediated through a cell surface G-protein coupled receptor

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which has been recently cloned from both retinoic acid differentiated human HL-60 cells (Yokomizo et al., 1997) and mouse eosinophil (Huang et al., 1998) cDNA libraries, respectively. Most recently, the BLT receptor has been found to be a co-receptor for the entry of human immunodeficiency virus-1 into CD4-positive cells (Owman et al., 1998). This receptor was first identified as the orphan receptor CMKRL1 (Owman et al., 1996) and subsequently misidentified as a new member of the P2Y group of purinoceptors (Akbar et al., 1996), since it was shown that ATP does not bind to the receptor (Herold et al., 1997).

Leukotriene B_4 has been implicated in a number of allergic and inflammatory diseases such as asthma, allergic rhinitis, allergic encephalomyelitis, psoriasis, rheumatoid arthritis, inflammatory bowel disease and otitis media. In the case of asthma, however, a potent antagonist of the BLT receptor, LY293111 (Marder et al., 1995), had no effect on allergen-induced early or late-phase responses in asthmatics (Evans et al., 1996), suggesting that inhibition of the BLT receptor is not a viable therapeutic approach for the treatment of bronchial asthma. The guinea pig has been extensively used as an in vivo model of allergic asthma (Chabot-Fletcher et al., 1995), experimental allergic encephalomyelitis (Fretland et al., 1991), middle ear inflammation (Sutbeyaz et al., 1996), tissue eosinophilia (Newsholme et al., 1994), skin inflammation and inflammatory bowel disease (Fretland et al., 1990).

Guinea pig models have been partially validated using in vitro radioligand binding assays to determine ligand affinities. Leukotriene B_4 binding sites have been studied primarily in guinea pig alveolar macrophages (De Brum-Fernandes et al., 1990) and eosinophils (Ng et al., 1991). Here, we describe the cloning and functional characterization of a guinea pig BLT receptor isolated from a lung cDNA library and compare its binding and functional properties with the recently cloned human BLT receptor. The recombinant guinea pig BLT receptor will provide another system to support these in vivo models.

2. Materials and methods

2.1. Chemicals

Tritiated leukotriene B_4 (205 Ci/mmol) was from NEN Life Science Products (Boston, MA); leukotriene B_4, leukotriene C_4, leukotriene D_4 and leukotriene E_4 were synthesized by the Department of Medicinal Chemistry at the Merck Frosst Centre for Therapeutic Research; 20-COOH-leukotriene B_4, 20-OH-leukotriene B_4,12(*S*)-HETE [(5*Z*,8*Z*,10*E*,12(*S*)14*Z*)-12-Hydroxyeicosatetraen-1-oic acid], 12(*R*)-HETE [(5*Z*,8*Z*,10*E*,12(*R*)14*Z*)-12-Hydroxyeicosatetraen-1-oic acid] and U75302 [6-(6-(3-hydroxy-1*E*,5*Z*-undecadienyl)-2-pyridinyl)-1,5-hexanediol] were from Biomol (Plymouth Meeting, PA); LipofectAMINE™ PLUS reagent and all tissue culture media were from GIBCO-BRL Canada (Burlington, Ontario); all other reagents were of analytical grade.

2.2. Cloning of the guinea pig BLT receptor

Three degenerate oligomers, designed from the human BLT receptor sequence, were synthesized (Research Genetics, Cambridge, MA), B4-1 5′GTSTGCGSRSTCAG-CATGTACGCCAGCGTSC-3′, B4-3 5′-AGGTTSA-CYRCGTGGTAGGGSRSCCA-3′ and B4-4 5′-GCACSG-GGTTSACGCTRSWRCTSARGAAGGC-3′ in order to screen by polymerase chain reaction a custom guinea pig lung cDNA library (Invitrogen, Carlsbad, CA). The polyA + RNA was prepared from guinea pig lung tissue and size selected on gel ($\geq$ 1 kb). The cDNA synthesis allowed for unidirectional cloning (*Bst*XI/*Not*I) in the expression vector pcDNA3.1(+) (Invitrogen). Transformation of Top10F′ *E. coli* resulted in 3.65×10^6 primary recombinants. DNA from pools of approximately 300 colonies recovered from Luria–Broth agar plates was prepared (Qiawell 96 ultra plasmid kit, Qiagen) for subsequent analysis using the polymerase chain reaction. Polymerase chain reactions were performed using a DNA thermal cycler 480 (Perkin Elmer Cetus) using the primer pairs B4-1 and B4-3 or B4-1 and B4-4 with Taq polymerase (Boehringer Mannheim) under the following conditions: denaturation: 95°C for 60 s, annealing 55°C for 30 s and extension 72°C for 30 s for 35 cycles. Polymerase chain reaction products were analyzed on agarose gel (Nu Sieve 3:1, FMC). Glycerol cultures corresponding to DNA pools 107, 487 and 562 which showed the expected fragment size were plated for isolation of the positive clones by colony hybridization. A previously identified and sequenced guinea pig BLT receptor DNA fragment, obtained using the polymerase chain reaction from guinea pig lung cDNA using primers B4-1 and B4-3, was random prime labeled (T7 Quick Prime Kit, Pharmacia) to screen the colonies. Clones 107, 487 and 562 were isolated, sequenced using an ABI-373 stretch automated sequencer (Applied Biosystems), and analyzed using Sequencher software (Gene Codes). The DNA of clone 487 was digested with the restriction enzymes Asp718I and *Ava*I to remove some of its 3′ non-coding sequence and then cloned into the expression vector pCEP4 (Invitrogen) to give gpBLTR-pCEP4 and into pcDNA3 (Invitrogen) to give gpBLTR-pcDNA3.

2.3. Transfection of HEK 293-EBNA cells and preparation of cell membranes

Human embryonic kidney (HEK) 293-EBNA (Epstein–Barr Virus Nuclear Antigen) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin-G, 100 μg/ml streptomycin sulphate and 250 μg/ml active Geneticin™ (G418) at 37°C in a humidified atmosphere of 6% CO_2 in

air. Cells were transfected with gpBLTR-pCEP4 or human (h) BLTR-pCEP4 using the LipofectAMINE™ PLUS reagent (GIBCO-BRL) following the manufacturer's instructions. Cells were harvested 48 h post-transfection, washed in phosphate-buffered saline (pH 7.4) and resuspended in 10 mM HEPES/KOH (pH 7.4) containing 1 mM EDTA at $\sim 10^7$ cells/ml. Membrane preparations were done as previously described (Wright et al., 1998).

2.4. Tritiated leukotriene B_4 binding assays

Tritiated leukotriene B_4 binding assays were performed in a final incubation volume of 200 μl of 10 mM HEPES/KOH (pH 7.4) containing 1 mM EDTA, 10 mM $MgCl_2$ and 0.4 nM [^{3}H]leukotriene B_4. Non-specific binding was determined in the presence of 1 μM leukotriene B_4. Dimethylsulphoxide (Me_2SO_4) was added to competing ligands to a final concentration of 1% (v/v). Where leukotriene C_4 and leukotriene D_4 were used as competing ligands, the assay also contained 50 mM serine-borate and 20 mM L-penicillamine, respectively. Addition of 0.7 and 1.0 μg of membrane proteins, from HEK 293-EBNA cells expressing the guinea pig BLT receptor or the human BLT receptor, initiated the reaction, respectively, and the samples were incubated for 60 min at room temperature. The samples were then subjected to filtration and the bound [^{3}H]leukotriene B_4 quantified by liquid scintillation counting.

Tritiated leukotriene B_4 specific binding was calculated by subtracting non-specific binding. Total binding represented $\sim$15% of the radioligand added to the incubation media. Specific binding accounted for 90%–95% of the total binding and was linear with respect to the concentrations of radioligand and protein present in the incubation medium. Non transfected HEK293 (EBNA) cells did not contain [^{3}H]leukotriene B_4 specific binding sites.

2.5. Data calculation

2.5.1. Saturation analysis

Receptor binding assays were performed as described over a concentration range of 0.07–2.4 nM [^{3}H]leukotriene B_4. Non-specific binding was determined at each radioligand concentration using 2.4 μM leukotriene B_4. Equilibrium dissociation constants (K_d) and the number of detectable specific binding sites (B_{max}) were determined by transformation of specific binding saturation isotherms using Accufit Saturation Two-Site data analysis software (Beckman Instruments) based on non-linear least-squares regression analysis adapted from Feldman (1972).

2.5.2. Sigmoidal equilibrium competition curves

Sigmoidal equilibrium competition curves were analyzed by custom designed software employing a non-linear least-squares fitting routine. K_i values were calculated from $K_i = \text{inflection point}/1 + ([\text{radioligand}]/K_d)$.

2.6. Receptor signaling in Xenopus laevis melanophores

Cell culturing of *X. laevis* melanophore cells has been described elsewhere (Daniolos et al., 1990). Briefly, *Xeno-*

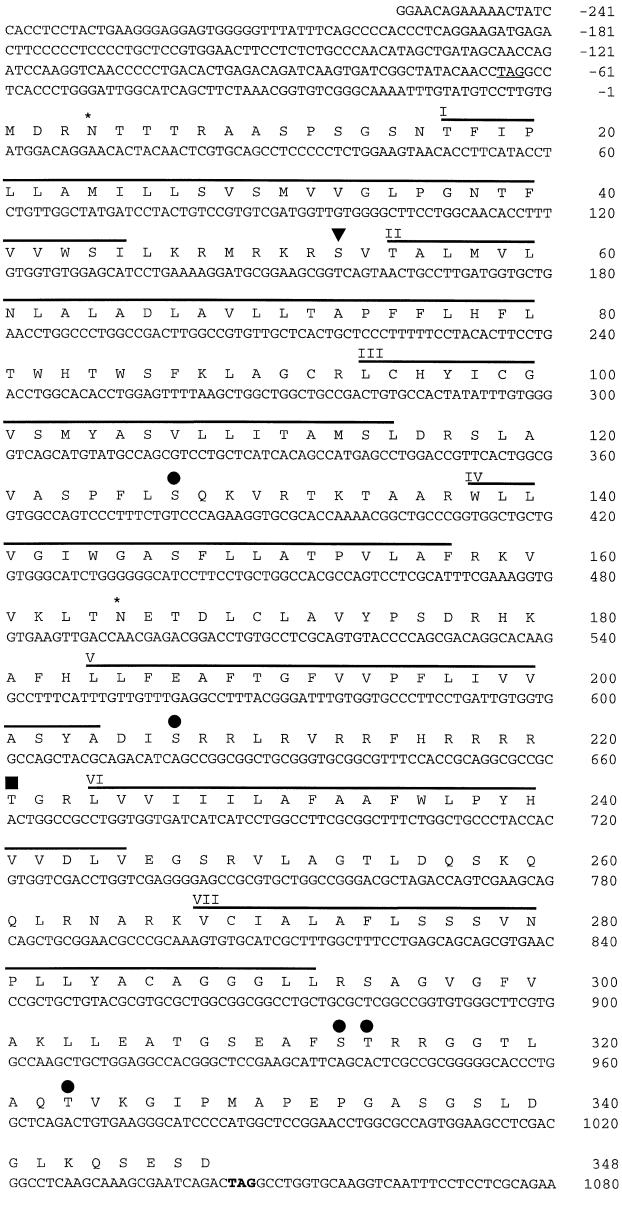


Fig. 1. Nucleotide and deduced amino acid sequence of guinea pig BLT receptor. The deduced amino acid sequence is shown above the nucleotide sequence. The positions of the putative transmembrane segments I–VII (based on the hydropathicity profile) are indicated by overlines above the amino acid sequence. An asterisk denotes a potential *N*-glycosylation sites, circles (●) denote potential protein kinase C (PKC) phosphorylation sites, inverse triangles (▼) denote potential protein kinase A (PKA) phosphorylation sites, and the square (■) denotes a potential PKC/PKA phosphorylation site. An upstream inframe stop codon and putative polyadenylation signal are underlined. The stop codon is in bold type. GenBank accession number AF134488.

pus fibroblast-conditioned growth medium was produced by incubation of fibroblasts at 27°C in 70% L-15 medium (Sigma), pH 7.3, supplemented with 20% heat inactivated fetal bovine serum (GIBCO), 100 μg/ml streptomycin, 100 units/ml penicillin and 2 mM glutamine. Transfection of melanophores is described in detail elsewhere (Kolakowski et al., 1998). Briefly, melanophores were incubated in fibroblast-conditioned growth medium for 1 h, detached by trypsinization (0.25% trypsin, JHR Bioscience) and resuspended to 2×10^6 cells/400 μl in 0.7 × PBS (v/v), pH 7.0 at 4°C. Two micrograms of guinea pig (gp) BLTR-pcDNA3 or human (h) BLTR-pCDNA3 were mixed with 2 μg of each of the internal control G-protein coupled receptors (pcDNA1 amp-cannabinoid 2 and pcDNA3-thromboxane A_2; Slipetz et al., 1995; Abramovitz, unpublished), and brought up to 24 μg total DNA with pcDNA3 plasmid. Mock-transfected melanophores were transiently transfected with 24 μg of pcDNA3 vector DNA. Mixtures were incubated on ice for 20 min with mixing every 7 min. Transient transfection of plasmid DNA into melanophores was performed by electroporation using a BTX ECM600 electroporator (Genetronics, San Diego, CA). Immediately following electroporation, cells were mixed with fibroblast-conditioned growth medium, plated onto flat bottom 96-well microtiter plates (NUNC, Rochester, NY) and incubated at 27°C. Medium was replaced the next day and the assay performed 48-h post-transfection.

Two independent assays were performed in the dark and at room temperature, one for G_i-coupled responses resulting in pigment aggregation, and one for G_s/G_q-coupled responses resulting in pigment dispersion. For G_i-coupled responses, monolayers in 96-well microtiter plates were incubated in the presence of 100 μl/well of supplemented 0.7 × L-15 medium (2% fibroblast-conditioned growth medium, 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin and 15 mM HEPES, pH 7.3), for 15 min to preset the cells to dispersion. Initial absorbance readings at 600 nm were measured prior to addition of ligand using a Bio-Tek El × 800 Microplate reader (ESBE Scientific). Serially diluted leukotriene B_4 was subsequently added to duplicate wells and after 1.5 h final absorbances were determined. For G_s/G_q-coupled responses, monolayers in 96-well microtiter plates were incubated in 100 μl 70% L-15 and 15 mM HEPES, pH 7.3 for 1 h, followed by the addition of melatonin (2 nM) for 1 h to induce pigment aggregation. Initial absorbance readings at 600 nm were then measured. Serially diluted leukotriene B_4 in dimethyl sulphoxide was then added to duplicate wells and after 1 h, the final absorbance was determined. Absorbance readings were converted to transmission values and pigment dispersion was quantitated using the following formula: $1 - T_f/T_i$, where T_i = initial transmission at 600 nm and T_f = final transmission at 600 nm.

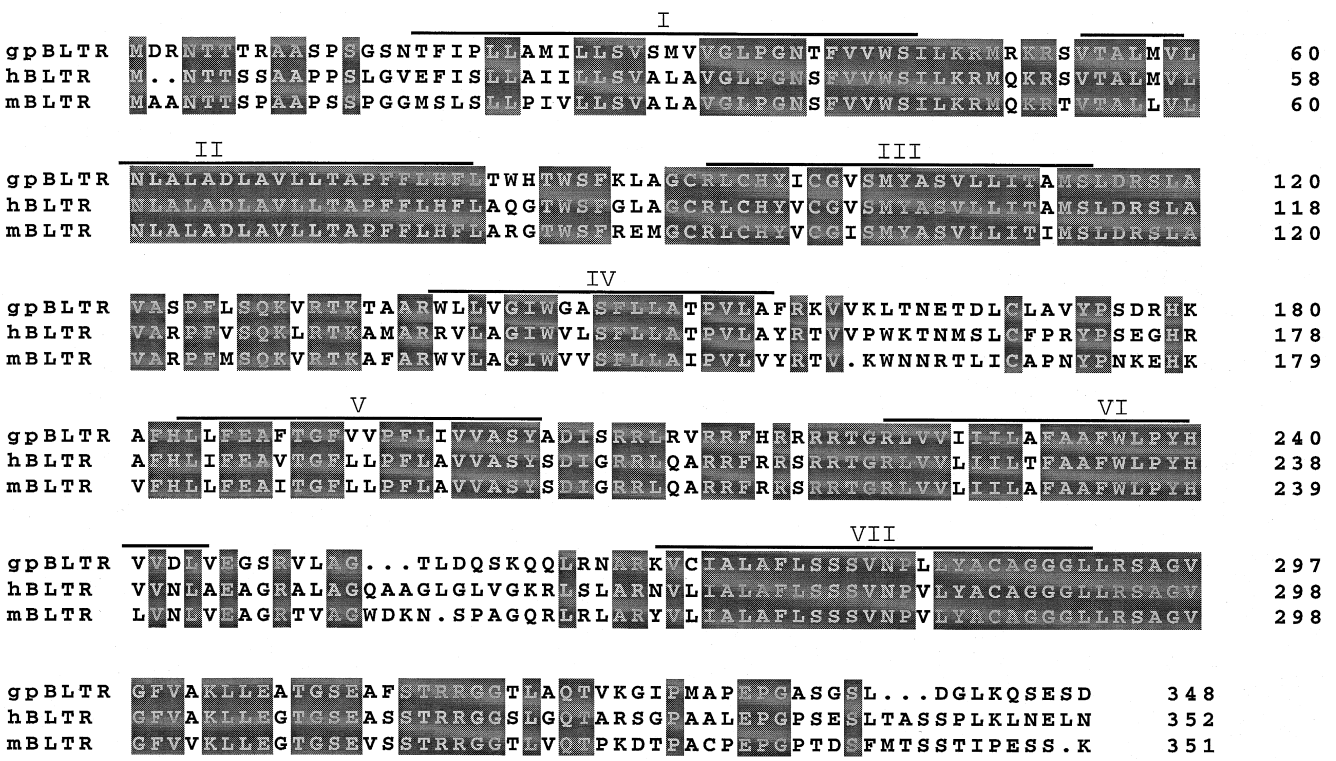


Fig. 2. A comparison of BLT receptor amino acid sequences. The deduced amino acid sequences of the guinea pig (gp), human (h) and mouse (m) BLT receptors are shown, aligned using GCG Wisconsin DNA software. Identical amino acids in all three sequences are shaded. Dots indicate gaps introduced in the sequences for alignment purposes. The positions of the putative transmembrane segments I–VII are indicated by overlines above the guinea pig BLT receptor amino acid sequence.

Table 1
Saturation analysis of [^{3}H]leukotriene B_4 specific binding to membranes from HEK293 (EBNA) cells expressing guinea pig BLT receptor or human BLT receptor. Receptor binding assays were performed as described in Section 2. Results are from two separate experiments

	K_d (nM)	B_{max} (pmol/mg membrane protein)
Guinea pig BLT receptor	0.39, 0.43	12.3, 11.5
Human BLT receptor	0.40, 0.34	9.1, 9.2

2.7. Aequorin luminescence assay

293-AEQ17 cells transfected transiently (as described above) with gpBLTR-pCEP4 or hBLTR-pCEP4 and $G_{\alpha 16}$-pcDNA3 plasmids (10 μg of each DNA per T75 flask) were used in the aequorin luminescence assay (Ungrin et al., 1999). Briefly, holo-aequorin was reconstituted in intact cells by charging 48 h post-transfection cultures for 1 h at 37°C in Ham's F12 (with 0.1% fetal bovine serum, 25 mM HEPES, pH 7.3) (GIBCO) containing 30 μM reduced glutathione (Sigma) and 8 μM of coelenterazine cp (Molecular Probes, Eugene, OR). After charging, the cells were washed from the growth surface by pipetting up and down, rinsed once and resuspended in Ham's F12 medium (modified as above) at 5×10^5 cells/ml. Experiments were performed using a Luminoskan RS plate reading luminometer (Labsystems, Needham Heights, MA) controlled by the Lskan Controller, custom software written in LabView (National Instruments, Austin, TX).

Test compounds in 2 μl Me_2SO were diluted in 98 μl modified Hank's balanced salt solution (HBSS) (with 25 mM HEPES, at pH 7.3) (GIBCO BRL) in a white cliniplate FB 96-well plate (Labsystems) and loaded into the luminometer. Wells were tested sequentially, starting at position A1, by rows. Cells (5×10^4 in 100 μl Ham's F12 medium, modified as above) were injected into the well and light emission was recorded over 30 s (''Peak 1'' recorded as a series of 60 half-second integrations). The cells were then lysed by injection of 25 μl of 0.9% Triton-X 100 solution in modified HBSS, and light emission measured for an additional 10 s (''Peak 2'', recorded as 20 half-second integrations). Serial drug dilutions were made with 10 duplicated data points per concentration–response curve, covering four and a half orders of magnitude. For the antagonist the plate was divided into seven series. Series 1 contained an leukotriene B_4 control curve into which transfected cells were injected in the absence of the antagonist, while series 2 through 7 contained leukotriene B_4 curves, into which cells were injected that had been preincubated in the presence of a given concentration of antagonist.

Peak integration values were obtained by summing the half-second integrations from the raw trace. Fractional luminescence for each well was determined by dividing the area under peak 1 by the total area under peaks 1 and 2. These calculations were performed using the Lskan Controller program, and a data file was generated containing both the raw traces, the calculated results for each well, drug concentrations, and the start time for each well. This data file was then subsequently analyzed using the LDAM software employing a modified version of the Levenberg–Marquardt four-parameter curve fitting algorithm to calculate EC_{50} values.

3. Results

3.1. Cloning of the guinea pig BLT receptor

In order to clone the guinea pig BLT receptor, degenerate oligomers based on the human BLT receptor sequence

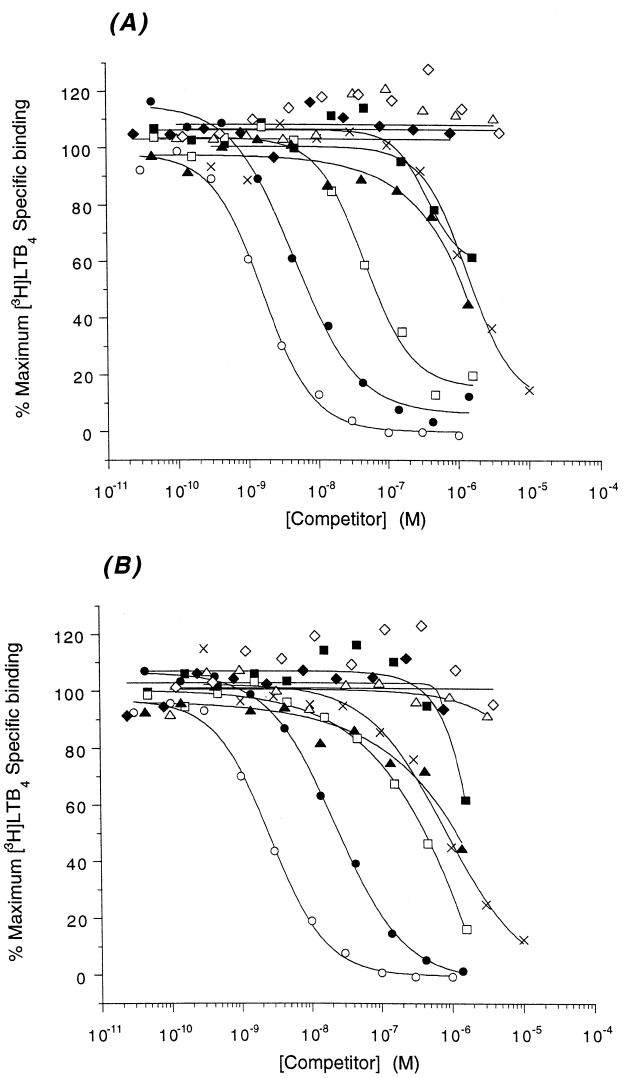


Fig. 3. Competition for [^{3}H]leukotriene B_4 specific binding to HEK 293-EBNA cell membranes expressing (A) guinea pig BLT receptor or (B) human BLT receptor. Receptor binding assays were performed as described in Methods. Each data point was determined in duplicate. ○: leukotriene B_4, ●: 20-OH-leukotriene B_4, □: 12(*R*)-HETE, ■: 12(*S*)-HETE, ◇: leukotriene D_4, ◆: leukotriene C_4, ∇: leukotriene E_4, ▲: 20-COOH-leukotriene B_4, ×: U75302.

(Yokomizo et al., 1997) were used to screen a guinea pig lung cDNA library. The initial identification of clones was done using the polymerase chain reaction on DNA pools, each pool extracted from ~300 bacterial library clone colonies. Three positive clones were isolated by colony hybridization for further sequence analysis. Clones 107 and 562 were identical with 257 bp of 5′ non-coding region compared to only 35 bp for clone 487 (Fig. 1). All the clone sequences ended with a polyA tail located 252 nucleotides after the stop codon. A purine rich region, approximately 120 bp in length, was found in the 3′ non-coding region which was removed when clone 487 was ligated into pCEP4. The sequence of all three clones revealed an identical 1047 bp open reading frame coding for a 348 amino acid protein with a calculated molecular mass of 38007 and a p*I* of 9.2. In vitro transcription translation of the guinea pig BLT receptor cDNA resulted in the production of an ~35 kDa protein in the absence of microsomes. Putative *N*-glycosylation sites, protein kinase C and cAMP phosphorylation sites, and the seven transmembrane domains are shown in Fig. 1. The nucleotide sequence of guinea pig BLT receptor shows 79% and 75% identity with the human and mouse BLT receptors, respectively, and overall the three receptors share 64% identity at the amino acid level (Fig. 2).

3.2. Radioligand binding studies using guinea pig and human BLT receptor-expressing HEK 293-EBNA cell membranes

Saturation analysis was performed to determine the equilibrium dissociation constants (K_d) and the maximum number of detectable specific binding sites (B_{max}) for [^{3}H]leukotriene B_4 specific binding to guinea pig and human BLT receptors (Table 1). Transformation of the deduced specific binding isotherms by saturation analysis demonstrated that [^{3}H]leukotriene B_4 specific binding in both species conformed to a single binding site model. The two receptor species showed similar high affinity for [^{3}H]leukotriene B_4 with K_d values of 0.3 to 0.4 nM. The level of expression of guinea pig and human receptors was also comparable with high B_{max} values ranging from 9 to 12 pmol/mg membrane protein.

A set of ligands were evaluated in equilibrium binding assays (Fig. 3) for their ability to compete for [^{3}H]-leukotriene B_4 specific binding to the guinea pig BLT receptor and the human BLT receptor (Table 2). In both cases, leukotriene B_4 was the most potent competitor displaying a K_i of 0.8 and 0.9 nM at the guinea pig and the human receptors, respectively. In contrast, other cysteinyl leukotrienes were inactive at concentrations ≥ 1 μM. The primary leukotriene B_4 metabolite 20-OH-leukotriene B_4 was the next most potent compound with a K_i of 4 and 11 nM at guinea pig and human BLT receptors, respectively, followed by the hydroxy-acid 12(*R*)-HETE (K_i = 35 and 193 nM, respectively). Finally, 20-COOH-leukotriene B_4, 12(*S*)-HETE and U75302, and analogue of leukotriene B_4, were significantly less potent with K_i values ranging from 0.4 to 0.9 μM at the guinea pig and human receptors.

3.3. Functional coupling of guinea pig and human BLT receptors in melanophores

Xenopus melanophore cells were transfected transiently with cDNAs for guinea pig, or human BLT receptor or mock transfected with pcDNA3 control vector alone. Transfected cells were pre-set in order to test for both G_i-coupled or G_q/G_s-coupled responses (see Section 2).

Cells transfected with guinea pig or human BLT receptor cDNAs and pre-set for pigment granule aggregation, responded in a dose dependent manner with EC_{50} values ranging from 0.04 to 0.15 nM or 0.006 to 0.02 nM, respectively, suggesting that the G_i-coupled signaling pathway was activated (Fig. 4). Mock-transfected *Xenopus* melanophores did not aggregate in the presence of increasing concentrations of leukotriene B_4 (see Fig. 4). In contrast, in melanophore cells transfected with guinea pig or

Table 2
Potencies (K_i and EC_{50}) of eicosanoids and a BLT receptor specific ''antagonist'' at the guinea pig and human BLT receptors. The K_i values were determined from competition of eicosanoids for [^{3}H]leukotriene B_4 specific binding to membranes from BLT receptor expressing HEK 293-EBNA cells. The EC_{50} values were determined from the concentration–response curves generated in the aequorin assay. The radioligand binding and functional aequorin assays were performed as described in Section 2. The number of experiments (n) ± standard deviation are shown for compounds tested more than twice

	K_i (nM)		EC_{50} (nM)	
	Guinea pig BLT receptor	Human BLT receptor	Guinea pig BLT receptor	Human BLT receptor
Leukotriene B_4	0.81 ± 0.06 (n = 3)	0.92 ± 0.21 (n = 3)	0.24 ± 0.21 (n = 6)	0.26 ± 0.09 (n = 5)
20-OH-leukotriene B_4	3.7	11.4	0.67 ± 0.31 (n = 3)	0.42 ± 0.25 (n = 4)
12(*R*)-HETE	35	193	116, 254	82, 242
12(*S*)-HETE	445	> 787	572, 987	700, 1337
20-COOH-leukotriene B_4	714	503	58, 213	25, 98
U75302	886	444	378 ± 366 (n = 3)	166
Leukotriene C_4	> 421	> 400	484	419
Leukotriene D_4	> 2030	> 1930	163	311
Leukotriene E_4	> 1740	> 1650	442	466

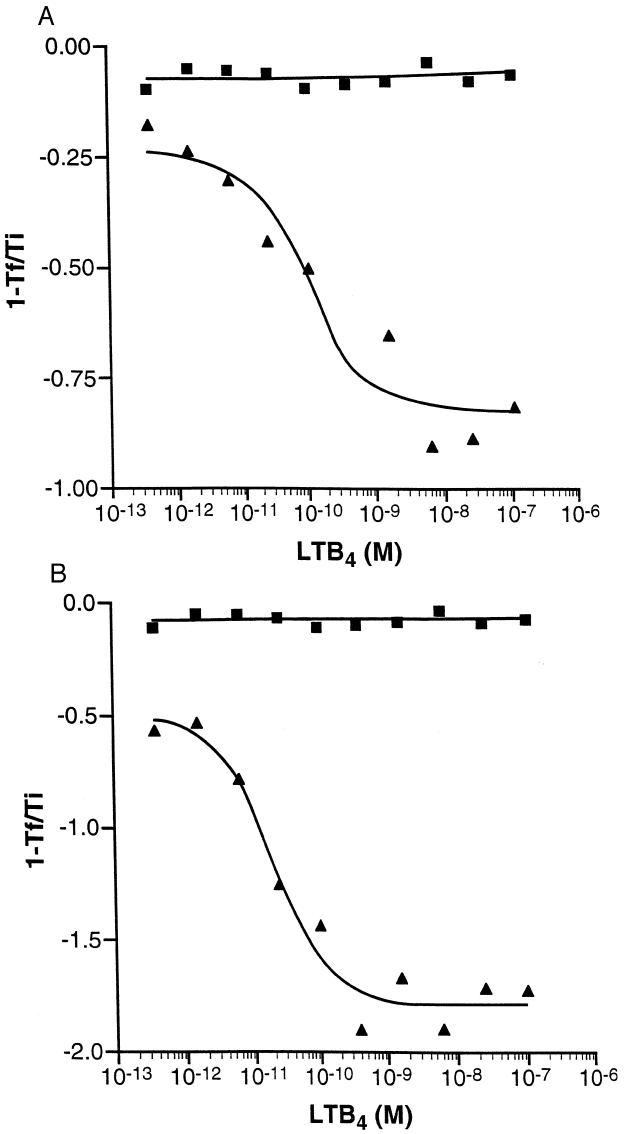


Fig. 4. Functional coupling of human and guinea pig BLT receptor in *Xenopus* melanophores. Pigment aggregation response in melanophores transfected with the human or guinea pig BLT receptor in response to leukotriene B_4 ligand stimulation as described in Section 2. Dose response curves of melanophores transiently transfected with pCDNA3 plasmid (■); and (A) guinea pig or (B) human BLT receptor (▲) and stimulated by the addition of serially diluted leukotriene B_4 ligand for 1.5 h at room temperature. Data are from a representative of three experiments with mean of duplicates plotted.

human BLT receptor or mock transfected with pcDNA3 alone, pigment granules did not disperse when pre-set for G_q or G_s-coupled responses (data not shown). These data suggest that guinea pig and human BLT receptors behave exclusively as G_i-coupled G-protein coupled receptors in the melanophore assay system.

3.4. Functional coupling of guinea pig and human BLT receptors in AEQ17-293 cells

An aequorin luminescence-based Ca^{2+} assay was used to evaluate the coupling of guinea pig and human BLT receptors to Ca^{2+} mobilization. Challenge of 293-AEQ17 cells expressing the guinea pig or human BLT receptor with leukotriene B_4 produced a concentration-dependent increase in the fractional luminescence response. This increase was small, however, $\leq$2-fold in the fractional luminescence response above background (Fig. 5). Recently, it has been reported that expression of $G_{\alpha 16}$ with the human BLT receptor in African Green Monkey kidney COS-7 cells caused an increase in the production of inositol phosphates by at least 10-fold over BLT receptor expressing cells alone (Gaudreau et al., 1998). Similarly, 293-AEQ17 cells co-transfected with guinea pig or human BLT receptor and $G_{\alpha 16}$ resulted in a robust fractional luminescence response, with a 3.5- to 6-fold increase above background in the Ca^{2+} mobilization response (Fig. 5). The same set of ligands evaluated in equilibrium binding assay were then tested in the aequorin assay using AEQ17-293 cells expressing $G_{\alpha 16}$ and the guinea pig or human BLT receptor and the concentration–response curves and EC_{50} values determined (Fig. 6; Table 2). Next to leukotriene B_4, 20-OH-leukotriene B_4 was the most potent activator in the functional assay with subnanomolar EC_{50} values followed by 20-COOH-leukotriene B_4 and 12(*R*)-HETE at both guinea pig and human receptors.

4. Discussion

We have cloned and characterized the guinea pig BLT receptor from a lung cDNA library. The deduced amino acid sequence shared slightly more identity with the human (Yokomizo et al., 1997) than the mouse (Huang et al., 1998) BLT receptor, 73% compared with 70%, respectively. Identity with other G-protein coupled receptor family 1 members (rhodopsin-like G-protein coupled receptors) was low ($\leq$30%), indicating that this receptor is a new subgroup within G-protein coupled receptor family 1.

Transient transfection of guinea pig or human BLT receptor cDNAs into HEK 293-EBNA cells resulted in a very high level of expression of both receptors (Table 1) with similar K_d values for [^{3}H]leukotriene B_4 in the subnanomolar range. The rank order of potency of leukotriene B_4 and related compounds in the radioligand binding assay (Table 2) was consistent with what has been previously reported for the BLT receptor characterized from guinea pig eosinophils (Ng et al., 1991) and similar for the human BLT receptor although the rank order of 12(*S*)-HETE, 20-COOH-leukotriene B_4 and U75302 was reversed (Table 2).

Leukotriene B_4 potently activated guinea pig or human BLT receptors expressed in *Xenopus* melanophore cells causing pigment aggregation, consistent with the receptor functionally coupling to a $G_{\alpha i}$ but not to a $G_{\alpha q}$ or $G_{\alpha s}$ signaling pathway. The endogenous BLT receptor has been shown to couple predominantly to $G_{\alpha i}$ in other cells including human leukocytes (Bomalaski and Mong, 1987) and HL60 cells (Fiore et al., 1993).

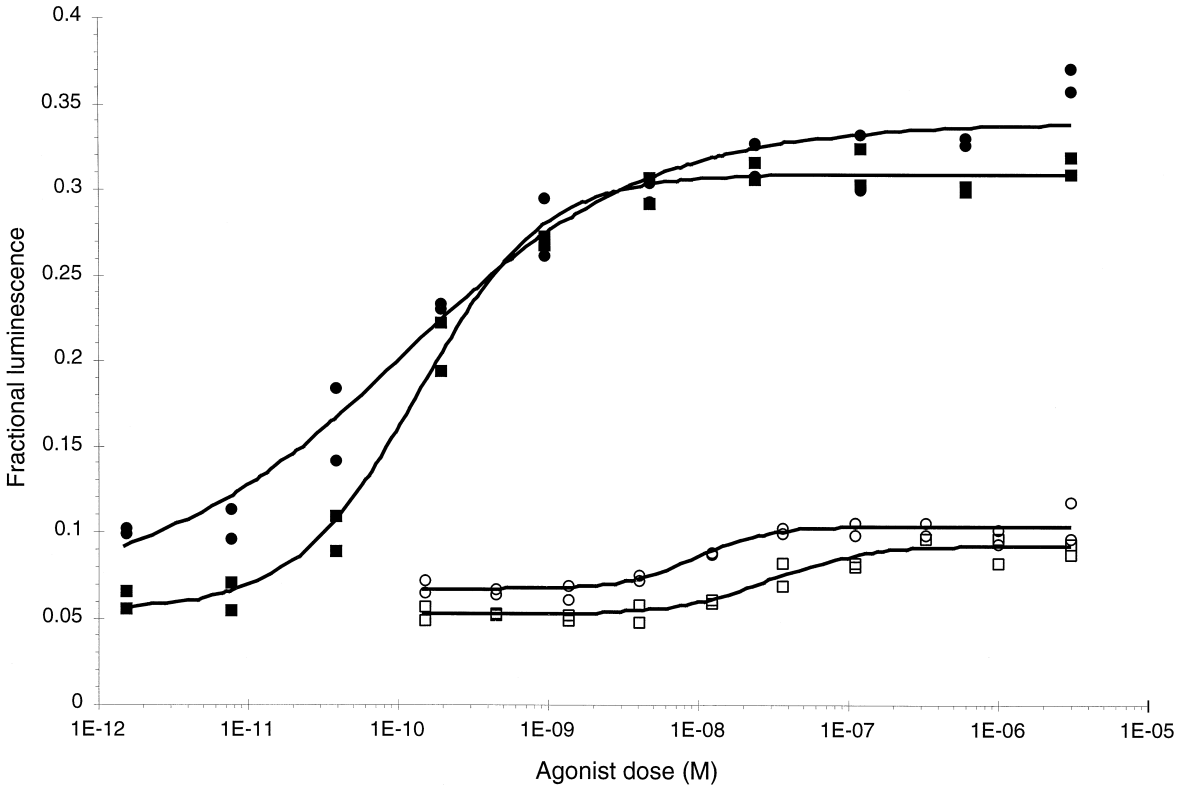


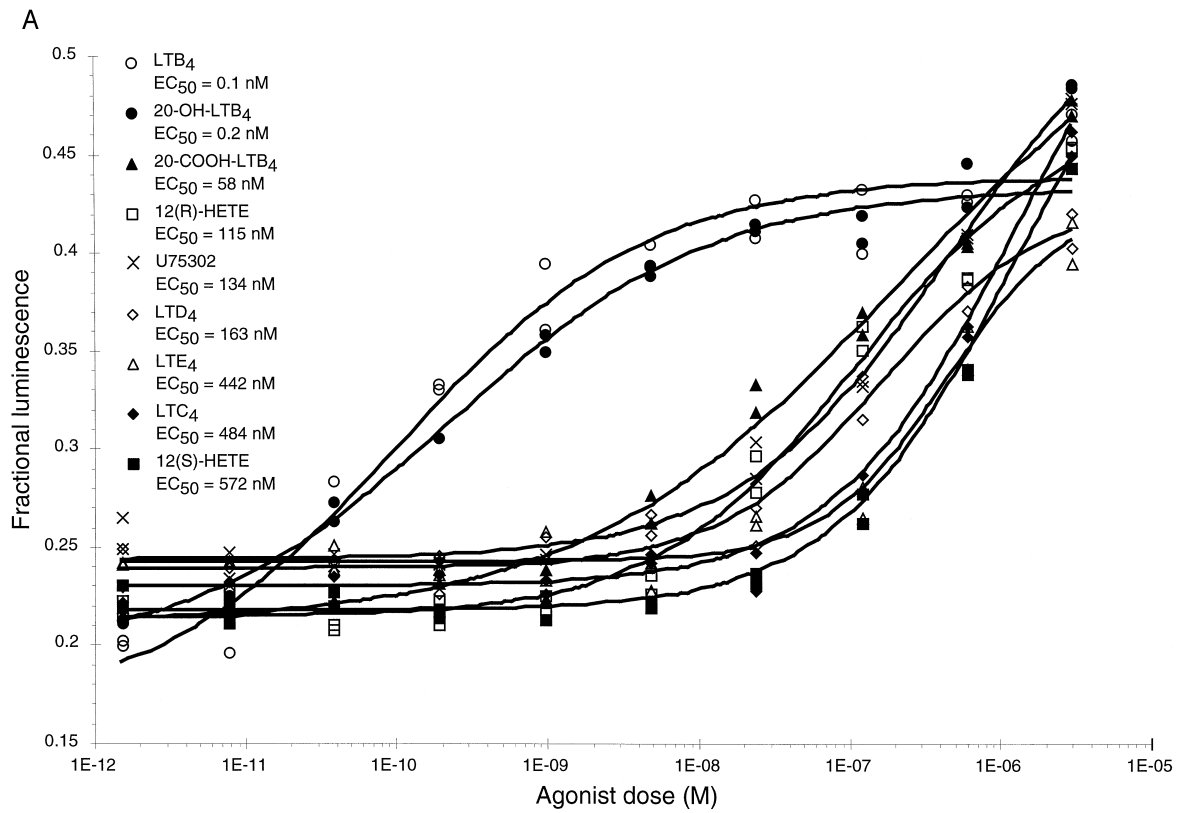
Fig. 5. Functional coupling of guinea pig and human BLT receptors expressed in 293-AEQ17 cells challenged with leukotriene B_4 in the presence (solid symbols) and absence (open symbols) of $G_{\alpha 16}$. Representative fractional luminescence responses of guinea pig (circles) and human (squares) BLT receptors to leukotriene B_4 are plotted as a function of the ligand concentrations. Duplicates for each sample are shown.

It has also been shown, however, that the recombinant human BLT receptor expressed in Chinese hamster ovary (CHO) cells can couple to a predominantly pertussis toxin insensitive Ca^{2+} mobilization pathway (Yokomizo et al., 1997). Guinea pig or human BLT receptors coupled poorly to Ca^{2+} mobilization when expressed in 293-AEQ cells. Upon co-transfection with $G_{\alpha 16}$, however, a robust response was detected. Other receptors, including the chemokine receptors, formyl-Met–Leu–Phe and C5a, have been shown to couple efficiently in HEK 293 cells co-expressing $G_{\alpha 16}$ (Amatruda et al., 1993; Jiang et al., 1996). In 293-AEQ cells, co-expressing the guinea pig or human BLT receptor and $G_{\alpha 16}$, and challenged with leukotriene B_4, the EC_{50} values were comparable to the K_d values from the radioligand binding study, showing that both receptors were efficiently coupled to $G_{\alpha 16}$ in these cells. Interestingly, in CHO cells, exogenous $G_{\alpha 16}$ was not required to detect a Ca^{2+} response (Yokomizo et al., 1997), but in this case the EC_{50} value was approximately 100-fold greater than the K_i suggesting that the receptor did not couple as efficiently to the endogenous G-proteins expressed in this cell line. The proof that the BLT receptor couples to Ca^{2+} pathways in vivo has not been demonstrated unequivocally, however, given that the BLT receptor and $G_{\alpha 16}$ are expressed in cells of hematopoetic lineage (Amatruda et al., 1991; Tenailleau et al., 1997; Yokomizo et al., 1997) these data and that of Gaudreau et al. (1998) suggest this coupling could occur through interaction with $G_{\alpha 16}$.

In general, most of the compounds tested were better activators then competitors at the two receptors (Table 2) when comparing data from the aequorin functional assay with that of the radioligand binding assay. This was most evident with the cysteinyl leukotrienes which were poor competitive inhibitors but better activators of the guinea pig and human receptors (Table 2). One possible reason for this may be that expression of recombinant $G_{\alpha 16}$ causes the receptor to be more susceptible to activation.

Fig. 6. Representative concentration response curves from 293-AEQ17 cells co-transfected with $G_{\alpha 16}$-pCDNA3 and either guinea pig (A) or human (B) BLT receptor-pCEP4 plasmids. The fractional luminescence responses to leukotriene B_4, ●: 20-OH-leukotriene B_4, □: 12(*R*)-HETE, ■: 12(*S*)-HETE, ◇: leukotriene D_4, ◆: leukotriene C_4, ∇: leukotriene E_4, ▼: 20-COOH-leukotriene B_4, ×: U75302 are plotted as a function of their concentrations. Sigmoidal curves were obtained by plotting fractional luminescence at each concentration and analyzed using a modified version of the Levenberg–Marquardt four parameter curve fitting algorithm to determine the EC_{50} values shown on the graph. Duplicates for each sample are shown.

U-75302, a structural analogue of leukotriene B_4, could also compete for receptor binding at both the guinea pig and human receptors with submicromolar K_i values. In the aequorin assay, U-75302 behaved as a full agonist when compared to leukotriene B_4, and was slightly more potent at activating the receptors than binding to them (see



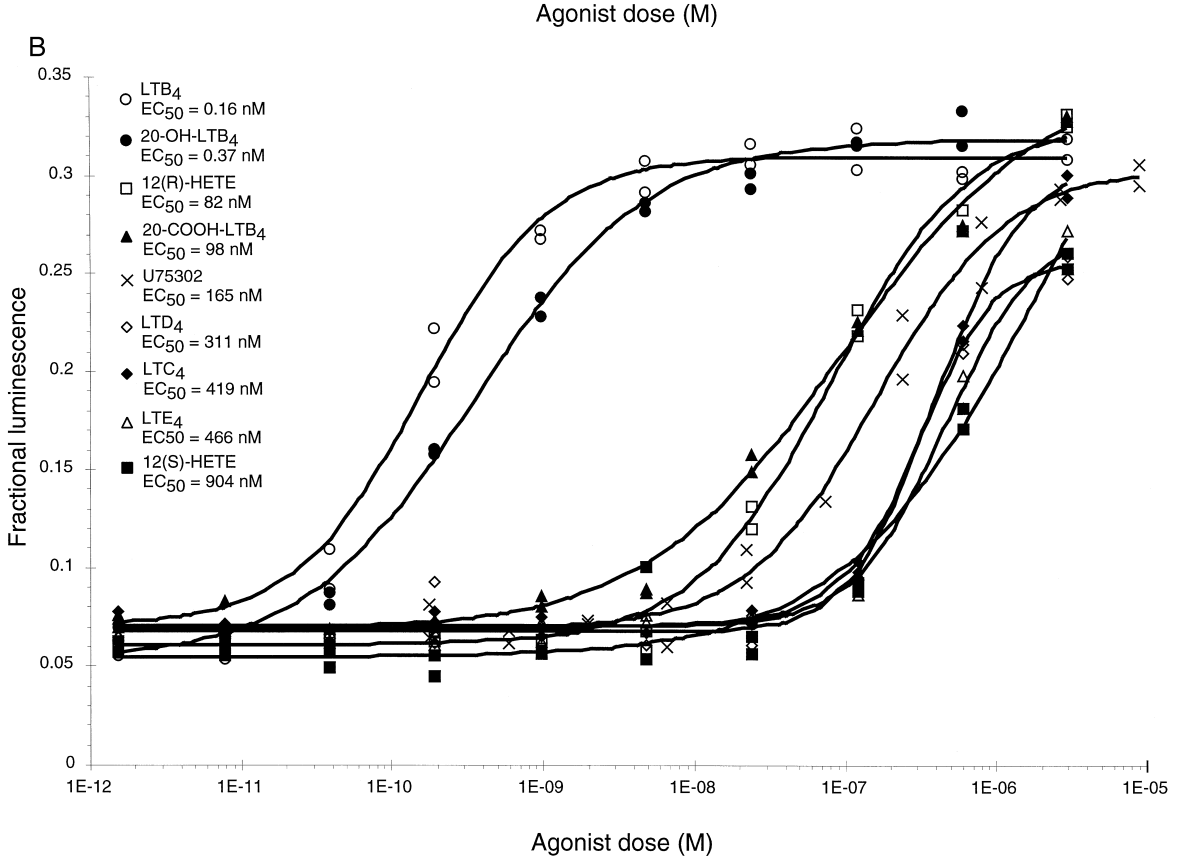


Table 2). Initially developed as an antagonist, U-75302 has also been reported to act as a partial agonist in a guinea pig lung parenchymal strip contraction assay (Lawson et al., 1989). It is also a potent chemotactic, but weak chemokinetic agent for guinea pig eosinophils (Tayor et al., 1991). In the aequorin assay, U-75302 did not display any antagonistic properties. It may be that under the conditions of a high receptor number its ability to act as a partial agonist or antagonist was masked. Alternatively, U-75302 may also be interacting with more than one receptor and that might explain the discrepancies in behavior observed using the various other assay systems.

In summary, we have characterized a guinea pig BLT receptor cloned from a lung cDNA library and compared its pharmacological profile and functional properties with the recently cloned human BLT receptor. The data is consistent with the guinea pig BLT receptor being the orthologue of human BLT receptor. The intrinsic potency of various leukotriene B_4 antagonists used in numerous guinea pig models for various inflammatory and allergic disorders can now be evaluated at the recombinant receptor.

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