methods

Fluorescent leukotriene B_4: potential applications

Alan Sabirsh,[1,*] Anders Wetterholm,[*] Jesper Bristulf,[†] Hakon Leffler,[§] Jesper Z. Haeggström,[*] and Christer Owman[†]

Department of Medical Biochemistry and Biophysics,* Division of Physiological Chemistry II, Karolinska Institute, Stockholm, Sweden; Department of Physiological Sciences,† Division of Molecular Neurobiology, Lund University, Lund, Sweden; and Department of Laboratory Medicine,§ Division of Microbiology, Immunology, and Glycobiology, Lund University, Lund, Sweden

Abstract **Leukotriene B_4 (LTB_4) is a potent lipid mediator of inflammation that acts primarily via a seven-transmembrane-spanning, G-protein-coupled receptor denoted BLT_1. Here, we describe the synthesis and characterization of fluorescent analogs of LTB_4 that are easy to produce, inexpensive, and without the disadvantages of a radioligand. Fluorescent LTB_4 is useful for labeling LTB_4 receptors for which no antibodies are available and for performing one-step fluorescence polarization assays conducive to high-throughput screening. We found that orange and green fluorescent LTB_4 were full agonists that activated the LTB_4 receptor BLT_1 with EC_{50} values of 68 and 40 nM, respectively (4.5 nM for unmodified LTB_4). Flow cytometric measurements and confocal imaging showed that fluorescent LTB_4 colocalized with BLT_1. Fluorescence polarization measurements showed that orange fluorescent LTB_4 bound to BLT_1 with a K_d of 66 nM and that this binding could be displaced by unlabeled LTB_4 and other BLT_1-specific ligands. Fluorescent LTB_4 analogs were also able to displace tritiated LTB_4. Orange fluorescent LTB_4 binding to enhanced green fluorescent protein-tagged BLT_1 could be observed using fluorescence resonance energy transfer. In addition to being a useful alternative to radiolabeled LTB_4, the unique properties of fluorescently labeled LTB_4 allow a variety of detection technologies to be used.**—Sabirsh, A., A. Wetterholm, J. Bristulf, H. Leffler, J. Z. Haeggström, and C. Owman. **Fluorescent leukotriene B_4: potential applications. *J. Lipid Res.* 2005.** 46: **1339–1346.**

Supplementary key words G-protein coupled receptor • pharmacology • method

Leukotriene B_4 (LTB_4) is a proinflammatory lipid mediator generated from arachidonic acid, stored in phospholipids of the nuclear membrane, by the sequential action of phospholipase A_2, 5-lipoxygenase, and LTA_4 hydrolase (1). In humans, LTB_4 is produced primarily by leukocytes of the myeloid lineage, and as a potent chemoattractant, it is important for directing leukocytes toward infected or inflamed tissues. Knockout experiments, deleting either LTA_4 hydrolase (2) or the high-affinity LTB_4 receptor BLT_1 (3, 4), have shown that in animals unable to produce or respond to LTB_4, death occurs after infections that would normally not be fatal and that this correlates with a lack of leukocyte infiltration into infected tissues. These data agree well with a recent report demonstrating that LTB_4, administered systemically to healthy volunteers promotes the production of antiviral cytokines (5). It has also been known for many years that LTB_4 plays a role in almost every pathophysiological condition involving inflammation, and this list has recently been expanded to include atherosclerosis as well (6–9). Recently, LTB_4 has also been found to modulate the behavior of lymphocytes, providing a link between the activation of the early innate immune system and the long-term adaptive immune responses (10).

The physiology of LTB_4 is complex, and this is partly because this mediator binds to several different receptor proteins in addition to BLT_1. Another G-protein-coupled receptor, BLT_2, also binds LTB_4, but with lower affinity (11–13). Unlike BLT_1, this receptor is expressed in many tissues, but its physiological role remains unclear. LTB_4 has also been shown to bind to and activate the vanilloid receptor VR1 (14) and the nuclear peroxisome proliferator-activating receptor α (15).

Radiolabeled LTB_4 is difficult to synthesize and is, at times, commercially unavailable, which is impeding molecular pharmacological studies of LTB_4 receptors. Furthermore, commercially available antibodies only recognize the human isoform of the LTB_4 receptor BLT_1 (16), making it impossible to assess LTB_4 receptor expression in other species. The current interest in LTB_4 as an immunomodulatory agent has also created a need for a method that would allow LTB_4 receptor expression to be easily evaluated in mouse tissues. A fluorescently labeled derivative of LTB_4, compatible with a range of fluorescence-based optical techniques, could potentially be useful for this pur-

Manuscript received 15 February 2005 and in revised form 17 March 2005.

Published, JLR Papers in Press, April 1, 2005.
DOI 10.1194/jlr.D500005-JLR200

[1] To whom correspondence should be addressed.
e-mail: alan.sabirsh@mbb.ki.se

pose. In addition, a fluorescent ligand would permit real-time measurements to be made at true equilibrium using fluorescence polarization and fluorescence resonance energy transfer (FRET) techniques. These measurements are made in homogenous mixtures requiring no further handling, which is ideal for high-throughput screening. This also facilitates experiments involving soluble LTB_4 binding proteins that are not compatible with methodologies that use filtration-based techniques to measure binding. Finally, by carefully choosing the fluorophores used to label LTB_4, it is possible to use FRET to evaluate ligand binding to receptors.

Here, we present the synthesis of fluorescent LTB_4 molecules. We show that these ligands bind to and activate BLT_1 and that they can be used successfully to study BLT_1 receptors using a variety of techniques incompatible with radiolabeled LTB_4, including flow cytometry, confocal microscopy, fluorescence polarization, and FRET. This will allow LTB_4 physiology to be studied at equilibrium, over time, and in tissues or biochemical systems that have previously been inaccessible.

MATERIALS AND METHODS

Chemicals and reagents

All reagents were purchased from Sigma, unless stated otherwise. RP69698 was obtained from Rhône-Poulenc/Aventis (Vitry Sur Seine, Cedex, France). LTB_4 antiserum was obtained from Cayman Chemical (Ann Arbor, MI) and Assay Designs (Ann Arbor, MI). Hydroxamate, RB 3040, and Kelatorphan were generous gifts from Prof. B. P. Roques (UFR des Sciences Pharmaceutiques et Biologiques). All leukotrienes were from Biomol (Plymouth Meeting, PA).

Anti-BLT_1 receptor antibody 7B1 (16) was obtained from Serotec. The molar antibody concentration was calculated using the molecular weight of the appropriate (mouse) immunoglobulin isotype obtained from the crystal structure (17). The concentration of protein in the antibody solution was determined using a protein assay according to the manufacturer's instructions (BCA Protein Assay™; Pierce, Rockford, IL). Antibody concentrations should be regarded as approximate because of uncertainties regarding their actual weight and the concentration of active antibody in antibody solutions.

Purified LTA_4 hydrolase was produced as described previously (18).

Labeling LTB_4 with Alexa Fluor™ dyes

Fluorophore-labeled LTB_4 was produced using LTB_4-aminopropylamide (LTB_4-APA; Biomol), and amine reactive succinimidyl esters of Alexa Fluor™ dyes (Molecular Probes, Eugene, OR) were dissolved in methanol to a concentration of 1 mM. LTB_4-APA was dried in a nitrogen atmosphere and redissolved in methanol to a concentration of 1.28 mM.

LTB_4-APA and Alexa Fluor™ dye solutions were mixed at a ratio of 1:2 and incubated for 60 min at room temperature with continuous stirring. The LTB_4-APA-Alexa Fluor™ product was isolated using reverse-phase HPLC with a Nova Pak C_{18} column (Waters, Milford, MA) and a mobile phase consisting of methanol-water-acetate at a ratio of 60:40:0.08 (pH set to 6.8 using NaOH).

The fractions containing LTB_4-APA and fluorescent LTB_4 were collected, and the HPLC mobile phase was removed by diluting each sample 1:1 with water and then filtering through Supel Clean™ LC-18 columns (Supelco/Sigma-Aldrich, Bellefonte, PA). Both LTB_4-APA and fluorescent LTB_4 were retained in the columns and were washed once with water before elution in methanol. The purified substances were dried in a nitrogen atmosphere and redissolved in methanol. Concentrations were determined using adsorbance at 270 nm for LTB_4-APA (extinction coefficient = 50,000) and 365 nm (the Alexa Fluor™ portion of the molecule absorbs light at 365 nm, whereas the unmodified LTB_4-APA does not). Using fluorescence emission, the concentration of fluorescent versions of LTB_4 could also be independently verified by comparison with a standard curve prepared using known quantities of each dye.

The various labeled versions of LTB_4 are referred to as "fluorescent LTB_4" or LTB_4-488 (for the green fluorescent LTB_4-APA-Alexa Fluor-488™ conjugate) and LTB_4-568 (for the orange fluorescent LTB_4-APA-Alexa Fluor-568™ conjugate) as necessary.

Cell culture and construction of HF1pBLT1 cell lines

Cultures of HeLa HF1pBLT1 luciferase reporter cells expressing BLT_1 or sham-transfected cells (HF1pSham) were established and maintained according to Kotarsky, Owman, and Olde (19). To create BLT_1 receptors that were C-terminally tagged with enhanced green fluorescent protein (EGFP), we used the mammalian expression vector pEAK12 (Edge Biosystems). This vector was used to construct the pEAK-HFTE vector containing a $(His)_{10}$ tag (H), a FLAG tag (F), and the recognition site for the tobacco etch virus (T) protease followed by EGFP (E). The HFTE cassette was assembled using PCR and cloned into pEAK12 between the *Eco*RI and *Not*I sites using standard methods. The open reading frame of the human BLT_1 receptor was generated using PCR with the following primers: 5 prime, ATATAAGCTTCCACCATGAACACTACATCTTCT (which includes a *Hind*III site and a Kozak consensus sequence); and 3 prime, CAGTGAATTCCCGTTCAGTTCGTTTAACTTGAGAG (this primer adds an *Eco*RI site and removes the natural stop codon). BLT_1 was then cloned into pEAK-HFTE between *Hind*III and *Eco*RI to generate pEAK-BLTR-HFTE, in which the open reading frame of BLT_1 was in frame with, and upstream of, the HFTE cassette such that all three tags and the protease site were added to the C-terminal end of the expressed receptor protein. The integrity of the construct was confirmed by sequencing with BigDye (Applied Biosystems). HeLa HF1 cells were stably transfected with pEAK-BLT1-HFTE to produce HF1pBLT1-EGFP cells.

Neutrophils were purified from buffy coats using density gradient centrifugation (Lymphoprep; Axis-Shield, Oslo, Norway) after dextran sedimentation of erythrocytes. Purity and viability were determined using May-Grunwald-Giemsa staining and trypan blue exclusion, respectively. The resulting cell cultures contained at least 95% neutrophils, and the cells were at least 99% viable.

Membrane preparation

Cell cultures were grown on tissue culture plates until confluent and then chilled to 4°C. The cell growth medium was removed and the cells were rinsed once with ice-cold PBS, before ice-cold Tris-HCl buffer (50 mM Tris base, 5 mM $MgCl_2$, and 1 mM EGTA, pH 7.5) was added to each plate. The cells were then scraped off and homogenized using a motorized Teflon pestle.

The cell homogenates were centrifuged at 1,000 *g* for 10 min at 4°C. The pellet was discarded, additional buffer was added, and the supernatant was rehomogenized. The resulting homogenate was centrifuged at 100,000 *g* for 60 min at 4°C, and the supernatant was discarded, additional buffer was added, and the pellet was rehomogenized. The membrane protein concentration was determined (BCA protein assay™), and the membrane isolates were portioned into aliquots before they were frozen at −80°C until use.

Confocal microscopy

For confocal microscopic studies, HF1pBLT1-EGFP and HF1pSham cells were seeded onto poly-D-lysine-coated cover slips at least 24 h before use. Just before staining, the cells were chilled to 4°C and rinsed with PBS. After addition of 100 nM LTB_4-568, the cells were incubated for 60 min at 4°C. The cells were then washed four times and fixed with 4% (w/v) paraformaldehyde for 5 min at room temperature. The expression of BLT_1-EGFP and LTB_4-568 binding were then examined using a TCS SP2 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with an argon laser (488 nm emission line) and a green He/Ne laser (543 nm emission line), respectively.

Flow cytometry

Flow cytometric analysis of HF1pBLT1 and HF1pSham cells was performed using a FACSVantage™ (Becton Dickinson, Franklin Lakes, NJ). After staining with 100 nM LTB_4-568, fluorescence was analyzed using a FACSArray bioanalyzer (Becton Dickinson) equipped with a green diode laser emitting 532 nm light.

Radioligand binding assays

Radioligand binding assays were performed using opaque white 96-well filter plates with FC glass fiber filters (model MAFCNOB, Multiscreen Assay System; Millipore, Bedford, MA). The plates were presoaked with binding buffer (0.02 M HEPES, 10 mM $CaCl_2$, and 10 mM $MgCl_2 \cdot 6H_2O$, pH 7.5), which was then exchanged for 75 μl of binding buffer containing 1.0 nM [^{3}H]LTB_4 and, if necessary, 2.0 μM unlabeled LTB_4 to determine nonspecific binding. The binding reaction was started by adding another 75 μl of binding buffer containing 1.0 μg of rehomogenized cell membrane and the appropriate concentration of any test substance. The membrane-ligand solution was incubated for 1 h at room temperature. The reaction was terminated by rapid filtration, and the filters were then washed three times with 200 μl volumes of ice-cold washing buffer (20 mM Tris-base and 0.5 g/l BSA). Excess washing buffer was removed by blotting, and the plates were dried at 40°C for 30 min. Twenty-five microliters of Microscint-O (Perkin-Elmer, Boston, MA) was then added to each well, and [^{3}H]LTB_4 binding was evaluated using a MicroBeta scintillation counter (Perkin-Elmer).

FRET analysis of fluorescent LTB_4 binding to BLT_1

HF1pSham, HF1pBLT1, and/or HF1pBLT1-EGFP cells were seeded onto black, clear-bottomed 96-well plates at least 48 h before use. Just before use, the cells were chilled to 4°C and gently washed three times in an ice-cold buffer solution (135 mM NaCl, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11 mM glucose, and 10 mM HEPES, pH 6.0, 7.5, or 9.0). Fifty microliters of cold buffer containing LTB_4-488 or LTB_4-568, or both, was added to appropriate wells, with or without excess unlabeled LTB_4. The cells were then incubated for 30 min at 4°C before they were washed three times with chilled buffer. Fluorescence and FRET were measured using a fluorometer (PolarStar™; BMG Labtech, Offenburg, Germany) chilled to <16°C. After fluorescence analysis, cell morphology was checked microscopically, the cells were counted, and the results were corrected for cell density. Cells on the same plate were also challenged with 100 nM LTB_4 to ensure that functional BLT_1 receptors were expressed. The resulting calcium release was visualized using FURA2-AM according to previously reported methods (20).

Fluorescence polarization assays of fluorescent LTB_4 binding

Fluorescence polarization assays of fluorescent LTB_4 binding to membrane preparations were performed in a total volume of 50 μl of binding buffer (0.02 M HEPES, 10 mM $CaCl_2$, and 10 mM $MgCl_2 \cdot 6H_2O$, pH 7.5) on black, 96-well, low-volume (50 μl/well) plates from Molecular Devices. For competition experiments, membrane preparations were added to each well (at a concentration of 10 μg protein/well) together with the appropriate competitor or 1 μM LTB_4 and 10 nM fluorescent LTB_4. The membranes were incubated at room temperature for 1 h before fluorescent ligand binding was analyzed using a fluorometer (PolarStar™) equipped to measure fluorescence polarization. For each competitor or LTB_4, the concentration that inhibited half of the specific binding (IC_{50}) of fluorescent LTB_4 was determined when possible from competitive binding curves using nonlinear regression (Prism; GraphPad Software, San Diego, CA). For measurements of ligand binding to purified soluble proteins, the procedure was essentially the same as described above, and molar concentrations were calculated, after protein determination, using molar weights obtained from crystallographic structures.

For measurements of fluorescent LTB_4 association and dissociation, a final concentration of 10 nM fluorescent LTB_4 was added to membrane preparations as described above, and repeated polarization measurements were made over 30 min before adding a final concentration of 1 μM LTB_4 and measuring polarization for a further 30 min. Saturation experiments were performed using up to 100 nM fluorescent LTB_4 with or without 10 μM LTB_4. Results are presented in millipolars calculated as $(\| - \perp)/(\| + \perp) \times 1{,}000$, where $\|$ is fluorescence emission polarized in parallel to the excitation source and $\perp$ is perpendicularly polarized fluorescence emission.

Fluorescence assays of fluorescent LTB_4 binding

Fluorescent LTB_4 binding to whole cells was examined using cells growing on black, clear-bottomed, 96-well plates. The protocol was similar to that used for fluorescence polarization, except that the final incubation volume was 100 μl and the entire procedure was performed at 4°C with an incubation time of 30 min. After the incubation, the cells were washed four times with PBS Dulbecco's, and then all fluid was removed from the wells. Fluorescence was measured using a fluorometer (Fluostar™; BMG).

Luciferase assay of BLT_1 activity

The assay for agonist-induced luciferase production was performed according to Kotarsky, Owman, and Olde (19). This assay is used here to measure the relative potencies and efficacies of LTB_4 and its fluorescent counterparts. Briefly, HF1pBLTR1 cells were seeded as described above onto white, clear-bottomed, 96-well plates (Corning Costar, Corning, NY) and grown until they were 80–90% confluent. PBS solutions containing ligands were then added (in a volume not exceeding 10 μl) to the wells (results are reported as final ligand concentrations). After a further 16 h of incubation, the cells were washed once with PBS and lysed. Luciferase activity was measured using a 96-well luminometer (Lumistar™; BMG). The half-maximum effective ligand concentrations (EC_{50}) were determined from the luciferase assay concentration response curves using nonlinear regression (Prism), and results are presented as mean EC_{50} values with 95% confidence intervals.

RESULTS

Synthesis of fluorescent LTB_4

The reaction between LTB_4-APA and the succinimidyl ester of the Alexa Fluor™ dye is simple (**Fig. 1**) and proceeds at room temperature, resulting in ~30% labeled LTB_4-APA after 1 h. The reaction mixture is slightly acidic (pH 6.0) and the yield was found to decrease significantly

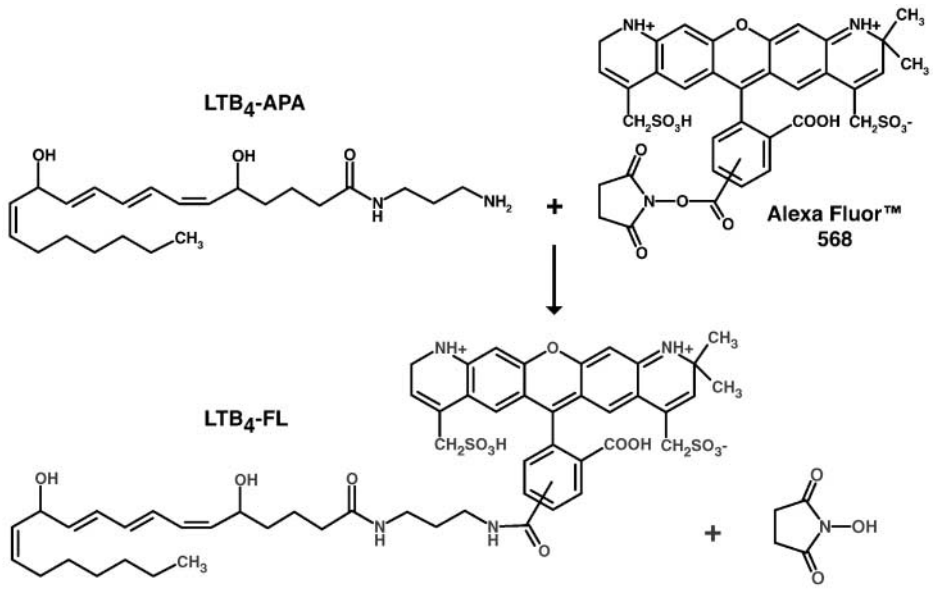

Fig. 1. The synthesis of fluorescent leukotriene B_4 (LTB_4-FL) from LTB_4-aminopropylamide (LTB_4-APA) and Alexa Fluor™ fluorophores. The free amino group of LTB_4-APA hydrolyzes the succinimidyl ester to form mixed isomers of LTB_4 Alexa Fluor™ 568 or 488 (fluorescent LTB_4).

if the pH was increased using sodium hydroxide. The unreacted Alexa Fluor™ dye passed through the column immediately followed by fluorescent LTB_4 and unreacted LTB_4-APA. The two reaction components and the product could be detected using 270 nm absorption. Some additional reaction products were also detected, particularly after the Alexa Fluor™ dye was stored (after solvation) for more than 5 months at −80°C, but these contaminants were easily distinguished from fluorescent LTB_4.

Fluorescence polarization measurements

We used a PolarStar™ (BMG) to measure the fluorescent anisotropy (polarization) of labeled LTB_4, and this machine was found, under our experimental conditions, to be sensitive in a linear manner over concentrations ranging from >100 nM to 1 nM. Although the concentration of fluorescent LTB_4 did not affect the average fluorescence polarization over this range, concentrations less than 5 nM approached the noise floor of the assay and tended to produce erratic polarization values. Solutions with increasing concentrations of glycerol were also used to evaluate the dynamic range of fluorescence polarization values that could be obtained using fluorescent LTB_4. Fluorescent LTB_4 was found to be sensitive to increasing glycerol concentrations, although not as sensitive as the free Alexa Fluor™ fluorophores, which had a larger dynamic range. The polarity shift of fluorescent LTB_4 was not sensitive to the addition of unlabeled LTB_4.

Fluorescent LTB_4 as a BLT_1 agonist

The ability of fluorescent LTB_4 to activate HF1pBLT1 luciferase reporter cells was studied by comparing unmodified LTB_4 with labeled LTB_4. The EC_{50} for LTB_4 was 4.5 nM (1.7–12) (means and 95% confidence intervals), whereas the EC_{50} for LTB_4-568 was found to be ~15 times higher at 68 nM (31–150), and the EC_{50} for LTB_4-488 was found to be ~10 times higher at 40 nM (25–78). Both ligands were full agonists. The EC_{50} for LTB_4 was not affected by the addition of free Alexa Fluor™ dye, even at concentrations as high as 10 μM.

Fluorescent LTB_4 and BLT_1 binding experiments

The binding of labeled LTB_4 to BLT_1 was examined using membranes prepared from HF1pBLT1 cells and measurements of fluorescence polarization. LTB_4-568 affinity for BLT_1 was calculated to be K_d = 66 nM (43–76) with a receptor concentration (Bmax) of 60 nM (31–101) according to the method of Prystay, Gosselin, and Banks (21). Accordingly, the association rate for LTB_4-568 was found to have an observed rate constant of 0.35 min^{-1} (0.33–0.37), so that specific binding of LTB_4-568 by BLT_1 was essentially complete after 20 min. LTB_4-568 is displaced quickly by unlabeled LTB_4, and it was impossible, for technical reasons, to make the first polarization measurement before a significant portion of the bound LTB_4-568 had been displaced. The specific LTB_4-568 binding could be displaced using unlabeled LTB_4, with an IC_{50} of 110 nM (78–160), and also by the LTB_4 antagonist RP69698 [IC_{50} = 1.60 μM (0.94–2.70)], the BLT_1-specific partial agonist U75302 [IC_{50} = 2.10 μM (0.14–3.0)], and a BLT_1 antibody [IC_{50} = 1.9 μM (0.1–3.6) approximate concentration; see Materials and Methods], but not by the chemically related LTC_4 or an isotype control antibody (**Fig. 2**). In competition experiments, there was no difference in the ability of unlabeled LTB_4 to displace labeled LTB_4, regardless of when the unlabeled ligand was added. No specific binding of fluorescent LTB_4 was observed when using sham-transfected HF1 cells or their membranes.

The interaction between fluorescent LTB_4 and BLT_1 was also examined using conventional nonequilibrium bind-

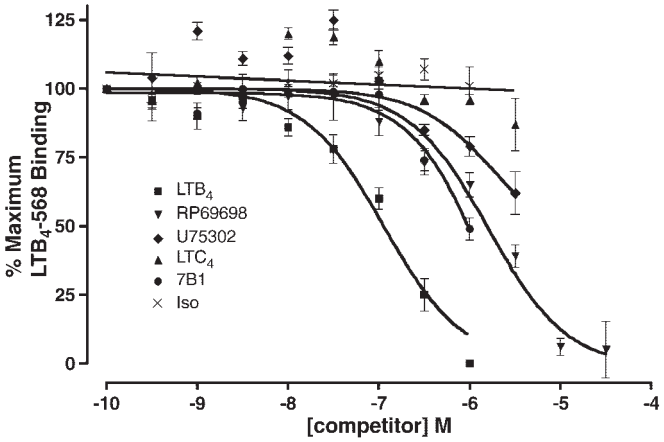

Fig. 2. Competition between LTB_4-568 and various ligands for BLT_1 binding. Displacement of LTB_4-568 (10 nM) binding to HF1pBLT1 cell membranes (10 μg/well) by unlabeled LTB_4, the LTB_4 antagonist RP69698, the BLT_1-specific partial agonist U75302, and the BLT_1 receptor antibody 7B1. Isotype control antibodies (Iso) and LTC_4 (which is not a ligand for BLT_1) did not displace LTB_4-568 binding. Data are presented as means ± SEM from triplicate wells in two separate experiments performed at 24°C.

ing experiments. Measuring fluorescence bound to whole cells expressing BLT_1, similar binding constants were obtained for LTB_4-568 affinity [K_d = 64 nM (27–102)] and LTB_4-568 displacement by LTB_4 [IC_{50} = 75 nM (36–156)]. The ability of fluorescently labeled LTB_4 to displace tritiated LTB_4 was also evaluated using membranes prepared from HF1pBLT1 cells. Tritiated LTB_4 could be displaced using unlabeled LTB_4 with a calculated IC_{50} of 3 nM (2.1–4.6), by LTB_4-488 [IC_{50} = 11 nM (5–26)], LTB_4-568 [IC_{50} = 22 nM (12–38)], and LTB_4-APA [IC_{50} = 180 nM (91–360)].

Colocalization of fluorescent LTB_4 and EGFP-tagged BLT_1

Confocal microscopy of HF1pBLT1 cells revealed that the cell surface expression of EGFP-tagged receptors and LTB_4-568 fluorescence overlapped (**Fig. 3C**). Flow cytometric analysis of cells expressing a C-terminal EGFP-tagged BLT_1 receptor and surface labeled with BLT_1 antibodies indicated that more than 90% of the expressed receptors could be found on the cell surface (Fig. 3A). This same population of cells bound LTB_4-568 specifically, and this binding could be displaced using unlabeled LTB_4 (Fig. 3B). Sham-transfected cells did not specifically bind LTB_4-568, and cells exposed to only Alexa Fluor 568™ could not be differentiated from unlabeled cells (Fig. 3B). To demonstrate that fluorescent LTB_4 could also label immunologically relevant cells, we also labeled neutrophils with LTB_4-568 or with LTB_4-568 and excess unlabeled LTB_4 to measure nonspecific binding. Nonspecific binding represented only ~15% of the total binding under these conditions. In other words, LTB_4-568 labeling made the neutrophils approximately six times brighter than background, demonstrating that fluorescent LTB_4 can be used to detect LTB_4 receptors even in cell populations that are not overexpressing the receptors.

We also performed a FRET analysis of LTB_4-568 binding to EGFP-tagged BLT_1 receptors. In contrast to EGFP, LTB_4-568 is poorly excited by blue light (485 nm). When LTB_4-568 was added to membrane preparations from cells expressing EGFP-tagged BLT_1, the yellow-green EGFP fluorescence emission was able to stimulate LTB_4-568 molecules in close proximity, which reemitted this light as red-orange (610 nm) fluorescence (**Fig. 4**). LTB_4-568 does emit some fluorescence at 610 nm even when stimulated with 485 nm light, but the effect is much larger in the presence of BLT_1-EGFP, indicating that the ligand and the receptor are in close proximity. Membranes from HF1pSham cells did not exhibit this effect. The observed energy transfer could also be disrupted by adding excess unlabeled LTB_4.

Fluorescent LTB_4 binding to enzymes and carrier proteins

LTB_4-568 binding to putative carrier proteins was also examined, and it was found that LTB_4-568 could bind to albumin. This binding was unique to albumin, but it could not be reversed by adding excess quantities of unlabeled LTB_4 (**Fig. 5**).

Similarly, we found that both LTB_4-488 and LTB_4-568 could bind to LTA_4 hydrolase, the enzyme responsible for LTB_4 production in tissues (data not shown). Over a range of concentrations (1–100 nM), fluorescent LTB_4 bound to LTA_4 hydrolase but could not be displaced by excess unlabeled LTB_4 or various inhibitors (RB 3040, Kelatorphan, or the substrate mimic hydroxamate), indicating that the observed binding was probably nonspecific.

LTB_4 and a fluorescence polarization immunoassay

We attempted to measure the concentration of free unlabeled LTB_4 by allowing it to compete with labeled LTB_4 for binding to a polyclonal rabbit anti-LTB_4 peroxidase antibody (Cayman Chemical) and a polyclonal rabbit anti-LTB_4 antibody (Assay Designs). Fluorescent LTB_4 was not recognized by either antibody solution, even at antibody concentrations 100 times those recommended by the manufacturer. Therefore, it will be necessary to produce antibodies specific for fluorescent LTB_4 before a one-step fluorescence polarization immunoassay can be produced.

DISCUSSION

Synthesis and activity of fluorescent LTB_4

Our fluorescent LTB_4 analogs can reversibly bind to the LTB_4 receptor BLT_1 and function as full agonists. Fluorescent LTB_4 bound specifically to BLT_1 because this binding could be displaced, not only by unlabeled LTB_4 but also by the BLT_1-specific partial agonist U75302, the LTB_4 antagonist RP69698, and a BLT_1 antibody that has been shown to interfere with LTB_4 binding (22). LTB_4-568, however, does not bind very tightly to BLT_1, and there is a discrepancy between the values obtained using polarization measurements, made in homogenous solutions, and radioligand binding experiments. Polarization measurements, however, are made at true equilibrium and are dependent on a significant amount of ligand depletion, which makes

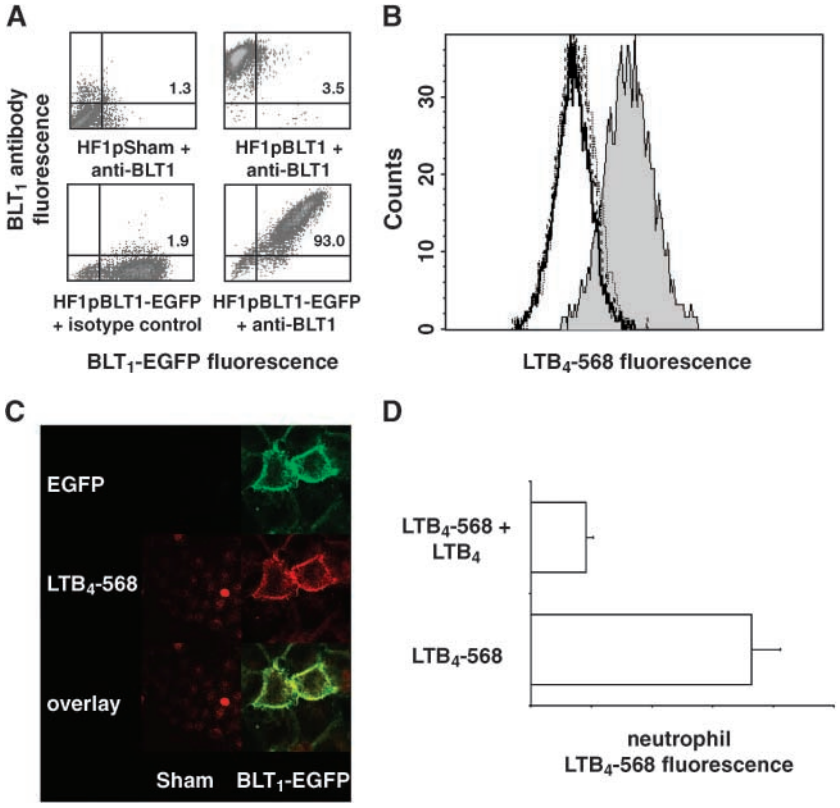

Fig. 3. Colocalization of LTB_4-568 and enhanced green fluorescent protein (EGFP)-tagged BLT_1. A: Flow cytometric analysis of HF1 cells expressing BLT_1 receptors that were C-terminally tagged with EGFP. The green fluorescence of the EGFP tag was used to measure the total number of receptors in each cell, and the red fluorescence of a BLT_1 antibody was used to measure surface expression. In combination, these measurements reveal that more than 90% of the receptor is on the cell surface (the percentage of double positive cells is given in the upper right quadrant of each scattergram). B: Representative curves showing that HF1pBLT1-EGFP cells bind 100 nM LTB_4-568 (grey shading) specifically. Also shown are curves depicting LTB_4-568 nonspecific binding (100 nM LTB_4-568 displaced using 10 μM LTB_4) and HF1pSham cells, which exhibited the same level of nonspecific binding and a lack of specific binding. C: LTB_4-568 staining of HF1pBLT1-EGFP cells and HF1pSham cells observed using confocal microscopy. Green EGFP fluorescence from tagged receptors in the cell membranes colocalized with the orange fluorescence from LTB_4-568. D: Neutrophils isolated from buffy coats were stained with either 10 nM LTB_4-568 (to determine total binding) or LTB_4-568 together with 1 μM LTB_4 (to determine nonspecific binding) before LTB_4-568 fluorescence was quantified using a plate fluorometer. The addition of unlabeled LTB_4 could displace almost 85% of the LTB_4-568 fluorescence. Data shown are means ± SEM from quadruplicate wells and two separate donors.

the results difficult to compare directly with those of traditional nonequilibrium competition experiments. In spite of this apparent low affinity, LTB_4-568 was surprisingly difficult to displace. Ligands that typically displace tritiated LTB_4 at concentrations in the nanomolar range (such as LTB_4 itself) required higher concentrations to displace fluorescent LTB_4. This may be because the fluorophore portion of fluorescent LTB_4 participates in the ligand-receptor interaction. Excess free fluorophore, however, did not affect the binding or function of fluorescent LTB_4, so the mechanism appears to be more complex and may depend on interaction between epitopes from both the leukotriene and the fluorophore. This is supported by the observation that fluorescent LTB_4 binds more strongly to BLT_1 than LTB_4-APA. Baneres and Parello (23) have shown that LTB_4 binding induces the dimerization of purified BLT_1 receptors, which in turn increases BLT_1 affinity for LTB_4. However, preincubating the receptors with either fluorescent LTB_4 or LTB_4 before adding LTB_4 or fluorescent LTB_4, respectively, did not affect the results. Nor are the apparently low ligand affinities dependent on active receptor conformations (and presumably G-protein coupling), as even antagonists (both small molecules and antibody peptides) bound more weakly than expected and were insensitive to the order in which they were added.

Fluorescent versions of LTB_4 bind to and activate BLT_1 specifically, but they are somewhat less potent agonists compared with unmodified LTB_4. They are not partial agonists, however, because the addition of labeled LTB_4 to LTB_4 solutions did not affect receptor activation by LTB_4. Exposure to labeled LTB_4 could also provoke equally large responses, provided the concentrations used were high enough.

LTB_4, modified such that the hydroxyl portion of the carboxyl group is exchanged for aminopropylamide, has previously been used to chemically label receptors for LTB_4

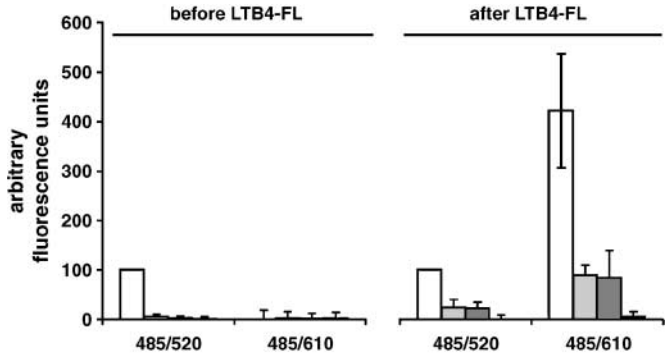

Fig. 4. Fluorescence resonance energy transfer (FRET) from EGFP-tagged BLT_1 to LTB_4-568. Membranes prepared from cells expressing EGFP-tagged BLT_1 were illuminated with 485 nm light, and fluorescence emission at 520 or 610 nm was recorded. FRET between BLT_1-EGFP and LTB_4-568 is revealed as a shift from 520 nm fluorescence emission to 610 nm emission. LTB_4-568 was added to wells containing membranes (white bars), membranes plus an excess of unlabeled LTB_4 (light gray bars), no membranes (dark gray bars), or a buffer control (no fluorophores; black bars). After the addition of LTB_4-568, much more orange-red (610 nm) fluorescence was produced by membranes containing EGFP that were stimulated with 485 nm (blue) light. Data have been normalized to the green fluorescence of EGFP and are shown as means ± SEM from quadruplicate wells in two separate experiments. The *x* axis shows excitation/emission wavelengths in nanometers.

(24), and this ligand retains agonistic activity. It also reacts readily with a variety of fluorophores that have been designed to include succinimidyl esters to facilitate labeling of the free amino groups found in proteins. Although we have focused on Alexa Fluor™ 488 and 568, any fluorophore coupled to a succinimidyl ester will react with similar chemistry, allowing the production of labeled LTB_4 molecules with emission maxima across the visible spectrum and into infrared.

The location of the fluorophore (Fig. 1) is fortuitous because in this position it allows the LTB_4 moiety to bind to and interact with BLT_1. Receptor modeling, ligand docking experiments, and point mutation of the receptor protein (our unpublished data) also support this idea.

Using the unique optical properties of fluorescent LTB_4

Using the optical properties of fluorescent LTB_4, we could locate LTB_4 molecules and LTB_4 receptors in living cells using a variety of techniques incompatible with radiolabeled LTB_4, including flow cytometry, fluorescence histochemistry, and the aforementioned fluorescence polarization.

We also used fluorescently labeled LTB_4 to label membranes from cells expressing BLT_1 receptors that were tagged at the C-terminal end with EGFP and observed both physical overlap between the two fluorophores and resonance energy transfer between the fluorescent ligand and the tagged receptor. The red shift in EGFP fluorescence after LTB_4-568 binding could also be reversed by the addition of unlabeled LTB_4, implying that this is not attributable to random interactions between EGFP and LTB_4-568 molecules in close proximity. Although we did observe binding to purified samples of the cytosolic LTA_4 hydrolase (see below), no cytosolic staining was observed in preparations of intact cells. This may be because, unlike leukocytes, parenchymal cells do not express LTA_4 hydrolase protein, or because fluorescent LTB_4 does not cross intact cell membranes.

Fluorescent LTB_4 binding to water-soluble proteins

One advantage of using fluorescent polarization is that there is no need to separate unbound ligand from bound ligand before measuring ligand binding. This in turn means that measuring fluorescent LTB_4 binding to soluble proteins is, a least theoretically, relatively straightforward. Molecules that bind fluorescent LTB_4 are interesting because many represent possible drug targets and could be used as platforms for high-throughput screening. If the displacement of fluorescent LTB_4 from LTA_4 hydrolase by specific enzymatic inhibitors could be mea-

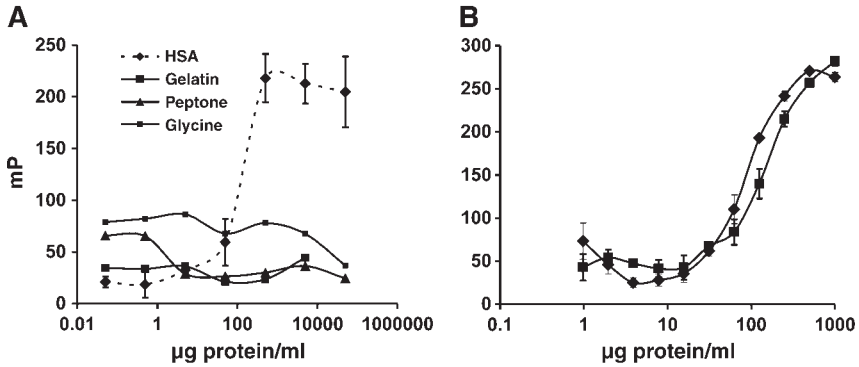

Fig. 5. LTB_4-568 binding to serum albumin. A: LTB_4-568 was added to solutions each containing one of three different polypeptides or the free amino acid glycine. Over a wide range of concentrations, only human serum albumin (HSA) bound LTB_4-568. B: LTB_4-568 (closed diamonds) was added to albumin solution with concentrations ranging from 1 µg/ml to 1 mg/ml, and binding was observed to increase in proportion to the albumin concentration. This binding, however, was not restricted to a site specific for the leukotriene moiety of LTB_4-568, because the addition of excess unlabeled LTB_4 (1 µM) displaced LTB_4-568 only slightly (closed squares). Data are presented as means ± SEM from quadruplicate wells in two separate experiments performed at 24°C. mP, millipolars.

sured using fluorescence polarization, this would create a one-step homogenous assay for screening enzymatic inhibitors. The binding of labeled LTB_4 to serum albumin showed that fluorescent LTB_4 can bind to this important serum protein. Unlike fluorescent LTB_4 binding to BLT_1, however, binding to serum albumin could not be displaced by unlabeled LTB_4, indicating that there are not specific binding epitopes for the LTB_4 moiety. Free Alexa Fluor™ 568 dye was also unable to displace serum albumin-bound LTB_4-568, suggesting that it was not binding of the fluorophore alone that was responsible for LTB_4-568 binding. Similar results were obtained using LTA_4 hydrolase, the enzyme responsible for the enzymatic production of LTB_4 and polyclonal antibodies specific for LTB_4. Although we did observe binding to LTA_4 hydrolase, this binding was not specific and could not be displaced by unlabeled LTB_4 or various LTA_4 hydrolase inhibitors. The two different antibodies we examined, which were designed for use in immunoassays, did not recognize (bind to) our fluorescent LTB_4 analogs.

Conclusions

Fluorescent LTB_4 has several advantages over native LTB_4 and tritiated LTB_4. Fluorescent LTB_4 is not radioactive and thus is safer to handle. Fluorescently labeled LTB_4 is also significantly cheaper than tritiated LTB_4, assuming that tritiated LTB_4 can be purchased at all. Fluorescent LTB_4 can also be used to label receptors for LTB_4 in tissues that are incompatible with currently available antibodies (at present, only antibodies against the human BLT_1 receptor are commercially available). The fact that fluorescent labels can be easily detected optically makes these LTB_4 analogs compatible with a range of techniques, including flow cytometry, microscopy, polarization, and resonance energy transfer. This in turn makes it possible to study LTB_4 binding proteins in new ways, in new tissues with fewer steps, at higher throughput, even in homogenous solutions.

This study was financially supported by the AFA Health Foundation, the Swedish Research Council (03X-10350), Konung Gustav V:s 80 årsfond, and European Commision FP6 funding (LSHM-CT-2004-005033). This publication reflects only the authors' views. The European Commission is not liable for any use that may be made of information herein.

REFERENCES

1. Funk, C. D. 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science.* **294:** 1871–1875.
2. Byrum, R. S., J. L. Goulet, J. N. Snouwaert, R. J. Griffiths, and B. H. Koller. 1999. Determination of the contribution of cysteinyl leukotrienes and leukotriene B4 in acute inflammatory responses using 5-lipoxygenase- and leukotriene A4 hydrolase-deficient mice. *J. Immunol.* **163:** 6810–6819.
3. Haribabu, B., M. W. Verghese, D. A. Steeber, D. D. Sellars, C. B. Bock, and R. Snyderman. 2000. Targeted disruption of the leukotriene B(4) receptor in mice reveals its role in inflammation and platelet-activating factor-induced anaphylaxis. *J. Exp. Med.* **192:** 433–438.
4. Tager, A. M., J. H. Dufour, K. Goodarzi, S. D. Bercury, U. H. von Andrian, and A. D. Luster. 2000. BLTR mediates leukotriene B(4)-induced chemotaxis and adhesion and plays a dominant role in eosinophil accumulation in a murine model of peritonitis. *J. Exp. Med.* **192:** 439–446.
5. Flamand, L., P. Borgeat, R. Lalonde, and J. Gosselin. 2004. Release of anti-HIV mediators after administration of leukotriene B4 to humans. *J. Infect. Dis.* **189:** 2001–2009.
6. Aiello, R. J., P. A. Bourassa, S. Lindsey, W. Weng, A. Freeman, and H. J. Showell. 2002. Leukotriene B4 receptor antagonism reduces monocytic foam cells in mice. *Arterioscler. Thromb. Vasc. Biol.* **22:** 443–449.
7. Subbarao, K., V. R. Jala, S. Mathis, J. Suttles, W. Zacharias, J. Ahamed, H. Ali, M. T. Tseng, and B. Haribabu. 2003. Role of leukotriene B4 receptors in the development of atherosclerosis: potential mechanisms. *Arterioscler. Thromb. Vasc. Biol.* **24:** 369–375.
8. Dwyer, J. H., H. Allayee, K. M. Dwyer, J. Fan, H. Wu, R. Mar, A. J. Lusis, and M. Mehrabian. 2004. Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. *N. Engl. J. Med.* **350:** 29–37.
9. Helgadottir, A., A. Manolescu, G. Thorleifsson, S. Gretarsdottir, H. Jonsdottir, U. Thorsteinsdottir, N. J. Samani, G. Gudmundsson, S. F. Grant, G. Thorgeirsson, et al. 2004. The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat. Genet.* **36:** 233–239.
10. Tager, A. M., S. K. Bromley, B. D. Medoff, S. A. Islam, S. D. Bercury, E. B. Friedrich, A. D. Carafone, R. E. Gerszten, and A. D. Luster. 2003. Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment. *Nat. Immunol.* **4:** 982–990.
11. Kamohara, M., J. Takasaki, M. Matsumoto, T. Saito, T. Ohishi, H. Ishii, and K. Furuichi. 2000. Molecular cloning and characterization of another leukotriene B4 receptor. *J. Biol. Chem.* **275:** 27000–27004.
12. Tryselius, Y., N. E. Nilsson, K. Kotarsky, B. Olde, and C. Owman. 2000. Cloning and characterization of cDNA encoding a novel human leukotriene B(4) receptor. *Biochem. Biophys. Res. Commun.* **274:** 377–382.
13. Wang, S., E. Gustafson, L. Pang, X. Qiao, J. Behan, M. Maguire, M. Bayne, and T. Laz. 2000. A novel hepatointestinal leukotriene B4 receptor. Cloning and functional characterization. *J. Biol. Chem.* **275:** 40686–40694.
14. Hwang, S. W., H. Cho, J. Kwak, S. Y. Lee, C. J. Kang, J. Jung, S. Cho, K. H. Min, Y. G. Suh, D. Kim, and U. Oh. 2000. Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. *Proc. Natl. Acad. Sci. USA.* **97:** 6155–6160.
15. Devchand, P. R., H. Keller, J. M. Peters, M. Vazquez, F. J. Gonzalez, and W. Wahli. 1996. The PPARalpha-leukotriene B4 pathway to inflammation control. *Nature.* **384:** 39–43.
16. Pettersson, A., A. Boketoft, A. Sabirsh, N. E. Nilsson, K. Kotarsky, B. Olde, and C. Owman. 2000. First-generation monoclonal antibodies identifying the human leukotriene B(4) receptor-1. *Biochem. Biophys. Res. Commun.* **279:** 520–525.
17. Harris, L. J., E. Skaletsky, and A. McPherson. 1998. Crystallographic structure of an intact IgG1 monoclonal antibody. *J. Mol. Biol.* **275:** 861–872.
18. Rudberg, P. C., F. Tholander, M. Andberg, M. M. Thunnissen, and J. Z. Haeggstrom. 2004. Leukotriene A4 hydrolase: identification of a common carboxylate recognition site for the epoxide hydrolase and aminopeptidase substrates. *J. Biol. Chem.* **279:** 27376–27382.
19. Kotarsky, K., C. Owman, and B. Olde. 2001. A chimeric reporter gene allowing for clone selection and high-throughput screening of reporter cell lines expressing G-protein-coupled receptors. *Anal. Biochem.* **288:** 209–215.
20. Sabirsh, A., J. Bristulf, and C. Owman. 2004. Exploring the pharmacology of the leukotriene B4 receptor BLT1, without the confounding effects of BLT2. *Eur. J. Pharmacol.* **499:** 53–65.
21. Prystay, L., M. Gosselin, and P. Banks. 2001. Determination of equilibrium dissociation constants in fluorescence polarization. *J. Biomol. Screen.* **6:** 141–150.
22. Sabirsh, A., A. Pettersson, Å. Boketoft, K. Kotarsky, and C. Owman. 2003. Differential inhibition of receptor activation by two mouse monoclonal antibodies specific for the human leukotriene B4 receptor, BLT1. *Int. Immunopharmacol.* **3:** 1829–1839.
23. Baneres, J. L., and J. Parello. 2003. Structure-based analysis of GPCR function: evidence for a novel pentameric assembly between the dimeric leukotriene B4 receptor BLT1 and the G-protein. *J. Mol. Biol.* **329:** 815–829.
24. Goldman, D. W., L. A. Gifford, R. N. Young, T. Marotti, M. K. Cheung, and E. J. Goetzl. 1991. Affinity labeling of the membrane protein-binding component of human polymorphonuclear leukocyte receptors for leukotriene B4. *J. Immunol.* **146:** 2671–2677.