

Identification of a Human cDNA Encoding a Functional High Affinity Lipoxin A₄ Receptor

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Summary

Lipoxin A₄ (LXA₄) triggers selective responses with human neutrophils that are pertussis toxin sensitive and binds to high affinity receptors ($K_d = 0.5 \pm 0.3$ nM) that are modulated by stable analogues of guanosine 5'-triphosphate (GTP). Here, we characterized [11,12-³H]LXA₄ specific binding with neutrophil granule and plasma membranes, which each display high affinity binding sites ($K_d = 0.7 \pm 0.1$ nM) that were regulated by GTP γ S. Since functional LXA₄ receptors are inducible in HL-60 cells, we tested orphan cDNAs encoding 7-transmembrane region receptors cloned from these cells for their ability to bind and signal with LXA₄. Chinese hamster ovary (CHO) cells transfected with the orphan receptor cDNA (pINF114) displayed specific ³H-LXA₄ high affinity binding (1.7 nM). When displacement of LXA₄ binding with pINF114-transfected CHO cells was tested with other eicosanoids, including LXB₄, leukotriene D₄ (LTD₄), LTB₄, or prostaglandin E₂, only LTD₄ competed with LXA₄, giving a K_i of 80 nM. In transfected CHO cells, LXA₄ also stimulated GTPase activity and provoked the release of esterified arachidonate, which proved to be pertussis toxin sensitive. These results indicate that pINF114 cDNA encodes a 7-transmembrane region-containing protein that displays high affinity for ³H-LXA₄ and transmits LXA₄-induced signals. Together, they suggest that the encoded protein is a candidate for a LXA₄ receptor in myeloid cells.

Lipoxygenase (LO)¹-derived eicosanoids are important lipid mediators (1). The 5-LO-derived products include leukotriene B₄ (LTB₄), a potent stimulus for phagocytic cells, and peptido-leukotrienes C₄, D₄, and E₄, which are potent bronchoconstrictors and are associated with the pathogenesis of asthma (1). Lipoxins (LX) are a newer class of bioactive LO-derived products that are generated by the interactions of either 5- and 12-LO and/or 15- and 5-LO followed by subsequent reactions (for a review see reference 2). The LXs are functionally distinct from leukotrienes and other eicosanoids and are primarily generated in human tissues during cell-cell interactions that are exemplified by leukocyte-platelet interactions (2). LXA₄ displays intriguing biological re-

sponses in several tissues (2), and with neutrophils they involve G protein-mediated signal transduction events (3-5). A LXA₄ receptor is induced in HL-60 cells upon differentiation, and it activates phospholipase D (5). LXA₄-induced lipid remodeling events are similar to those of other leukocyte stimuli (i.e., LTB₄ and FMLP), but specifically differ by triggering only selective responses (in the nanomolar range) without initiating aggregation or degranulation (3). In addition, LXA₄ modulates and inhibits neutrophil responses elicited by receptor-mediated stimuli including FMLP (6-8) and LTB₄ in vivo (9). Thus, LXA₄ has a selective profile of action of interest in multicellular responses.

Albeit some structural similarities between LXA₄ and LTB₄ exist (e.g., are identical between C1-C5), LXA₄ receptor interactions in neutrophils involve binding sites that are not recognized by LTB₄ (3, 4). Peptido-leukotrienes, the actions of which are antagonized by LXA₄ in vivo and in vitro (10-12), compete with ³H-LXA₄ binding in neutrophils (4). Interactions between LXA₄ and LTD₄ were also noted with human endothelial cells, where a putative LTD₄ receptor binds ³H-LXA₄ with an affinity ~20-fold lower than that observed with neutrophils (5). With the exception of endothelial and mesangial cells, where LXA₄ is blocked by a LTD₄ receptor antagonist (SKF104353), LXA₄ specific

¹ Abbreviations used in this paper: AMP-PNP, 5'-adenylylimidodiphosphate; CHO, Chinese hamster ovary cells; DPBS/PBS, Dulbecco's PBS; PBS²-without divalent cations; FPR, formyl peptide receptor; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); leukotriene B₄ (LTB₄), 5S,12R-dihydroxy-6,14-cis-8,10-trans-dihydroxyeicosatetraenoic acid; leukotriene D₄ (LTD₄), 5S-hydroxy-6R-(S-cysteinyl-glycynyl)-7,9-trans-11,14-cis-eicosatetraenoic acid; lipoxin A₄ (LXA₄), 5S,6R,15S-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid; lipoxin B₄ (LXB₄), 5S,14R,15S-trihydroxy-6,10,12-trans-8-cis-eicosatetraenoic acid; LO, lipoxygenase; PGE₂, 9-oxy-11 α ,15S-dihydroxy-5-cis-13-trans-prostadienoic acid; PT, Pertussis toxin holotoxin.

binding is not observed with other common cell types including red cells, platelets, or lymphocytic cell lines (5, 10). Thus, in addition to binding with specific leukocyte LXA₄ receptors, it appears that LXA₄ can also interact with LTD₄ receptor sites which, in certain tissues, may represent a subset or subtype of LTD₄ receptors (i.e., LTD₄/LXA₄-r).

These results, and the finding that functional LXA₄ receptors are inducible in promyelocytic lineages (HL-60 cells) (5), as is the case for other receptors (13–15), prompted us to investigate whether orphan 7-transmembrane receptor cDNAs recently isolated from myeloid lineages (16, 17) could encode for LXA₄ receptors or binding proteins.

Materials and Methods

Materials. Tritiated LXA₄ ([11,12-³H]LXA₄) (40 Ci/mmol) was obtained from a custom catalytic hydrogenation of 11,12-acetylenic LXA₄ methyl ester performed by New England Nuclear (NEN), DuPont Co. (Boston, MA) and purified as in references 4 and 5. [γ -³²P]GTP (30 Ci/mmol), ³H-FMLP (53.6 Ci/mmol), and ³H-arachidonate (100 Ci/mmol) were also from NEN, DuPont Co. *N*-(*p*-amylcinnamoyl)anthranilic acid and synthetic LXA₄, LXB₄, LTD₄, prostaglandin E₂ (PGE₂), and LTB₄ were obtained from Cascade Biochem Ltd. (Reading, Berkshire, England). Pertussis toxin (holotoxin) (PT) was purchased from List Biological Laboratories, Inc. (Campbell, CA). Dulbecco's PBS (DPBS) and cell culture reagents were from Whittaker M. A. Bioproducts (Walkersville, MD), and plasticware was from Marsh Biomedical Products, Inc. (Rochester, NY). GTP γ S, FMLP, and 4-*p*-bromophenacyl bromide were from Sigma Chemical Co. (St. Louis, MO), and silicon oil was from Hüls America (Bristol, PA).

Neutrophil Isolation and Subcellular Fractionation. Human neutrophils were obtained by the modified Bøyum method (18) from fresh heparinized blood after venipuncture of healthy normal volunteers. Cell suspensions in PBS were monitored for cell number and viability. To obtain plasma membrane- and granule-enriched fractions, neutrophil fractionation was carried out as in reference 4. Briefly, after suspension in HBSS²⁻ containing 0.1% albumin and diisopropyl fluorophosphate (5 mM) for 30 min at 37°C, neutrophils were adjusted to 4 × 10⁷ cells/ml of HBSS²⁻ with added MgCl₂ (2.5 mM). Next, cells kept at 4°C throughout the procedure were sonicated four times using an ice-cold probe (100 W, 15 s). Crude sonicates were supplemented with EDTA (2.5 mM final) and sequentially centrifuged at 150 (10 min), 18,000 (30 min), and 100,000 g (1 h). Granule- and plasma membrane-enriched fractions were recovered in the 18,000 and 100,000 g pellets, respectively, as indicated by the monitoring of marker enzymes (cf. 4).

Expression of Orphan Receptors in Chinese Hamster Ovary Cells. Cells were grown in 100-mm petri dishes incubated in a 5% CO₂ atmosphere at 37°C in α MEM supplemented with adenosine, deoxyadenosine, and thymidine in addition to serum and antibiotics. Chinese hamster ovary (CHO) cells were transfected using the DEAE-dextran procedure (19) for transient expression of plasmid DNAs for orphan receptors (i.e., denoted pINNF114, pINNF154) and formyl peptide receptor (FPR) (16, 17). The sequences of cDNA for pINNF114 (17) and pINNF154 (16) and deduced amino acid sequences have been reported. 48 h after transfection (10 μ g DNA/dish), cells were detached using PBS²⁻ containing EDTA (5 mM) (3 min, 20°C) and centrifuged (200 g, 10 min) after addition of complete α MEM (2:1 vol/vol). CHO cell permeabilization was achieved with two cycles of freezing (dry ice-acetone bath) fol-

lowed by thawing at room temperature. All cell preparations were resuspended in PBS²⁺ before being used in binding assays.

Ligand Binding Assays. [³H]LXA₄ binding was performed as in (4, 5). Briefly, centrifugation at high speed (30 s, 12,000 g) through silicon oil was used with intact cell suspensions (2–5 × 10⁶ cell/0.5-ml aliquots), and filtration through microspin filter units (0.45 μ m cellulose acetate; PGC Scientifics, Gaithersburg, MD) was used with both subcellular fractions and permeabilized cell suspensions. Incubations were performed at 4°C for the indicated duration in the presence or absence of excess unlabeled homo- or hetero- ligands (1–3 log excess) to determine total and specific binding. Both pellets and filters were next resuspended in scintillation cocktail and radioactivity determined by a Wallac 1409 β -counter (Pharmacia-Wallac Oy, Turku, Finland). Results obtained were analyzed with the Ligand program (Biosoft Elsevier).

GTPase Assays. GTPase activity was determined in transfected CHO cells by a modification of the method described by Cassel and Selinger (20). CHO cells were harvested 72 h after transfection with pINNF114 or a mock vector. Adherent cells were detached from culture plates using PBS²⁻ (5 mM EDTA). Cells were washed with PBS and resuspended (10⁷ cells/ml) in ice-cold buffer (containing 138 mM KCl, 25 mM Tris-HCl, 1 mM EGTA, 1 mM MgCl₂, 0.4 mg/ml creatine kinase, 5 mM phosphocreatine, 0.8 mM 5'-adenylylimidodiphosphate [AMP-PNP], 0.1 mM ATP, 0.1 μ M GTP, and 0.5 μ M [γ -³²P]GTP). Cells in suspension were transferred to electroporation cuvettes kept in ice and were permeabilized with one discharge of 875 V/cm from a 250 μ F capacitor (cat. no. 165-2098; Bio-Radiations, Bio-Rad Laboratories, Richmond, CA). Uptake of ³²P by cells was monitored and averaged ~12% of total [γ -³²P]GTP in solution. Samples were transferred to a 30°C water bath and incubated for the indicated times in the presence of agonists. Aliquots (100 μ l) were removed at indicated times and added to 750 μ l of ice-cold NaH₂PO₄ (10 mM, pH 2.0), containing 5% (wt/vol) charcoal and 0.1% Triton X-100, and vortexed. After incubating on ice for at least 15 min, samples were centrifuged for 3 min at 3,000 g, and radioactivity in 400 μ l of supernatant was determined by liquid scintillation counting. The rate of agonist-dependent GTPase activity was determined by subtracting the amount of free ³²P_i at time zero from values at designated intervals and calculating individual slopes. The rates of GTP hydrolysis in pINNF114 CHO cells were equivalent to mock transfected cells with vehicle and PGE₂, whereas, with LXA₄, the rates in pINNF114 cells were more than three times higher. Vehicle added to mock transfected CHO cells typically gave values of 0.06 pmol/min/10⁶ cells.

Release of Esterified ³H-Arachidonate from CHO Cells. 48 h after transfection, CHO cells were incubated for 3 h at 37°C in complete α MEM (5 ml/dish) containing 0.1 μ Ci of ³H-arachidonate/ml. Esterification of ³H-arachidonate represented 71.0 ± 12.5% of added material. Phospholipid class distribution was resolved by two-dimensional TLC (3) (with ~81.1% of esterified ³H-arachidonate in the total phospholipid fractions), and the individual classes were phosphatidylserine/phosphatidylinositol, 27%; phosphatidylcholine, 24.2%; and phosphatidylethanolamine, 29.9% of the esterified label. Next, cells were washed twice and resuspended in PBS²⁻ (5 mM EDTA). After resuspension in PBS²⁺ (2 × 10⁶ cells/ml), cells transfected with either FPR- or pINNF114-carrying vectors were incubated at 37°C with selected putative ligand. LXA₄-induced (10⁻⁹ M) ³H-arachidonate release from pINNF114 transfected CHO cells was 2.4–11.3% of incorporated ³H-arachidonate. This was equivalent to 24.4–39.7% of the maximal release obtained using the calcium ionophore A₂₃₁₈₇ (2.5 μ M). Background values obtained with mock and pINNF114 transfected CHO

cells exposed to vehicle (EtOH 0.1%) were $\sim 1.0\%$ of the esterified ^3H -arachidonate. In parallel determinations, cells were exposed to two commonly used phospholipase A_2 inhibitors, 4-*p*-bromophenacyl bromide or *N*-(*p*-amylcinnamoyl)anthranilic acid, 10 min before agonist additions. At indicated time intervals, aliquots (1 ml) were layered on a cushion of silicon and centrifuged (30 s, 12,000 *g*). The radiolabel content of individual supernatants (750- μl aliquots) was determined (21).

Northern Blot Analysis. Multiple human tissue Northern blots (Clontech, Palo Alto, CA) containing ~ 2.0 μg /lane poly(A⁺) RNA were probed using a pIN114 open reading frame that had been labeled with [α - ^{32}P]dCTP by random priming, as described (13). Hybridization was done at 42°C for 18 h. After washing (as described in legend to Fig. 6), the blot was exposed to X-Omat AR5 film at -70°C overnight with an intensifying screen, after which they were stripped and reprobed with a ^{32}P -labeled human actin (Clontech) probe using identical conditions.

Constructs. FLAG-FPR and FLAG-pIN114 were constructed employing an octapeptide (DYKDDDDK) encoding sequence termed FLAG as in (22). All constructs were inserted into the EcoRI site of pRc/CMV that had been mutated so as to have a single EcoRI site located within the cloning site (22).

Results

Specific binding of [11,12- ^3H]LXA₄ in human neutrophils gives a K_d of 0.5 ± 0.3 nM and is distributed in plasma membrane- ($\sim 42\%$), granule- (34.5%), and nuclear- (23.3%) enriched fractions. ^3H -LXA₄ binding with intact neutrophils and plasma membrane is modulated by GTP stable analogs (4). To determine whether the interaction of ^3H -LXA₄ with different subcellular fractions gives similar characteristics, [11,12- ^3H]LXA₄ specific binding with granule membrane was compared with that observed with plasma membrane-enriched fractions. Isothermic binding was performed at 4°C with ^3H -LXA₄ (0.1–15 nM) in the presence or absence of a 3 log excess of unlabeled LXA₄. Results from Scatchard analyses show that ^3H -LXA₄ binds neutrophil granule membrane-enriched fractions with comparable K_d (0.8 nM) but larger B_{max} (4.1×10^{-11} M) than with plasma membranes (K_d 0.7 nM, B_{max} 2.1×10^{-11} M) (Fig. 1). In addition, exposure of granule membrane fractions to GTP γ S, a stable analog of GTP, reduced the B_{max} (2.7×10^{-11} M) to values similar to those observed for ^3H -LXA₄ specific binding with plasma membranes (Fig. 1). These findings suggest that ^3H -LXA₄ binding to intact neutrophils and granule membrane-associated fractions involves one class of binding sites. Therefore, a higher abundance of LXA₄ receptors is likely for total cellular copies than that calculated solely on the basis of cell surface receptor expression ($\sim 1,800$ /cell in neutrophils, cf. 4). This granule membrane-associated fraction of LXA₄ binding sites may represent a reserve store as documented for other neutrophil receptors (23).

Since LXA₄ specific and functional receptors present in neutrophils are induced upon differentiation in HL-60 cells (5), we assessed orphan cDNAs recently cloned from libraries derived from differentiated myeloid lineages. Several orphan receptor cDNAs have been isolated that are members of the 7-transmembrane domain G protein-coupled receptor family and also display general sequence homology to the FMLP

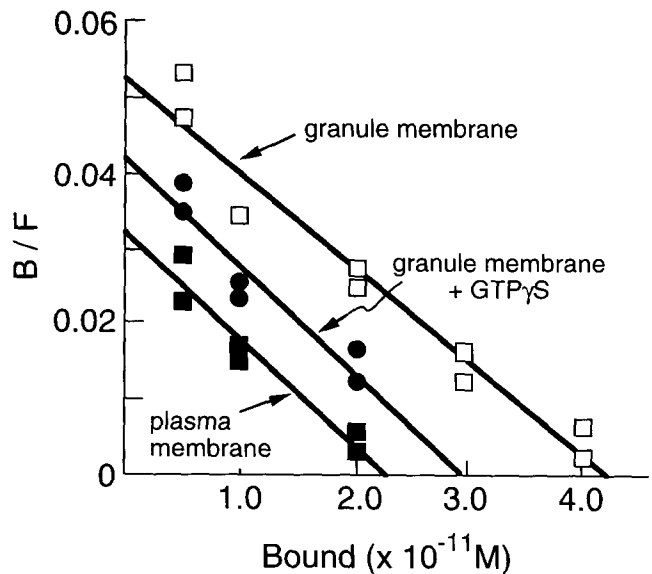


Figure 1. Scatchard plots of ^3H -LXA₄ binding with isolated neutrophil granule and plasma membranes: modulation by GTP γ S. Neutrophil subcellular fractions were obtained as in the Materials and Methods. Increasing concentrations of ^3H -LXA₄ (0.3–15 nM) with or without a 3 log order of magnitude excess of unlabeled LXA₄ were added to isolated granule membranes or plasma membranes (50 μg protein/determination), and specific binding was determined (4°C, 10 min). Parallel experiments with granule membranes were performed after incubation with GTP γ S (20 μM , 3 min). Results represent the average values of duplicate determinations obtained with neutrophils from two separate donors.

receptor (16, 17). High affinity ligands have not been identified for these sequence-related putative receptors, and they were coined FMLP homologous orphan receptors (16) or related FMLP receptors (RFP; 17). CHO cells were transfected with these cDNAs and tested for their ability to bind [11,12- ^3H]LXA₄ (48 h after transfection). As shown in Fig. 2 (left), CHO cells transfected with pIN114 display specific binding with LXA₄ ($K_d \sim 5$ nM), and, for purposes of direct comparison, those transfected with FPR (right) displayed high affinity binding for its ligand FMLP ($K_d \sim 5$ nM). Nonspecific binding was comparable with both ligands (Fig. 2). ^3H -LXA₄ did not display high affinity binding with several other related orphan 7 transmembrane region receptors transfected in CHO cells including pIN154 (see 16, 17) (data not shown). Albeit a low abundance of $\sim 2,000$ LXA₄ receptors per cell was observed with intact cells (Fig. 2), the receptor abundance may reflect, in part, transfection efficiency that is usually 20–30% with DEAE-dextran transfection. Also, posttranscriptional modification and/or intracellular trafficking could lead to partitioning among cellular membranes other than the surface plasma membrane, which may give lower receptor B_{max} with transfected cells. To test this hypothesis, we examined permeabilized cells after transfection for specific binding with both ligands. Experiments with ^3H -FMLP ($n = 4$) and ^3H -LXA₄ ($n = 7$) indicate that K_d values were unaltered after permeabilization of transfected cells (intact 5.6 nM versus permeabilized 5.0 nM for FMLP; and intact 6.5 nM versus permeabilized 7.3 nM for LXA₄). In contrast, the

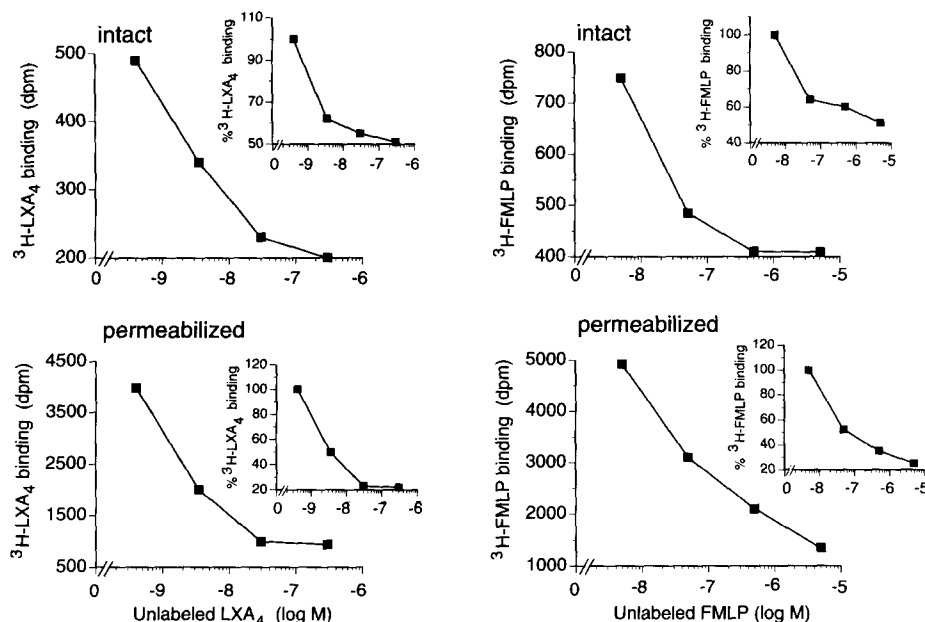


Figure 2. Ligand binding with transfected CHO cells. After transfection (48 h) with either FPR or pINF114, CHO cells were harvested (PBS²⁻ EDTA 5 mM), washed twice with PBS²⁺ (1,100 rpm, 10 min) and adjusted to 10⁷ cells/ml. Aliquots (0.5 ml containing 5 × 10⁶ cells) were incubated with either ³H-FMLP (5 nM, FPR transfected CHO, *right*) or ³H-LXA₄ (0.5 nM, pINF114 transfected CHO, *left*), in the absence or presence of increasing quantities of unlabeled homoligands (1–3 log order molar excess). Parallel determinations were obtained for intact (*top*) or permeabilized (*bottom*) cell suspensions. Results are the average of duplicate determinations and are representative of more than three separate experiments with each transfectant. (*Insets*) ³H-ligand binding displacement curves reported as percent remaining after addition of unlabeled ligand (mean of *n* = 3 separate experiments for each ligand).

B_{max} values obtained with permeabilized cells were about five- to sevenfold higher than those with intact cells (2.3×10^{-10} M vs. 4.9×10^{-11} M for FMLP and 3.6×10^{-10} M versus 5.2×10^{-11} M for LXA₄) (see Fig. 2, *bottom*). Next, pINF114 cDNA was modified at the corresponding NH₂-terminus sequence to contain a FLAG peptide-encoding sequence (22). After transfection, FLAG-pINF114-expressing cells were harvested (48 h) and fractionated, and individual enriched fractions for nuclei, organelle, and plasma membrane were electrophoresed by polyacrylamide gel. Western blot

analysis with a mouse mAb recognizing the FLAG peptide portion showed the distribution of pINF114 construct associated with plasma membranes as ~35%, organelle pellet ~52%, and nuclei ~13%.

Given the higher B_{max} obtained with permeabilized cell suspensions and the finding that >50% of pINF114-encoded protein was associated with intracellular fractions, permeabilized transfected CHO cells were used to further charac-

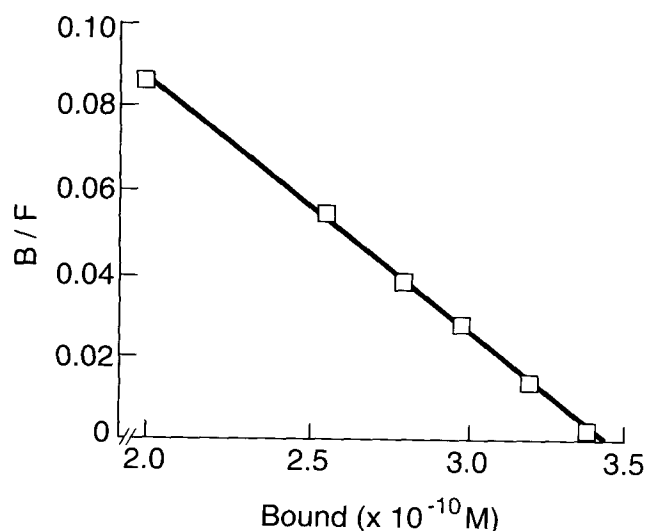


Figure 3. Scatchard plot of ³H-LXA₄ binding to pINF114-transfected CHO cells. pINF114-transfected CHO cells (10⁷ cells/0.5 ml) were permeabilized and incubated (4°C, 10 min) with an increasing concentration of ³H-LXA₄ (0.3–15 nM) with or without 3 log order excess of unlabeled LXA₄ (*n* = 8). Results are from computer-assisted analysis of isothermic binding data analyzed using the Ligand program.

Table 1. Eicosanoid Competition of ³H-LXA₄ Binding with pINF114-transfected CHO Cells

Compound	K_i
	<i>nM</i>
LXA ₄	5.6
LXB ₄	NS*
LTD ₄	79.9
LTB ₄	NS*
PGE ₂	NS*

After transfection with pINF114 (48 h), intact CHO cells were harvested in PBS²⁻ (5 mM EDTA), washed twice in PBS²⁺ and adjusted to 4–10 × 10⁷ cells/ml. Aliquots (200 μl) were added to microcentrifuge tubes containing 800 μl of PBS²⁺ and ³H-LXA₄ (0.3 nM final) alone, or in the presence of increasing concentrations of indicated compounds (3–300 nM). All solutions were kept at 4°C. Cells were incubated for 5 min followed by layering aliquots (0.5 ml) from each experimental point on top of a silicon oil cushion (density = 1.013). Samples were centrifuged and pellet radioactivity measured by scintillation counting. K_i values reported are obtained from evaluating displacement curves via the Ligand program. Results are the means of three separate experiments.

* Ligand program analysis of data failed linear regression for competition in the concentration range tested (3–300 nM).

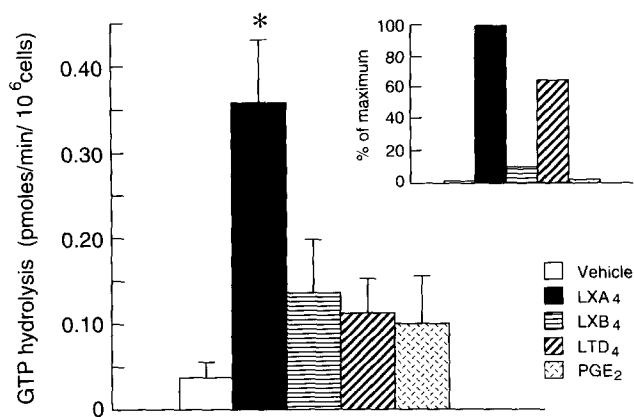


Figure 4. Structure–function relationship of ligand-induced GTPase activity in pINF114-transfected CHO cells. Rates of [γ -³²P]GTP hydrolysis were determined by calculating the linear regression of ³²P release in the initial 30 s after ligand addition (10^{-7} M) to electroporated, pINF114-transfected CHO cells (\pm SEM, $n = 3$). (*) Significantly higher than vehicle and other ligands by Student's t test ($p < 0.05$). (Inset) GTPase activity of transfected CHO cells minus activity obtained with mock transfected CHO cells, expressed as percent LXA₄-induced GTPase activity; results are from one experiment ($d = 2$) representative of three separate experiments.

terize ³H-LXA₄ binding to the pINF114 product. Time course of label association at 4°C gave results similar to those obtained with neutrophils, and equilibrium binding was obtained within 5 min (data not shown). Analysis of isothermic binding of ³H-LXA₄ (1–15 nM, 5 min at 4°C) to pINF114 transfected CHO cells gave a K_d of 1.7 ± 0.8 nM and a B_{max} of $3.8 \pm 0.5 \times 10^{-10}$ M (Fig. 3). These values are in agreement with the K_d values obtained for both neutrophil and retinoic acid differentiated HL-60 cells (5).

LXA₄ binding with neutrophils and HL-60 cells is stereoselective in that neither LTB₄ nor LXB₄ competes for LXA₄ binding (4). Structural requirements for ³H-LXA₄ binding with pINF114-transfected CHO cells were examined with LXB₄, LTD₄, LTB₄, or PGE₂. Only LTD₄ proved effective in displacing tritiated LXA₄ binding with pINF114 transfected CHO cells ($K_i \sim 80$ nM, Table 1). Results are consistent with those obtained with neutrophils (4, 5) where, among the compounds examined, only LTD₄ competed with ³H-LXA₄ binding ($K_i \sim 70$ nM). These findings indicated that specific LXA₄ binding with pINF114-transfected CHO cells is selective, prompting the assessment of transmembrane signaling.

Does pINF114 Transduce LXA₄ Signals? As shown in Fig. 4, after transfection of CHO cells with pINF114, LXA₄ specifically induced GTPase activity. The maximum rate triggered by LXA₄ was reached within 30 s and proved to be concentration dependent (data not shown). LXB₄ and PGE₂ gave significantly lower levels of GTPase activity than LXA₄. LTD₄ also stimulated GTPase activity in pINF114 transfectants when activity associated with mock transfectants assayed in parallel was subtracted (Fig. 4, inset). These results suggest that the structure–function relationship for

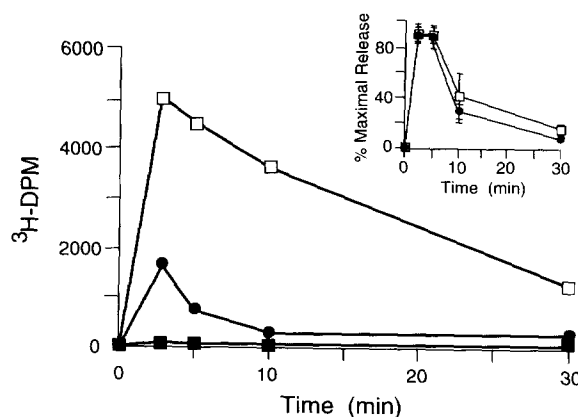


Figure 5. Time course of LXA₄-induced release of ³H-arachidonate from transfected CHO cells. After labeling with ³H-C20:4 (37°C, 3 h), transfected CHO cells were kept at 37°C and exposed to either LXA₄ (10^{-9} M: mock transfected [■]; pINF114 transfected [□]) or FMLP (5×10^{-7} M, FPR transfected [●]). At the indicated intervals, aliquots (2×10^6 cells) were layered onto a cushion of silicon oil and centrifuged. The ³H-arachidonic acid content released into the supernatants was determined. Parallel determinations were performed with ligands and vehicle in transfected and mock transfected CHO cells. Values obtained with vehicle alone (EtOH, 0.1% final) in mock and transfected CHO cells were subtracted from those obtained with respective ligands. Results are representative of three separate experiments with duplicate determinations. (Inset) The profile as percentage of maximal release. Results are the mean \pm SEM of three separate experiments.

stimulating GTPase activity is similar to that obtained for ³H-LXA₄ binding competition (Table 1).

LXA₄ stimulates arachidonate release (2, 3), and CHO cells can mobilize arachidonate in response to ligand-specific stimulation of transfected receptors (21). Next, pINF114-CHO and mock transfected CHO cells were labeled with ³H-arachidonate and exposed to LXA₄ (10^{-9} M) to determine whether this receptor can stimulate release. A specific, LXA₄-dependent release of esterified ³H-arachidonic acid was obtained with pINF114 transfected CHO cells (Fig. 5). Time course experiments showed maximal release in the 3–5 min interval with a subsequent decline. A similar profile was also observed for FMLP-induced release of esterified ³H-arachidonic acid with FPR transfected CHO cells, reported for purposes of direct comparison (Fig. 5 and its inset). Both LXA₄ (10^{-9} M) and FMLP (10^{-7} M)-induced arachidonate release proved sensitive to phospholipase inhibitors *N*-(*p*-amylcinnamoyl)anthranilic acid (100 μ M) ($n = 3$) and 4-*p*-bromophenacyl bromide ($n = 1$; 50 μ M) (data not shown). Selective inhibition of LXA₄-induced release of ³H-arachidonate from pINF114 transfected CHO cells was observed when labeled cells were exposed to PT (4–6 h, 37°C) before agonist addition (Table 2). To ensure that PT treatment did not simply impact in the agonist's temporal response, time course of ³H-arachidonate release was monitored at 0-, 5-, and 10-min intervals (data not shown). Thus, CHO cells transfected with pINF114 specifically bind and give responses with LXA₄.

Preliminary studies on the distribution of mRNA for this receptor showed that the most abundant levels for the spe-

Table 2. Impact of PT Treatment on LXA₄-induced Arachidonate Release in Transfected CHO Cells

PT	Percentage of ³ H-Arachidonate release			
	Mock CHO cells		pINF114 CHO cells	
	A ₂₃₁₈₇ (2.5 μM)	LXA ₄ (10 ⁻⁹ M)	A ₂₃₁₈₇ (2.5 μM)	LXA ₄ - (percent inhibition) (10 ⁻⁹ M)
(ng/ml)				
0	100.0*	0.0	100.0*	36.5 - (0.0%)
10	100.0	0.0	100.0	3.7 - (89.9%)
100	100.0	0.0	100.0	0.0 - (100.0%)

After ³H-arachidonate labeling (37°C, 3 h), cells were exposed to increasing concentrations (0–100 ng/ml) of PT (37°C, 6 h). At 5 min after additions, ³H-arachidonate release was measured as described in the legend to Fig. 5. Results are the average of duplicate determinations from a representative of four separate experiments.

* Data are reported as percentages obtained with each addition relative to A₂₃₁₈₇ (2.5 μM) stimulation after subtraction of values obtained with vehicle alone.

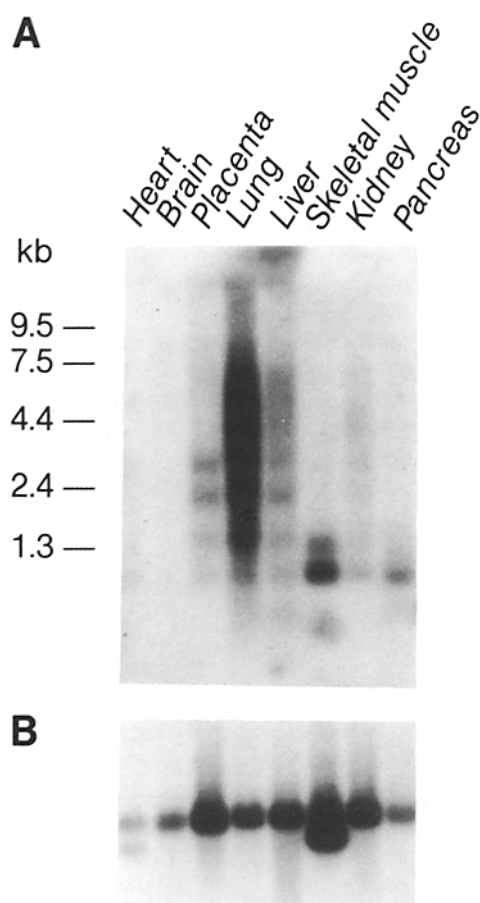


Figure 6. Tissue distribution of LXA₄ receptor mRNA. Multiple human tissue blot was probed with ³²P-pINF114 and ³²P-actin. (A) pINF114 probe. (B) Actin probe. After hybridization, blot was washed at room temperature (2× SSC, 0.1% SDS) for 40 min followed by a second (40 min) wash in 0.1× SSC, 0.1% SDS at 50°C.

cies of interest (i.e., ~1.8–2.0 kb) were associated with human lung, followed by placenta (Fig. 6), tissues known to have a relatively high degree of phagocytic cell infiltrates. Other bands were observed that crosshybridize with this receptor (Fig. 6). The 1.0-kb band is unknown. The 1.4-kb band corresponds to the FMLP receptor size (24). The 2.4–2.6-kb band is also FMLP-like, but distinct from LXA₄ receptor. Bands of higher molecular size may represent presplicing forms of these receptors. Both the FMLP and the LXA₄ receptor genes possess a 5.0-kb intron (24) that is spliced to generate the open reading frame.

Discussion

The present results establish that the interactions of LXA₄ with cells expressing pINF114, a previously isolated orphan receptor of the 7-transmembrane region class (16, 17), meet the criteria commensurate with ligand–receptor interactions and transmembrane signaling (25). Namely, transfected cells display specific binding with ³H-LXA₄ that is displaced with excess unlabeled ligand (Fig. 2). Binding was selective for LXA₄ in that other eicosanoids including LXB₄, LTB₄, and PGE₂ did not displace LXA₄ specific binding (Table 1). CHO cells possess the components required for transmembrane signaling with activation of phospholipases after expressing rogue 7-transmembrane spanning receptors and addition of appropriate ligands (21, 26). In the present experiments, CHO cells transfected with pINF114 transduced signal in response to LXA₄, by both activating GTPase (Fig. 4) and releasing arachidonic acid (Fig. 5). Thus, the product encoded by pINF114 specifically binds ³H-LXA₄ and transduces signals with LXA₄, indicating that pINF114 is a candidate for a functional LXA₄ receptor. This does not, however, preclude the existence of other LXA₄ binding sites or

other receptors in addition to the product of pINF114 that can transduce LXA₄ signals. To date, neither LTD₄ receptors nor other receptors for LO-derived products have been cloned. LTD₄ did displace ³H-LXA₄ from transfected CHO cells (*K_i*, 79.9 nM) and gave ~65–70% of the GTPase activity when compared with equimolar amounts of LXA₄ (*inset*, Fig. 3). LTD₄ competes for ³H-LXA₄ binding (5), and, in certain tissues, LXA₄ and peptido-leukotrienes appear to share a common site of action (5, 10–12). Thus, the present findings suggest that certain types or subclasses of peptido-leukotriene receptors may be structurally related to the pINF114 encoded receptor.

pINF114 and pINF154 were originally sequenced as FMLP-related receptors and coined “related formyl peptide receptors” (RFP) (16, 17). FPR transduces signal with FMLP (22), and FMLP clearly activates leukocytes; however, the endogenous ligands for these receptors have been questioned earlier (23). FMLP is a synthetic analogue and is thought to be a sur-

rogate of *N*-formylated, bacterial-derived proteins (23). An endogenous extracellular ligand for the FMLP receptor is not established. Although Carp (27) noted that *N*-formyl methionyl proteins from mitochondria of damaged cells are chemoattractant for PMN and that this response is blocked with a FMLP receptor antagonist, it still remains unclear as to whether *N*-formylated proteins are unique ligands for the class of receptors (23). The present results indicate that at least one FPR receptor-related sequence is a receptor for a lipid-derived ligand. In this regard, it is of interest that the pINF114 gene has been mapped to chromosome 5a, as have the genes for the complement component 5a receptor (16) and the recently identified thromboxane A₂ receptor gene (28). The present results will now permit further analysis of LX site(s) of action, the mechanism underlying LXA₄ responses such as inhibition of neutrophil function (6–9), and elucidation of components involved in LX signal transduction.

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