Mutations of Serine 236−237 and Tyrosine 302 Residues in the Human Lipoxin A_4 Receptor Intracellular Domains Result in Sustained Signaling†

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ABSTRACT: Lipoxin A_4 (LXA_4) is a potent negative modulator of the inflammatory response. The antiinflammatory activities of LXA_4, such as inhibition of agonist-induced polymorphonuclear cell (PMN) chemotaxis and upregulation of β-2 integrins, require the expression of a G-protein-coupled, high-affinity LXA_4 receptor (LXA_4R). We now report that stimulation of PMN with proinflammatory agonist N-formyl peptides (FMLP), calcium ionophore A_{23187}, or phorbol mirystate acetate (PMA) is followed by marked downregulation of LXA_4 binding (B_{max} decrease of ∼45%) and decreased activation of phospholipases A_2 (PLA_2) and D (PLD). Elucidation of the mechanisms underlying these effects was addressed by structure−function analyses of the intracellular domains of LXA_4R. Mutant molecule, S236/S237 → A/G (LXA_4R^{pk}) and Y302 → F (LXA_4R^{tk}) were obtained by site-directed mutagenesis to yield receptors lacking the putative targets for serine/threonine kinase- or tyrosine kinase-dependent phosphorylation. Expression of wild-type and mutated LXA_4R sequences in CHO and HL-60 cells was used to examine LXA_4 ligand−receptor interactions and signal transduction events. Results indicated that cells expressing LXA_4R^{pk} or LXA_4R^{tk} displayed sustained activation of PLA_2 and PLD in contrast to the transient ones obtained with LXA_4R^{wt} (peak activation at 2−3 min). Moreover, inhibition of LXA_4-dependent PLA_2 activity by PMA in LXA_4R^{wt} transfected CHO cells was not observed in cells expressing LXA_4R^{pk}. Phosphopeptide immunoblotting revealed that the functional differences between wild-type and mutant LXA_4 receptors are accompanied by distinct changes in the receptor protein phosphorylation pattern. Further characterization of these and related LXA_4R intracellular domains will help to better understand specific events that regulate the antiinflammatory activities of LXA_4.

The molecular studies of G-protein-coupled heptahelical receptors have identified in the intracellular domains several serine, threonine, and tyrosine-containing motifs that are subject to phosphorylation, resulting in modulation of receptor affinity, internalization and desensitization (*1*). We and others have previously characterized in human polymorphonuclear cells (PMN)[1] two highly homologous receptors, the formyl peptide receptors (FPR) and LXA_4R, members of the formyl peptides family of heptahelical receptors (*2*, *3*). Activation via these two receptors leads to opposite biological effects, with FPR mediating PMN activation via high-affinity binding of N-formylated peptides, while binding of LXA_4R by its cognate endogenous lipid ligand LXA_4 inhibits PMN responses (*4*, *5*). Because no significant cross-binding is observed (*5*), LXA_4 counter-regulation of FMLP-induced PMN activation is likely to involve cross-talk between intracellular signaling pathways. Substantial evidence indicates that interaction of LXA_4 with its cognate receptor results in potent antiinflammatory activities in in vitro and in vivo models (*6*−*10*). Phosphorylation of heptahelical receptor targets residues located in the third intracellular loop (i3) and C-terminus and involves the activation of G-protein-coupled receptor kinases (GRKs), protein kinase A (PKA), protein kinase C (PKC), and tyrosine kinases (*1*, *11*). The functional impact of these modifications has been characterized in several heptahelical receptors including β-adrenergic and dopaminergic receptors (*1*, *11*).

We undertook the study of LXA_4R expression and functional regulation by kinase-induced phosphorylation. We have identified amino acid motifs that are potential targets for serine/threonine kinase or tyrosine kinase phosphorylation common to both FPR and LXA_4R. These motifs could account for the previously reported homologous and heterologous mechanisms of receptor sequestration/internalization

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[1] Abbreviations: DPBS, Dulbecco's phosphate buffer; FMLP, *N*-formyl-Met−Leu−Phe peptide; LXA_4, lipoxin A_4, 5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; LXA_4R, lipoxin A_4 receptor; LXA_4R^{wt}, lipoxin A_4 receptor, wild-type in pcDNA3 expression vector; LXA_4R^{pk}, lipoxin A_4 receptor, S236/S237 → to A/G mutant in pcDNA3 expression vector; LXA_4R^{tk}, lipoxin A_4 receptor, Y302 → to F mutant in pcDNA3 expression vector; αLXA_4R, rabbit serum raised against the lipoxin A_4 receptor; αphos-ser, polyclonal sera raised against phosphorylated serine; αphos-tyr, monoclonal antibody against phosphorylated tyrosine.

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and desensitization (*1*, *12*). The results here presented indicate that mutation of serine 236−237 and tyrosine 302 of LXA_4R markedly affected signaling by its endogenous ligand LXA_4.

MATERIAL AND METHODS

Tissue culture media and supplements were obtained from Biowhittaker (Walkersville, MD). Fine chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. NP-40 and proteinase inhibitors cocktail were from Boehringer Mannheim Biochemicals (Indianapolis, IN). ^{3}H-arachidonic acid, ^{3}H-palmitate (DuPont-NEN, Boston, MA), and LXA_4, (Cayman Chemicals, Ann Arbor, MI) were all resuspended in ethanol (EtOH) and used at a final concentration <0.1%. Thin-layer chromatography (TLC) supplies were obtained from Fisher Scientific (Pittsburgh, PA). Chemiluminescence analysis employed horseradish peroxidase (HRP)-labeled protein A (Transduction Laboratories, Lexington, KY), polyclonal anti-phosphoserine, and HRP-labeled anti-phosphotyrosine monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit sera raised against LXA_4R was obtained from Research Genetics (Huntsville, AL).

Cell Culture. Chinese hamster ovary (CHO) cells, DUK^- strain, were maintained in α-MEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, 400 μM L-glutamine, 100 μM adenosine, 100 μM 2′-deoxyadenosine, and 100 μM thymidine (at 37 °C in humidified atmosphere with 5% CO_2). HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 400 μM L-glutamine.

Mutagenesis of LXA_4R. Transient Transfections and Establishment of Stably Transfected HL-60 Cell Lines. Site-directed mutagenesis was adopted to generate S236/S237 → to A/G (LXA_4R^{pk}) and Y302 → to F (LXA_4R^{tk}) mutants based on the Genbank LXA_4R cDNA sequence X63819. Briefly, a PCR strategy using a high fidelity Pfu-polymerase (Stratagene, La Jolla, CA) was used to introduce the desired mutations. To generate LXA_4R^{pk}, a forward primer containing a *Bsu*36I restriction site (5′-GGC ACC CCT GAG GAG AGG CT, nt 560−579) and a reverse primer containing a *Xho*I site (5′-TAG ATG CAT GCT CGA GCG GC, nt 1797−1778) were used separately in a first PCR reaction each containing one of two complementary primers with the necessary base mutations (5′-AGG GAC GGC **C**GG **C**TT TAA TCA TGC C, nt 717−693; and 5′-GGC ATG ATT AAA **G**CC **G**GC CGT CCC T, nt 693−717). A 1:1 mix of the amplimers obtained in the first PCR round were amplified again using the two external primers containing the restriction sites. The same strategy was used for LXA_4R^{tk}, using complementary primers containing the desired point mutation (5′-CAC AAA GAC GAA AAG CA**T** GGG GTT G, nt 915−891; and 5′-CAA CCC C**A**T GCT TTT CGT CTT TGT G, nt 891−915). The obtained PCR amplimers were cloned into pcDNA3 expression vector (Invitrogen, Carlsbad, CA) containing the contiguous LXA_4R cDNA fragments and fully sequenced to confirm the full ORF. Wild-type and mutated LXA_4R pcDNA3 plasmids were then used for transient transfection of CHO cells by dextran sedimentation. Cells were next incubated for 48 h prior their use in bioassays. In alternative, HL-60 cells were transfected by electroporation with linearized plasmids, and clonal populations were isolated by two sequential limit dilution steps. Clones expressing wild-type or selected LXA_4R mutations were expanded in neomycin containing media for three months, followed by determination of the expression of transfected sequences by RT-PCR assay (see Results).

Ligand Binding Assay. ^{3}H-FMLP (DuPont-NEN) and ^{3}H-LXA_4 (*3*) were added to cultures in the presence of increasing concentrations of homologous or competing ligand and incubated at 37 °C. Computer-aided analysis (Ligand, Biosoft Elsevier, Oxford, UK) was used for the determination of LXA_4 B_{max}. Incubations were stopped by transferring cells in tubes containing silicon oil cushion, followed by centrifugation at 12000*g* for 1 min. After discarding supernatants, cell pellets were resuspended in scintillation fluid for β counting.

Phospholipase D (PLD) Activation Assay. Cells transfected with LXA_4R^w, LXA_4R^{pk}, LXA_4R^{tk}, or empty vector (mock) were labeled with ^{3}H-palmitic acid (5 μCi/mL) at 37 °C for 2 h. Samples were then centrifuged twice (1200 rpm for 10 min), and cells were resuspended with Dulbecco's phosphate buffer (DPBS). Cell aliquots (2×10^6 cells/sample) were added to DPBS containing agonists with or without 0.5% EtOH. After the indicated periods, incubations were stopped by adding a methanol/chloroform solution, and phospholipids were extracted by a modified Bligh and Dyer procedure and spotted on TLC plates (*13*). Phosphatidyl ethanol (PEt), a PLD-catalyzed trapping product, was resolved as previously reported (*14*). PEt bands identified by comigration with synthetic standard (Biomol) and characteristic R_f values (= 0.36) were scraped from the TLC plates, and their tritium content was measured by β scintillation counting. PEt amounts for each sample were normalized by the percent recovery of ^{14}C-chloramphenicol added as internal standard before phospholipid extraction.

Phospholipase A_2 (PLA_2) Activation Assay. Cells transfected with LXA_4R^w, LXA_4R^{pk}, or empty vector (mock) were labeled with ^{3}H-arachidonic acid at 37 °C for 1 h. At the end of the incubation, cells were centrifuged (1200 rpm × 10 min), washed, and resuspended in DPBS. Cell aliquots (2×10^6 cells/sample) were added to DPBS-containing agonists and incubated for indicated periods. Samples were next layered onto silicon oil cushions and centrifuged (12000*g* × 1 min). Supernatant aliquot (850 μL) were used for measuring tritium content by β scintillation counting.

Immunoprecipitation and Western Blot. Western blot analysis of LXA_4R expression in transfected cells was performed by standard techniques. Briefly, LXA_4, PMA, or calcium ionophore A_{23187} were added at the indicated concentrations to cultures of mock, LXA_4R^w, LXA_4R^{pk}, or LXA_4R^{tk} -transfected CHO cells. Cells were then centrifuged (1200 rpm for 5 min), and pellets were resuspended in ice-cold lysis buffer. LXA_4R was immunoprecipitated with a rabbit polyclonal antisera, as described previously (αLXA_4R) (*5*). After determining protein content by the Bradford method, equal amounts of immunoprecipitated material was resolved on gradient SDS−PAGE. Proteins were then transferred to PVDF membranes (Millipore Corporation, Bedford, MA) by semi-dry blotting, and the phosphorylated LXA_4R was visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ) using polyclonal anti-phosphoserine (αphos-ser) and monoclonal anti-phos-

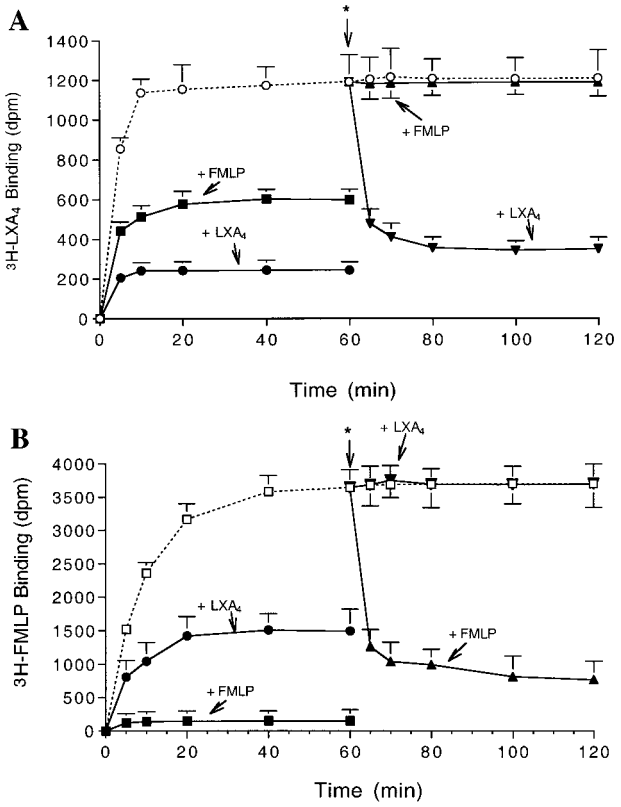


FIGURE 1: LXA_4 and FMLP cross-talk in pre- or post-equilibrium binding of tritiated homoligands. Human PMN isolated from peripheral blood were resuspended in DPBS (10^7 cells/mL) and incubated at 37 °C before performing binding assays. Unlabeled LXA_4 or FMLP were added either simultaneously (LXA_4 ●, FMLP ■) to ^{3}H-LXA_4 (○) or ^{3}H-FMLP (□) or, as indicated by arrows, after reaching equilibrium binding (LXA_4 ▼, FMLP ▲). (A) Unlabeled LXA_4 (1 nM) or FMLP (0.5 μM) were added to the culture medium to compete ^{3}H-LXA_4 specific binding with PMN. (B) Unlabeled LXA_4 and FMLP were added to the culture medium to compete ^{3}H-FMLP specific binding with PMN. Results are the mean ± SEM of triplicate determinations from three separate experiments.

photyrosine (αphos-tyr) antibodies (both from Transduction Laboratories). Equal protein loading and transfer to nylon membranes was confirmed, after completing the chemiluminescence studies, by densitometric analysis of Coomassie Blue-stained blots with a P1000 Bio-Rad imaging system (Hercules, CA).

Statistical Analysis. Paired Student's *t* test was used to determine statistical significance of differences between samples, unless otherwise noted.

RESULTS AND DISCUSSION

It was previously reported that no direct cross-binding occurs between LXA_4 and FMLP in CHO cells transfected with either FPR or LXA_4R (*5*). However, we found cross-interactions between these ligands in PMN, a cell type that expresses both receptors (*13*). As indicated in Figure 1, a 50−70% reduction of ^{3}H-FMLP binding by LXA_4 and a similar reduction of ^{3}H-LXA_4 binding by FMLP was only observed when the heterologous competing ligands are added before reaching the homoligands equilibrium binding. These results with PMN indicate that the FMLP/LXA_4 cross-interactions are not due to receptor binding competition since no displacement is observed when the heteroligands are added after reaching equilibrium binding (Figure 1).

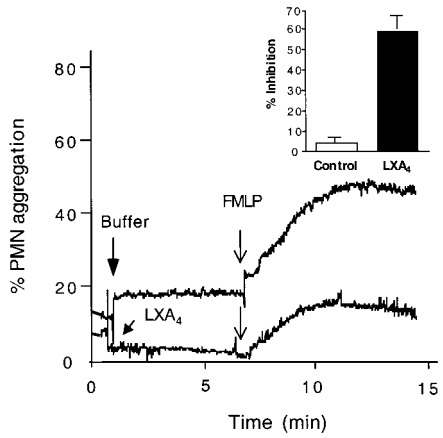


FIGURE 2: LXA_4 inhibition of FMLP-induced PMN aggregation. PMN aggregation was performed as previously reported. PMN (10^7 cells/mL) were stirred at ~800 rpm in a nefelometric vial (37 °C) before adding equal volumes of DPBS buffer with (lower tracing) or without (upper tracing) LXA_4 (1 nM). After 5−7 min of incubation, FMLP (5×10^{-7} M) was added to the cell suspensions. Light transmittance was continuously monitored, and the percent aggregation scale was normalized to the maximum aggregation effect obtained with 5×10^{-6} M FMLP (plateau at ~5−7 min). Results are from one representative experiment of five separate ones.

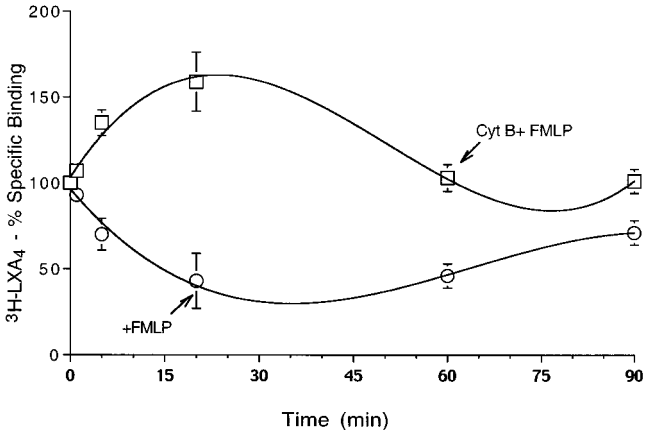


FIGURE 3: Effects of FMLP and cytochalasin B ^{3}H-LXA_4 B_{max} in PMN. FMLP (5×10^{-7} M), in the presence or absence of cytochalasin B (pretreatment at 37 °C, 15 min, 5 μg/mL) was added to PMN cell suspensions simultaneously to ^{3}H-LXA_4. At the indicated time points, cell aliquots were tested for ^{3}H-LXA_4 specific binding and B_{max} was determined. Downregulation of the LXA_4R B_{max} was obtained with cells challenged with FMLP alone, while in the presence of cytochalasin B a reciprocal increase was observed. LXA_4R regulation by FMLP were transitory with a return to basal values of B_{max} by ~60 min.

The functional consequences of LXA_4 and FMLP cross-interactions are shown in Figure 2. These results indicate that exposure of PMN to nanomolar concentrations of LXA_4 results in marked inhibition of FMLP-induced cell aggregation. We have previously demonstrated that inhibition of FMLP-induced PMN aggregation by LXA_4 depends on signaling through LXA_4R, and involves LXA_4-dependent inhibition of β2 integrin up-regulation on the PMN surface (*5*). In a similar fashion, we observed that PMN exposure to FMLP modifies LXA_4 cell interactions. As shown in Figure 3, at concentrations optimal to stimulate cell aggregation, chemotaxis, degranulation, and Ca^{2+} mobilization, FMLP caused up to 50% reduction in ^{3}H-LXA_4 specific binding to PMN. In contrast, binding was upregulated when PMN were treated with cytochalasin B (5 μg/mL), an inhibitor of

Table 1: Impact of Cell Preexposure to FMLP, A_{23187}, and PMA on the ^{3}H-LXA$_4$ B_{max} in PMN[a]

	PMN (fmol)	
	20 min	60 min
control	29.2 ± 5.1	29.4 ± 6.9
FMLP (5×10^{-7} M)	10.6 ± 2.9	20.6 ± 6.2
FMLP (5×10^{-7} M) + cyt B	38.5 ± 5.6	30.8 ± 3.9
A_{23187} (5 μM)	17.3 ± 6.3	30.1 ± 16.4
PMA (50 nM)	14.6 ± 3.0	4.5 ± 1.9

[a] Polymorphonuclear cells (2×10^7/mL) were incubated for 20 min at 37 °C in the presence or absence of agonists at the indicated concentrations (cytochalasin B 2.5 μg/mL when present). Next, aliquots (1×10^7 cells) were used in binding assays with ^{3}H-LXA$_4$ (0.3 nM) in the presence of increasing concentrations of unlabeled LXA$_4$ (3−300 nM). Results (specific binding, fmol/10^7 cells) represent the mean ± SD of three separate experiments.

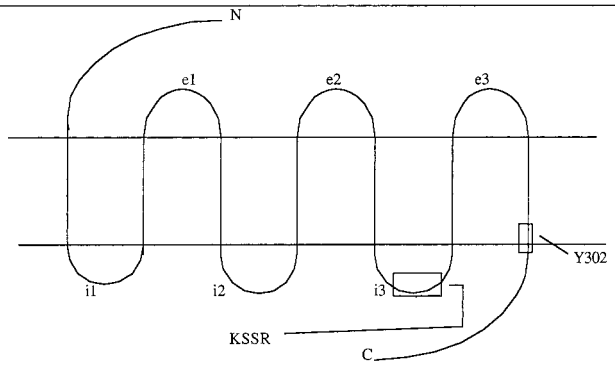
cytoskeleton assembly, for 20 min at 37 °C, before challenge with FMLP (Figure 3). Cytochalasin B reversal of FMLP-dependent modification of LXA$_4$ specific binding and the cyclic profile of ^{3}H-LXA$_4$ binding suggest that mechanisms of sequestration/internalization are involved in regulating the availability of LXA$_4$R on the cell surface in a manner similar to that previously described for LTB$_4$ binding sites in PMN (*15*). In fact, a negative regulation of LTB$_4$ receptor B_{max}, similar to that shown in Figure 3, is obtained after exposing PMN to exogenous LTB$_4$ and is accompanied by a decrease of plasmalemma associated binding and a concomitant increase in cytosol/ER enriched subcellular fractions (*15*).

In PMN, only a limited fraction of the total binding sites for LTB$_4$, FMLP, and LXA$_4$ is expressed on the cell surface. Large quantities of these binding sites are present instead in the membranes of cytoplasmic granules (*13*). For example, cell activation results in recycling of LTB$_4$ receptors between the surface and the membranes of intracellular organelles (*15*). Physical removal of receptor complexes from the cell surface is noted for many heptahelical receptors (*16*). In particular, examination of the β-adrenergic receptor has identified a highly conserved tyrosine residue at the junction of the VII transmembrane with the C-terminus domain that is the target of agonist-induced phosphorylation (*17*). The phosphorylation state of this residue activates intracellular mechanisms leading to receptor internalization (*17*).

Signaling via activated heptahelical receptors is also modulated by modifying the coupling to downstream signaling molecules such as the heterotrimeric G-protein complex and arrestins (*16*, *18*−*20*). These mechanisms lead to receptor desensitization, and, as for the internalization processes, phosphorylation of critical residues in the i3 loop and C-terminus of heptahelical receptors appear to be involved (*1*, *11*). Distinct serine/threonine kinases have been implicated in carrying out ligand-induced phosphorylation of heptahelical receptors, and it has been previously shown that similar mechanisms are operative for FPR (*12*). We were, therefore, interested in examining the potential role of these kinases in the modulation of LXA$_4$R function. On the basis of the high degree of homology between FPR and LXA$_4$R, we identified residues in LXA$_4$R that could be targeted by kinase-dependent phosphorylation. Site-directed mutagenesis was then used to introduce conservative substitutions of the following residues: S236/S237 → to A/G (LXA$_4$R^{pk}) and Y302 → to F (LXA$_4$R^{tk}), as shown in Table 2. After verification of mutations by DNA sequencing, we expressed the wild-type and mutated LXA$_4$R constructs in CHO and HL-60 cells. First, the expression of LXA$_4$R mRNA in transfected cells was confirmed by RT-PCR. As shown in Figure 4, equivalent expression of LXA$_4$R mRNA and protein levels were noted in transiently transfected CHO cells and in stably transfected HL-60 cells.

Table 2: Comparison of Nucleotide and Amino Acid Sequences of Wild-Type and Mutant LXA$_4$R Constructs with FPR[a]

LXA$_4^{wt}$:	693	G M I K S S R P GGC ATG ATT AAA TCC AGC CGT CCC T	K S S P AAG TCC AGT CCT :FPR
LXR$_4^{pk}$:	693	G M I K *A* *G* R P GGC ATG ATT AAA GCC GGC CGT CCC T	
LXA$_4^{wt}$:	891	N P M L Y V F V C AAC CCC ATG CTT TAC GTC TTT GTG	L Y V F CTC TAT GTC TTC :FPR
LXR$_4^{tk}$:	891	N P M L *F* V F V C AAC CCC ATG CTT TTC GTC TTT GTG	



[a] Sequence of wild-type and mutated LXA$_4$R cDNA. Conservative substitution of serine and tyrosine residues expressed in both LXA$_4$R and FPR receptor proteins were obtained as described in Material and Methods. On the LXA$_4$R short i3 loop (16 aa) the selected target residues for mutagenesis were identified on the basis of the consensus for serine/threonine kinase phosphorylation. Tyrosine 302 is a highly conserved residue among heptahelical receptors and has been indicated as the target for agonist-induced phosphorylation leading to intracellular sequestration of the receptor complex (*16*). Nucleotides and amino acids targeted for mutagenesis are indicated by bold type for original sequences or italic and bold type for mutated ones.

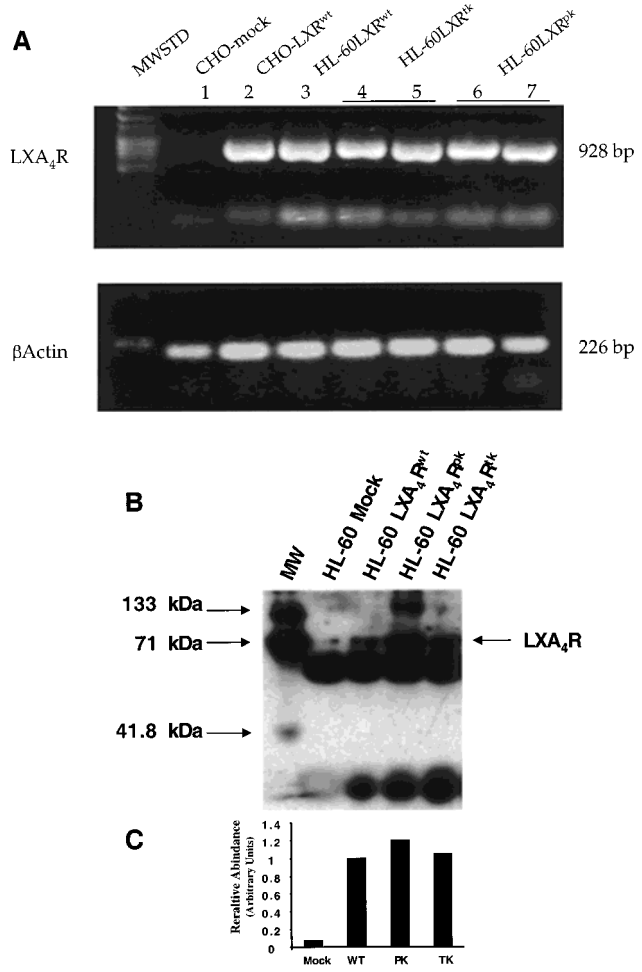


FIGURE 4: RT-PCR and Western blot analysis of LXA_4R expression in cells transfected with wild-type or mutant constructs. Mock, wild-type, and two mutated LXA_4R sequences were transfected in CHO or HL-60 cells, as described in Materials and Methods. (A) Total RNA was isolated from cells, subjected to DNAse treatment and expression of LXA_4R mRNA determined by RT-PCR analysis. Equal PCR loading was monitored by amplification of β-actin. (B) HL-60 cells stably transfected with mock, wild-type, or mutated LXA_4R sequences were lysed. Cell lysates were electrophoresed and transferred onto nylon membranes for Western blot with αLXA_4R. (C) Densitometric profile for the 71 kDa LXA_4R band after normalization of protein loading. Representative results are shown.

Transfected cells have been used previously in functional assays to characterize the signaling induced by ligand activation of LXA_4R (*3*, *13*, *21*, *22*). The results shown in Table 3 indicate that HL-60 cells stably transfected with wild-type or mutant LXA_4R cDNA had equivalent expression of functional LXA_4R protein on the cell surface. The B_{max} determined by ^{3}H-LXA_4 specific binding in stably transfected cells was only slightly higher than that in retinoic acid-differentiated HL-60 cell. We have previously reported that neutrophilic differentiation of HL-60 cells induces expression of functional LXA_4R as well as FPR, which are not present in undifferentiated cells (*5*). We found here that ^{3}H-LXA_4 B_{max} was decreased by FMLP only in retinoic acid-differentiated HL-60 cells but not in undifferentiated HL-60 cells stably transfected with wild-type or mutant LXA_4R constructs (Table 3). Since undifferentiated HL-60 cells lack FPR expression, these results confirm the notion that cross-interactions between LXA_4 and FMLP in PMN, shown in Figures 1−3, involve intracellular cross-talk mechanisms downstream of receptor occupancy.

Table 3: Impact of FMLP on the ^{3}H-LXA_4 B_{Max} in Differentiated or Stably Transfected HL-60 Cells[a]

	control	FMLP	
	20 min	20 min	60 min
mock transfected HL-60	ND	ND	ND
differentiated HL-60	207.4 ± 19.3	145.1 ± 22.3*	235.1 ± 47.8
HL-60/LXA_{4R}^{wt}	295.6 ± 32.5	273.1 ± 28.6	271.5 ± 31.8
HL-60/LXA_{4R}^{pk}	312.2 ± 35.9	283.2 ± 25.3	291.6 ± 23.1
HL-60/LXA_{4R}^{tk}	264.2 ± 39.4	271.1 ± 15.8	260.1 ± 17.7

[a] Cells were incubated in the presence or absence of FMLP (5×10^{-7} M) for the indicated periods. Aliquots (1×10^7 cells) were used in binding assays with ^{3}H-LXA_4 (0.3 nM) and increasing concentrations of unlabeled LXA_4 (3−300 nM). ND, not detectable; *$P < 0.02$ as compared to control. Results (fmol/10^7 cells) are the mean± SD of three separate experiments.

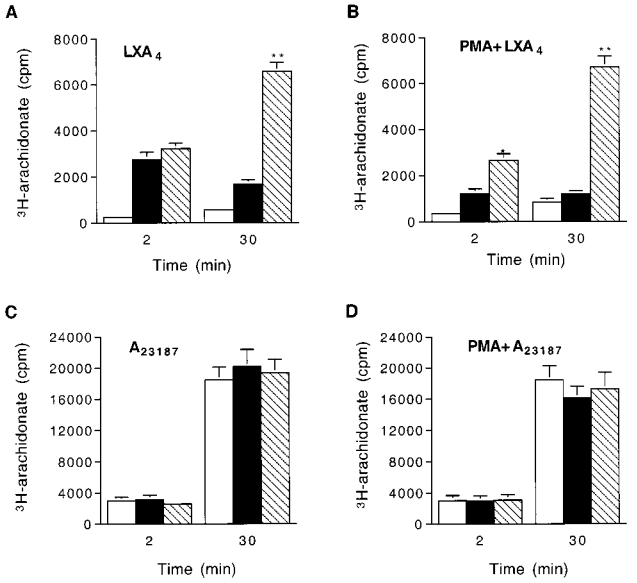


FIGURE 5: LXA_4-dependent PLA2 activation via $LXA4R^{wt}$ or $LXA4R^{pk}$ signaling: modulation by PMA. CHO cells were transiently transfected with mock (white bars), LXA_4R^{wt} (black bars), or LXA_4R^{pk} plasmids (hatched bars) and incubated for 48 h before labeling with ^{3}H-arachidonic acid (0.5 μCi/mL at 37 °C for 30 min). After removing excess unesterified ^{3}H-AA, samples (10^7 cells/mL) were incubated in the presence or absence of PMA (50 nM) before adding 10^{-9} M LXA_4 (panels A and B) or 5 μM A23187 (panels C and D). At the indicated time points, the tritium content in supernatant aliquots (850 μL) was measured by β counting, as described in Materials and Methods. Results represent the mean ± SD of three separate experiments with duplicate determinations.

Although lacking expression of LXA_4R and FPR, undifferentiated HL-60 cells do express functional PLD (*14*). This allowed us to examine the functional consequences of LXA_4-induced signaling also in undifferentiated HL-60 cells stably transfected with LXA_4R^{pk}, LXA_4R^{tk}, or LXA_4R^{wt}, in addition to transiently transfected CHO cells. In this latter cell type, we compared LXA_4-induced PLA_2 activation in cells expressing LXA_4R^{pk} or LXA_4R^{wt}. The results revealed distinct differences in LXA_4-induced PLA_2 activation between LXA_4R^{wt} and LXA_4R^{pk} -transfected cells (Figure 5). As previously reported (*3*), rapid activation of PLA_2 via LXA_4R^{wt} (maximum signal at ~2 min) was transient and was followed by abatement of the response at later time points, with only a ~3-fold increase of ^{3}H-AA release 30 min after LXA_4 addition (Figure 5, panel A). In striking

contrast, in LXA_4R^{pk}-transfected cells, the early peak of PLA_2 activation at 2 min was followed by a sustained effect resulting in a ~10-fold increase of ^{3}H-AA release 30 min after LXA_4 addition (Figure 5, panel A). The difference in LXA_4-induced PLA_2 activation between LXA_4R^{wt} and LXA_4R^{pk} -transfected cells was even greater when cells were pretreated with PMA for 20 min (Figure 5, panel B). In fact, PMA sharply decreased the PLA_2 response to LXA_4 only in CHO cells transfected with LXA_4R^{wt}, while no change was observed with LXA_4R^{pk}-transfected cells (Figure 5, panel B). In contrast to LXA_4, the PLA_2 responses were similar with LXA_4R^{wt}- or LXA_4R^{pk} -transfected cells treated with A_{23187}, which bypasses receptor-mediated signaling (Figures 5, panels C and D).

These results indicate that the KSSR sequence in the i3 loop region of LXA_4R is a target for phosphorylation by PKC or related serine/threonine kinases such as GRKs. The functional consequence of second messenger-dependent kinase (i.e., PKC) activation is held to constitute a critical event for the heterologous receptor desensitization, while GRKs activation is implicated in the homologous desensitization (*1*). In light of its PKC agonist role, an enzyme also target for LXA_4 direct activation (*23*), the impact of PMA on LXA_4R signaling is of particular interest. The convergence of LXA_4 receptor-mediated signaling and direct PKC activation could lead to significant modifications in leukocyte responsiveness and has been previously suggested to determine the bi-phasic dose−response profile of LXA_4-dependent PLD activation (*13*). Since direct interactions of LXA_4 with PKC are observed only at concentrations in >100-fold excess of those used in our study to activate LXA_4R signaling, these two pathways appear to regulate in opposite manner leukocyte responsiveness to LXA_4. Examples of events potentially dependent from LXA_4R-dependent PKC activation are the LXA_4R^{wt} desensitization observed in Figure 5, as well the potential cross-desensitization with the homologous FPR receptor suggested in Figure 2. The direct LXA_4 interaction with PKC and its functional consequence is also suggested by previous work where staurosporine, a PKC inhibitor, modifies LXA_4R-dependent PLD activation elicited in leukocytes by LXA_4 (*13*). The combination of these two distinct mechanisms of PKC activation are ultimately contributing to the appearance of a distinct bi-phasic profile in the LXA_4 dose response observed for many of its biological activities in PMN as well as synovial fibroblasts (*7*, *13*). In fact, the bi-phasic profile of LXA_4-induced responses is not present in these cell types when a methyl-ester LXA_4 is used rather than the free acid form since the former cannot activate PKC directly (*22*, *23*).

Next, we examined LXA_4-induced PLD activation in HL-60 cells stably expressing LXA_4R^{pk}, LXA_4R^{tk}, or LXA_4R^{wt}. This promyelocytic cell line was chosen because of its biological and functional similarities to mature leukocytes and because here LXA_4R and FPR expression and function were previously characterized (*3*, *13*). PLD activation and PEt formation in both LXA_4R^{pk}- and LXA_4R^{tk}-expressing HL-60 cells was significantly altered as compared to LXA_4R^{wt}-expressing cells (Figure 6). LXA4 induced a characteristic transient activation of PLD using HL-60 LXA_4R^{wt} cells, while a sustained response was obtained with HL-60 LXA_4R^{pk} and LXA_4R^{tk}cells. In particular, a ~2-fold

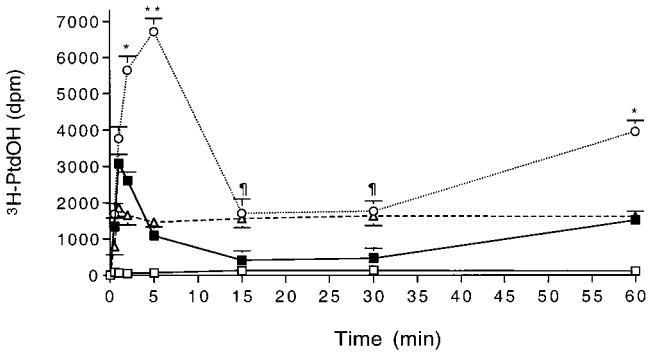


FIGURE 6: LXA_4-dependent PLD activation via LXA_4R^{wt} or LXA_4R^{pk} or LXA_4R^{tk} signaling. Undifferentiated HL-60 cells (□) or HL-60 cell lines stably transfected with LXA_4R^{w} (■), LXA_4R^{tk} (△), or LXA_4R^{pk} (○) were labeled with ^{3}H-palmitic acid (0.5 μCi/mL) at 37 °C for 120 min. Cells were then resuspended in DPBS (2×10^6 cells/mL) and LXA_4 (1 nM) was added. Incubations were stopped at the indicated times by addition of ice-cold MeOH/CHCl3. Phospholipids were extracted by a modified Bligh and Dyer method and resolved on TLC. ^{3}H-PEt ($R_f = 0.36$) was scraped, and silica was suspended in scintillation cocktail for β counting. Statistical significance analysis of PEt signals between LXA_4R^{pk} and LXA_4R^{wt} gave P values < 0.05 (*) and < 0.01 (**), respectively, at 2 and 5 min after agonist addition. A P value <0.1 (¶) was obtained comparing LXA_4R^{tk} and LXA_4R^{pk} to LXA_4R^{w} at 15 and 30 min. The results are the mean ± SEM of three separate experiments in duplicate determinations

increase in maximum PLD activity was obtained with LXA_4R^{pk} as compared to LXA_4R^{wt}, followed by a significantly upregulated, yet similar, cyclical profile of the response (Figure 6). A strikingly different profile of PLD activity was induced by LXA_4 in HL-60 cells expressing LXA_4R^{tk}. In these cells, a partial reduction of the maximum PEt signal was followed by a constant level of PLD activity without cyclical regulation (Figure 6). These results do not appear to be due to altered receptor expression in these cells (see Figure 4 and Table 3); rather, they may reflect mechanisms of desensitization and signal-transduction coupling in heptahelical receptors. For example, desensitization mechanisms, such as GRKs-dependent phosphorylation of target residues, has been shown to markedly affect early stages of receptor activation (*11*), while subsequent regulatory steps are involved in receptor internalization followed by downregulation and/or resensitization of receptor complexes (*24*).

To study the correlation between the molecular events described above and the functional variations observed in PLA_2 and PLD activities in cells expressing mutated LXA_4R we established the pattern of ligand-induced LXA_4R phosphorylation. For this purpose, HL-60 cells stably expressing wild-type or mutated LXA_4R were lysed, and LXA_4R was immunoprecipitated with anti-LXA_4R antisera, followed by Western blotting with αphos-ser and αphos-tyr mAb. The results clearly indicated that whereas LXA_4R^{pk} and LXA_4R^{tk} did not display phosphorylation of serine and tyrosine residues, a time-dependent increase in phosphorylation of LXA_4R^{wt} was observed (Figure 7). These findings indicate that preserving Y302 in the LXA_4R^{pk} mutant permits tyrosine phosphorylation signal comparable to that seen with LXA_4R^{wt}. As a result, LXA_4R^{pk} shares with LXA_4R^{wt} a cyclic profile of PLD activation, although LXA_4R^{pk} displays a significant delay in the decrease of the PEt signal. The cyclic profile of PLD activation with LXA_4R^{pk} could be explained by a

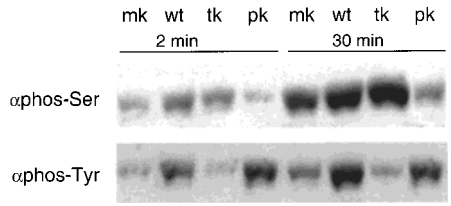


FIGURE 7: LXA_4-induced phosphorylation of LXA_4R: comparison of wild-type vs mutated constructs. Control cells or stably transfected HL-60 cells expressing LXA_4R^{wt}, LXA_4R^{pk}, or LXA_4R^{wt} were resuspended in DPBS (10^7 cells/mL) and incubated at 37 °C. Next, LXA_4 (10^{-9} M) was added and at indicate time points cells aliquots were harvested and centrifuged, and the pellet was lysed. LXA_4R was immunoprecipitated with anti-LXA_4R antiserum and subjected to Western blotting, as described in Materials and Methods. Intensity of tyrosine and serine phosphorylation signals was determined by chemiluminescence of Western blots with HRP conjugated αphos-tyrosine and αphos-serine antibodies. Results from a representative experiment are shown ($n = 5$).

normal receptor internalization process dependent from Y302 phosphorylation. The delay by which PLD activity decreases after receptor activation is probably a consequence of the mutant lack of agonist-dependent serine phosphorylation and linked desensitization mechanisms that would otherwise lead the early termination of the functional responses [as shown in Figures 6 and 7 and ref *1*]. Similarly, studies with LXA_4R^{tk} indicate that impairment of Y302 phosphorylation is accompanied by loss of the cyclic profile of LXA_4-induced PLD activation (Figures 6 and 7). In agreement with previous literature, the impairment of internalization mechanisms in heptahelical receptors is also linked to loss of receptor resensitization (*17*). The alteration of this latter mechanism based on the dephosphorylation of receptor molecules is only possible in the presence of acidic pH found in the endosomal vesicles, after molecules are sequestered (*24*) and could explain the lower PLD response observed with LXA_4R^{tk} (Figure 6).

Overall, these results support the notion that intracellular cross-talk downstream of receptor occupancy underlies the reciprocally antagonistic effects of LXA_4 and FMLP on leukocyte activation. We identified S236, S237, and Y302 as critical residues that function as molecular switches signaling receptor internalization and desensitization, resulting in the modulation of LXA_4R function. Future studies are warranted to establish if these cross-interactions constitute a general signaling model leading to the antiinflammatory activities that LXA_4 displays against proinflammatory agonists interacting with heptahelical receptors other than FPR, such as PAF and LTB_4 (*6*, *9*). Likewise, the LXA_4R mutant constructs described here might help elucidate the mechanisms by which LXA_4 regulates cytokine-mediated effects in leukocytes and other cells (*7*, *8*).

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