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Involvement of BLT1 Endocytosis and Yes Kinase Activation in Leukotriene B_4-Induced Neutrophil Degranulation[1]

Eric Gaudreault, Charles Thompson, Jana Stankova, and Marek Rola-Pleszczynski[2]

One of the important biological activities of human neutrophils is degranulation, which can be induced by leukotriene B_4 (LTB_4). Here we investigated the intracellular signaling events involved in neutrophil degranulation mediated by the high affinity LTB_4 receptor, BLT1. Peripheral blood neutrophils as well as the promyeloid PLB-985 cell line, stably transfected with BLT1 cDNA and differentiated into a neutrophil-like cell phenotype, were used throughout this study. LTB_4-induced enzyme release was inhibited by 50–80% when cells were pretreated with the pharmacological inhibitors of endocytosis sucrose, Con A and NH_4Cl. In addition, transient transfection with a dominant negative form of dynamin (K44A) resulted in ~70% inhibition of ligand-induced degranulation. Pretreating neutrophils or BLT1-expressing PLB-985 cells with the Src family kinase inhibitor PP1 resulted in a 30–60% inhibition in BLT1-mediated degranulation. Yes kinase, but not c-Src, Fgr, Hck, or Lyn, was found to exhibit up-regulated kinase activity after LTB_4 stimulation. Moreover, BLT1 endocytosis was found to be necessary for Yes kinase activation in neutrophils. LTB_4-induced degranulation was also sensitive to inhibition of PI3K. In contrast, it was not affected by inhibition of the mitogen-activated protein kinase MEK kinase, the Janus kinases, or the receptor tyrosine kinase epidermal growth factor receptor or platelet-derived growth factor receptor. Taken together, our results suggest an essential role for BLT1 endocytosis and Yes kinase activation in LTB_4-mediated degranulation of human neutrophils. *The Journal of Immunology,* **2005, 174: 3617–3625.**

Leukotriene B_4 (LTB_4)[3] is a potent lipid mediator of inflammation synthesized predominantly by cells of myeloid origin such as monocytes and neutrophils (1). LTB_4 is the agonist for two membrane G protein-coupled receptors (GPCR), namely a high affinity receptor, BLT1 (2–7) and a low affinity receptor, BLT2 (8–12). BLT1 is principally expressed on cells of the myeloid lineage such as monocytes and on neutrophils (13–15), but also on eosinophils, T cells, and NK cells.

For many GPCRs, receptor endocytosis is seen as the starting event for the activation of intracellular signaling proteins such as MAPKs (16–20). However, receptor internalization-independent kinase activation has also been observed for other receptors, including μ- and δ-opioid receptors (21) as well as the platelet-activating factor receptor (PAFR) (22).

Human neutrophils express several tyrosine kinase members of the Src family including c-Src, Fgr, Hck, Yes, Fyn, and Lyn (23). The involvement of tyrosine kinase of the Src family in neutrophil biological activities has been reported. Src family kinase activity was shown to be important in fMLP-induced superoxide production in human neutrophils (24). These cells also use Fgr and Hck for adhesion-dependent lactoferrin release (25). Moreover, LTB_4 has been shown to activate the Src kinase Lyn in eosinophils, and inhibition of Src kinases attenuated both superoxide generation and chemotaxis (26).

Because Src kinases can be associated with GPCR endocytosis (16, 27), we investigated whether both events could, in a dependent or independent manner, be involved in neutrophil degranulation in response to LTB_4. Degranulation is one of the most important biological activities of neutrophils. Many stimuli can lead to neutrophil degranulation including LTB_4 (28). At this time, the only characterized BLT1-dependent intracellular signaling events leading to degranulation have been reported using the RBL-2H3 rat basophilic leukemia cell line stably transfected with wild-type BLT1 cDNA (29). These intracellular signaling events include calcium influx and PI3K activation. In this study, we used human peripheral blood neutrophils as well as a human neutrophil-like cellular model, the human promyeloid leukemia PLB-985 cell line stably transfected with BLT1 cDNA. These BLT1-transfected cells were differentiated with DMSO to a neutrophil-like phenotype that allowed us to study BLT1-dependent intracellular signaling events leading to degranulation in a cellular model closely related to human neutrophils.

Here we show that both BLT1 endocytosis and Yes kinase activation are important events in LTB_4-induced neutrophil degranulation.

Materials and Methods

Reagents

RPMI 1640, MEM, and geneticin (G418) were from Invitrogen Canada. FBS, cytochalasin B, protein A-Sepharose beads, octylphenyl-polyethylene glycol, enolase from rabbit muscle, leupeptin, 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, soybean trypsin inhibitor, and Na_3VO_4 were from Sigma-Aldrich Canada. DMSO and paraformaldehyde were from Fischer Scientific. Dextran, Ficoll-Paque PLUS, anti-rabbit HRP-conjugated Ab, and ECL kit were from Amersham Biosciences. U75302, 4-amino-5-(4methylphenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1),

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[3] Abbreviations used in this paper: LTB_4, leukotriene B_4; PLB-BLT, PLB-985 cells stably transfected with BLT1 cDNA; GPCR, G protein-coupled receptor; PAFR, platelet-activating factor receptor; G418, geneticin; PTX, *Bordetella pertussis* toxin; RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; PP1, 4-amino-5-(4methylphenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine; AG490, *N*-benzyl-3,4-dihydroxylbenzylidenecyanoacetamide; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; PD98059, 2′-amino-3′-methoxyflavone; AG1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; AG1296, 6,7-dimethoxy-3-phenylquinoxaline.

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N-benzyl-3,4-dihydroxylbenzylidenecyanoacetamide (AG490), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), 2′-amino-3′-methoxyflavone (PD98059), 6,7-dimethoxy-3-phenylquinoxaline (AG1296), LY294002, and *p*-nitrophenyl *N*-acetyl-β-glucosaminide were from BIOMOL. FURA-2AM and 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) were from Calbiochem. Anti-Src2, anti-Fgr, Anti-c-Src, anti-Lyn, anti-Hck, and anti-Yes Abs were from Santa Cruz Biotechnology. LTB_4 was from Cayman Chemicals. BSA was from BIO MEDIA Canada. [γ-^{32}P]ATP was from PerkinElmer.

Cell culture and transfection

PLB-985 cells (a kind donation of Dr. Patrick MacDonald, Université de Sherbrooke, Sherbrooke, Canada) were grown in RPMI 1640 supplemented with 10% FBS and gentamicin sulfate (40 μg/ml). RBL-2H3 cells (a kind donation of Dr. Sylvain Bourgoin, Université Laval, Quebec, Canada) were grown in MEM supplemented with 10% FBS and gentamicin sulfate (40 μg/ml). All cells were cultured at 37°C in a humidified 5% CO_2 incubator.

PLB-985 cells were stably transfected with pcDNA3 vector containing a construct encoding for a myc-tagged BLT1 sequence (30). In short, 30×10^6 cells were electroporated at 320 V using 30 μg of pcDNA3-BLT. Cells were then cultured for 2 wk in medium containing G418 at a concentration of 800 μg/ml. After 2 wk of G418 selection, cells were sorted twice using a FACSVantage cell sorter (BD Biosciences). These cells are referred to as PLB-BLT.

PLB-BLT and PLB-985 cells were cultured in medium supplemented with 1.25% DMSO for 3 days before each experiment to induce cell differentiation into neutrophil-like phenotype, unless mentioned otherwise.

Nucleofection

Differentiated PLB-BLT cells (10×10^6 cells), loaded in nucleofection buffer, were nucleofected with 10 μg of plasmid containing either wild-type or dominant negative dynamin 1A (a kind donation of Dr. Marc Caron, Duke University) using nucleofector program *U02* (Amaxa Biosystems). For degranulation experiments, cells were used 8 h posttransfection. For in vitro kinase assay, after transfection, cells were resuspended in medium containing 10% FBS for 8 h followed by overnight serum starvation (0.1%).

RBL-2H3 (5×10^6 cells) in nucleofector buffer were nucleofected with 3 μg of plasmid containing Myc-BLT1 in combination with 7 μg of empty vector or plasmids containing either wild-type or dominant negative dynamin 1A using nucleofector program *U02*. Degranulation experiments were performed 24 h posttransfection

Isolation of human neutrophils

Neutrophils were obtained from peripheral blood of healthy medication-free volunteers after informed consent in accordance with an Internal Review Board-approved protocol, as described previously (31). Briefly, peripheral blood leukocytes were enriched by dextran sedimentation, layered over a Ficoll-Hypaque cushion, and centrifuged at $4000 \times g$ for 20 min. Mononuclear leukocytes were collected at the interface, whereas neutrophils were obtained from the pellet. Neutrophils were depleted of erythrocytes by osmotic shock, then washed and resuspended in PBS until used.

β-Hexosaminidase release assays

Degranulation was determined by measuring the release of a granule marker, β-hexosaminidase, as described previously by Ali et al. (32), with some modifications. For PLB-BLT cells, after 3 days of differentiation, 2.5×10^5 cells were washed once with 1 ml of PBS. For human neutrophils, 4×10^6 cells were used. For transiently transfected RBL-2H3 cells, measurement of β-hexosaminidase release was performed with 1×10^5 cells. When necessary, cells were incubated with inhibitors for indicated times at 37°C. Cell pellets were resuspended in 250 μl of PBS containing cytochalasin B (4.8 μg/ml) and incubated for 5 min. Cytochalasin B was used to facilitate degranulation without priming cells with other reagents that might interfere directly in intracellular signaling pathways. Cells were then stimulated with LTB_4 (100 nM) or other stimuli for 10 min. After stimulation, β-hexosaminidase activity was measured in 50 μl of cell-free supernatant by spectrophotometric analysis using 50 μl of 2 mM *p*-nitrophenyl-*N*-acetyl-β-glucosaminide as chromogenic substrate. Cell supernatant and substrate were incubated for 1 h at 37°C. The reaction was stopped by adding 150 μl of a 0.1M Na_2CO_3-$NaHCO_3$ buffer at pH 9.5. OD was then read at 405 nm using a spectrophotometer (BioRad). Values were expressed as percentages of total β-hexosaminidase, which was determined in cells lysed with 0.1% Triton X-100. All percentages were corrected by subtracting spontaneous β-hexosaminidase release in cell supernatants (19.8 ± 0.78% for PLB-BLT cells). All assays were performed in triplicate, and OD was read three times.

Receptor endocytosis

Receptor endocytosis was visualized by flow cytometry. Cells (5×10^5) were stimulated with LTB_4 (100 nM) for the indicated times. After stimulation, cells were fixed in a solution of 2% paraformaldehyde and incubated on ice for 15 min. Cells were then washed and resuspended in 50 μl of PBS, and 50 μl of anti-Myc Ab hybridoma supernatant (9E10 hybridoma; American Tissue Culture Collection) were added before incubation at room temperature for 30 min. Cells were then resuspended in 100 μl of PBS-2% BSA containing FITC-conjugated goat anti-mouse IgG + IgM Ab (BioCan Scientific) at 1/1000 dilution and incubated in the dark at room temperature for 30 min. After incubation, cells were washed and resuspended in 100 μl of PBS. Receptor expression was assessed by cytometric analysis using FACScan flow cytometer (BD Biosciences). Endocytosis levels were calculated using mean fluorescence intensity which is in direct correlation with the level of receptor expression at the surface of the cell.

Cytofluorometric analysis

Endogenous expression of BLT1 and BLT2 by PLB-985, PLB-BLT cells, and human neutrophils was determined by flow cytometry. PLB-985 and PLB-BLT were cultured in medium supplemented with 1.25% DMSO for up to 4 days, and BLT1 expression was analyzed every day during cell differentiation. BLT2 expression was determined after PLB-BLT cells were differentiated for 3 days. BLT1 and BLT2 expression was also assessed on neutrophils freshly isolated from peripheral blood. Cells (2.5×10^6) were placed in 100 μl of a PBS-paraformaldehyde (2%) solution and incubated at room temperature for 15 min. After one wash with PBS, cells were placed in the presence of 100 μl of PBS, 0.1% saponin solution and incubated at room temperature for 15 min. Cells were then washed once and resuspended in 20 μl of human IgG solution (100 μg/ml) and incubated at room temperature for 20 min. Cells were then resuspended in 150 μl of PBS-2% BSA, and a 50-μl aliquot of this cellular suspension was used with control rabbit or mouse isotypic Ab (BioCan Scientific), mouse monoclonal anti-BLT1 Ab (clone AD5), or rabbit polyclonal anti-BLT2 (Cayman Chemicals) at a dilution of 1/1000 in PBS, 2% BSA and incubated for 30 min at room temperature. After one wash, 100 μl of PBS, 2% BSA containing FITC-conjugated anti-rabbit or anti-mouse Ab were added and cells were incubated in the dark at room temperature for 30 min. After a last wash, cells were resuspended in 100 μl of PBS, and receptor expression was assessed by cytometric analysis using a FACScan (BD Biosciences).

Calcium mobilization assay

Cells (5×10^6/ml PLB-BLT or PLB-985 cells, 10×10^6/ml human neutrophils) in Fura loading buffer (120 mM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4 \cdot 6H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$, 5.56 mM glucose, 25 mM HEPES) were loaded with a DMSO solution of Fura 2/AM (1 mM) at a final concentration of 3 μM for 1 h. Changes in fluorescence were recorded using a spectrofluorimeter (SPF 500C; SLM Aminco) in a slow time-based acquisition mode. $[Ca^{2+}]_i$ was calculated using the formula $[Ca^{2+}]_i = K_D [(F - F_{min})/(F_{max} - F)]$ after calibration with Triton X-100 and EGTA. When indicated, cells were pretreated with the BLT1-specific antagonist U75302 (10 μM) for 10 min before stimulation.

Immunoprecipitation

Differentiated PLB-BLT cells were serum starved (0.1% FBS) for 12–24h. After pretreatment with or without specific inhibitors followed by a stimulation with LTB_4 for the indicated times, cells (5×10^6) were lysed in 400 μl of radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1% octylphenyl-polyethylene glycol, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM Na_3VO_4). Samples were precleared for 1 h at 4°C using protein A-Sepharose. Supernatants were collected and incubated for 90 min or overnight with 2 μg of appropriate Abs. The mixture was then incubated for 2 h at 4°C with BSA-treated protein A-Sepharose beads. Protein A-Sepharose was pelleted by brief centrifugation and washed 3 × 15 min with 1 ml radioimmunoprecipitation assay buffer.

Src kinase activity assay

Src kinase assay was performed as described before by Flint et al. (33) with some modifications. Immunoprecipitation was performed as described above. Acid-denatured enolase from rabbit muscle was prepared by adding

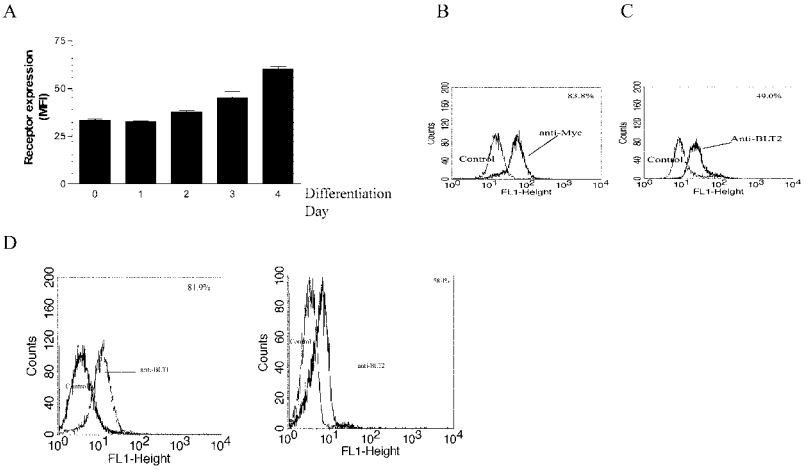

FIGURE 1. BLT1 and BLT2 expression on PLB-985 cells, differentiated PLB-BLT cells, and human neutrophils. *A,* PLB-985 cells were differentiated from 0 to 4 days using DMSO (1.25%) and endogenous BLT1 expression was assessed by cytofluorometry using anti-BLT1 Ab. Results are means of two independent experiments performed in duplicate. MFI, mean fluorescence index. *B* and *C,* PLB-985 cells stably transfected with a c-myc-tagged BLT1 construct were differentiated for 3 days, and cytofluorometric analysis was performed using either anti-Myc (*B*) or anti-BLT2 (*C*) Ab. FL, Fluorescence. *D,* Cell surface expression of BLT1 and BLT2 on freshly isolated human neutrophils. Values are percentages of positive cells and are representative of two independent experiments performed in duplicate.

100 μl of 50 mM acetic acid to 100 μl of an enolase solution, incubating at 30°C for 10 min, neutralizing with 50 μl of 1 M HEPES (pH 7.5), and then diluting with 450 μl of kinase buffer (10 mM HEPES (pH 7.0), 5 mM $MnCl_2$). Immunoprecipitates were washed twice with kinase buffer, and 30 μl of enolase solution was added. When necessary, PP1 (10 μM) was added with subsequent incubation at 37°C for 15 min. The kinase reaction was started by adding 5 μl of [γ-^{32}P]ATP (0.1 mCi/ml in kinase buffer) and stopped after incubation at 30°C for 30 min by the addition of 10 μl of Laemmli SDS-PAGE loading buffer four times and boiling for 5 min. Proteins were separated by reducing SDS gel electrophoresis on a 12% polyacrylamide gel and transferred onto nitrocellulose membrane. Phosphorylated enolase was detected by autoradiography. For protein loading assessment, membranes were blocked with Tween-Tris-buffered saline, 10% milk for 1 h and incubated either with anti-Src2, anti-Fgr, anti-c-Src, anti-Yes, anti-Hck, or anti-Lyn Abs (dilution 1/1000) for 90 min at room temperature. Washes 4 × 5 min were performed with Tween-Tris-buffered saline, and membranes were blotted with secondary anti-rabbit HRP-conjugated Ab (Amersham Biosciences). Protein detection was performed using ECL detection system (Amersham Biosciences).

Statistical analyses

Data were analyzed by one-tailed ANOVA followed by a Newman-Keuls post hoc test using PRISM3 software. Paired Student's *t* test was performed where indicated. Differences were considered significant at $p \leq 0.05$ for $n \geq 3$.

Results

PLB-BLT cells is a model for BLT1 signaling studies in neutrophils

We first wanted to examine the expression of the two LTB_4 receptors (BLT1 and BLT2) on PLB-985 cells during the differentiation process or on 3-day differentiated PLB-BLT cells. During the first day of differentiation, PLB-985 cells expressed very little endogenous BLT1 (Fig. 1*A*). Fully differentiated PLB-985 cells did express BLT1 but showed very low levels of calcium mobilization after stimulation with LTB_4 (Fig. 2*A*). This calcium mobilization was totally abrogated when cells were pretreated with the specific BLT1 antagonist U75302 (34) at a concentration of 10 μM. For those reasons, PLB-985 cells were stably transfected with BLT1 cDNA and used at differentiation day 3 throughout this study. This allowed us to observe little endogenous BLT1 expression (Fig. 1*A*) accompanied by high transfected BLT1 expression

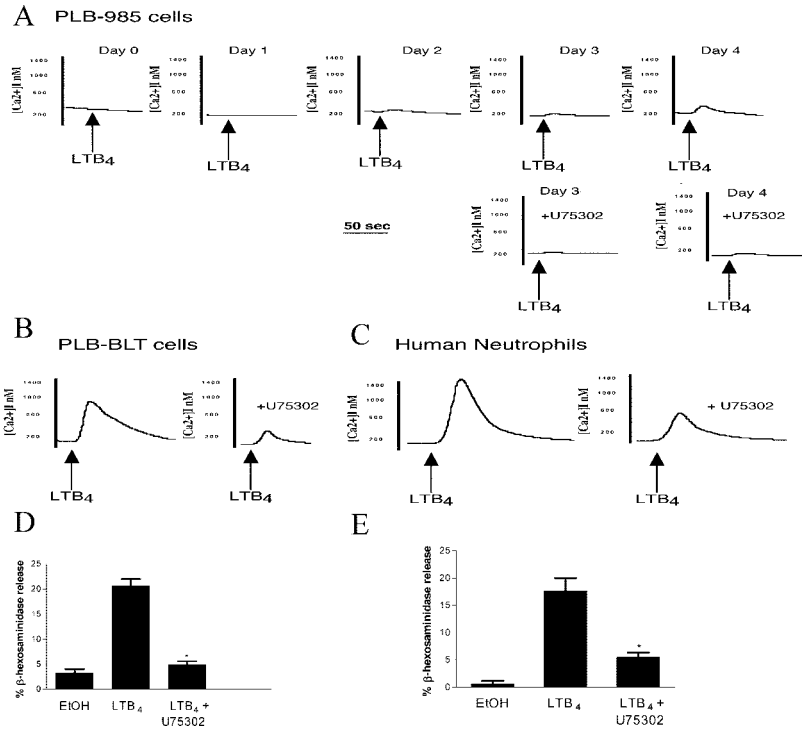

FIGURE 2. LTB_4-induced calcium mobilization and degranulation by PLB-985 cells, PLB-BLT cells, and human neutrophils. PLB-985 cells (5×10^6 cells) differentiated with DMSO from 0 to 4 days (*A*), PLB-BLT cells (5×10^6 cells) differentiated for 3 days (*B*) or human neutrophils (10×10^6 cells) isolated from peripheral blood (*C*) were loaded for 1 h at room temperature with FURa 2/AM (3 μM) followed by incubation with or without U75302 (10 μM) for 10 min before LTB_4 stimulation (100 nM). Fluorescence intensities were then recorded. Data are representative of at least three independent experiments, each done in duplicate. Differentiated PLB-BLT cells (2.5×10^5 cells) (*D*) or human neutrophils (4×10^6 cells) (*E*) were pretreated or not with U75302 (10 μM) at 37°C for 30 min before stimulation for 10 min with 100 nM LTB_4 or its appropriate vehicle (ethanol; EtOH). After stimulation, β-hexosaminidase release was assessed as described in *Materials and Methods*. *, $p < 0.05$ as compared with LTB_4 treatment alone. Data represent percentages of β-hexosaminidase release compared with total cellular enzyme concentration calculated by cell lysis in 0.1% Triton. Values are means ± SEM of three independent experiments performed in triplicate.

on cell surface, reaching 83.8% of positive cells (Fig. 1*B*), which is similar to BLT1 expression on human neutrophils (Fig. 1*D*), and calcium mobilization reaching similar levels as with human neutrophils (Fig. 2, *B* and *C*). Calcium mobilization was also inhibited to similar levels when PLB-BLT cells or human neutrophils were pretreated for 10 min with U75302 before LTB_4 stimulation (Fig. 2, *B* and *C*). However, total inhibition in calcium signaling using U75302 was not observed, possibly due to additional BLT2 signaling. BLT2 expression was also detected on PLB-BLT cells at differentiation day 3 using a specific Ab directed against human BLT2 (Fig. 1*C*). To verify whether this endogenous BLT2 expression could play a role in degranulation after LTB_4 stimulation, we conducted degranulation experiments after pretreatment with U75302 (10 μM). Degranulation was completely inhibited when PLB-BLT cells were pretreated with U75302, indicating that LTB_4-triggered enzyme release was totally BLT1 dependent in our cellular model (Fig. 2*D*). When the same degranulation experiment was performed with human neutrophils isolated from peripheral blood, pretreatment with U75302 did not completely abolish LTB_4-mediated degranulation, suggesting partial BLT2 involvement in neutrophil degranulation (Fig. 2*E*).

BLT1 internalization is crucial for LTB_4-induced degranulation

Receptor internalization has been demonstrated to be important as a first event leading to activation of multiple intracellular signaling pathways for some GPCRs (17). As shown in Fig. 3A, using cytofluorometric analysis, BLT1 was internalized in our PLB-BLT cellular system after stimulation with LTB_4 (100 nM). Cells were stimulated with agonist and fixed on ice; receptor expression on the cell surface was measured using an Ab directed against the Myc-tagged BLT1. Myc-BLT1 internalization was statistically significant at 2 min after stimulation and with a tendency to last up to 60 min after LTB_4 administration.

To assess the importance of BLT1 internalization in LTB_4-induced degranulation, a first set of experiments was initiated using chemical blockers of receptor endocytosis. Pretreatments with 0.45 M sucrose (20 min), Con A 0.25 mg/ml (20 min), or 10 mM NH_4Cl (10 min) led to, respectively, 84.8 $\pm$ 4.6, 80.6 $\pm$ 16.7, and 47.9 $\pm$ 4.7% inhibition of PLB-BLT degranulation after stimulation with 100 nM LTB_4 (data not shown). Due to the possibility of nonspecific activity of those pharmacological inhibitors, we transiently transfected PLB-BLT cells with cDNAs of either dynamin 1A or a dominant negative mutant of dynamin (K44A) known to block receptor-mediated dynamin-dependent endocytosis. We recently showed that dynamin K44A successfully inhibits BLT1 internalization (35). As shown in Fig. 3*B,* transfection with the dominant negative form of dynamin resulted in inhibition of LTB_4-induced degranulation reaching 75.9 $\pm$ 15.4% as opposed to no inhibition when PLB-BLT cells were transfected with wild-type dynamin 1A or empty vector.

To clarify whether BLT1 endocytosis-dependent cellular degranulation was specific to PLB cells, we transiently transfected BLT1 cDNA with either dynamin 1A or dynamin K44A cDNAs into the rat basophilic leukemia RBL-2H3 cell line, a cellular model often used to study degranulation events (29, 36–40). Levels of transfection were comparable with those of PLB-BLT. No significant inhibition of degranulation was observed in cells transfected with dynamin K44A as compared with cells transfected with dynamin 1A (Fig. 3*C*) or the empty vector, suggesting that the requirement for BLT1 endocytosis in LTB_4-induced degranulation may be cell or species specific.

A pertussis-sensitive $G\alpha_i$ protein subunit and PI3K are important for degranulation induced by LTB_4

We and others have previously shown that BLT1 can signal through $G\alpha_i$ protein subunit (41, 42). To test the involvement of

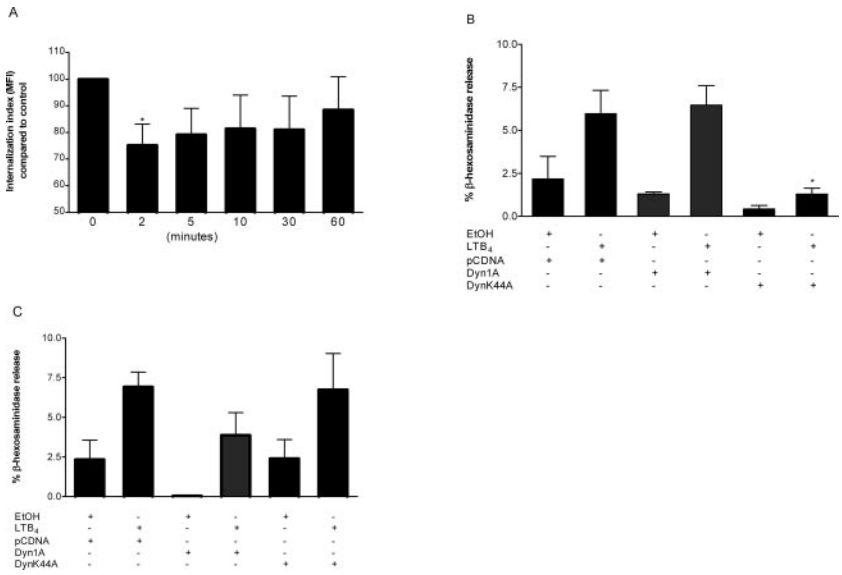

FIGURE 3. Effect of receptor endocytosis blockade on BLT1-mediated degranulation in PLB-BLT and RBL-2H3 cells. *A,* Receptor endocytosis was determined by cytofluorometric analysis. PLB-BLT cells were stimulated for indicated times with 100 nM LTB_4. After stimulation, cells were fixed with a 2% paraformaldehyde solution for 15 min on ice. Samples were then analyzed by cytofluorometry using anti-Myc Ab. *, $p < 0.05$ as compared with unstimulated cells. *B,* PLB-BLT cells were nucleofected with either empty vector or vectors containing either wild-type dynamin (Dyn) 1A or dominant negative dynamin (K44A). Eight hours posttransfection, cells (2.5×10^5 cells) were stimulated with 100 nM LTB_4 or its appropriate vehicle (ethanol; EtOH) for 10 min. After stimulation, β-hexosaminidase release was assessed. *, $p < 0.05$ as compared with transfection with empty vector. *C,* RBL-2H3 cells were transiently nucleofected with BLT1 cDNA and with either empty vector or vectors expressing dynamin 1A or dominant negative dynamin K44A. Twenty-four hours after transfection, 10^5 cells were stimulated with 100 nM LTB_4 for 10 min. After stimulation, β-hexosaminidase release was assessed. Values are means $\pm$ SEM of three independent experiments performed in triplicate.

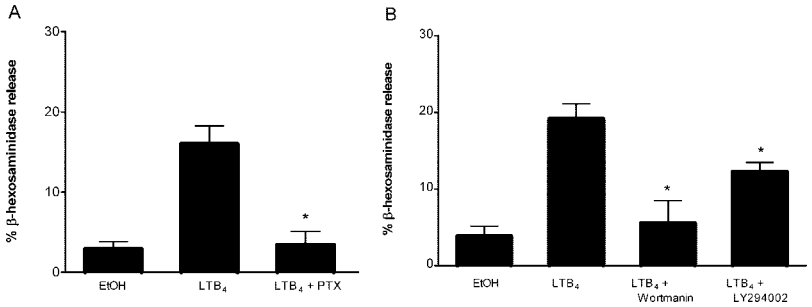

FIGURE 4. Effects of $G\alpha_i$ protein subunit and PI-3 kinase blockade on BLT1-mediated degranulation. *A,* PLB-BLT cells were pretreated or not with *Bordetella pertussis* toxin (50 ng/ml) at 37°C for 6 h. After incubation with PTX, 2.5×10^5 cells were stimulated with 100 nM LTB_4 or its appropriate vehicle (ethanol; EtOH) for 10 min. After stimulation, β-hexosaminidase release was assessed. *B,* PLB-BLT cells (2.5×10^5 cells) were pretreated or not with wortmannin (1 μM) or LY294002 (10 μM) at 37°C for 1 h. After incubation with inhibitors, cells were stimulated with 100 nM LTB_4, its appropriate vehicle (ethanol) for 10 min. After stimulation, β-hexosaminidase release was assessed. Values are means ± SEM of three independent experiments performed in triplicate.

$G\alpha_i$ protein subunit in LTB_4-induced degranulation of PLB-BLT cells, we used pertussis toxin (PTX), an inhibitor of $G\alpha_i$ and $G\alpha_0$ proteins. PLB-BLT cells were pretreated for 6 h with PTX (50 ng/ml) or vehicle, followed by 10 min of stimulation with 100 nM LTB_4. PTX treatment resulted in an 80.4 ± 7.0% inhibition of degranulation (Fig. 4*A*).

BLT1-mediated degranulation in RBL-2H3 cells stably transfected with BLT1 cDNA was shown to be partially dependent on PI3K activity (29). To verify whether this was the case in our PLB-BLT model, the specific PI3K inhibitors LY294002 (10 μM) and wortmannin (1 μM) were used. Preincubation for 1 h with LY294002 followed by stimulation with LTB_4 led to a 40.9 ± 5.5% inhibition of β-hexosaminidase release, whereas pretreatment with wortmannin (1 μM) led to 72.7 ± 12.3% inhibition of LTB_4-mediated degranulation (Fig. 4*B*). These results suggest the involvement of PI3K in BLT1-mediated degranulation in PLB-BLT cells.

Src family kinases, but not JAK2, p38 kinase, or MEK/ERK kinases, are important in BLT1-mediated degranulation

Receptor endocytosis and $G\alpha_i$ protein activation have already been demonstrated as important contributors for the activation of different kinases, including Src kinases, by different GPCRs (16, 18, 20, 43). Moreover, Src family kinases have been shown to mediate certain neutrophil functions. To assess the requirement for Src kinase activation in the phenomenon of granule secretion mediated by BLT1, PLB-BLT cells were preincubated for 1 h with a specific Src kinase family inhibitor, PP1 (10 μM), before stimulation with 100 nM LTB_4 for 10 min. PP1-treated cells showed a 44.5 ± 7.0% reduction in degranulation as compared with cells stimulated with LTB_4 alone (Fig. 5*A*), suggesting a partial involvement of Src kinases in LTB_4-induced granule release. In addition, human neutrophils pretreated with 30 μM PP1 (PP1 concentration showing maximal inhibition) showed a 27.6 ± 4.2% inhibition of degranulation after LTB_4 stimulation (Fig. 5*B*). Moreover, PP1 inhibition of degranulation in PLB-BLT cells was concentration dependent, reaching 62.8 ± 1.6% at the inhibitor concentration of 50 μM (Fig. 5*C*). Pretreatment with PP1 diluent (DMSO) at a concentration of 0.1% in solution did not significantly affect LTB_4-mediated degranulation, showing that the inhibition of PLB-BLT degranulation by PP1 was specific to the inhibitor even at high concentrations.

To examine whether other signaling pathways could be involved in LTB_4-mediated degranulation in our neutrophilic cellular model, degranulation assays were performed using specific inhibitors for JAK2, p38 kinase, and MEK/ERK kinases. When PLB-BLT cells were pretreated for 1 h with the JAK2 inhibitor AG490 (10 μM), the p38 kinase inhibitor SB203580 (10 μM), or with the MEK/ERK kinase inhibitor PD98059 (10 μM), no statistically significant reduction in degranulation was observed compared with PLB-BLT cells stimulated with LTB_4 alone (Fig. 6*A*). As already published by Fisher et al. (44), LTB_4-mediated neutrophil degranulation (calculated by elastase release), as opposed to PLB-BLT cells, can be mediated by p38 kinase. To confirm this finding, we performed degranulation experiments using specific inhibitors for JAK2, p38 kinase, and MEK/ERK kinases. LTB_4-mediated neutrophil degranulation was not affected by treatments with inhibitors of JAK2 and MEK/ERK kinases, but a statistically significant inhibition of 34.1 ± 3.6% (Fig. 6*B*) was observed when neutrophils were preincubated with SB203580 followed by stimulation with LTB_4, showing a difference between our neutrophil-like model and human neutrophils. This difference might be due to high mitogenic activity in PLB-BLT cells that becomes very difficult to block with pharmacological inhibitors. To verify inhibitor specificity, we performed degranulation experiments with human neutrophils preincubated with SB203580 and stimulated with fMLP. As Mocsai et al. had

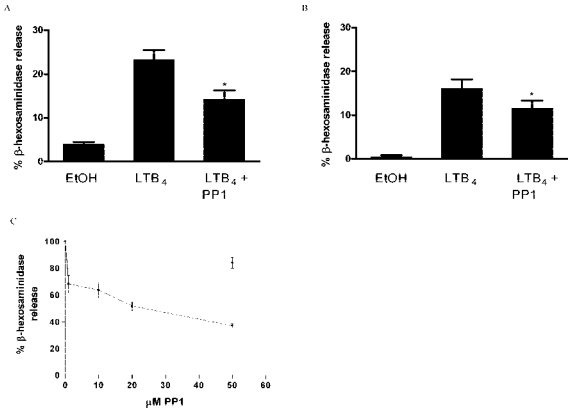

FIGURE 5. Effects of Src family kinase inhibition on BLT1-mediated degranulation in PLB-BLT cells and human neutrophils. *A,* PLB-BLT cells (2.5×10^5) were pretreated with Src family kinases inhibitor PP1 (10 μM) for 1 h at 37°C and were then stimulated with LTB_4 (100 nM) or its vehicle (ethanol; EtOH) for 10 min. After stimulation, β-hexosaminidase release was assessed. *B,* Human neutrophils (4×10^6 cells) isolated from peripheral blood were pretreated or not with PP1 (30 μM) for 1 h at 37°C and were then stimulated with LTB_4 (100 nM) or its vehicle (ethanol) for 10 min. After stimulation, β-hexosaminidase release was assessed. *C,* Concentration-dependent inhibition of degranulation using PP1 (0–50 μM). Values are means ± SEM of three independent experiments performed in triplicate.

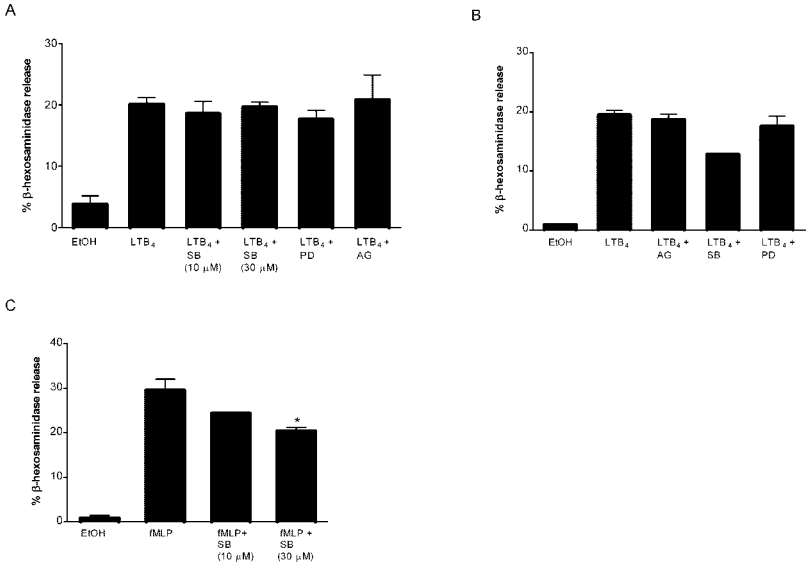

FIGURE 6. Effects of Jak, p38, and Mek/ERK1/2 kinase inhibition on LTB_4-mediated degranulation of PLB-BLT cells and human neutrophils. PLB-BLT cells (2.5×10^5 cells) (*A*) or human neutrophils (*B*) were pretreated with a p38 kinase inhibitor (SB203580 (SB), 10 or 30 μM), an MEK/ERK1/2 kinase inhibitor (PD98059 (PD), 10 μM) or a Jak kinase inhibitor (AG490 (AG), 10 μM) for 1 h at 37°C followed by stimulation with LTB_4 (100 nM) or its vehicle (ethanol; EtOH) for 10 min. After stimulation, β-hexosaminidase release was assessed. *C,* Human neutrophils were pretreated or not with SB203580 (10 or 30 μM) for 1 h at 37°C followed by stimulation with fMLP (100 nM) or its vehicle (EtOH) for 10 min. After stimulation, β-hexosaminidase release was assessed. Values are means ± SEM of three independent experiments performed in triplicate.

already demonstrated (45), preincubation with a higher SB203580 concentration (30 μM) led to a statistically significant 32.5 ± 5.9% inhibition of fMLP-mediated neutrophil degranulation.

Yes kinase is activated by LTB_4

Because Src family kinases were found to be involved in BLT1-mediated neutrophil degranulation, it was of interest to examine whether Src kinases were directly activated by LTB_4 treatment and if so, which member(s) of the family was involved in this cellular activity. Using anti-Src2, an Ab recognizing different members of the Src kinase family, Src kinases were immunoprecipitated followed by an in vitro kinase assay using enolase as a phosphorylation substrate. Src family kinases were activated between 2 and 5 min after LTB_4 stimulation, with peak activation at 3 min (data not shown).

Because the Ab used for immunoprecipitation can detect different Src kinases, we wanted to investigate which kinase in particular was activated by BLT1. We targeted c-Src, c-Fgr, c-Yes, c-Hck, and c-Lyn which are known to be present and can have a role in neutrophil biological activities (23). Three minutes of stimulation was used before immunoprecipitation followed by the in vitro kinase assay. As shown in Fig. 7*A,* Yes kinase, but not c-Src Fgr, Hck, or Lyn could be activated by LTB_4. This activation was blocked by a 15-min pretreatment with PP1 (10 μM) before the kinase assay, showing the specificity of the response.

To examine whether BLT1 endocytosis played a role in LTB_4-induced Yes kinase activation, differentiated PLB-BLT cells were nucleofected with either an empty vector or a vector containing the dominant negative dynamin K44A. After stimulation followed by immunoprecipitation of Yes, in vitro kinase activity was assessed. As shown in Fig. 7*B,* when mock transfected PLB-BLT cells were stimulated with LTB_4, a significant increase in Yes kinase activation was observed (ratio of 2, compared with vehicle stimulation). In contrast, cells transfected with dynamin K44A showed a reduced Yes kinase activation after LTB_4 stimulation, compared with mock transfected cells (ratio of 0.74 for dynamin K44A transfection). These findings suggest a direct relationship between BLT1 endocytosis and subsequent Yes kinase activation.

Receptor tyrosine kinase (RTK) trans *activation is not involved in BLT1-mediated degranulation*

Cross-talk between certain GPCRs and RTKs can lead to *trans* activation of the latter and activation of additional signaling pathways. This has been identified for some RTKs such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) in signaling from the angiotensin II receptor, M1 and M2 muscarinic acetylcholine receptor, ET-1 receptor, and lysophosphatidic acid receptor, to name a few (46–49). Also, by means of *trans* activation, RTKs can activate proteins such as Src family kinases that can be used in GPCR-mediated signaling pathways. We wanted to assess whether EGFR and PDGFR *trans* activation was involved in LTB_4-induced degranulation. We used a specific EGFR inhibitor, AG1478 (100 nM), and a specific PDGFR inhibitor, AG1296 (10 μM). Pretreatment of cells with AG1478 (Fig. 8*A*) and AG1296 (Fig. 8*B*) for 1 h followed by stimulation for 10 min with 100 nM LTB_4 did not lead to any statistically significant reduction in degranulation when compared with cells stimulated with LTB_4 alone. These results suggest that RTK *trans* activation is not a necessary event leading to degranulation in neutrophils stimulated with LTB_4.

Discussion

In this study, we investigated the intracellular signaling events in BLT1-mediated neutrophil degranulation. The conclusions drawn from this study are 4-fold: (1) the neutrophil-like cellular model of PLB-BLT is suitable for studies involving BLT1-mediated intracellular signaling events; (2) receptor endocytosis is essential for LTB_4-mediated enzyme release in neutrophil-like cells; (3) Src family kinase member Yes is activated by LTB_4 and can be partially involved in BLT1-mediated degranulation; and (4) LTB_4-mediated enzyme release does not seem to involve EGFR or PDGFR *trans* activation.

The promyeloid PLB-985 cell line (50) is known for its capacity to differentiate into neutrophil- or monocyte-like cells. Pedruzzi et al. (51) recently used differentiated PLB-985 cells to study fMLP-mediated degranulation. PLB-985 cells were also used by Kaldi et al. (52) to show that fMLP-mediated degranulation was dependent on the presence of cholesterol in the cell membrane. Because the only report on BLT1-mediated signaling events involved in degranulation was studied using rat RBL-2H3 cells stably transfected with BLT1 cDNA (29), it was of great interest to use a human cellular model such as PLB-985 cells to study human neutrophil degranulation.

Several reports state the importance of GPCR endocytosis as an initial and essential event for the activation of protein kinases such

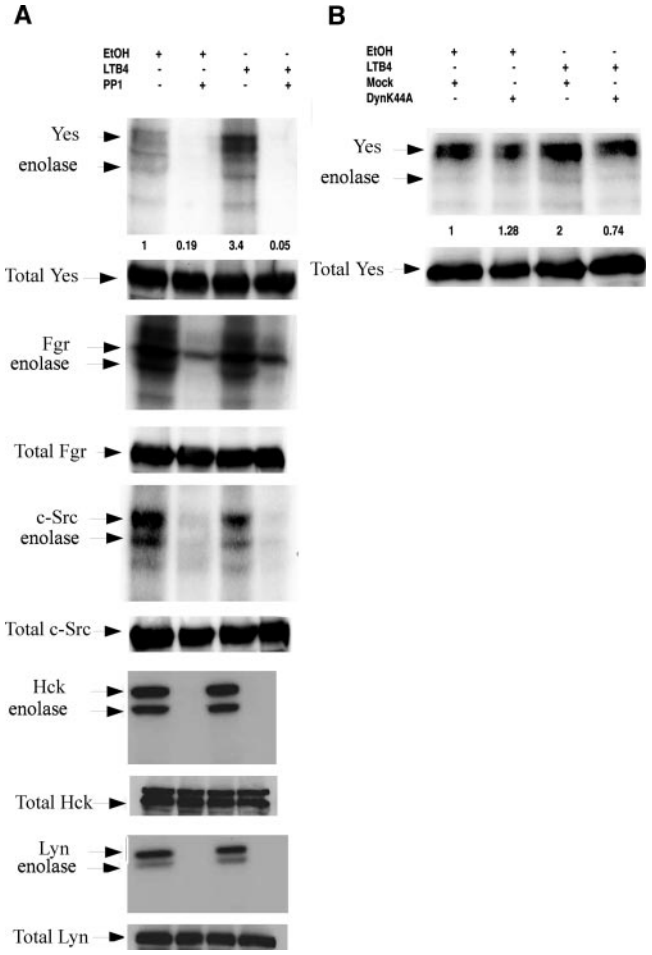

FIGURE 7. Effect of LTB_4 on Src kinase activity in PLB-BLT cells. *A* and *B,* PLB-BLT cells were differentiated for 3 days followed by overnight starvation in medium without FBS. After starving, cells were stimulated with 100 nM LTB_4 or its vehicle (ethanol; EtOH) for 3 min. Immunoprecipitation was then performed using anti-Yes, anti-Fgr, anti-c-Src, anti-Hck, or anti-Lyn Abs. Immunoprecipitates were incubated or not with PP1 (10 μM) for 15 min at 37°C before the beginning of an in vitro kinase assay. Enolase was used as a phosphorylation substrate for the kinases. Total protein loading was assessed by Western blotting using appropriate Abs. *B,* Differentiated PLB-BLT cells were nucleofected with either empty vector or vector containing dynamin (Dyn) K44A. After transfection, cells were put in medium with 10% FBS for 8 h followed by overnight serum deprivation. Cells were then stimulated with LTB_4 (100 nM) or its vehicle for 3 min. Immunoprecipitation was then performed using anti-Yes Ab followed by an in vitro kinase assay. Total protein loading was assessed by Western blotting. Numbers represent densitometric ratios (enolase/total src) adjusted to 1 for vehicle stimulation (ethanol). Blots are one representative of at least three independent experiments yielding similar results.

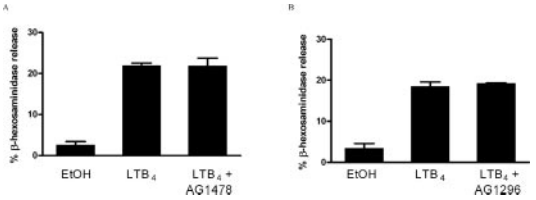

FIGURE 8. Effect of EGFR and PDGFR inhibition on BLT1-mediated degranulation in PLB-BLT cells. *A* and *B,* 2.5×10^5 cells were pretreated with or without an inhibitor of EGFR action, AG1478 (100 nM) (*A*) or with an inhibitor of PDGFR action, AG1296 (10 μM) (*B*), for 1 h at 37°C followed by a 10-min stimulation with LTB_4 or its vehicle (ethanol; EtOH). After stimulation, β-hexosaminidase release was assessed. Values are means ± SEM of three independent experiments performed in triplicate.

as MAPKs. Daaka et al. demonstrated the importance of lysophosphatidic acid receptor and β_2-adrenergic receptor endocytosis for MAPK activation using transfection of dominant negative forms of β-arrestin 1 and dynamin in HEK293 cells (17). Ignatova et al. (53) also showed the importance of opioid receptor internalization for ERK activation in COS-7 cells transiently transfected with dominant negative dynamin. Luttrell et al. (16) showed the importance of lysophosphatidic acid, thrombin, and bombesin receptor internalization in rat 1a fibroblasts for ERK 1/2 activation. Moreover, GPCR internalization has also been linked with other cellular activities. Yang et al. (54) showed that IL-8-mediated chemotaxis was blocked in HEK293 cells cotransfected with CXCR2 and the dominant negative K44A mutant of dynamin. Interestingly, Barlic et al. (43) demonstrated the importance of β-arrestin for IL-8-mediated activation of Src family kinase Hck and subsequent granule release in granulocytes. However, other GPCRs have been shown to activate intracellular signal pathways independently of receptor internalization. We recently showed that PAFR stimulation leads to ERK 1/2 activation independently of receptor internalization in HEK293 cells (22). Internalization-deficient mutants of PAFR were used in addition to transient transfections with dominant negative forms for arrestins 2 and 3 and dynamin to demonstrate this phenomenon. Moreover, Vines et al. (38) demonstrated that the termination of fMLP-mediated degranulation in transfected RBL-2H3 cells was dependent on receptor phosphorylation and arrestin binding but independent of receptor internalization. We demonstrate here that BLT1 does internalize, and by different strategies we show the importance of BLT1 endocytosis for PLB-BLT cell degranulation. We also show the relation between receptor internalization and Yes kinase activation using transient transfection with the dominant-negative K44A mutant of dynamin.

Src family kinases have already been connected to different cellular activities in human neutrophils, such as degranulation. Mocsai et al. (25) demonstrated that adhesion-dependent neutrophil lactoferrin release requires Fgr and Hck activity using Src kinase blockade with PP1 and neutrophils deficient in Fgr and Hck. Mocsai et al. (45) also showed that fMLP-induced neutrophil secretion of primary and secondary granules is mediated by p38 MAPK which is activated by Src family kinases. Because our results with pharmacological inhibitors indicate a lack of involvement of MAPK in BLT1-dependent degranulation but show Src kinase involvement, we studied the direct role of Src kinases in this cellular process. Yes kinase activation is an important event for different cell types of the myeloid origin; Yes kinase is activated when neutrophils are stimulated with granulocyte-macrophage CSF (55). Moreover, in macrophages, Yes kinase was shown to be activated in response to *Aspergillus candidus* spores independently of macrophage superoxide production (56). In this report, we demonstrate the particular activation of Yes kinase, but not c-Src, Fgr, Hck or Lyn, in neutrophil-like cells stimulated with LTB_4.

RTK *trans* activation by GPCRs is a receptor cooperation event in which EGFR and PDGFR have been shown as the principal protein tyrosine kinases to be activated. Linseman et al. (57) demonstrated PDGFR *trans* activation by angiotensin II receptor in rat smooth muscle cells in 1995. In 1996, Daub et al. (58) showed a link between EGFR- and GPCR-mediated activation of MAPK pathways for lysophosphatidic acid, thrombin, and ET-1 receptors in rat-1 fibroblasts. MAPKs are not the only kinases activated by RTK *trans* activation. Src family kinases can also be effector kinases activated by this receptor cooperation. Pierce et al. (59) demonstrated the involvement of Src family kinases in EGFR *trans*

activation by α_{2A}-adrenergic receptor in transfected COS-7 cells. In contrast, an example of direct RTK *trans* activation-independent events leading to intracellular kinase activation has also been reported. Kramer et al.(60) showed that δ-opioid receptor-dependent activation of ERK isoforms was independent of EGFR, PDGFR, and insulin receptor *trans* activation. It was thus important to test the possibility of RTK *trans* activation leading to Yes kinase activation by LTB_4 and subsequent neutrophil degranulation. Our results tend to rule out this possibility because pretreatment with specific inhibitors of EGFR and PDGFR kinases did not have any effect on LTB_4-mediated degranulation.

In this study, we provide a new cellular system for the study of BLT1-mediated events in neutrophils. We also demonstrate the importance of receptor endocytosis and the partial involvement of Src family kinases, potentially Yes kinase, in LTB_4-mediated degranulation. Receptor endocytosis and Yes kinase activation also seem to be related events that may lead to degranulation. Finally, degranulation mediated by BLT1 does not seem to involve any RTK *trans* activation event.

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Disclosures

The authors have no financial conflict of interest.

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