Leukotriene B₄ (LTB₄) is a potent lipid mediator involved in host defense and inflammatory responses. It causes chemotaxis, generation of reactive oxygen species, and degranulation. However, only little is known of the molecular mechanisms by which LTB₄ induces these biological activities. To analyze the intracellular signaling pathways to mediate lysosomal enzyme release through the cloned LTB₄ receptor (BLT1), we transfected BLT1 to rat basophilic leukemia cells (RBL-2H3). LTB₄ dose-dependently released β-hexosaminidase, and the release was mostly inhibited when the cells were pretreated with pertussis toxin, indicating that the degranulation is mediated by Gᵢ proteins. LTB₄ activated phosphatidylinositol 3-kinase (PI3-K) through Gᵢ, and inhibition of PI3-K by wortmannin or LY290042 inhibited degranulation. Granulocytes from PI3-K−/− mice showed reduced LTB₄-induced degranulation, suggesting that this isozyme of PI3-K is involved in the degranulation. LTB₄ also caused calcium release from intracellular stores and calcium influx from the outside milieu through Gᵢ, but only the calcium influx is critical for the lysosomal enzyme release. Calcium influx and PI3-K activation are both downstream events of Gᵢ proteins, since they were inhibited by pertussis toxin. These two events are in essence independent each other, because calcium depletion did not affect PI3-K, and inhibition of PI3-K did not attenuate calcium influx significantly. Thus, our results have clearly shown that LTB₄ binds BLT1 and activates Gᵢ-like protein, and both PI3-K activation and a sustained calcium elevation by calcium influx are necessary for enzyme release in these cells.

Leukotriene B₄ (LTB₄), a metabolite of arachidonic acid, is one of the most potent chemotaxants for neutrophils. LTB₄ activates leukocytes, leading to chemotaxis, release of lysosomal enzyme, and production of superoxide anions, thus playing important roles in host defense (1, 2). Overproduction of LTB₄ is involved in various inflammatory diseases including psoriasis (3), bronchial asthma (4), rheumatoid arthritis (5), inflammatory bowel diseases (6), and ischemic renal failure (7). LTB₄ binds to a specific G protein-coupled receptor named BLT1, which was cloned in our laboratory (8). Signaling to activate leukocytes through BLT1 is poorly understood, in part because most of the previous studies have been done using peripheral leukocytes, especially granulocytes. The lifetime of these cells is short (several days), which makes it difficult to perform studies using various drugs or gene transfection. We have previously analyzed signaling pathway from BLT1 using Chinese hamster ovary cells stably expressing human BLT1 (8). In these cells, LTB₄ increases intracellular IP3 levels by Gᵢ-like protein(s), inhibits adenylyl cyclase activity, and activates chemotaxis through Gᵢ-like protein(s). In hematopoietic cells, however, the intracellular events leading to release of lysosomal enzymes and generation of oxygen species are poorly understood. We generated rat basophilic leukemia (RBL-2H3) cells overexpressing BLT1 and examined the pathway to release lysosomal enzyme through BLT1.

The major pathway of activating lysosomal enzyme release in mast cell involves the aggregation of high affinity receptors (FcεRI) for IgE by corresponding antigens (9, 10). This activation of FcεRI induces histamine release following the activation of tyrosine kinases ( Lyn, Syk, Btk, etc.), phosphatidylinositol 3-kinase (PI3-K) (11–14), and calcium increase (15). Although G protein-coupled receptor-mediated activation of PI3-K was also found in fMLP-stimulated human neutrophils and thrombin-stimulated human platelets (16–19), the physiological role of PI3-K in these cells and its relation to calcium increase are somewhat uncertain. Here we demonstrate that LTB₄-induced enzyme release requires a PI3-K activation and calcium influx by the interaction of BLT1 and Gᵢ.
was from Funakoshi (Tokyo, Japan). Wortmannin was from Seikagaku Co. Ltd. (Tokyo, Japan). 1,2-Bis(O-alkyl)phenyloxathiane (N,N,N',N')-racetamide acid acetoxyethyl ester (BAPTA/AM) and Fura-2/AM were from Dojin (Kumamoto, Japan); LY294002, staurosporine, calphostin C, genistein, herbbimycin A, PD98056, and SKF86365 were from Calbiochem (San Diego, CA); and fatty acid-free bovine serum albumin fraction V (BSA) was from Bayer (Kankakee, IL). LTβ was kindly provided by Otsuka Pharmaceutical Company (Osaka, Japan).

**Cell Culture and Transfection—**RBL-2H3 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a humidified 5% CO2 incubator. The open reading frame of the human LTβ receptor (BLT1) was subcloned into a mammalian expression vector pcDNA3 (Invitrogen). RBL-2H3 cells (106 cells in a 10-cm dish) were transfected by lipofection with 30 μl of Transfectam (Biosepra, Marlborough, MA) and 10 μg of the expression vector. Clones resistant to G418 (1 mg/ml; Invitrogen) were isolated by limiting dilution, tested for the receptor expression by binding assay, and maintained in the presence of 0.3 mg/ml G418. A binding assay for LTβ in RBL-2H3 cells was performed as essentially described previously (20). Briefly, cells on a 24-well culture plate were incubated for 1 h at 25 °C with various concentrations of [3H]LTβ, in the presence or absence of 10 μM unlabeled LTβ, in HEPES-Tyrode's BSA buffer (25 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1.5 mM CaCl2, 12 mM NaHCO3, 5.6 mM Na2-Ascorbate, 0.49 mM MgCl2, and 0.37 mM Na2HPO4) containing 1% (v/v) BSA. The cells were washed three times with HEPES-Tyrode's BSA buffer and lysed in 1% Triton X-100. The radioactivity of the cell lysate was determined by liquid scintillation counting. Ligand binding assay showed a specific binding for LTβ (Kd = 1 nM) comparable with the previous reports on LTβ binding in human neutrophils (21,22).

**β-Hexosaminidase Release Assays—**Degranulation was determined by measuring the release of a granule marker, β-hexosaminidase, as described previously (23). The cells were seeded in 24-well collagen-coated plates at 5 × 104 cells in 1 ml of complete Dulbecco's modified Eagle's medium and serum-starved for 12 h. Adherent cells were washed twice in prewarmed PBS. Cells were then treated with LTβ, and the activity of β-hexosaminidase was quantified in the supernatants and the cell lysates by spectrophotometric analysis using p-nitrophenyl N-acetyl-p-D-glucosaminide as a substrate (24). Values for β-hexosaminidase released in the medium were expressed as the percentages of the total β-hexosaminidase, which was determined in the cells lysed in 0.1% Triton X-100.

**Fluorometric Imaging of Intracellular Calcium Concentrations in RBL Cells—**Cells were cultured on glass bottom dishes (Matsunami, Osaka, Japan) for 12 h and loaded with 5 μM Fura-2/AM in HEPES-Tyrode's BSA buffer at 37 °C for 1 h and further incubated at room temperature for 1 h. Cells were stimulated with ligands at room temperature. For depletion of intracellular calcium, cells were incubated at 25 °C for 1 min in the presence of 40 μM BAPTA/AM before stimulation. Fluorometric cell images were recorded with an ICCD camera/image analysis system (ARGUS-50, Hamamatsu Photonics, Hamamatsu, Japan), and the ratios (340/380 nm) were determined.

**Measurement of PI3-K Activity—**Phosphatidylinositol 3,4,5-triphosphate (P(3,4,5)P3) production was measured according to the method described by Traynor-Kaplan et al. (17). RBL cells were cultured at a density of 106 cells/ml in a glass tube and serum-starved for 12 h. Medium was changed to the labeling buffer consisting of phosphate-free hanks' balanced saline solution, 0.05% BSA (fraction V; Sigma), and 5 μg/ml cytochalasin B (Sigma) were preincubated at 37 °C for 10 min with or without wortmannin, and 50 μl of the ligand solution was added to initiate the reaction. After incubation for 10 min at 37 °C, the reaction was terminated by placing the plate on ice. After centrifugation at 2,300 × g for 5 min at 4 °C, 20 μl of the supernatants were transferred to a new 96-well plate. The remaining cells were lysed by adding 20 μl of lysis buffer (1 × Hanks' balanced saline solution, 5% Triton-X). The MPO activities of the supernatants and cells were measured in MPO assay buffer (50 mM potassium phosphate buffer, pH 6.0, 0.157 mg/ml o-dianisidine (Sigma), and 0.0005% hydrogen peroxide). Commercially available MPO (Calbiochem) in serial dilution was used as a standard.

**Calcium Mobilization in Mouse Granulocytes—**Mouse granulocytes were incubated with 3 μM Fura-2/AM (Dojin, Kumamoto, Japan) in HEPES-Tyrode's BSA buffer at 37 °C for 30 min, washed, and resuspended in HEPES-Tyrode's BSA buffer at a density of 4 × 106/ml. One-half ml of the cell suspension was applied to a CAF-100 system (Jasco, Tokyo, Japan), and 5 μl of LTβ in MeSO was added. Change in intracellular Ca2+ concentration was measured by the ratio of emission fluorescence of 500 nm, by excitation at 340 and 380 nm.**

**RESULTS**

**Involvement of PI3-K in LTβ-induced Enzyme Release—**RBL-BLT1 cells showed LTβ-dependent release of β-hexosaminidase, a mast cell lysosomal enzyme, whereas mock-transfected cells did not (Fig. 1). The EC50 value of LTβ was 24 ± 8 nM (n = 3 ± S.D.). We then examined the intracellular signals leading to the release of β-hexosaminidase. To examine the effects of PTX or depletion of conventional protein kinase C on ligand-induced β-hexosaminidase release, cells were treated overnight with 50 ng/ml PTX or 100 ng/ml PMA. LTβ-induced β-hexosaminidase release in RBL-BLT1 cells was mostly blocked by PTX pretreatment but was not affected by PMA pretreatment (Fig. 2A). These results indicate that in RBL-BLT1 cells, LT1 couples to PTX-sensitive G protein(s), and conventional protein kinase C is not required for LTβ-induced degranulation. Next, the effects of various inhibitors on LTβ-induced β-hexosaminidase release were examined. Neither a broad kinase inhibitor (staurosporine), a protein kinase C inhibitor (calphostin C), tyrosine kinase inhibitors (genistein and herbbimycin A), nor a mitogen-activated protein kinase kinase inhibitor (PD98059) inhibited the LTβ-induced enzyme release (Fig. 2B). Two kinds of PI3-K inhibitors, wortmannin and LY294002, inhibited LTβ-induced enzyme release with IC50 values of 70 ± 32 nm and 0.103 ± 0.041 mm (n ± S.D.), respectively (Figs. 2B and
Thus, in RBL-BLT1 cells, LTB4-induced enzyme release requires the activation of PI3-K.

**Activation of PI3-K following LTB4 Stimulation**—We then examined whether or not PIP3 was generated in RBL-BLT1 cells by LTB4 stimulation. As shown in Fig. 4A, cellular PIP3 level reached 2–3-fold basal level after 15 s of LTB4 stimulation. LTB4-induced PIP3 production was transient and dose-dependent (Fig. 4, A–D). The increase in PIP3 was abolished by treatment of the cells with either wortmannin or LY294002 or by PTX pretreatment (Fig. 4E). These results suggest that LTB4 utilizes PTX-sensitive G protein(s) to activate PI3-K. The PIP3 production was independent of calcium increase, since neither BAPTA/AM nor EGTA inhibited its production (Fig. 4E; see below).

**Requirement of Calcium Influx for LTB4-induced Enzyme Release**—BLT1 is known to couple with Gq-like proteins to activate phospholipase C in Chinese hamster ovary cells (8). In RBL-BLT1 cells, LTB4 induced a rapid increase of intracellular calcium concentration, and pretreatment of cells with PTX almost completely abolished the calcium response (Fig. 5, A and B). This result shows that BLT1 couples to PTX-sensitive G protein(s) to activate PI3-K. The PIP3 production was independent of calcium increase, since neither BAPTA/AM nor EGTA inhibited its production (Fig. 4E; see below).

**Independent Process of Calcium Elevation and PI3-K Activation**—We examined whether the activation of PI3-K is dependent on the intracellular calcium increase. In the presence of 1 mM EGTA or 50 μM SKF96365, a calcium ion channel blocker, LTB4-induced enzyme release was completely blocked (Fig. 6A), suggesting that extracellular calcium is required to release enzyme. Loading cells with an intracellular calcium chelator BAPTA/AM is a common method to prevent initial rapid elevations of intracellular calcium induced by various ligands (28, 29). Preloading of the cells with BAPTA/AM did not inhibit LTB4-induced enzyme release, but this enzyme release was delayed for 2–3 min compared to the untreated cells (Fig. 6B). Next, we examined the time course of calcium mobilization by LTB4 in the presence or absence of BAPTA/AM. In the absence of BAPTA/AM, LTB4-induced calcium concentration peaked at 1 min after stimulation (Fig. 7A), whereas in BAPTA/AM-loaded cells, it was observed after 2–3 min of LTB4 stimulation (Fig. 7B). In the presence of 1 mM EGTA or 50 μM SKF96365, LTB4 induced an initial calcium transient, but a subsequent sustained calcium mobilization was not observed (Fig. 7, C and D).

Compared with the results of Fig. 6, a calcium influx and a sustained calcium elevation are required for the enzyme release rather than the initial transient calcium mobilization from internal stores.

**Fig. 2. Effects of various treatments on LTB4-induced β-hexosaminidase release in RBL-BLT1 cells.** A, serum-depleted cells were preincubated with 50 ng/ml PTX or 100 ng/ml PMA overnight and stimulated with 100 nM LTB4 at 37 °C for 15 min. B, serum-depleted cells were preincubated with various inhibitors at 37 °C for 15 min and stimulated with 100 nM LTB4 at 37 °C for 15 min. Each data point is the mean ± S.D. of triplicates. Shown are representative data from three independent experiments with similar results.

**Fig. 3. Inhibition of LTB4-induced β-hexosaminidase release in RBL-BLT1 cells by wortmannin and LY294002.** Serum-depleted cells were pretreated with various doses of wortmannin (A) and LY294002 (B) at 37 °C for 15 min prior to stimulation with 100 nM LTB4. Each data point is the mean ± S.D. of triplicates. Presented are typical data from three independent experiments with similar results.
was abolished (Fig. 7, C and D). Even in the presence of EGTA or BAPTA/AM, LTB$_4$-induced PIP$_3$ production was not affected (Fig. 4E), suggesting that neither the initial nor a sustained calcium increase is required for PI3-K activation and production of PIP$_3$ in these cells. These results show that LTB$_4$-induced PI3-K activation is independent of calcium mobilization. Conversely, we examined the effects of PI3-K inhibition on calcium responses. Neither wortmannin nor LY294002 inhibited calcium responses significantly (Fig. 8). All of these data suggest that LTB$_4$ activates G$_i$-like protein, and both PI3-K and calcium influx are pivotal for the enzyme release from RBL-BLT1 cells.

**LTB$_4$- or fMLP-dependent MPO Release from Mouse Peritoneal Granulocytes**—To examine whether PI3-K activation plays crucial roles in degranulation of native cells, we measured LTB$_4$- and fMLP-induced myeloperoxidase (MPO) release from granulocytes of wild-type and PI3-K$_\gamma$-deficient mice (26). We chose MPO as a marker of degranulation, since MPO, but not $\beta$-hexosaminidase, is quite abundant in granulocytes and commonly used to examine stimulation-dependent degranulation. LTB$_4$ and fMLP dose-dependently induced MPO release in these cells, and LTB$_4$ was more potent than fMLP (Fig. 9A). As is the case of RBL cells, wortmannin dose-dependently inhibited LTB$_4$- and fMLP-induced MPO release (Fig. 9A). In PI3-K$_\gamma$-deficient granulocytes, LTB$_4$-induced MPO release was reduced to 20–25% of wild-type granulocytes (Fig. 9B), suggesting that the $\gamma$ isozyme of PI3-K plays a dominant role in LTB$_4$-induced degranulation. We measured LTB$_4$-induced calcium mobilization in wild-type and PI3-K$_\gamma$-deficient granulocytes (Fig. 9C). PI3-K$_\gamma$-deficient granulocytes showed a similar calcium increase toward 100 nM LTB$_4$, showing that the reduced MPO release in PI3-K$_\gamma$-deficient granulocytes is not due to the reduced calcium increase.

**DISCUSSION**

Enzyme release is one of the most important biological responses of inflammatory cells by LTB$_4$ stimulation, leading to inflammation and host defense against infection (2). To study
The effects of EGTA, BAPTA/AM, and extracellular calcium concentrations on LTB₄-induced enzyme release. A, serum-depleted cells were stimulated with 100 nM LTB₄ at 37 °C for 15 min. Cells were loaded with 40 μM BAPTA/AM at 37 °C for 10 min. For EGTA, the medium was replaced with calcium-free medium containing 1 mM EGTA just prior to LTB₄ application. For SKF96365, cells were incubated with 50 μM SKF96365 for 10 min before LTB₄ application. B, time course of LTB₄-induced β-hexosaminidase release and the effect of BAPTA/AM. Each data point is the mean ± S.D. of triplicates. These are representative data from three independent experiments with similar results.

Signaling for the enzyme release, we used rat basophilic leucemia (RBL) cells, which contain the same functional G proteins reported in human neutrophils (30). Expression of LTB₄ receptor in RBL cells allowed us to examine the signaling pathway to LTB₄-dependent enzyme release.

LTB₄ plays its functions via specific G protein-coupled receptors named BLT1 and BLT2 (8, 31), and we transfected BLT1, a high affinity and blood cell-specific LTB₄ receptor to RBL cells. LTB₄ is reported to activate the Gᵢ and Gₒ classes of G proteins (32) to inhibit adenylyl cyclase and to activate phospholipase C, respectively. Saito et al. (33) reported that PTX-sensitive Gᵢ protein mediates thrombin- and compound 48/80-induced histamine release in mast cells and basophils. In this study, we showed that a series of signaling including Gi, calcium influx, and PIP3 production are necessary for the ultimate enzyme release through LTB₄-BLT1 interaction.

First, as shown in Fig. 2A, the degranulation is totally sensitive to PTX treatment, suggesting that Gᵢ-like molecules are related to enzyme release. Next, we examined the effects of various inhibitors and toxins on LTB₄-dependent enzyme release. We found that LTB₄-triggered enzyme release was inhibited by wortmannin and LY294002 (Figs. 2B and 3), suggesting that PI3-K is deeply involved in degranulation. We then examined whether LTB₄ can indeed activate PI3-K and found that PIP3 production was enhanced by 2–3-fold upon LTB₄ stimulation (Fig. 4, A–D). Pretreatment of the cells with PTX completely abolished LTB₄-dependent PIP3 production, suggesting that Gᵢ-like molecules are required for LTB₄-dependent PI3-K activation (Fig. 4E). Pretreatment of the cells with various kinase inhibitors did not affect LTB₄-induced enzyme releases (Fig. 2B). Depletion of conventional protein kinase C by overnight PMA treatment did not inhibit enzyme releases (Fig. 2A). GF109203X at higher concentrations showed inhibitory effects on the enzyme release (data not shown). GF109203X is an inhibitor for both conventional protein kinase C and atypical protein kinase C (34). Therefore, it is possible that atypical protein kinase C is involved in either step for degranulation (35, 36). Haribabu et al. (37) reported that the chemotaxis was blocked by PTX, showing that BLT1 couples to PTX-sensitive Gᵢ-like proteins in RBL-BLT1 cells. They also showed that BLT1-mediated calcium increase and enzyme release were almost insensitive to PTX or wortmannin treatment. In our study, BLT1-mediated calcium increase, enzyme release, and PIP3 production were sensitive to PTX (Figs. 2A, 4E, and 5). We do not have clear explanations for this discrepancy, but the experimental conditions and stable cell lines with different expression levels of BLT1 can cause the apparent difference.

Calcium mobilization by FcεRI cross-linking is essential to antigen-induced enzyme release in RBL cells (15). Thus, we examined the relationship between calcium mobilization and enzyme release. Calcium mobilization from internal stores and subsequent calcium entry from the extracellular space are the two major components of calcium increase following activation of cell surface receptors (38). In our experiments, EGTA or SKF96365, a calcium channel blocker, inhibited LTB₄-induced enzyme release, showing the critical role of calcium influx in enzyme release. Calcium depletion in these cells by BAPTA/AM loading resulted in the delayed increase of intracellular calcium (Fig. 7B). BAPTA/AM treatment caused the similar delay in LTB₄-induced enzyme release (Fig. 6B). The local high calcium concentrations by calcium influx or a sustained calcium elevation with a critical duration might be necessary for the enzyme release.

Finally, we have performed a series of experiments to iden-
tify the relationship between calcium influx and PI3-K activation. Neither EGTA nor BAPTA/AM treatment affected LTB4-depended PIP3 production (Fig. 4E), showing that PI3-K activation does not require calcium increase. Conversely, we determined whether inhibition of PI3-K affects calcium mobilization or calcium influx, since several investigators have reported that PI3-K regulates phospholipase C/H253/H254 activation with FcRI activation (39, 40). We found that treatment of cells with wortmannin or LY294002 did not significantly change the calcium response by LTB4 stimulation (Fig. 8). In three independent lines of PI3-K/H253 null mice, calcium mobilization in neutrophils was examined using various chemoattractants. fMLP (26, 41), C5a, platelet-activating factor, or IL-8 (42) induced similar calcium increase in neutrophils from wild-type and PI3-K/H253 null mice. We also confirmed that LTB4-induced calcium mobilization was not reduced in PI3-K/H253 null granulocytes (Fig. 9C).

It is an important future issue how G1 activates PI3-K in RBL-2H3 cells. The role of the βγ subunit of G protein might be the most important, since PI3-K activation by the βγ subunit of G proteins induces respiratory burst in hematopoietic cells (43, 44). Neutrophils of PI3-K/H253 null mice impaired chemotractant-induced respiratory burst and chemotaxis (26, 42). Our present data (Fig. 9) showed clearly that PI3-K is the most important PI3-K isozyme in LTB4-dependent degranulation. Signals to calcium influx from G1 molecules are another concern for future studies. They are either due to direct activation of calcium channels or due to the capacitance calcium entry as proposed (45–47).

In summary, using RBL-2H3 cells expressing BLT1, we found that PTX-sensitive G1 molecules are important for the degranulation process. G1 then activates PI3-K and calcium influx, and both are required for enzyme release in inflammatory cells. We further demonstrated that calcium influx and PI3-K might be processes that are independent from each other, although both are downstream events of G1 activation. Although these two signals are required for degranulation, other downstream molecules of G1 can also play roles in the enzyme release.

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