

The Immediate Phase of *c-kit* Ligand Stimulation of Mouse Bone Marrow–derived Mast Cells Elicits Rapid Leukotriene C₄ Generation through Posttranslational Activation of Cytosolic Phospholipase A₂ and 5-Lipoxygenase

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Summary

c-kit ligand (KL) activated mouse bone marrow–derived mast cells (BMMC) for the dose- and time-dependent release of arachidonic acid from cell membrane phospholipids, with generation of leukotriene (LT) C₄ in preference to prostaglandin (PG)D₂. KL at concentrations of 10 ng/ml elicited half-maximal eicosanoid generation and at concentrations of >50 ng/ml elicited a maximal generation of ~15 ng LTC₄ and 1 ng PGD₂ per 10⁶ cells, with 20% net β-hexosaminidase release 10 min after stimulation. Of the other cytokines tested, none, either alone or in combination with KL, elicited or modulated the immediate phase of mediator release by BMMC, indicating strict specificity for KL. Activation of BMMC in response to KL was accompanied by transient phosphorylation of cytosolic phospholipase A₂ and reversible translocation of 5-lipoxygenase to a cell membrane fraction 2–5 min after stimulation, when the rate of arachidonic acid release and LTC₄ production were maximal. BMMC continuously exposed to KL in the presence of IL-10 and IL-1β generated LTC₄ in marked preference to PGD₂ over the first 10 min followed by delayed generation of PGD₂ with no LTC₄ over several hours. Pharmacologic studies revealed that PGD₂ generation in the immediate phase depended on prostaglandin endoperoxide synthase (PGHS)-1 and in the delayed phase on PGHS-2. Thus, KL provided a nonallergic stimulus for biphasic eicosanoid generation by mast cells. The immediate phase is dominated by LTC₄ generation with kinetics and postreceptor biosynthetic events similar to those observed after cell activation through the high affinity IgE receptor, whereas the delayed phase of slow and selective PGD₂ production is mediated by induction of PGHS-2.

The ability of the stromal cytokine, *c-kit* ligand (KL)¹ to directly activate mouse bone marrow–derived mast cells (BMMC) for prostanoid generation during 2–10 h and to prime these cells for IgE-dependent prostanoid and leukotriene (LT) generation after 1 d reveals biochemically separate responses over time (1, 2). Furthermore, the finding that BMMC can be stimulated directly by cytokines to generate prostanoids extends the recognition of the involvement of mast cells in non-IgE–dependent biologic processes. KL in the presence of IL-1β and/or IL-10 induced the transcription and translation of prostaglandin endoperoxide synthase (PGHS;

or cyclooxygenase)-2 leading to delayed PGD₂ generation (1). In contrast, the immediate response of these mast cells to IgE-dependent activation was the generation of LTC₄ in marked preference to PGD₂ (3), which was derived via PGHS-1 even when PGHS-2 was present and functional through cytokine treatment (1). BMMC treated with KL plus appropriate accessory cytokines for >1 d were primed for increased IgE-dependent acute PGD₂ synthesis caused by up-regulation of PGHS-1, cytosolic phospholipase A₂ (cPLA₂), and hematopoietic PGD₂ synthase (2). KL has previously been reported to initiate exocytosis of mast cells (4, 5) at concentrations similar to those needed for proliferation (6), differentiation (7, 8), survival through inhibition of apoptosis (9), adhesion (10), and chemotaxis (11). Because of the importance of identifying non-IgE–dependent mechanisms for mast cell activation and elaboration of the cysteinyl LTs, we have now analyzed the immediate response of BMMC to KL in terms of eicosanoid biosynthesis.

¹ Abbreviations used in this paper: BMMC, bone marrow–derived mast cells; cPLA₂, cytosolic phospholipase A₂; FLAP, 5-lipoxygenase–activating protein; KL, *c-kit* ligand; 5-LO, 5-lipoxygenase; LT, leukotriene; MAP kinase, mitogen-activated protein kinase; MIP-1α, macrophage inflammatory protein-1α; MCP-1, monocyte chemoattractant protein-1; NGF, nerve growth factor; PGHS, PG endoperoxide synthase; TNP, trinitrophenyl.

The initial step in arachidonic acid metabolism is the release of free arachidonic acid from cell membrane phospholipids by phospholipase A₂ (PLA₂). Among the several characterized mammalian PLA₂ enzymes, 85-kD cPLA₂ and 14-kD type II secretory PLA₂ are implicated in supplying arachidonic acid to downstream biosynthetic enzymes (12). cPLA₂ is rapidly activated by translocation from the cytosol to a cell membrane compartment in response to an increase in cytoplasmic Ca²⁺ concentration (13) and by phosphorylation by mitogen-activated protein (MAP) kinase (14) in response to a wide variety of stimuli including cross-linking of FcεRI on mast cells (15). Type II PLA₂ has been implicated in the mechanism of degranulation (16) and in making arachidonic acid available for prostanoid biosynthesis (17, 18), but it has not been implicated in LT biosynthesis (18). After crosslinking of FcεRI, 5-lipoxygenase (5-LO) is activated by reversible Ca²⁺-dependent translocation to the perinuclear membrane (19), where 5-LO-activating protein (FLAP) presents arachidonic acid for sequential conversion to 5-hydroperoxyeicosatetraenoic acid and then to LTA₄ (20). LTA₄ is conjugated with reduced glutathione to form LTC₄ by LTC₄ synthase, an integral perinuclear membrane protein that has significant homology with FLAP (21). PGHS, which occurs in two integral membrane isoforms, catalyzes the oxygenation of arachidonic acid to PGH₂. Only PGHS-1 is constitutively expressed, and the expression of the two isoforms is differently regulated by particular growth factors and cytokines (1, 2, 22–30). The conversion of PGH₂ to PGD₂ is regulated in mast cells by a glutathione-dependent, cytosolic, hematopoietic PGD₂ synthase (2, 31).

We now report that KL elicits the rapid generation of LTC₄ from BMMC in a 10–15-fold excess over PGD₂. Transient phosphorylation of cPLA₂ and transient translocation of 5-LO occurred in parallel with the release of arachidonic acid and the generation of LTC₄, respectively. The immediate phase of KL-initiated eicosanoid release is not influenced by the presence of accessory cytokines and is followed by a delayed, selective generation of PGD₂ via PGHS-2 that requires IL-10 and/or IL-1β as accessory cytokines. The finding that KL, a tissue-derived factor, directly activates BMMC for eicosanoid generation at a level comparable to IgE-dependent stimulation reveals a likely alternative route for the appearance of lipid mediators in allergic and inflammatory processes. Furthermore, the KL-dependent regulation of arachidonic acid metabolism in BMMC can be divided into three sequential phases: immediate, delayed, and priming.

Materials and Methods

Materials. Recombinant mouse KL and IL-10 were acquired through expression in baculovirus, and their concentrations were determined as previously described (1, 2). Recombinant mouse IL-1β, IL-3, IL-4, IL-6, GM-CSF, TGF-β1, macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), IFN-γ, and TNF-α were purchased from Genzyme Corp. (Cambridge, MA). 2.5 S nerve growth factor (NGF) and indomethacin were purchased from Sigma Immunochemicals (St. Louis, MO). Recombinant mouse IL-9 (32) was provided by C. Uyttenhove and

J.-C. Renauld (Ludwig Institute for Cancer Research, Brussels, Belgium). Rabbit antiserum to human cPLA₂ was provided by J. D. Clark (Genetics Institute, Cambridge, MA), rabbit antiserum to 5-LO was from J. F. Evans (Merck Frosst, Quebec, Canada), and the PGHS-2 inhibitor, NS-398 (33) was from J. Trzaskos (Merck DuPont, Wilmington, DE).

Activation of BMMC with KL. Bone marrow cells from male BALB/cJ mice (Jackson Laboratory, Bar Harbor, ME) were cultured for 3–6 wk in 50% enriched medium (RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% FCS/50% WEHI-3 cell (American Type Culture Collection, Rockville, MD)-conditioned medium as described (1, 2). After 3 wk, >97% of the cells in culture were BMMC as assessed by staining with toluidine blue or with alcian blue and safranin. BMMC, washed once with enriched medium, were stimulated by resuspension with KL in enriched medium at a cell density of 5 × 10⁶ cells per ml and were incubated for various periods. The concentration of KL used in typical experiments was 100 ng/ml. In separate experiments, BMMC were incubated for 10 min with IL-1β (5 ng/ml), IL-3 (100 U/ml), IL-4 (1.5 ng/ml), IL-6 (5 ng/ml), IL-9 (100 U/ml), IL-10 (10 U/ml), GM-CSF (100 U/ml), TGF-β1 (2.5 ng/ml), NGF (500 ng/ml), MIP-1α (10⁻⁷ M), MCP-1 (10⁻⁷ M), IFN-γ (100 U/ml), or TNF-α (500 U/ml) either alone or in combination with 100 ng/ml KL. The reaction was stopped by centrifugation of the cells at 120 g for 5 min at 4°C, and the supernatants were retained for assay of mediator release. The cell pellets were suspended in enriched medium and disrupted by freeze-thawing. β-Hexosaminidase, a marker of mast cell degranulation, was quantitated in the supernatants and pellets by spectrophotometric analysis of the hydrolysis of *p*-nitrophenyl-β-D-2-acetamido-2-deoxyglucopyranoside (34). The percent release of β-hexosaminidase was calculated by the formula $[S/(S + P)] \times 100$, where *S* and *P* are the β-hexosaminidase contents of equal portions of each supernatant and cell pellet, respectively. PGD₂ and LTC₄ were assayed by radioimmunoassay (Amersham Corp., Arlington Heights, IL).

For comparison to IgE-dependent activation, BMMC were suspended at 1 × 10⁷ cells per ml in WEHI-3 cell-conditioned medium and sensitized with 10 μg/ml monoclonal IgE anti-trinitrophenyl (TNP) for 30 min. After being washed twice with enriched medium, the cells were resuspended in enriched medium at 5 × 10⁶ cells per ml and were incubated at 37°C for 10 min with 100 ng/ml TNP-BSA or with 100 ng/ml KL. Mediator release was assessed as described above.

In certain experiments, the effects of indomethacin and NS-398 on immediate PGD₂ release were assessed. BMMC were cultured with 100 ng/ml NS-398, 100 ng/ml indomethacin, or in culture medium without inhibitors, together with 10 μg/ml IgE anti-TNP, at a density of 1 × 10⁷ cells per ml for 2 h. The cells were then washed and activated with 100 ng/ml TNP-BSA or 100 ng/ml KL at 5 × 10⁶ cells/ml for 10 min. To assess the effect of these inhibitors on delayed PGD₂ release, replicate cells were cultured at a density of 1 × 10⁶ cells per ml for 2 h in medium containing 100 ng/ml KL, 10 U/ml IL-10, and 5 ng/ml IL-1β, and then for 3 h more with these cytokines in the absence or presence of each inhibitor. At the end of each experiment, the cells were pelleted and the PGD₂ released into the supernatant was measured by radioimmunoassay.

Measurement of Arachidonic Acid Release from Activated BMMC. BMMC were suspended in 50% WEHI-3 cell-conditioned medium at 1 × 10⁶ cells per ml and were incubated with 1 μCi/ml [³H]arachidonic acid (100 Ci/mmol) (New England Nuclear, Boston, MA)

at 37°C for 12 h. The cells were then washed three times with enriched medium and stimulated with KL as described above. The [³H]arachidonic acid associated with the cell pellet and that released into the supernatant were quantitated in a liquid β -scintillation counter (Beckman Scientific Instruments, Palo Alto, CA). The percent release of [³H]arachidonic acid was calculated by the formula $[S/(S + P)] \times 100$, where *S* and *P* are the cpm of equal portions of supernatant and cell pellet, respectively. Alternatively, the total lipids in replicate samples of cells and supernatants were extracted as described (17) and were developed by thin layer chromatography on silica gel plates (Sigma) with a solvent system of chloroform/methanol/acetic acid (65:25:10 vol/vol). The spots of each phospholipid and neutral lipid were visualized by exposure to I₂ vapor and scraped from the plates; the radioactivity was then counted in a liquid β -scintillation counter.

Phosphorylation of cPLA₂. BMMC (2×10^6 cells/ml) were prelabeled for 1.5 h at 37°C with [³²P]orthophosphate (500 μ Ci/ml) (Amersham) in phosphate-free Eagle's MEM (Sigma) supplemented with 5% FCS, 50 U/ml IL-3, and in the case of IgE/Ag stimulation, 10 μ g/ml IgE anti-TNP. After being washed, the cells were resuspended in 800 μ l of enriched medium at a density of 5×10^6 cells per ml, and activated with 100 ng/ml KL or with 100 ng/ml TNP-BSA as described above. The cells were pelleted by centrifugation at 10,000 *g* for 20 s at 4°C and were immediately lysed by 10 min incubation at 4°C in 200 μ l of buffer comprising 10 mM sodium phosphate buffer, pH 7.4, 5 mM Na₃VO₄, 50 μ g/ml leupeptin (Sigma), 1.5 μ M pepstatin A (Sigma), 1 mM PMSF (Sigma), and 0.1% NP-40 (Boehringer Mannheim GmbH, Mannheim, Germany). The lysate was centrifuged at 10,000 *g* for 20 s at 4°C. The resulting supernatant was mixed with 20 μ l of a 50% (vol/vol) suspension of protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) in lysis buffer that had been preincubated with 5 μ l of antiserum to cPLA₂ for 1 h at 4°C. After incubation for 2 h at 4°C, the beads were sedimented by brief centrifugation at 10,000 *g* for 20 s at 4°C and washed three times with the lysis buffer. The proteins bound to the beads were solubilized by boiling for 5 min, applied to 10% SDS-PAGE (Schleicher & Schuell, Inc., Keene, NH), and electrophoresed. The separated proteins were electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) in a transfer buffer consisting of 20 mM Tris, 150 mM glycine, and 20% methanol with a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 250 V for 1.5 h. Immunoprecipitated, phosphorylated protein was visualized by autoradiography at -80°C with XAR-5 film (Eastman Kodak Co., Rochester, NY). The membrane was then sequentially treated with the following: 5% nonfat milk in 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.1% Tween 20 (Bio-Rad) (TBS-Tween) for 1 h; antiserum against cPLA₂ at a dilution of 1:2,000 in TBS-Tween for 1 h; TBS-Tween for three washes; and horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) (1:8,000 dilution) in TBS-Tween for 1 h. After five washes, the protein bands were visualized with a chemiluminescent technique using an ECL Western blot analysis system (Amersham). In preliminary experiments, cPLA₂ in cell lysates (4×10^6 cells equivalents) was completely and quantitatively precipitated by 5 μ l of the antiserum against cPLA₂ under the conditions described (data not shown).

5-LO Translocation. Before and after treatment with cytokines, BMMC (5×10^6 cells) were pelleted by centrifugation at 10,000 *g* for 20 s at 4°C and were immediately frozen in dry ice/ethanol. Rapid freezing is reported to be essential for the detection of membrane-bound 5-LO in BMMC after IgE/Ag stimulation (19). Then 0.5 ml of a buffer composed of 50 mM Tris-HCl, pH 7.4,

5 mM Na₃VO₄, 50 μ g/ml leupeptin, 1.5 μ M pepstatin A, and 1 mM PMSF, precooled at 4°C, was added, and the cells were disrupted by sonication (Branson sonifier; 60% work cycle, setting 6; Branson Sonic Power, Danbury, CT). The lysate was centrifuged at 100,000 *g* for 1 h at 4°C. The resulting pellet was reconstituted in 100 μ l of the same buffer. 10- μ l portions of the 100,000 *g* supernatant and pellet were resolved in 10% SDS-PAGE, electroblotted to a nitrocellulose membrane, and immunoblotted with rabbit antiserum against 5-LO at a dilution of 1:5,000 as described above. Translocation of 5-LO was defined by the immunodetection of 5-LO in the 100,000 *g* pellet.

Results

Time Course, Dose Dependence, and Cytokine Specificity of Eicosanoid Release from BMMC Activated with KL. When BMMC were resuspended in enriched medium containing 100 ng/ml KL, the release of β -hexosaminidase was detectable by 1 min ($5.1 \pm 0.6\%$ release, mean \pm SEM, *n* = 3) and reached a near maximum of $25.4 \pm 1.7\%$ (*n* = 3) by 5 min. [³H]arachidonic acid release into the supernatant from prelabeled BMMC was evident by 1 min ($1.5 \pm 0.3\%$, *n* = 5) and reached a maximum of $12.1 \pm 2.1\%$ (*n* = 5) by 5 min. LTC₄ release was barely detectable for the first 2 min, reached half-maximum at 5 min, and plateaued at a maximum of 13.9 ± 1.1 ng per 10^6 cells (*n* = 3) by 10 min. PGD₂ release was apparent by 2 min and increased to a maximum of 1.0 ± 0.2 ng per 10^6 cells (*n* = 3) at 10 min (Fig. 1).

The dependence of mediator release from BMMC on the concentration of KL was examined after 10 min of stimulation (Fig. 2). β -Hexosaminidase, arachidonic acid, LTC₄, and PGD₂ were released in a dose-dependent manner; all reached maximum levels with a KL concentration of 50–100 ng/ml. However, interpolation from the dose-response curves revealed an EC₅₀ of ~ 10 ng/ml for the release of [³H]arachidonic acid, LTC₄, and PGD₂ compared to an EC₅₀ of ~ 25 ng/ml for β -hexosaminidase release, reflecting greater sensitivity of arachidonic acid metabolism than of exocytosis to the action of KL.

Because the time course of KL-stimulated mediator release was comparable to that of IgE-dependent activation, the two processes were compared for product release at 10 min, which represented a plateau for each reaction. BMMC sensitized with hapten-specific IgE and stimulated with specific Ag released twofold more β -hexosaminidase, 1.5-fold more arachidonic acid, 2.9-fold more LTC₄, and 2.3-fold more PGD₂ than BMMC stimulated with 100 ng/ml KL (Table 1). Thus, the product profiles were similar in amount and distribution with LTC₄/PGD₂ ratios of 11:1 and 9:1, respectively, for IgE-dependent and KL-initiated activation. KL-dependent release of each mediator was markedly reduced when BMMC were activated in the absence of extracellular Ca²⁺ (Table 1).

Among the cytokines evaluated, IL-3 (Fig. 3), IL-4, IL-6, IL-9, IL-10, IL-1 β , GM-CSF, TGF- β 1, NGF, MIP-1 α , MCP-1, IFN- γ , and TNF- α (*n* = 4; data not shown) did not directly induce mediator release from BMMC during 10 min incubation at the concentrations noted in Materials and Methods.

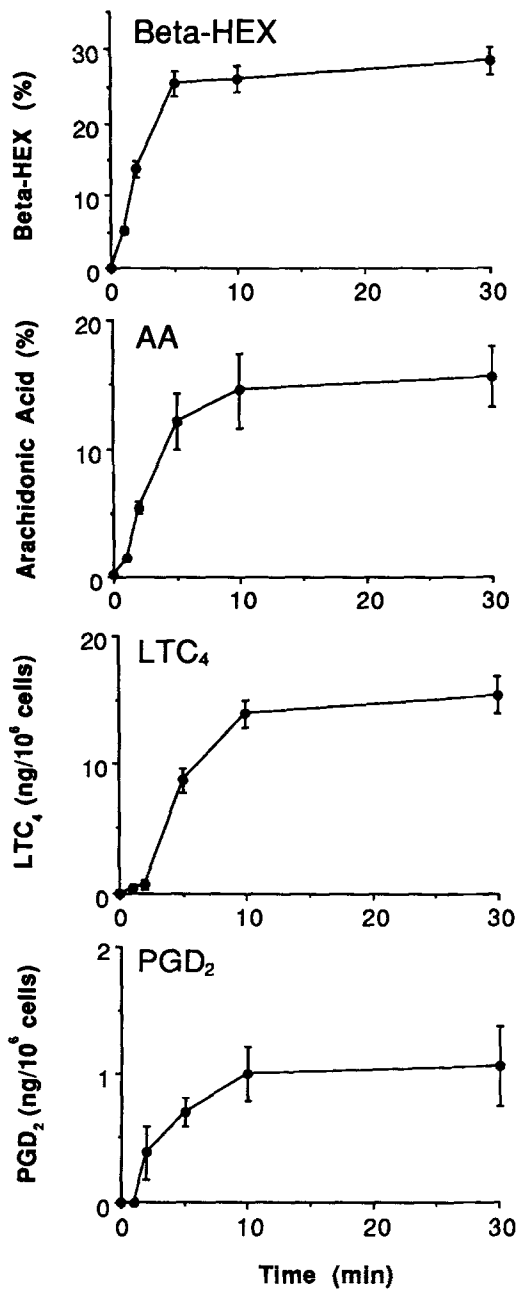


Figure 1. Time course of the release of β -hexosaminidase (*Beta-HEX*), arachidonic acid (*AA*), LTC_4 , and PGD_2 from BMMC treated with KL. BMMC were incubated with 100 ng/ml KL for the indicated periods, and the release of each mediator was assessed as described in Materials and Methods. The values are the mean \pm SEM of three to five independent experiments.

These cytokines neither enhanced nor suppressed mediator release from BMMC activated by 100 ng/ml KL for 10 min or by a suboptimal concentration of 10 ng/ml KL ($n = 2$; data not shown). Furthermore, the combination of KL + IL-10 + IL-1 β , which is optimal for induction of PGHS-2-dependent PGD_2 generation during 2–10 h (1), did not elicit any greater PGD_2 generation during 10 min, the interval used for assessing the immediate response, than did KL alone

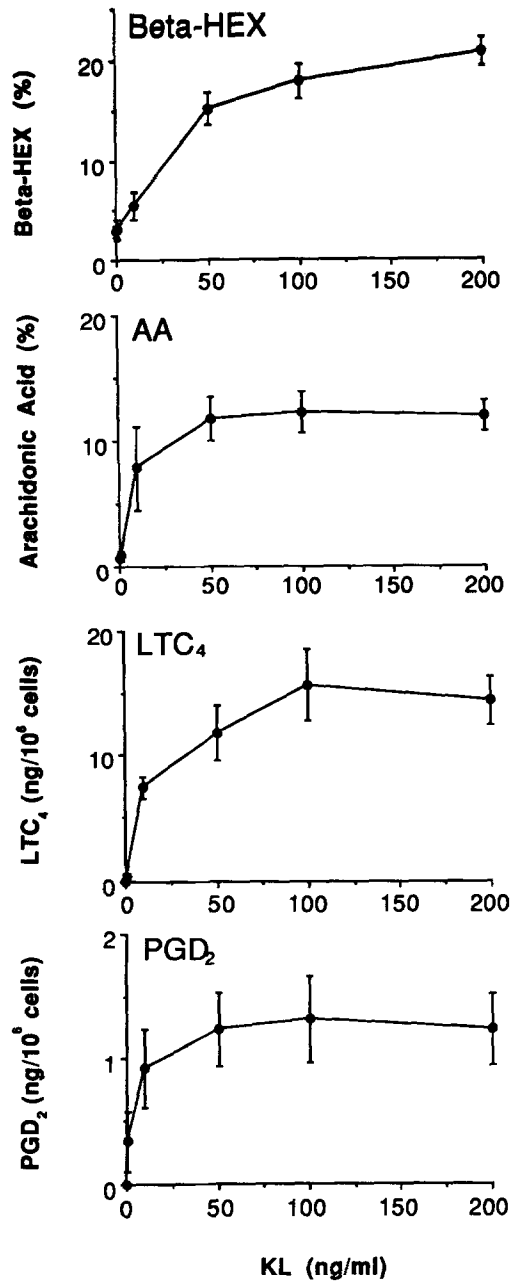


Figure 2. Dose-dependent effect of KL on immediate mediator release from BMMC. BMMC were incubated for 10 min with the indicated concentrations of KL, and the release of each mediator was assessed as described in Materials and Methods. The values are the mean \pm SEM of four independent experiments.

(Fig. 3). Thus, BMMC treated with KL in combination with IL-10 and IL-1 β as accessory cytokines exhibited predominant generation of LTC_4 with a small amount of PGD_2 during the immediate response, followed by a delayed generation of PGD_2 extending from 2–10 h with no appreciable generation of LTC_4 (Fig. 3). Exocytosis did not accompany the latter process (data not shown).

Activation of cPLA₂ in BMMC Treated with KL. After a 12-h preincubation of BMMC with [3H]arachidonic acid,

Table 1. Immediate Mediator Release from BMMC Activated with KL or with IgE/Ag

Treatment	Ca ²⁺	β -Hexosaminidase (%)	Arachidonic acid (%)	LTC ₄ (ng/10 ⁶ cells)	PGD ₂ (ng/10 ⁶ cells)
KL	+	18.0 ± 6.7	14.5 ± 1.3	15.3 ± 2.2	1.7 ± 0.2
KL	-	6.3 ± 1.3*	2.4 ± 0.2*	0.4 ± 0.4*	0.3 ± 0.3*
IgE/Ag	+	36.0 ± 3.1*	21.0 ± 4.0*	43.0 ± 3.7 [†]	3.7 ± 0.7 [†]

BMMC were treated for 10 min with KL or with IgE/Ag in the presence or absence of Ca²⁺, as described in Materials and Methods. Values represent the mean ± SEM of three experiments.

* $P < 0.05$ and [†] $P < 0.01$ vs KL in the presence of Ca²⁺.

99% of the radioactivity was consistently incorporated into phospholipid pools, mainly into phosphatidylethanolamine (55.0%) and phosphatidylcholine (31.6%), followed by phosphatidylinositol/phosphatidylserine (11.2%) and other phospholipids (1.4%). After incubation with 100 ng/ml KL for 10 min, free arachidonic acid increased 12-fold to 13.6%, accompanied by decreases in the percent of total [³H]arachidonic acid remaining in phosphatidylethanolamine and phosphatidylcholine without any appreciable changes in other phospholipids (Table 2). In terms of total counts, the decrement from these two phospholipids of 9.1 and 3.8% for a total of 12.9% matched the net 12.5% increase in free arachidonic acid.

To assess the activation of cPLA₂ in terms of phosphorylation, BMMC were preincubated with [³²P]orthophosphate, sensitized with IgE, activated with either KL or with Ag for 2 min, lysed, and immunoprecipitated with antiserum against cPLA₂. The immunoprecipitates were resolved on SDS-PAGE, transferred to a nitrocellulose membrane, and visualized by autoradiography. The immunoprecipitates contained a single major phosphorylated protein with a molec-

ular mass of ~100 kD (Fig. 4 A), which was identified as cPLA₂ by immunoblotting with the same anti-cPLA₂ Ab (data not shown). Increased incorporation of ³²P into cPLA₂ protein was demonstrated in KL-treated BMMC as compared to BMMC treated with IL-3 as a negative control, and was comparable with that observed in BMMC activated with IgE/Ag (Fig. 4 A). Kinetic experiments revealed that phosphorylation of cPLA₂ in response to KL was detectable at 1 min, reached a maximum at 2–5 min, a time when the rate of arachidonic acid release was maximal, and thereafter declined (Fig. 4 B).

Reversible Translocation of 5-LO in BMMC Treated with KL. The translocation of 5-LO from the cytosol to a cell membrane compartment was assessed after activation of BMMC with 100 ng/ml KL. SDS-PAGE/immunoblotting was performed with the 100,000 g supernatants and pellets of the lysates of BMMC that were unstimulated or stimulated with KL for 2–10 min (Fig. 5). 5-LO protein in the membrane fractions increased in KL-treated cells relative to that in IL-3-treated cells after 2 min, although most of the 5-LO remained in the 100,000 g supernatants (Fig. 5 A). The increase in 5-LO protein in the 100,000 g pellet was maximal

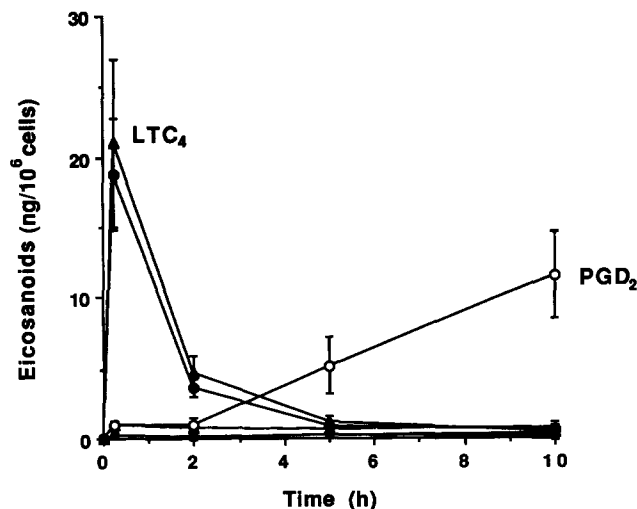


Figure 3. Biphasic cytokine-initiated eicosanoid generation. BMMC were incubated with IL-3 (squares), KL (triangles), or KL + IL-10 + IL-1 β (circles) for the indicated periods, and the release of PGD₂ (open symbols) and LTC₄ (closed symbols) was assessed as described in Materials and Methods. The values are the mean ± SEM of four independent experiments.

Table 2. Liberation of Arachidonic Acid from Phospholipids after Activation of BMMC with KL

Lipids	[³ H]Arachidonic acid incorporated (% of total)	
	No stimulus	KL
Phosphatidylethanolamine	55.0 ± 0.6	45.9 ± 3.0*
Phosphatidylcholine	31.6 ± 0.4	27.8 ± 1.5*
Phosphatidylinositol/ phosphatidylserine	11.2 ± 0.1	11.8 ± 1.6
Other phospholipids	1.4 ± 0.2	1.1 ± 0.1
Free arachidonic acid	1.1 ± 0.1	13.6 ± 0.2*

[³H]Arachidonic acid-labeled BMMC were treated with 100 ng/ml KL for 10 min, and lipids were extracted and separated as described in Materials and Methods. The values are mean ± SEM of three experiments.

* $P < 0.05$ vs no stimulus.

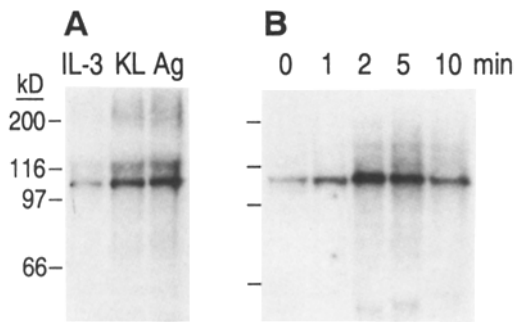


Figure 4. Phosphorylation of cPLA₂. (A) BMMC pre-labeled with ³²P were treated for 2 min with 100 U/ml IL-3, or 100 ng/ml KL, or were sensitized with 10 μg/ml IgE anti-TNP and activated for 2 min with 100 ng/ml TNP-BSA. (B) BMMC pre-labeled with ³²P were stimulated with 100 ng/ml KL for the indicated periods. The cells were lysed, immunoprecipitated with anti-cPLA₂, resolved on SDS-PAGE, transferred to a nitrocellulose membrane, and visualized by autoradiography, as described in Materials and Methods.

after 2–5 min of treatment with KL, a time when the rate of LTC₄ generation was maximal, and returned to near basal levels by 10 min (Fig. 5 B).

Effect of PGHS Inhibitors on KL-stimulated Mediator Release from BMMC. When BMMC were treated for 3 h with NS-398, a reagent that selectively and irreversibly inactivates PGHS-2 (33), and then were stimulated with KL or with IgE/Ag, there was no inhibition of the generation of PGD₂ during the first 10 min (Table 3). In contrast, the generation of PGD₂ by replicate cells that were cultured with KL, IL-10 and IL-1β for 5 h was markedly suppressed (~82% inhibition) by NS-398. By comparison, treatment of BMMC for 3 h with indomethacin (Table 3) or aspirin (data not shown), which are nonspecific but more selective inhibitors of PGHS-1 than PGHS-2 (35, 36), markedly inhibited each of the three reactions by >90%.

Discussion

The demonstration that the 5-LO pathway to LTC₄ generation can be directly activated in mast cells by a tissue-derived cytokine, KL, reveals an alternative non-IgE-dependent mechanism for activation of this pathway for lipid mediator synthesis. The biochemical steps used by KL for arachidonic acid release and processing are those also recruited by IgE-dependent activation; and the kinetics, the amounts, and the ratio of LTC₄/PGD₂ elaborated are comparable. The associated finding that continuous KL stimulation in the presence of two accessory cytokines, IL-1β and IL-10, leads to delayed selective generation of PGD₂ at 2–10 h (1) demonstrates sequential direct cytokine effects on the same target cell for eicosanoid biosynthesis with different major products in the immediate and delayed phases. Taken together, these direct and time-dependent responses to KL of an effector cell of allergic inflammation, the mast cell, reveal previously unexplored pathways for providing membrane-derived bioactive mediators.

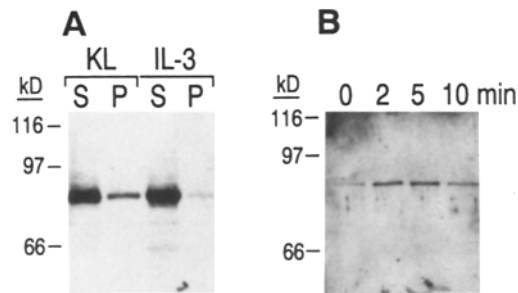


Figure 5. Translocation of 5-LO. (A) BMMC were treated for 2 min with either 100 ng/ml KL or 100 U/ml IL-3. (B) BMMC were treated with 100 ng/ml KL for the indicated periods. The distribution of 5-LO in the 100,000 g supernatant (S) and pellet (P) was assessed by SDS-PAGE/immunoblotting as described in Materials and Methods.

When BMMC were exposed to KL, there was rapid mobilization of arachidonic acid from cell membrane phospholipids, followed by substantial generation of LTC₄ in preference to PGD₂ accompanied by exocytosis of β-hexosaminidase (Fig. 1). Exocytosis was apparent within the first minute and reached a maximum to a plateau at 5 min. Eicosanoid generation occurred more slowly than exocytosis and was preceded by the release of arachidonic acid from cell membrane phospholipids. Thus, arachidonic acid release was seen within 1 min of exposure to KL and was followed by detectable release of LTC₄ and PGD₂ by 2–5 min, reaching a plateau by 10 min (Fig. 1). The dose-response studies revealed that the release of arachidonic acid and the synthesis of LTC₄ and PGD₂ were more sensitive than exocytosis to the action of KL, with EC₅₀ values of ~10 ng/ml KL for arachidonic acid metabolism as compared to ~25 ng/ml KL for exocytosis (Fig. 2). As each event was elicited through the same receptor, the different sensitivity to the action of KL presumably lies in the different postreceptor events leading to either arachidonic acid metabolism or exocytosis. A near maximal response was observed at ~50 ng/ml KL (Fig. 2), a concentration similar to that which induces proliferation of BMMC (2, 6) and

Table 3. Effect of NS-398 and Indomethacin on PGD₂ Generation

Treatments	PGD ₂ (ng per 10 ⁶ cells)		
	No inhibitor	NS-398	Indomethacin
IgE/Ag (10 min)	2.3 ± 0.3	2.8 ± 0.3	<0.2*
KL (10 min)	1.3 ± 0.2	1.4 ± 0.3	<0.2*
KL + IL-10 + IL-1β (5 h)	3.8 ± 0.5	0.7 ± 0.3†	<0.2*

BMMC were activated in the presence or absence of inhibitors as described in Materials and Methods. The values are the mean ± SEM of five experiments.

* *P* < 0.01 and † *P* < 0.05 vs no inhibitor.

cytosis of human skin mast cells and rodent serosal mast cells (4, 5). The dose dependence of immediate LTC₄ generation from BMMC in response to KL is also comparable with that observed for the delayed phase of cytokine-induced PGD₂ generation via PGHS-2 (1) and for priming of BMMC for enhanced IgE-dependent PGD₂ and LTC₄ generation (2).

KL-initiated immediate activation of BMMC has kinetics similar to those of IgE/Ag activation. The product profile elicited by KL is also comparable to that elicited by IgE/Ag with the generation of LTC₄ in preference to PGD₂ in a molar ratio of ~10:1 (Table 1). Previous studies, in which activation of BMMC through FcγRIII was compared to that through FcεRI (37), also revealed that the ratio of products acutely generated via the 5-LO and the cyclooxygenase pathways is determined by the cell phenotype rather than the ligand. KL and IgE/Ag fall into a group of mast cell activators that are dependent on extracellular Ca²⁺ (Table 1), thereby distinguishing them from G protein-coupled agonists, such as substance P and compound 48/80, which elicit mast cell activation independent of extracellular Ca²⁺ (38). Moreover, both KL and IgE/Ag trigger mast cell exocytosis (2–5), adhesion (10, 39), and proliferation (6, 40); stimulate tyrosine phosphorylation leading to activation of the *ras*/MAP kinase pathway (41, 42); and induce the transcription of immediate early genes, such as *c-jun* and *c-fos* (43, 44). Thus, both receptors, irrespective of their divergent structures, activate several common signal transducing molecules to elicit similar responses in mast cells.

To identify the biochemical steps leading to rapid lipid mediator generation in KL-treated BMMC, the posttranslational modification of the individual enzymes involved in postreceptor metabolism of arachidonic acid to LTC₄ and PGD₂ was assessed. Our studies reveal that the events after signal transduction that lead to immediate generation of LTC₄ by KL include phosphorylation of cPLA₂ (Fig. 4) and translocation of 5-LO (Fig. 5). cPLA₂ was phosphorylated transiently after activation of BMMC by KL and reached a maximum at 2 min, when the rate of arachidonic acid release was maximal (Fig. 4). Dephosphorylation of cPLA₂ at 10 min coincided with the loss of further arachidonic acid release. Because both KL and IgE/Ag stimulate MAP kinase via a *ras*-dependent postreceptor pathway (41, 42), it is likely that the phosphorylation of cPLA₂ observed in both systems (15) is mediated by MAP kinase. Furthermore, both KL and IgE/Ag increase intracellular Ca²⁺ concentrations (4, 45), which is a critical event in the activation of cPLA₂.

The translocation of 5-LO to the membrane fraction 2–5 min after activation of BMMC with KL, when the rate of LTC₄ generation was maximal, was transient and was reversed by 10 min (Fig. 5). IgE-dependent activation of mast cells for LTC₄ generation is also associated with reversible translocation of 5-LO to the membrane fraction, whereas the continuous elevation of cytosolic Ca²⁺ associated with ionophore stimulation causes permanent translocation to the membrane leading to inactivation of the enzyme (19). Thus the transient Ca²⁺ influx initiated by KL (4) facilitates activation and translocation of both cPLA₂ and 5-LO, thereby allow-

ing the released arachidonic acid to be sequentially metabolized by 5-LO and LTC₄ synthase at the perinuclear membrane (21, 46). Other mechanisms, such as the interaction of 5-LO with Grb2 and cytoskeletal proteins through its SH3-binding motif (47), might also be involved in 5-LO translocation.

The other cytokines that were studied had no effect on immediate mediator release either alone or in combination with KL. The lack of effect of NGF and β-chemokines is of particular note because these cytokines are each reported to stimulate mediator release from certain populations of mast cells or basophils. Both NGF and KL stimulate exocytosis from rat peritoneal mast cells (5, 48), sustain viability of these cells in culture by inhibiting apoptosis (9, 49), and induce maturation of BMMC toward the connective tissue mast cell phenotype (7, 8, 50). MIP-1α and MCP-1 not only act as potent histamine-releasing factors for human basophils but also induce histamine release from mouse connective tissue mast cells in vitro and in vivo (51, 52). Whereas there are no ancillary cytokine requirements for immediate eicosanoid generation in response to KL, accessory cytokines are important for the delayed response to KL (1) and for the priming by KL for IgE-dependent activation of BMMC (2). The KL-initiated, PGHS-2-dependent, delayed phase of PGD₂ generation requires IL-10 and/or IL-1β as accessory cytokines (1); and priming of BMMC by KL for increased IgE-dependent PGD₂ generation is augmented by IL-3, IL-9, or IL-10 (2). Thus, the immediate activation of BMMC by KL is unique in terms of its apparent specificity to a single cytokine and the lack of enhancement by other cytokines.

Incubation of BMMC with the triad of KL + IL-10 + IL-1β revealed two phases of eicosanoid biosynthesis. The immediate phase was characterized by the generation of LTC₄ in preference to PGD₂ and was followed 2–10 h later by the release of PGD₂ in the absence of detectable LTC₄ generation (Fig. 3). Inhibition by aspirin and indomethacin but not by NS-398, together with the observation that BMMC constitutively express PGHS-1 but not PGHS-2 when maintained in IL-3 alone (1, 2), indicate that PGHS-1 is the isoform that mediates rapid prostanoid biosynthesis in response to KL (Table 3), as in the case of IgE/Ag stimulation (1, 2). This contrasts with the dependence of the delayed phase of PGD₂ generation on PGHS-2 (1) (Table 3).

Various physiologic, nonimmunologic mast cell secretagogues have been characterized. Many of these, such as substance P (38), C5a (53), and chymase (54), have been shown to elicit degranulation and PGD₂ generation from human skin mast cells and rat peritoneal mast cells, which are poor sources of LTC₄. Adenosine selectively primes BMMC for degranulation in response to IgE/Ag with no effect on LT generation (55). Thrombin elicits degranulation of BMMC in the absence of LTC₄ synthesis (56). Thus, of the other nonimmunologic activators of mast cells, none has been shown to elicit substantial LT generation, and in no instance have the postreceptor biosynthetic events leading to eicosanoid synthesis been examined.

Our current studies reveal that KL regulates eicosanoid

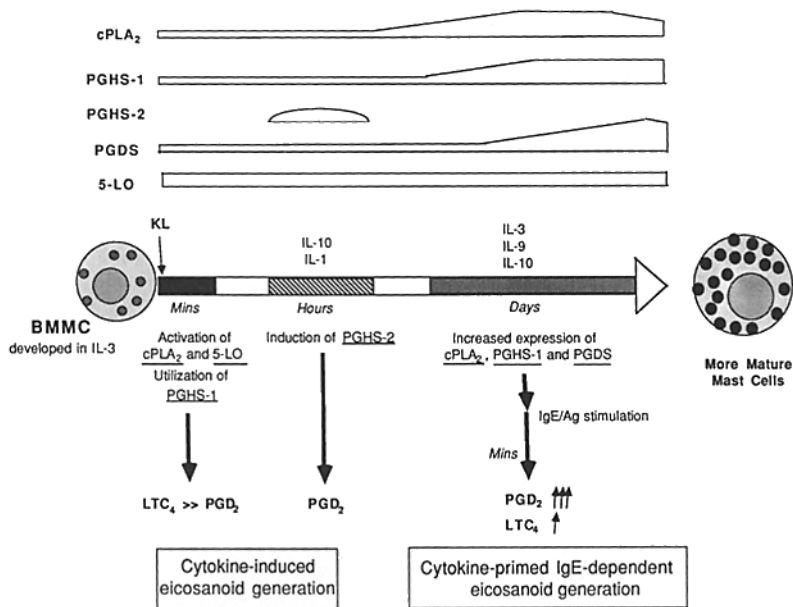


Figure 6. Diagram of KL regulation of arachidonic acid metabolism in BMMC. When BMMC are treated with KL with the appropriate accessory cytokines, the expression of each enzyme involved in arachidonic acid metabolism and its product is changed as illustrated. In the first phase, KL rapidly activates cPLA₂ and 5-LO, leading to immediate generation of LTC₄ in preference to PGD₂, via PGHS-1, within minutes. In the second phase, PGHS-2 is induced in response to KL in combination with IL-10 and/or IL-1 β , accompanied by a delayed phase of PGD₂ generation over 2–10 h. LTC₄ is not generated. In the third phase, the expression of cPLA₂, PGHS-1, and hematopoietic PGD₂ synthase (PGDS) is increased by KL from 1 d to 1 wk, leading to priming for increased generation of PGD₂ in response to IgE/Ag over 10 min. IL-3, IL-9, and IL-10 each augment the expression of PGHS-1, thereby further enhancing the priming effect of KL. The top portion of the figure schematically depicts the changes in expression of each enzyme relative to the starting amount of that particular protein determined by SDS-PAGE/immunoblotting during the three phases of the KL-dependent cellular response.

generation in BMMC in at least three different and sequential phases as illustrated in Fig. 6. The first phase represents an immediate response to KL alone, leading to generation of LTC₄ in preference to PGD₂ through posttranslational activation of cPLA₂ and 5-LO, and utilization of PGHS-1. The second phase is characterized by PGHS-2-dependent delayed PGD₂ generation over several hours, with a requirement for

IL-10 and/or IL-1 β as accessory cytokines (1). In the third phase, after 1 d, KL primes BMMC for increased IgE-dependent PGD₂ synthesis through increased expression of cPLA₂, PGHS-1, and hematopoietic PGD₂ synthase, with IL-3, IL-9, or IL-10 acting to further augment PGHS-1 expression (2).

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