Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 4803–4807, April 1999
Biochemistry

Cytosolic phospholipase A_2 is essential for both the immediate and the delayed phases of eicosanoid generation in mouse bone marrow-derived mast cells

HIROSHI FUJISHIMA*†, RENÉ O. SANCHEZ MEJIA†§, CLIFTON O. BINGHAM, III*†, BING K. LAM*†, ADAM SAPIRSTEIN¶‖, JOSEPH V. BONVENTRE*§**, K. FRANK AUSTEN*†,††, AND JONATHAN P. ARM*†,†††‡‡

Departments of *Medicine and ¶Anesthesia and Critical Care, Harvard Medical School, Boston, MA 02115; †Division of Rheumatology, Immunology, and Allergy and ††Partners' Asthma Center, Brigham and Women's Hospital, Boston, MA 02115; **Medical and ‖Anesthesia Services, Massachusetts General Hospital, Boston, MA 02114; and §Harvard–Massachusetts Institute of Technology Division of Health Sciences, Boston, MA 02115

Contributed by K. Frank Austen, February 24, 1999

ABSTRACT **We have used mice in which the gene for cytosolic phospholipase A_2 ($cPLA_2$) has been disrupted to demonstrate the absolute requirement for $cPLA_2$ in both the immediate and the delayed phases of eicosanoid generation by bone marrow-derived mast cells. For the immediate phase, quantitative analysis of the products of the 5-lipoxygenase pathway showed that gene disruption of $cPLA_2$ prevented the provision of arachidonic acid substrate for biosynthesis of proximal intermediates. By analogy, we conclude that arachidonic acid substrate was also not available to prostaglandin endoperoxide synthase 1 in the immediate phase of prostaglandin (PG) D_2 generation. These defects occurred with two distinct stimuli, stem cell factor and IgE/antigen, which were, however, sufficient for signal transduction defined by exocytosis of β-hexosaminidase. Whereas $cPLA_2$ is essential for immediate eicosanoid generation by providing arachidonic acid, its role in delayed-phase PGD_2 generation is more complex and involves the activation-dependent induction of prostaglandin endoperoxide synthase 2 and the supply of arachidonic acid for metabolism to PGD_2.**

Leukotrienes and prostaglandins, derived from the oxidative metabolism of arachidonic acid (1, 2), are potent lipid mediators of tissue inflammation (3, 4). The first step in the generation of these lipid mediators, collectively known as eicosanoids, is the liberation of esterified arachidonic acid from the *sn*-2 position of cell membrane glycerophospholipids by the action of phospholipase A_2 (PLA_2) (5). The family of mammalian PLA_2 enzymes includes the 85-kDa group IV cytosolic PLA_2 ($cPLA_2$); a number of low molecular weight, cysteine-rich PLA_2 enzymes, among which are the group IIA and group V PLA_2 species; and calcium-independent species of PLA_2 (6). The contribution of different PLA_2 enzymes, especially $cPLA_2$ as opposed to group IIA and group V PLA_2, to the provision of arachidonic acid for eicosanoid generation remains unclear (7–9).

Two phases of eicosanoid generation have been defined in mouse bone marrow-derived mast cells (BMMC) (10, 11) and in the MMC34 mast cell line (12, 13). The immediate phase, elicited by cross-linking the high-affinity Fc receptor for IgE (FcεRI) or by ligation of c-*kit* with stem cell factor (SCF), is characterized by the rapid generation of prostaglandin (PG) D_2, which depends on the action of constitutively expressed prostaglandin endoperoxide synthase (PGHS) 1, and leukotriene (LT) C_4 (12, 14). The delayed phase, elicited by SCF in combination with IL-1β and IL-10 (10) or by antigen activation after sensitization with hapten-specific IgE, with (11) or without (13) cytokine priming, is characterized by the generation of PGD_2 in the absence of leukotrienes. Importantly and distinctively, delayed-phase PGD_2 generation depends on the induced expression of PGHS-2 (10–14).

The species of PLA_2 supplying arachidonic acid in each phase of eicosanoid generation is not resolved. $cPLA_2$ was implicated in the immediate phase by its phosphorylation within 2 min of activation through FcεRI or c-*kit* (14) and the inhibition of the immediate response by preincubation of BMMC with methyl arachidonyl fluorophosphate (MAFP), a $cPLA_2$ inhibitor (12). Whereas Reddy and colleagues (15) also implicated group V PLA_2 in the immediate generation of PGD_2 by inhibition with antisense DNA, we found that scalaradial, a relatively preferential inhibitor of the low molecular weight species of PLA_2, did not inhibit the immediate phase of either PGD_2 or LTC_4 generation (16). By contrast, in our studies, the delayed phase of PGD_2 generation was inhibited by heparin, which released a PLA_2 activity from the cell into the culture medium, and by scalaradial (16). Although heparin binds strongly to the group IIA PLA_2 (17) and has been used as an agent to implicate that enzyme in eicosanoid biosynthesis (18), both immediate and delayed PGD_2 generation were intact in BMMC derived from mice in which the gene for group IIA PLA_2 was naturally disrupted (15, 16). We therefore concluded that a low molecular weight PLA_2 species distinct from the group IIA enzyme participated in the delayed response. However, Reddy and Herschman (12) demonstrated that the delayed phase of PGD_2 generation, like the immediate, was inhibited by MAFP, thereby implicating $cPLA_2$ in both phases.

To clarify the role of $cPLA_2$ in the immediate and the delayed phases of eicosanoid generation in the mast cell, we turned from pharmacologic approaches to the analysis of BMMC derived from mice in which the gene for $cPLA_2$ has been disrupted ($cPLA_2-/-$) (19). We now demonstrate that although $cPLA_2$ is required for both the immediate and the delayed phases of eicosanoid generation, its role in the delayed phase is complex. The observation that BMMC derived from $cPLA_2-/-$ mice respond to treatment with cytokines and exogenous arachidonic acid with induction of PGHS-2 and delayed generation of PGD_2, but do not respond to treatment with cytokines alone, reveals a dual role for $cPLA_2$. That role includes both the amplification of the induction of PGHS-2 and the supply of arachidonic acid as substrate to PGHS-2 in the delayed phase of PGD_2 generation.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Abbreviations: 5-LO, 5-lipoxygenase; BMMC, bone marrow-derived mast cells; $cPLA_2$, cytosolic phospholipase A_2; FcεRI, high-affinity Fc receptor for IgE; LT, leukotriene; MAFP, methyl arachidonyl fluorophosphate; PG, prostaglandin; PGHS, prostaglandin endoperoxide synthase; PLA_2, phospholipase A_2; SCF, stem cell factor; TNP, trinitrophenyl.

‡‡To whom reprint requests should be addressed at: Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital, Smith Research Building, Room 638B, 1 Jimmy Fund Way, Boston, MA 02115. e-mail: jarm@rics.bwh.harvard.edu.

In the immediate phase, $cPLA_2$ is required to provide substrate to both 5-lipoxygenase (5-LO) and PGHS-1.

MATERIALS AND METHODS

Materials. Mouse recombinant IL-1β (Genzyme), WEHI-3 cells (American Type Culture Collection), arachidonic acid, and PGE_2 (Cayman Chemicals, Ann Arbor, MI) were purchased. Mouse recombinant IL-3, IL-9, and IL-10 were produced by expression in insect cells, and their concentrations were determined as described (10, 20). William Smith (Michigan State University, East Lansing, MI) supplied rabbit polyclonal antiserum to PGHS-2, and Jim Clark (Genetics Institute, Boston, MA) supplied rabbit polyclonal antiserum to $cPLA_2$.

Culture of BMMC. Bone marrow cells from $cPLA_2-/-$ mice, strain-matched $cPLA_2+/+$ control mice, and BALB/c mice (The Jackson Laboratory) were cultured for 3–7 weeks in 50% enriched medium (RPMI 1640 medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% fetal calf serum)/50% WEHI-3 cell-conditioned medium as described (21).

Activation of BMMC. To assess IgE- and SCF-dependent immediate eicosanoid generation, BMMC were resuspended at a concentration of 10^7 cells per ml in 50% WEHI-3 cell-conditioned medium and sensitized with a 1:100 dilution of monoclonal mouse IgE anti-trinitrophenyl (TNP) ascites at 37°C overnight (conditions for optimal sensitization of BMMC for FcεRI-dependent activation). The cells were washed in enriched medium, resuspended at a concentration of 5×10^6 cells per ml in enriched medium, and stimulated at 37°C in a dose-dependent manner with 1–300 ng/ml TNP-BSA to establish an optimal concentration or with 100 ng/ml SCF for 15 min at 37°C. The reaction was stopped by centrifugation of cells at $120 \times g$ at 4°C for 5 min, and the supernatants were retained for assay of PGD_2 generation and percentage of β-hexosaminidase release (21, 22). For reverse-phase HPLC analysis of leukotriene generation the same protocol was followed except that BMMC were activated at a concentration of 10^7 cells per ml.

To assess cytokine-dependent delayed-phase generation of PGD_2, BMMC were resuspended at a concentration of 10^6 cells per ml in enriched medium containing 100 ng/ml SCF, 10 units/ml IL-10, and 5 ng/ml IL-1β (10). After 1 h of culture at 37°C, the cells were centrifuged at $200 \times g$ for 5 min to remove the products of the immediate phase of eicosanoid generation and resuspended in fresh medium with fresh cytokines. The cells were then cultured for an additional 7 h and centrifuged at $200 \times g$ for 5 min at 4°C. The cell pellets were retained for SDS/PAGE immunoblot analysis and the supernatants for assay of PGD_2. In selected experiments, arachidonic acid or PGE_2 was added to BMMC in a dose-dependent manner.

β-Hexosaminidase was quantitated by spectrophotometric analysis of the hydrolysis of *p*-nitrophenyl-β-D-2-acetamido-2-deoxyglucopyranoside (22). PGD_2 was measured by using RIA (Amersham Pharmacia). Leukotriene release in the immediate phase was measured by using reverse-phase HPLC (23).

SDS/PAGE Immunoblot Analysis. The expression of $cPLA_2$ and PGHS-2 was analyzed by SDS/PAGE immunoblot with rabbit polyclonal antisera at 1:3,000 and 1:5,000 dilutions, respectively (10, 20). Proteins were visualized with an enhanced chemiluminescence detection system (Pierce). The induced expression of PGHS-2 in BMMC from $cPLA_2+/+$ mice and $cPLA_2-/-$ mice was quantitated by densitometry with IMAGEQUANT software.

Data Analysis. Each experiment was performed at least three times. Pooled data are expressed as mean ± SEM and were analyzed by Student's *t* test for unpaired data.

RESULTS

Development of BMMC. By 4 weeks of culture with WEHI-3 cell-conditioned medium as the source of IL-3, >98% of cells derived from both $cPLA_2-/-$ and $cPLA_2+/+$ mice were mast cells as indicated by metachromatic staining with toluidine blue. However, significantly more BMMC were obtained from $cPLA_2-/-$ mice than from $cPLA_2+/+$ mice. From 10^7 starting bone marrow cells, $5.9 \pm 1.5 \times 10^6$ BMMC and $1.3 \pm 0.2 \times 10^6$ BMMC were obtained at 3 weeks from $cPLA_2-/-$ mice and $cPLA_2+/+$ mice, respectively ($n = 9$, $P = 0.008$). The absence of $cPLA_2$ in BMMC derived from $cPLA_2-/-$ mice was confirmed by using SDS/PAGE immunoblot analysis. A ≈110-kDa protein was detected by antiserum to $cPLA_2$ in extracts of BMMC derived from $cPLA_2+/+$ and BALB/c mice but not in extracts of BMMC derived from $cPLA_2-/-$ mice (Fig. 1).

Immediate-Phase Mediator Generation. Immediate-phase mediator generation in response to antigen stimulation of IgE-sensitized BMMC was evaluated in BMMC derived from 9 BALB/c mice on 9 occasions, in BMMC from 7 $cPLA_2+/+$ mice on 11 occasions, and in BMMC from 9 $cPLA_2-/-$ mice on 17 occasions. β-Hexosaminidase, PGD_2, and leukotriene products were released by $cPLA_2+/+$ BMMC, and β-hexosaminidase was released by $cPLA_2-/-$ BMMC in a dose-dependent manner in response to TNP-BSA after sensitization with IgE anti-TNP. Maximal mediator generation occurred in response to 100 ng/ml TNP-BSA, consistent with previous data (14).

BMMC from BALB/c, $cPLA_2+/+$, and $cPLA_2-/-$ mice released $16.5 \pm 3.1\%$, $19.0 \pm 5.2\%$, and $30.4 \pm 3.5\%$ β-hexosaminidase, respectively, 15 min after activation with 100 ng/ml TNP-BSA (Fig. 2*A*); the release by BMMC from $cPLA_2-/-$ mice was significantly greater than the release by BMMC from $cPLA_2+/+$ mice ($P = 0.03$) and BALB/c mice ($P = 0.008$). In contrast, PGD_2 generation was undetectable in BMMC from $cPLA_2-/-$ mice, although BMMC from BALB/c mice and $cPLA_2+/+$ mice generated 2.4 ± 0.8 ng of PGD_2 per 10^6 cells and 3.8 ± 0.7 ng of PGD_2 per 10^6 cells, respectively, in response to 100 ng/ml TNP-BSA (Fig. 2*B*) ($P = 0.0001$). Similarly, the generation of LTC_4 and other 5-LO pathway metabolites was undetectable in IgE-sensitized BMMC from $cPLA_2-/-$ mice in response to antigen even though the response for each metabolite and for their sum appeared to be augmented in BMMC from strain-matched $cPLA_2+/+$ mice compared with BMMC derived from BALB/c mice (Table 1).

Immediate-phase mediator generation in response to 100 ng/ml SCF was evaluated in BMMC derived from 7 BALB/c mice on 7 occasions, in BMMC from 6 $cPLA_2+/+$ mice on 7 occasions, and in BMMC from 7 $cPLA_2-/-$ mice on 7 occasions. BMMC from BALB/c, $cPLA_2+/+$, and $cPLA_2-/-$ mice released $11.6 \pm 3.0\%$, $6.1 \pm 1.5\%$, and $23.6 \pm 7.7\%$ β-hexosaminidase, respectively, 15 min after activation with 100 ng/ml SCF (Fig. 2*C*). Secretory granule exocytosis in response to SCF was significantly greater in the BMMC derived from $cPLA_2-/-$ mice than in BMMC derived from $cPLA_2+/+$ mice ($P = 0.04$). In contrast, PGD_2 generation was undetectable in BMMC from

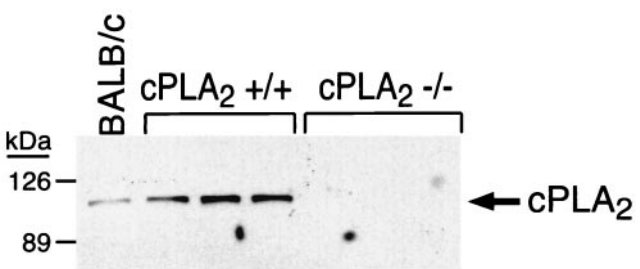

FIG. 1. Expression of $cPLA_2$ in BMMC. SDS/PAGE immunoblot analysis of the expression of $cPLA_2$ in BMMC derived from a BALB/c mouse, three $cPLA_2+/+$ control mice, and three $cPLA_2-/-$ mice. Extracts from 2×10^5 cells were applied to each lane.

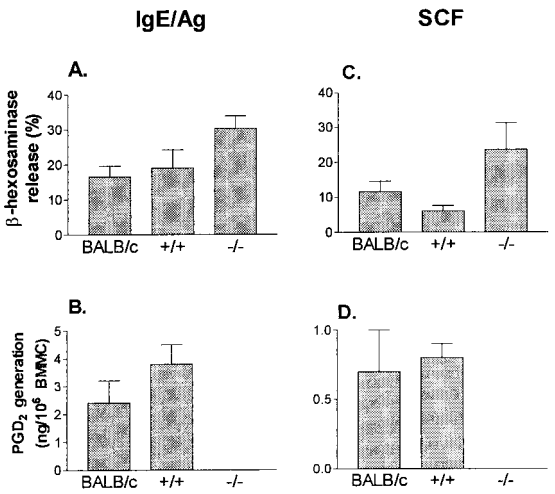

FIG. 2. Immediate-phase β-hexosaminidase release and PGD_2 generation. Immediate β-hexosaminidase release (%) (*A* and *C*) and PGD_2 generation (ng per 10^6 BMMC) (*B* and *D*) were measured in BMMC derived from BALB/c, $cPLA_2+/+$, and $cPLA_2-/-$ mice sensitized with IgE anti-TNP and activated with either TNP-BSA (IgE/Ag; *A* and *B*) or SCF (*C* and *D*) for 10 min.

$cPLA_2-/-$ mice, although BMMC from BALB/c and $cPLA_2+/+$ mice generated 0.7 ± 0.3 ng PGD_2 per 10^6 cells ($P = 0.0001$) and 0.8 ± 0.1 ng PGD_2 per 10^6 cells ($P = 0.004$), respectively, in response to 100 ng/ml SCF (Fig. 2*D*). Similarly, the generation of LTC_4 and other lipoxygenase metabolites was undetectable in BMMC from $cPLA_2-/-$ mice in response to SCF (Table 1).

Delayed-Phase PGD_2 Generation. The PGHS-2-dependent delayed-phase generation of PGD_2 in response to SCF + IL-10 + IL-1β was determined in BMMC from 5 BALB/c mice on 5 occasions, in BMMC from 6 $cPLA_2+/+$ mice on 6 occasions, and in BMMC from 7 $cPLA_2-/-$ mice on 11 occasions. To eliminate the contribution of immediate-phase PGD_2 generation by PGHS-1, BMMC were washed 1 h after activation, resuspended in fresh medium and cytokines, and examined for PGD_2 generation 7 h later. Delayed-phase PGD_2 generation was undetectable in BMMC derived from $cPLA_2-/-$ mice. In comparison, BMMC from BALB/c and $cPLA_2+/+$ mice released 3.8 ± 0.8 ng of PGD_2 per 10^6 cells and 2.0 ± 0.4 ng of PGD_2 per 10^6 cells, respectively, 8 h after activation with SCF + IL-10 + IL-1β ($P < 0.0001$) (Fig. 3*A*). Importantly, the induced expression of PGHS-2 in BMMC from $cPLA_2-/-$ mice was $36.4 \pm 6.4\%$ that observed in BMMC from $cPLA_2+/+$ mice 8 h after cytokine-dependent activation (Fig. 3*B*).

These data indicate that $cPLA_2$ may contribute to delayed-phase PGD_2 generation by supplying arachidonic acid to PGHS-2 and/or by facilitating the induction of PGHS-2. Although PGE_2 is not an eicosanoid product of BMMC, it was reported to induce PGHS-2 in the MC3T3-E1 mouse osteoblast cell line (24). To investigate the possible roles of $cPLA_2$ both in inducing PGHS-2 and in supplying arachidonic acid to the induced enzyme, the dose-dependent effects of exogenous arachidonic acid and PGE_2 on the induction of PGHS-2 and delayed-phase PGD_2 generation were assessed in BMMC from $cPLA_2-/-$ mice. Arachidonic acid (0.1–10 μg/ml) or PGE_2 (0.1–10 μg/ml) was added to the culture medium at the time of cytokine stimulation. To eliminate the contribution of immediate-phase PGD_2 generation by PGHS-1, BMMC were washed 1 h after activation as described above and were resuspended in fresh medium containing fresh cytokines and either arachidonic acid or PGE_2. The addition of PGE_2 to the culture medium led to a marked increase in PGHS-2 even at 0.1 μg/ml, in BMMC from $cPLA_2-/-$ mice (Fig. 4*B*). However, the increment in PGHS-2 was accompanied by only minimal delayed-phase PGD_2 generation of 0.3 ± 0.1 ng PGD_2 per 10^6 cells 8 h after cytokine stimulation at the highest PGE_2 concentration of 10 μg/ml (Fig. 4*A*). Therefore, the lack of delayed-phase PGD_2 generation in BMMC from $cPLA_2-/-$ mice was not due simply to impaired induction of PGHS-2, suggesting that $cPLA_2$ also is needed to provide arachidonic acid to the induced PGHS-2 in the delayed phase. Consistent with this suggestion, stimulation with SCF + IL-10 + IL-1β in the presence of arachidonic acid led to a concentration-dependent restoration of delayed-phase PGD_2 generation (Fig. 4*C*) in BMMC derived from $cPLA_2-/-$ mice accompanied by a modest and plateau increase in the expression of PGHS-2 (Fig. 4*D*). At a concentration of 10 μg/ml arachidonic acid, delayed-phase PGD_2 generation reached 2.5 ± 0.7 ng of PGD_2 per 10^6 cells ($P = 0.001$; $n = 9$), almost an order of magnitude more than the amount obtained with 10 μg/ml PGE_2 ($n = 6$; $P = 0.0001$), even though induction of PGHS-2 was less.

DISCUSSION

We have demonstrated in BMMC from mice with disruption of the $cPLA_2$ gene that $cPLA_2$ is absolutely required for immediate-phase generation of the leukotrienes, LTC_4 and LTB_4, and the prostanoid, PGD_2, in response to signaling through c-*kit* or FcεRI. We have further found that $cPLA_2$ is also critical for delayed-phase PGHS-2-dependent PGD_2 generation acting to amplify the cytokine-dependent induction of PGHS-2 as well as to supply arachidonic acid to the induced enzyme.

BMMC were derived from cPLA2−/− mice and from $cPLA_2+/+$ mice in WEHI-3 cell-conditioned medium according to established protocols. By 4 weeks of culture, >98% of cells derived from both types of mice were mast cells as indicated by metachromatic staining with toluidine blue. Thus, $cPLA_2$ is not required for normal IL-3-dependent development of BMMC from bone marrow. Furthermore, BMMC from $cPLA_2-/-$ mice exhibited intact, or even augmented, signal transduction-dependent exocytosis to perturbation of either c-*kit* or FcεRI, indicating that signal transduction through these characteristic mast cell receptors is intact. SDS/PAGE immunoblotting confirmed the presence of $cPLA_2$ in BMMC from $cPLA_2+/+$ mice and its absence in BMMC from $cPLA_2-/-$ mice (Fig. 1). PCR analysis confirmed that the gene for the group IIA enzyme was disrupted in BMMC from both $cPLA_2+/+$ and $cPLA_2-/-$ mice (data not shown) derived from 129 ES cells on a C57BL/6 background; this is consistent with the observation that the gene for group IIA PLA_2 is disrupted in 129 and C57BL/6 mice (16, 25, 26). Reverse transcription–PCR analysis established the presence of transcripts for the group V enzyme in BMMC from both $cPLA_2+/+$ and $cPLA_2-/-$ mice (data not shown), consistent with studies in BMMC derived from C57BL/6 and AKJ mice (15).

The immediate phase of both leukotriene and PGD_2 generation, in response to SCF or to IgE and antigen, was completely ablated in BMMC derived from $cPLA_2-/-$ mice, whereas BMMC from $cPLA_2+/+$ mice provided PGD_2 and all of the pathway intermediates of leukotriene generation (Fig. 2 and Table 1). The absence of the intermediates, 5-hydroxyeicosatetraenoic acid (5-HETE; derived from 5-hydroperoxyeicosatetraenoic acid) and 6-*trans*-LTB_4 diastereoisomers (derived by the nonenzymatic hydrolysis of LTA_4), in reverse-phase HPLC analyses of products from $cPLA_2-/-$ BMMC indicates that the biosynthetic failure is to provide arachidonic acid to 5-LO in concert with 5-LO-activating protein. This results in an absence of LTA_4 substrate for the terminal enzymes, LTC_4 synthase and LTA_4 hydrolase. By analogy, there would also be no arachidonic acid for PGHS-1 to convert

Table 1. Generation of leukotrienes and pathway intermediates in BMMC derived from BALB/c, $cPLA_2+/+$, and $cPLA_2-/-$ mice

		BMMC		
Stimulus	Product	BALB/c (n = 5)	$cPLA_2+/+$ (n = 3)	$cPLA_2-/-$ (n = 3)*
IgE/Ag	LTC_4	16.4 ± 4.0	73.6 ± 4.0	0
	LTB_4	8.0 ± 2.8	17.0 ± 2.3	0
	5-HETE	8.7 ± 3.0	34.4 ± 2.0	0
	6-*t*-LTB_4	5.0 ± 2.9	30.6 ± 3.2	0
SCF	LTC_4	11.6 ± 4.8	14.5 ± 0.7	0
	LTB_4	7.0 ± 1.9	11.3 ± 1.0	0
	5-HETE	9.0 ± 3.5	1.9 ± .01	0
	6-*t*-LTB_4	4.7 ± 2.6	3.7 ± 1.7	0

Leukotriene (LT) pathway products (ng per 10^6 cells) were measured by using RP-HPLC in supernatants of BMMC sensitized with IgE anti-TNP and activated with 100 ng/ml TNP-BSA (IgE/Ag) or with 100 ng/ml SCF. 5-HETE, 5-hydroxyeicosatetraenoic acid; 6-*t*-LTB_4, 6-*trans*-LTB_4 diastereoisomers. *, $P < 0.01$ compared to total leukotriene generation from $cPLA_2+/+$ BMMC for both agonists.

to PGH_2, the substrate for the terminal enzyme, glutathione-dependent, hematopoietic PGD synthase. These findings are compatible with our earlier studies that showed phosphorylation of $cPLA_2$ (14) and failure of an inhibitor of the low molecular weight PLA_2 species to attenuate immediate eicosanoid generation (16).

These data are consistent with the observation that in peritoneal macrophages isolated from $cPLA_2-/-$ mice immediate LTC_4, LTB_4, and PGE_2 generation in response to the calcium ionophore A23187 is markedly inhibited (19, 27). However, the inclusion of analyses for the proximal products of 5-LO/5-LO-activating protein metabolism of arachidonic acid pinpoints the disruption to the provision of arachidonic acid *per se*, thereby eliminating any substrates for the terminal pathway enzymes. These data are in contrast to those obtained by using inhibition approaches with human monocytes in which the 5-LO pathway and prostanoid pathways were segregated, with the former requiring a low molecular weight PLA_2 and the latter requiring $cPLA_2$ (28, 29). Marshall and colleagues (29) also demonstrated that the immediate generation of LTC_4, but not PGD_2, was inhibited in BMMC treated with the low molecular weight PLA_2 inhibitor, SB203347, and activated with IgE and antigen. The uncoupling of leukotriene and prostanoid pathways for the human monocyte and mouse BMMC (28, 29), but not mouse peritoneal macrophages (19, 27) or mouse BMMC from $cPLA_2-/-$ mice (16) (Table 1, Fig. 2), could reflect a lack of specificity of inhibition approaches or a difference in experimental design. Our current studies with BMMC (Table 1), like those with mouse peritoneal macrophages (19, 27), reveal an absolute requirement for $cPLA_2$ in the immediate generation of both prostanoid and leukotriene pathway products.

Reddy and Herschman (12) demonstrated that treatment of BMMC with MAFP, an inhibitor of $cPLA_2$, 10 min before (but not at the time of) activation through FcεRI inhibited immediate PGD_2 generation. LTC_4 generation was not examined. In contrast to the findings of Marshall and colleagues (29), they also demonstrated inhibition of immediate phase PGD_2 generation in BMMC through the action of SB203347 (12) and in the MMC-34 mast cell line through antisense inhibition of the group V PLA_2 (15). Our data indicate an absolute requirement for $cPLA_2$ in the immediate phase of generation of both PGD_2 and LTC_4 and reveal that group V PLA_2 alone is not sufficient. The data from Reddy and colleagues could indicate a cooperative and/or amplifying action of group V PLA_2 in the immediate phase of PGD_2 generation or may reflect the lack of specificity of pharmacologic and antisense approaches. A cooperative action of $cPLA_2$ with the group V and group IIA low molecular weight enzymes has also been shown in trans-

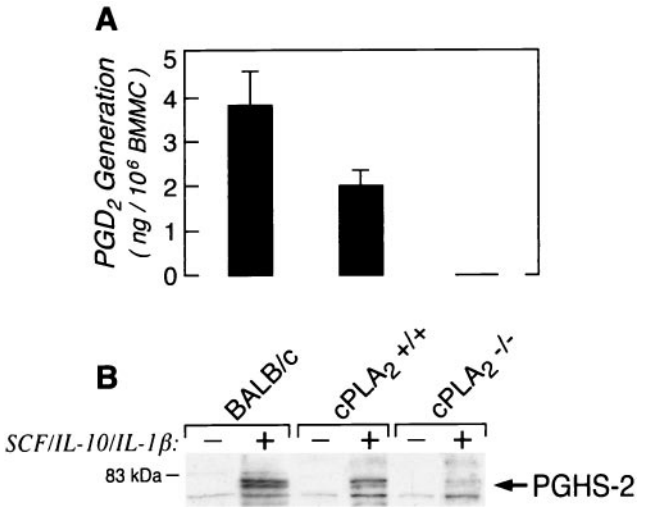

FIG. 3. Delayed-phase PGD_2 generation. Delayed-phase PGD_2 generation (*A*) was determined in BMMC derived from BALB/c, $cPLA_2+/+$, and $cPLA_2-/-$ mice. BMMC were stimulated with SCF + IL-10 + IL-1β for 1 h, washed, and incubated for 7 h more with the same cytokines. PGD_2 released into the supernatant was measured by using RIA. PGHS-2 induction (*B*) was assessed by SDS/PAGE immunoblotting 8 h after the initial activation with SCF + IL-10 + IL-1β (+) or after continued culture in WEHI-3 cell-conditioned medium (–).

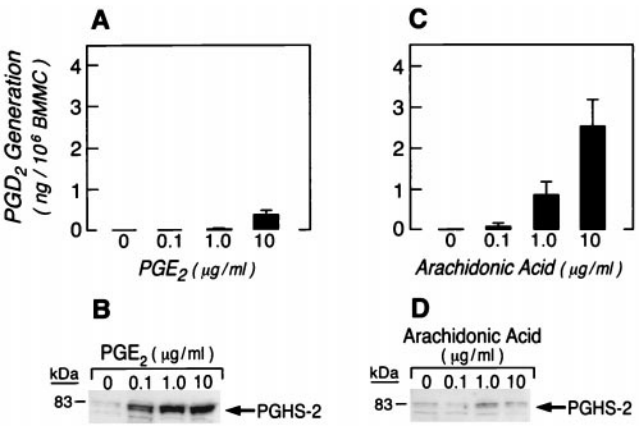

FIG. 4. Delayed-phase PGD_2 generation and induction of PGHS-2 in the presence of arachidonic acid or PGE_2. BMMC were stimulated with SCF + IL-10 + IL-1β in the presence of increasing concentrations of PGE_2 or arachidonic acid for 1 h, washed, and incubated for 7 h more with cytokines and PGE_2 or arachidonic acid to assess delayed-phase PGD_2 generation by RIA (*A* and *C*) and PGHS-2 induction by SDS/PAGE immunoblotting (*B* and *D*).

fected 293S cells and CHO cells (30), which have a small basal expression of $cPLA_2$ and release minimal arachidonic acid in response to A23187. When these cell lines were transfected with either the group IIA or the group V PLA_2, they released a substantial amount of arachidonic acid in response to A23187 that was inhibited by MAFP, suggesting that endogenous $cPLA_2$ was required for the action of the low molecular weight enzymes. Such cooperation also has been described in $P388D_1$ macrophages primed with lipopolysaccharide for 1 h and stimulated with platelet-activating factor (31) to release intracellular arachidonic acid, which facilitates the action of the group V PLA_2 to provide arachidonic acid for conversion to PGE_2 via PGHS-2 over 10 min (31–33).

Our studies also show that $cPLA_2$ is required for the delayed phase of PGD_2 generation in BMMC (Fig. 3*A*), apparently both to supply arachidonic acid and to facilitate the ligand-dependent induction of PGHS-2 (Fig. 3*B*). In previous studies, we have shown that delayed-phase PGD_2 generation is deficient in BMMC derived from 129 mice because of failure of induction of PGHS-2 (16). However, in the present series of experiments, delayed-phase generation of PGD_2 was intact in BMMC from the littermate controls, no doubt because of the contribution of the C57BL/6 background, although it was somewhat less than in BMMC from BALB/c mice (Fig. 3*A*). Delayed-phase generation of PGD_2 was seen in each set of BMMC from $cPLA_2+/+$ mice and in none of the BMMC from $cPLA_2-/-$ mice. Thus, differences in the genetic background are unlikely to account for the absence of delayed-phase PGD_2 generation in BMMC from $cPLA_2-/-$ mice or the diminished induction of PGHS-2. PGHS-2 expression was restored by the supply of either arachidonic acid or PGE_2 (Fig. 4). However, only arachidonic acid restored the delayed phase of PGD_2 generation in the presence of membrane signals via SCF + IL-10 + IL-1β. These observations suggest that $cPLA_2$ is important not only for the amplification of PGHS-2 but also in the supply of arachidonic acid in the delayed phase. A role for $cPLA_2$ in augmenting the induced expression of PGHS-2 has been reported in the MC3T3-E1 osteoblast cell line, in which delayed-phase PGE_2 generation in response to tumor necrosis factor α and IL-1β depends on $cPLA_2$ and PGHS-2 (24). Inhibitors of $cPLA_2$ or PGHS-2 reduced the expression of both $cPLA_2$ and PGHS-2, and expression of both enzymes was restored by the addition of exogenous arachidonic acid or PGE_2.

That a cooperative action of $cPLA_2$ (Fig. 4) with a low molecular weight enzyme (16) is important in delayed-phase PGD_2 generation by nontransformed BMMC is supported by transfection studies in other cells. A delayed phase of arachidonic acid release and PGE_2 generation in 293 or CHO cells, stimulated by IL-1β and fetal calf serum, depended on PGHS-2, was augmented by transfection with $cPLA_2$, group IIA PLA_2, or group V PLA_2 (30), and was inhibited by MAFP (30). Because we washed the BMMC 1 h after activation with the cytokine triad, the delayed PGD_2 generation associated with PGHS-2 induction and function would require that any participating low molecular weight PLA_2 species be firmly retained with the cell. Inasmuch as our BMMC from $cPLA_2-/-$ mice were also naturally disrupted for the group IIA PLA_2 gene, the current candidate for a low molecular weight enzyme supporting the function of $cPLA_2$ would be group V PLA_2. Our previous findings that exogenously added heparin, which binds cationic PLA_2, or scalaradial, an inhibitor of low molecular weight PLA_2 enzymes, each inhibited delayed-phase PGD_2 generation (16) are compatible with a role for two PLA_2 enzymes in this pathway. The requirement that exogenous arachidonic acid be supplied with the cytokine triad to $cPLA_2-/-$ BMMC containing group V PLA_2 implies a concerted interaction to induce PGHS-2 and provide its substrate.

We thank Richard P. Goddeau and Chioma Nwankwo for technical assistance. This work was supported by National Institutes of Health Grants HL36110, AI22531, AI31599 (to K.F.A.), DK02493 (to A.S.), MERIT-DK39773, DK38452, NS10828 (to J.B.), and ES06105 (to B.K.L.); by an International Research Grant from the Japan Eye Bank and by the Kowa Life Science Foundation (to H.F.); by a grant from the Arthritis Foundation and a President's Grant-in-Aid award from the American Academy of Allergy Asthma and Immunology (to C.O.B.); by American Cancer Society Grant RPG-97-001-01-BE and a Burroughs Wellcome Fund Developing Investigator Award (to J.P.A.); and by a grant from the Hyde and Watson Foundation.

1. Samuelsson, B. (1983) *Science* **220,** 568–575.
2. Smith, W. L. (1992) *Am. J. Physiol.* **263,** F181–F191.
3. Henderson, W. R. (1994) *Ann. Intern. Med.* **121,** 684–697.
4. Goetzl, E. J., An, S. & Smith, W. L. (1995) *FASEB J.* **9,** 1051–1058.
5. Dennis, E. A. (1994) *J. Biol. Chem.* **269,** 13057–13061.
6. Dennis, E. A. (1997) *Trends Biochem. Sci.* **22,** 1–2.
7. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. & Knopf, J. L. (1991) *Cell* **65,** 1043–1051.
8. Glover, S., Bayburt, T., Jonas, M., Chi, E. & Gelb, M. H. (1995) *J. Biol. Chem.* **270,** 15359–15367.
9. Tischfield, J. A. (1997) *J. Biol. Chem.* **272,** 17247–17250.
10. Murakami, M., Matsumoto, R., Austen, K. F. & Arm, J. P. (1994) *J. Biol. Chem.* **269,** 22269–22275.
11. Murakami, M., Bingham, C. O., III, Matsumoto, R., Austen, K. F. & Arm, J. P. (1995) *J. Immunol.* **155,** 4445–4453.
12. Reddy, S. T. & Herschman, H. R. (1997) *J. Biol. Chem.* **272,** 3231–3237.
13. Kawata, R., Reddy, S. T., Wolner, B. & Herschman, H. R. (1995) *J. Immunol.* **155,** 818–825.
14. Murakami, M., Austen, K. F. & Arm, J. P. (1995) *J. Exp. Med.* **182,** 197–206.
15. Reddy, S. T., Winstead, M. V., Tischfield, J. A. & Herschman, H. R. (1997) *J. Biol. Chem.* **272,** 13591–13596.
16. Bingham, C. O., III, Murakami, M., Fujishima, H., Hunt, J. E., Austen, K. F. & Arm, J. P. (1996) *J. Biol. Chem.* **42,** 25936–25944.
17. Murakami, M., Nakatani, Y. & Kudo, I. (1996) *J. Biol. Chem.* **271,** 30041–30051.
18. Murakami, M., Kudo, I. & Inoue, K. (1993) *J. Biol. Chem.* **268,** 839–844.
19. Bonventre, J. V., Huang, Z., Taheri, M. R., O'Leary, E., Li, E., Moskowitz, M. A. & Sapirstein, A. (1997) *Nature (London)* **390,** 622–625.
20. Murakami, M., Matsumoto, R., Urade, Y., Austen, K. F. & Arm, J. P. (1995) *J. Biol. Chem.* **270,** 3239–3246.
21. Razin, E., Stevens, R. L., Akiyama, F., Schmid, K. & Austen, K. F. (1982) *J. Biol. Chem.* **257,** 7229–7236.
22. Robinson, D. & Stirling, J. L. (1968) *Biochem. J.* **107,** 321.
23. Lam, B. K., Xu, K., Atkins, M. B. & Austen, K. F. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11598–11602.
24. Murakami, M., Kuwata, H., Amakasu, Y., Shimbara, S., Nakatani, Y., Atsumi, G. & Kudo, I. (1997) *J. Biol. Chem.* **272,** 19891–19897.
25. MacPhee, M., Chepenik, K. P., Liddell, R. A., Nelson, K. K., Siracusa, L. D. & Buchberg, A. M. (1995) *Cell* **81,** 957–966.
26. Kennedy, B. P., Payette, P., Mudgett, J., Vadas, P., Pruzanski, W., Kwan, M., Tang, C., Rancourt, D. E. & Cromlish, W. A. (1995) *J. Biol. Chem.* **270,** 22378–22385.
27. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J. & Shimizu, T. (1997) *Nature (London)* **390,** 618–622.
28. Roshak, A., Sathe, G. & Marshall, L. A. (1994) *J. Biol. Chem.* **269,** 25999–26005.
29. Marshall, L. A., Bolognese, B., Winkler, J. D. & Roshak, A. (1997) *J. Biol. Chem.* **272,** 759–765.
30. Murakami, M., Shimbara, S., Kambe, T., Kuwata, H., Winstead, M. V., Tischfield, J. A. & Kudo, I. (1998) *J. Biol. Chem.* **273,** 14411–14423.
31. Balsinde, J. & Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 6758–6765.
32. Balboa, M. A., Balsinde, J., Winstead, M. V., Tischfield, J. A. & Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 32381–32384.
33. Balsinde, J., Balboa, M. A. & Dennis, E. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7951–7956.