

Detection of secretory phospholipase A₂s related but not identical to type IIA isozyme in cultured mast cells

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Abstract We previously reported that BALB/cJ mouse-derived bone marrow-derived mast cells (BMMC) exhibited two sequential phases of prostaglandin D₂ (PGD₂) generation in response to Fc_ε receptor I (Fc_εRI) crosslinking and cytokine stimulation, the late phase of which was suppressed by an antibody raised against type IIA secretory phospholipase A₂ (sPLA₂). Here we report that BMMC derived from C57BL/6J mice, which are genetically deficient in type IIA sPLA₂, display both immediate and delayed PGD₂ generation normally. Lysates of C57BL/6J-derived BMMC contained a Ca²⁺-dependent PLA₂ that was absorbed to a column conjugated with anti-type IIA sPLA₂ antibody and had a similar molecular mass of 14 kDa, as assessed by immunoblotting. Therefore we speculate that a sPLA₂ similar to, but distinct from, type IIA sPLA₂ would compensate for type IIA sPLA₂ deficiency in C57BL/6J-derived BMMC. We found that the two type IIA-related sPLA₂ family members, type V and type IIC sPLA₂s, were expressed in BMMC as well as in rat mastocytoma RBL-2H3 cells.

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Key words: Phospholipase A₂; Mast cell; Prostaglandin D₂; Cytokine (mouse)

1. Introduction

We have previously reported that BALB/cJ mouse-derived bone marrow-derived mast cells (BMMC) stimulated by Fc_εRI crosslinking via IgE and antigen in the presence of specific accessory cytokines show two sequential prostaglandin D₂ (PGD₂) biosynthetic responses over time [1–6]. Immediate PGD₂ generation, occurring within a few minutes in response to a transient increase in cytoplasmic Ca²⁺ concentration, is associated with transient perinuclear translocation and phosphorylation of cytosolic phospholipase A₂ (cPLA₂) [3,7,8]. The constitutively expressed cyclooxygenase (COX) isoform, COX-1, is the dominant enzyme involved in this rapid response, which converts arachidonic acid to PGH₂, which in turn is metabolized to PGD₂ via glutathione-dependent PGD₂ synthase [1,2,4,5]. Delayed PGD₂ generation, occurring over several hours of culture, is associated with the de novo induction and function of COX-2 [1,4,5]. The segregated processing of arachidonic acid implies a distinct supply or different intracellular conditions for the metabolism of arachidonic acid by the alternative COX isoforms. Based upon

observations that delayed PGD₂ generation is accompanied by the induction of type IIA secretory PLA₂ (sPLA₂) transcript and is suppressed efficiently by an antibody against this enzyme, we speculated that type IIA sPLA₂ is functionally linked to COX-2-dependent delayed PGD₂ generation [6,9]. Moreover, earlier findings that histamine release from activated mast cells was sensitive to several inhibitors directed against type IIA sPLA₂ led us to suggest that type IIA sPLA₂ is also involved in exocytosis [10].

However, the recent finding that several inbred mouse strains with natural disruption of the type IIA sPLA₂ gene do not exhibit any apparent abnormality [11,12] has raised confusion about the role played by type IIA sPLA₂ in mast cell activation. Here we show that BMMC derived from the C57BL/6J strain, possessing a disrupted type IIA sPLA₂ gene, have the ability to elicit immediate and delayed PGD₂ generation as well as exocytosis to a level comparable with that of BMMC derived from BALB/cJ strain, which expresses normal type IIA sPLA₂. Importantly, lysates of C57BL/6J-derived BMMC contained sPLA₂ immunoreactive with the anti-type IIA sPLA₂ antibody, suggesting that sPLA₂ related, but not identical, to type IIA sPLA₂ would compensate for the function of type IIA sPLA₂ in mast cells. RT-PCR analysis has revealed that cultured mast cells also express type V and IIC sPLA₂s.

2. Materials and methods

2.1. Materials

Recombinant mouse interleukin (IL)-1β was purchased from Genzyme. Recombinant mouse *c-kit* ligand (KL), IL-10 and type IIA sPLA₂ were obtained by expression in a baculovirus system as described previously [1]. A rabbit polyclonal antibody against mouse type II sPLA₂ was prepared as described previously [6]. A rabbit polyclonal antibody against mouse COX-2 was purchased from Cayman Chemical. Monoclonal IgE anti-trinitrophenyl (TNP) and TNP-conjugated bovine serum albumin as an antigen were provided by Dr. J.P. Arm and H. Katz (Harvard Medical School, Boston, MA). Aspirin was purchased from Sigma.

2.2. Preparation and activation of BMMC

BMMC were obtained from either BALB/cJ or C57BL/6J mouse bone marrow cells by culturing with 50% (v/v) WEHI-conditioned medium as a source of IL-3, as described previously [1]. Cells (10⁷ cells/ml) were sensitized with 10 μg/ml IgE anti-TNP for 30 min at 37°C, washed, and suspended in enriched medium [6] containing 100 units/ml IL-10, 5 ng/ml IL-1β, 100 ng/ml KL and 20 ng/ml antigen at 37°C. After various incubation periods, the supernatants were assayed for PGD₂ and PGE₂ using a PGD₂ radioimmunoassay kit (Amersham) and a PGE₂ enzyme immunoassay kit (Cayman), respectively, and for β-hexosaminidase as a marker of exocytosis [2]. The cells were analyzed for protein expression. To assess only the delayed response, BMMC were pretreated with 10 μg/ml aspirin for 2 h to inactivate COX-1, which is essential for immediate PGD₂ generation, washed, and then activated as described previously [4].

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Abbreviations: BMMC, bone marrow-derived mast cells; cPLA₂, cytosolic phospholipase A₂; sPLA₂, secretory phospholipase A₂; COX, cyclooxygenase; Fc_εRI, Fc_ε receptor I; PGD₂, prostaglandin D₂; IL, interleukin; KL, *c-kit* ligand; TNP, trinitrophenyl

2.3. Immunoaffinity column

BMMC were suspended in 10 mM Tris-HCl (pH 7.4) containing 1 M NaCl and 1 mM EDTA at 1×10^7 cells/ml, disrupted by sonication and centrifuged for 1 h at $100\,000 \times g$ at 4°C. The pooled supernatants were applied to a formyl cellulofine (Seikagaku Kogyo) column that had been conjugated with the anti-mouse type IIA sPLA₂ antibody, as described previously [6]. The bound sPLA₂ was eluted with 50 mM glycine-HCl buffer (pH 2.2).

2.4. SDS-PAGE/Immunoblotting

Samples were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes (Schleicher and Schuell) using a semi-dry blotter (MilliBlot-SDE system; Millipore), probed with antibodies against type IIA sPLA₂ or COX-2, and visualized using an enhanced chemiluminescence Western blot analysis system (Amersham), as described previously [1,13].

2.5. sPLA₂ assay

The standard reaction mixture consisted of 100 mM Tris-HCl, pH 9.0, 4 mM CaCl₂, 2 μM 1-palmitoyl-2-[¹⁴C]linoleoyl-phosphatidylethanolamine (Amersham) and an aliquot of the samples. After incubation for 30 min at 37°C, the released linoleic acid was extracted as described [13] and counted by a β-scintillation counter.

2.6. Recombinant type IIC sPLA₂

The cDNA for type IIC sPLA₂, obtained by RT-PCR from testis as described previously [6,14], was subcloned into pVL1393 (Pharmin-gen) and transfected into Sf9 cells (Invitrogen) using a baculovirus system [1]. One week after the third transfection, the supernatants were collected and used as recombinant type IIC sPLA₂.

2.7. RT-PCR

Specific primers for PCR for three sPLA₂s were synthesized (Greiner Japan). Type IIA sPLA₂ primers used were 5'-ATG AAG GTC CTC CTC CTG CTA G-3' and 5'-TCA GCA TTT GGG CTT CTT CC-3' [13], type IIC sPLA₂ primers were 5'-ATG GAC CTC CTG GTC TCC TCA GG-3' and 5'-CTA GCA ATG AGT TTG TCC CTG C-3' [6], and type V sPLA₂ primers were 5'-CAG GGG GCT TGC TAG AAC TCA A-3' and 5'-AAG AGG GTT GTA AGT CCA GAG G-3' [15]. The RT-PCR was carried out using a RNA PCR kit (AMV) version-2 (TaKaRa), according to the manufacturer's instructions, using 1 μg of total RNA from BMMC or rat mastocytoma RBL-2H3 as a template. Equal amounts of each RT product were PCR-amplified with *ex Taq* polymerase (TaKaRa) for 30 cycles consisting of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C. The amplified cDNA fragments were resolved on 1.5% (w/v) agarose gels and visualized by ethidium bromide.

3. Results

3.1. Mediator release from C57BL/6J-derived BMMC

To assess the immediate response, BMMC derived from C57BL/6J and BALB/cJ mice (type IIA sPLA₂ (–) and (+), respectively) were each sensitized with IgE and activated with antigen for 10 min. C57BL/6J- and BALB/cJ-derived BMMC generated 4.5 ± 1.5 and 3.1 ± 0.7 ng of PGD₂ per 10^6 cells and released $33 \pm 8\%$ and $28 \pm 5\%$ of β-hexosaminidase, respectively ($n = 2$, mean $\pm$ S.D.).

To assess the delayed response, these BMMC were sensitized with IgE for 2 h in the presence of aspirin to irreversibly inactivate preexisting COX-1 [4], and then activated with antigen in the presence of KL, IL-10 and IL-1β, thus eliciting maximal delayed PGD₂ generation without accompanying immediate PGD₂ generation. As shown in Fig. 1, BMMC from both strains produced PGD₂ over 2–10 h, reaching a maximal level of ~ 10 ng/ 10^6 cells at 10 h, in parallel with the induction of COX-2 expression. PGE₂ was produced only in minimal amounts by both strains. Thus, despite the lack of type IIA sPLA₂, C57BL/6J-derived BMMC were capable of both immediate and delayed PGD₂ generation and exocytosis to a

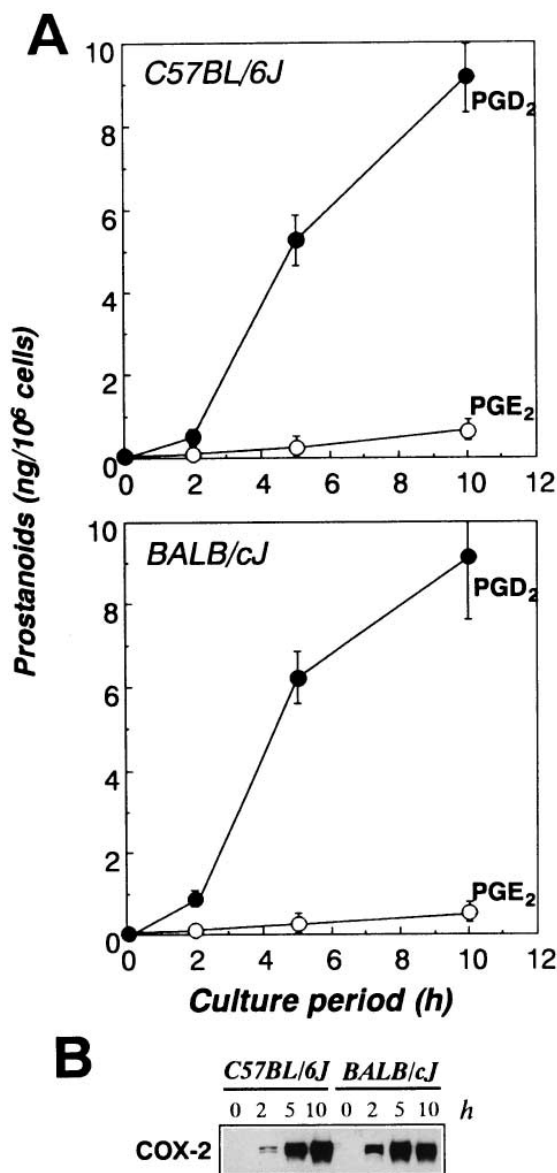


Fig. 1. Delayed PGD₂ and PGE₂ generation and COX-2 protein expression by BMMC derived from C57BL/6J (left) and BALB/cJ (right). BMMC (1×10^6 cells/ml) sensitized with IgE in the presence of aspirin were washed and then activated for the indicated periods with antigen in combination with the cytokine triad, KL, IL-10 and IL-1β. Supernatants and cells were taken for PGD₂ (closed symbols) and PGE₂ (open symbols) assays (mean $\pm$ S.D.; $n = 2$) (A) and immunoblotting for COX-2 (B), respectively.

level comparable with BALB/cJ-derived BMMC, which express type IIA sPLA₂ normally.

3.2. Detection of immunoreactive sPLA₂ in C57BL/6J-derived BMMC

In order to search for the sPLA₂-like activity in BMMC, $100\,000 \times g$ supernatants of cell lysates were prepared from both strains and applied to an immunoaffinity column conjugated with anti-mouse type IIA sPLA₂ antibody. Although the PLA₂ activity hydrolyzing 2-linoleoyl-phosphatidylethanolamine was undetectable in the flow-through fractions (data not shown), significant PLA₂ activity, which was comparable to that in BALB/cJ-derived BMMC, was recovered

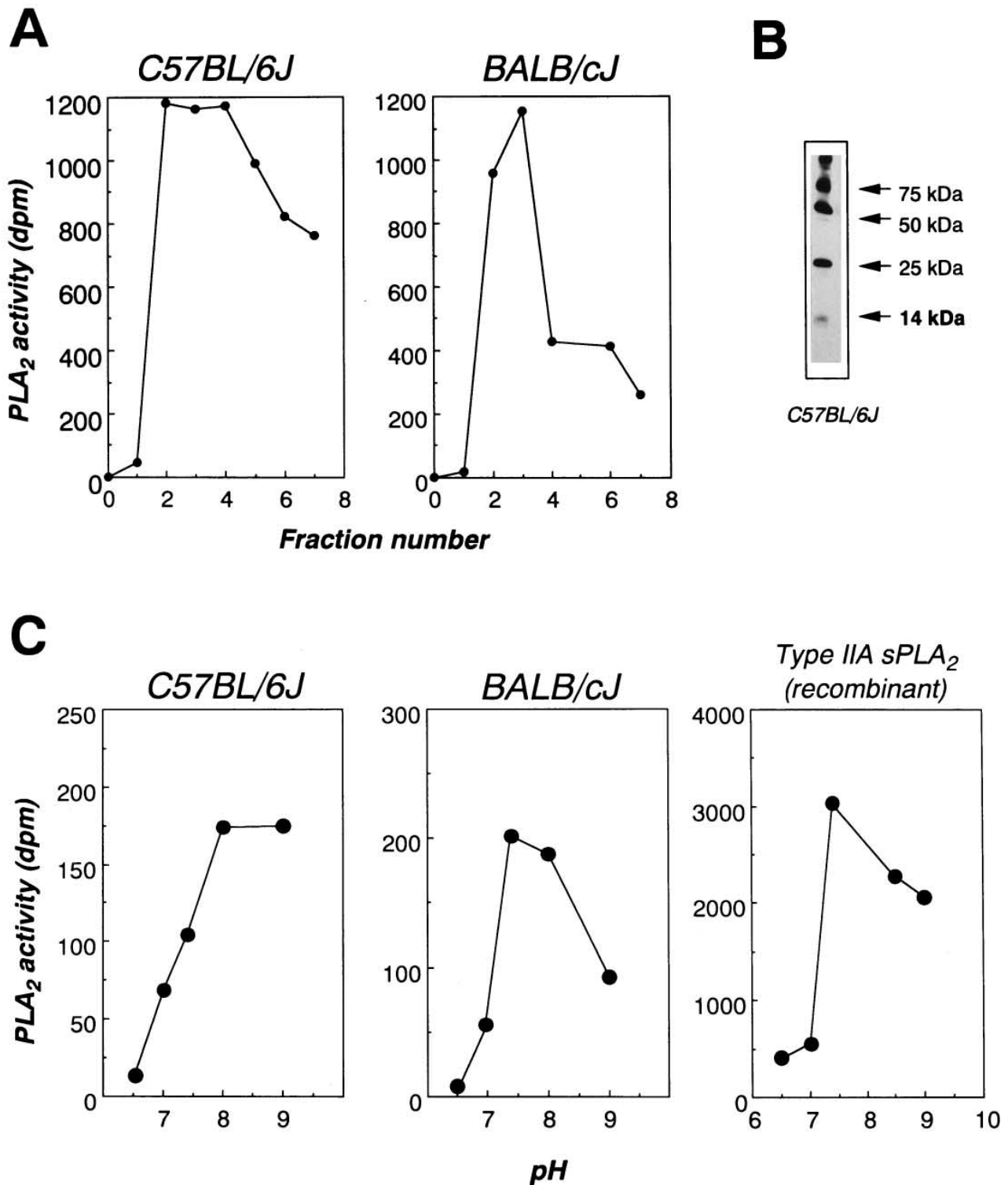
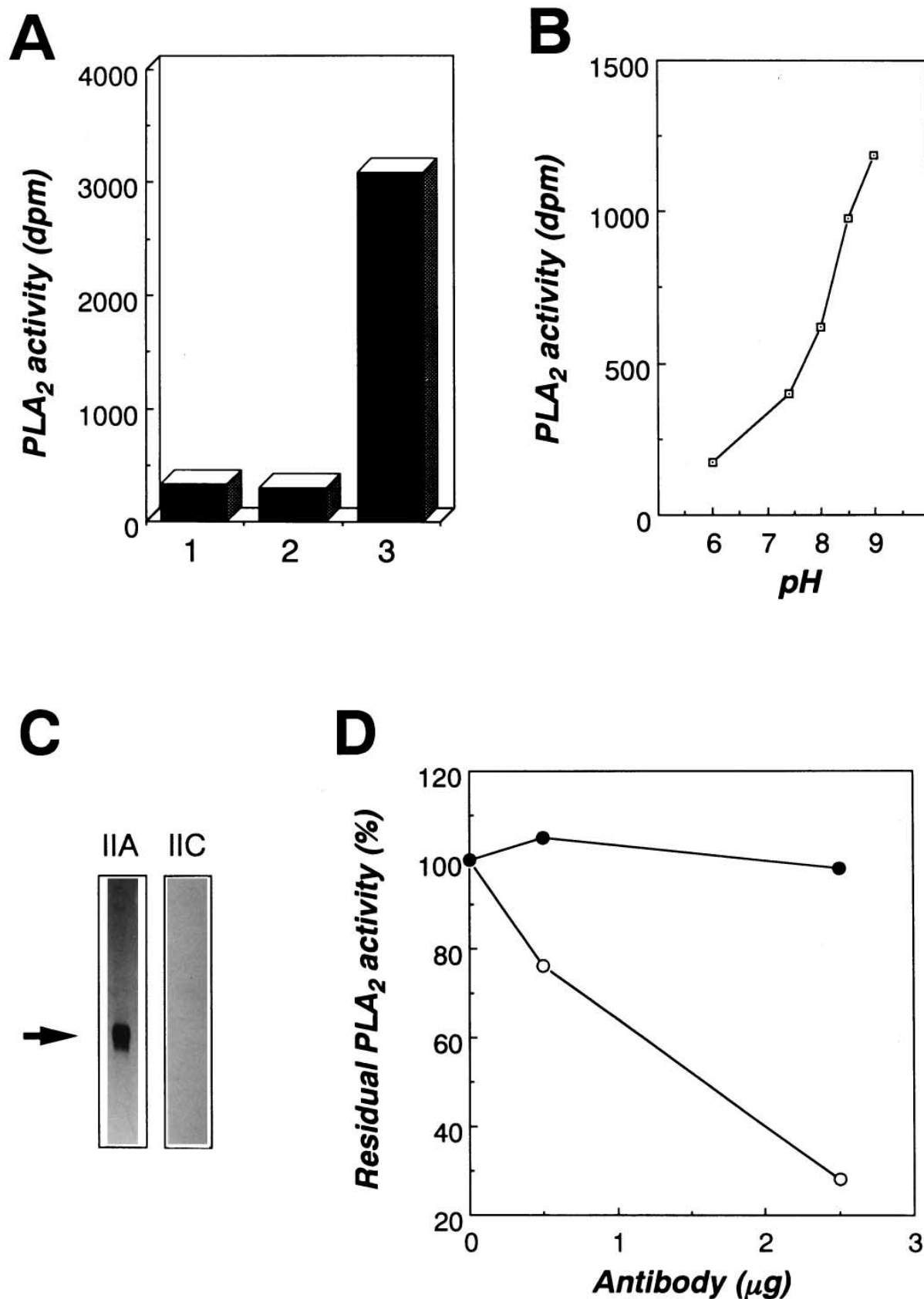


Fig. 2. Characterization of immunoreactive sPLA₂s in BALB/cJ- and C57BL/6J-derived BMMC. (A) Immunoaffinity column chromatography. The 100 000×g supernatants of lysates of C57BL/6J (left) or BALB/cJ (right) BMMC (5×10⁷ cell equivalents) were applied to an anti-type IIA sPLA₂ antibody-conjugated cellulofine column, and sPLA₂ activity bound to the column was eluted with 50 mM glycine-HCl buffer, pH 2.2. PLA₂ activity after elution with glycine-HCl buffer is shown. (B) Immunoblotting. Fraction 13 in panel (A) of C57BL/6J BMMC was neutralized and incubated with anti-type IIA sPLA₂ antibody-conjugated beads for 1 h at room temperature. The immunoprecipitate was subjected to SDS-PAGE/immunoblotting using anti-type IIA sPLA₂ antibody. Visualized bands with molecular masses of approximately 25, 50, and 75 kDa correspond to IgG light chain, heavy chain and their complex, respectively. (C) pH dependence of immunoreactive sPLA₂ activities. sPLA₂ activities of C57BL/6J and BALB/cJ BMMC [fraction 12 in panel (A)] were assayed under different pH conditions. A representative result of three independent experiments is shown.



from C57BL/6J-derived BMMC after elution of the bound proteins with an acidic buffer (Fig. 2A). The PLA₂ immuno-

reactive with the anti-type IIA sPLA₂ antibody in C57BL/6J-

derived BMMC (Fig. 2B) showed a molecular mass of approximately 14 kDa as visualized by immunoblotting. Both immunoreactive PLA₂ activities in BALB/cJ- and C57BL/6J-

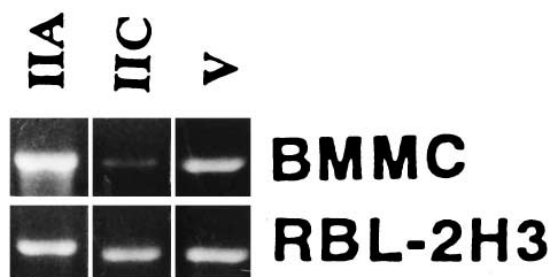


Fig. 4. Detection of transcripts for various sPLA₂s in cultured mast cells by RT-PCR. Expression of type IIA, IIC and V sPLA₂s in BALB/cJ-derived BMMC and RBL-2H3 cells were detected after 30 cycles of RT-PCR amplification. The fragment sizes (~430 bp) were consistent with the predicted sizes of each sPLA₂ cDNA. Hybridization with the specific cDNA probes confirmed that these bands indeed corresponded to the respective sPLA₂s (data not shown).

derived BMMC required millimolar order Ca²⁺ for enzymatic activity (data not shown). Thus, the ability to hydrolyze 2-linoleoyl-phosphatidylethanolamine, immunochemical property, molecular mass and mM Ca²⁺ requirement all suggest that the PLA₂ detected in C57BL/6J-derived BMMC is sPLA₂-like enzyme. The pH profiles of the sPLA₂ activities in both strains, in comparison to that of recombinant mouse type IIA sPLA₂, is shown in Fig. 2C. sPLA₂ detected in BALB/cJ-derived BMMC, as well as recombinant mouse type IIA sPLA₂, showed optimal activity at pH 7.4, whereas that in C57BL/6J-derived BMMC had an optimal pH of 8–9. This difference, together with the genetic evidence [11,12], implies that the immunoreactive sPLA₂ detected in C57BL/6J-derived BMMC is distinct from type IIA sPLA₂.

3.3. Comparison with type IIC sPLA₂

We have recently reported that BALB/cJ-derived BMMC express the type IIC sPLA₂ transcript, as assessed by RT-PCR [6]. In order to clarify whether the immunoreactive sPLA₂ detected in C57BL/6J-derived BMMC was identical to the type IIC enzyme, we prepared recombinant type IIC sPLA₂ using a baculovirus system (Fig. 3A) and compared its properties with those of C57BL/6J-derived BMMC sPLA₂. The activity of the baculovirus-expressed type IIC sPLA₂ was Ca²⁺-dependent, requiring millimolar order Ca²⁺ (data not shown), and was optimal at pH 9 (Fig. 3B). The specific activity of this enzyme was about one eighth that of recombinant type IIA sPLA₂ (data not shown). However, the anti-type IIA sPLA₂ antibody did not crossreact with recombinant type IIC PLA₂, as demonstrated by immunoblotting (Fig. 3C) and immunoabsorption (Fig. 3D). Therefore most sPLA₂ detected in C57BL/6J-derived BMMC may differ from type IIC sPLA₂.

3.4. Detection of type V sPLA₂ in cultured mast cells

To assess the expression of type IIA-like sPLA₂s in

BMMC, RT-PCR was carried out using specific primers for type IIA, IIC or V sPLA₂s. After 30 cycles of amplification, the expression of type IIA, IIC and V sPLA₂s was clearly detected in BALB/cJ-derived BMMC (Fig. 4). Similarly, type IIA, IIC and V sPLA₂s were detected in rat mastocytoma RBL-2H3 cells (Fig. 4), indicating that mast cells generally contain more than one sPLA₂. While this experiment was in progress, the expression of type V, but not type IIC, sPLA₂ has been shown in C57BL/6J-derived BMMC [16]. We also observed the similar expression pattern of these sPLA₂s in C57BL/6J-derived BMMC (data not shown). Thus, the expression of each sPLA₂ appears to differ among mast cells obtained from different sources.

4. Discussion

We have previously reported the cloning of mouse type IIA sPLA₂ from cytokine-stimulated BALB/cJ BMMC and its possible participation in COX-2-dependent delayed PGD₂ generation on the basis of its marked suppression by an antibody against recombinant type IIA sPLA₂ [6]. However, our present finding that type IIA sPLA₂-deficient C57BL/6J-derived BMMC displayed PGD₂ generation to an extent comparable with BALB/cJ-derived BMMC argues against the role of type IIA sPLA₂ in mast cell activation. Bingham et al. [9] have also recently observed a similar phenomenon, and concluded that a sPLA₂ distinct from type IIA sPLA₂ may be responsible for delayed PGD₂ generation.

Detailed analysis of the PLA₂ activity in BMMC lysates revealed that C57BL/6J-derived, type IIA sPLA₂-deficient BMMC contained a sPLA₂ immunochemically close to type IIA sPLA₂ (Fig. 2A and B). The level of this enzyme activity was comparable with that of immunoreactive sPLA₂ activity detected in BALB/cJ-derived BMMC (Fig. 2A), consistent with the fact that BMMC derived from both strains exhibited PGD₂ generation to a similar extent (Fig. 1). Although sPLA₂s in C57BL/6J- and BALB/cJ-derived BMMC were immunochemically indistinguishable, they differed significantly in their pH-dependence; the former preferred alkaline pH and the latter was most active at neutral pH (Fig. 2C). Neutral pH preference was also observed in recombinant type IIA sPLA₂, consistent with its expression in BALB/cJ-derived BMMC. We previously reported that rat mastocytoma RBL-2H3 cells expressed at least three PLA₂ enzymes, including cPLA₂, type IIA sPLA₂ and an unknown PLA₂ with chromatographic behaviors similar to type IIA sPLA₂ [17]. Fonteh et al. [18] also demonstrated the presence of two type IIA sPLA₂-like enzymes in BMMC derived from CBA/J mice. Collectively, these results suggest that C57BL/6J-derived BMMC express the sPLA₂-like enzyme, which can substitute for type IIA sPLA₂ to regulate mast cell function, thereby reflecting the redundancy of sPLA₂ family members. Alternatively, the sPLA₂-like enzyme, rather than type IIA

Fig. 3. Characteristics of recombinant type IIC sPLA₂ expressed by Sf9 cells. (A) Release of type IIC sPLA₂ activity from baculovirus-infected Sf9 cells. Columns 1, 2, and 3 correspond to cells without transfection, cells transfected with control vector, and cells transfected with type IIC sPLA₂, respectively. The supernatants were collected and assayed for PLA₂ activity. (B) pH profile of type IIC sPLA₂. (C) Supernatants of Sf9 cells expressing type IIA (lane 1) and type IIC (lane 2) sPLA₂s were immunoblotted with the anti-type IIA sPLA₂ antibody. (D) Supernatants of Sf9 cells expressing type IIA (open circles) and type IIC (closed circles) sPLA₂s were incubated with the indicated amounts of anti-type IIA sPLA₂ antibody for 1 h. After precipitation with protein-A Sepharose (Pharmacia), PLA₂ activities remaining in the supernatants were measured. A representative result of three independent experiments is shown.

sPLA₂, may be a true effector of arachidonate metabolism even in BMMC expressing type IIA sPLA₂.

Type IIA, IIC and V sPLA₂s are closely related to one another and map to the same chromosome locus [14,19], implying their functional redundancy. In view of our previous finding that BALB/cJ-derived BMMC express mRNA for type IIC sPLA₂ [6] (Fig. 4), we examined the identity of C57BL/6J-derived BMMC sPLA₂ with the recombinant type IIC enzyme. Although type IIC sPLA₂ showed Ca²⁺ and pH dependence similar to that of C57BL/6J-derived BMMC sPLA₂, no crossreactivity of the anti-type IIA sPLA₂ antibody with type IIC sPLA₂ was observed (Fig. 3), indicating that C57BL/6J-derived BMMC sPLA₂ may be distinct from type IIC sPLA₂. This conclusion is further supported by the observation that type IIC sPLA₂ transcript was detectable only minimally in C57BL/6J-derived BMMC (data not shown) [16] and its expression in BALB/cJ-derived BMMC was not correlated with PGD₂ generation [6].

We have found that type V sPLA₂ was expressed in BMMC (Fig. 4). During the course of this study, Reddy et al. [16] have also reported that type V sPLA₂ is expressed in various mouse mast cells, including those derived from type IIA sPLA₂-deficient C57BL/6J mice, and is crucial for the regulation of arachidonate metabolism. Although the contribution of type V sPLA₂ was confined to immediate, but not delayed, PGD₂ generation in the mast cell lines they used [16], it is at present unclear whether the delayed PGD₂ generation observed in primary BMMC, which Bingham et al. [9] and our group have studied, also depends on type V sPLA₂. Of note, whereas Reddy et al. [16] failed to detect the transcripts for type IIA and IIC sPLA₂s in their mast cells, these transcripts were readily detectable in our BALB/cJ-derived BMMC (Fig. 4). Moreover, these three sPLA₂s were also detected in rat mastocytoma RBL-2H3 (Fig. 4). The distinct profiles of sPLA₂ expression might reflect the heterogeneity of mast cells, which is highly affected by microenvironments during culture. Indeed, type IIA, but not IIC, sPLA₂ expression was greatly induced in BALB/cJ-derived BMMC when cultured with KL in combination with IL-10 and IL-1β [6,9]. Although these observations suggest that sPLA₂ detected in C57BL/6J-derived BMMC in this study is identical to type V sPLA₂, the final conclusion will have to be awaited until the enzymatic properties of recombinant type V sPLA₂, such as immuno-reactivity and pH dependence, are compared with the present results. Also, it will be of interest to examine the cytokine regulation of type V sPLA₂ expression in BMMC. Nevertheless, type V sPLA₂ has also been implicated in PGE₂ generation in P388D₁ mouse macrophage-like cells, in which type

IIA sPLA₂ expression is barely detectable [18], and therefore is emerging as a novel effector involved in arachidonate-mediated signal transduction.

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