Redundant and Segregated Functions of Granule-Associated Heparin-Binding Group II Subfamily of Secretory Phospholipases A_2 in the Regulation of Degranulation and Prostaglandin D_2 Synthesis in Mast Cells[1]

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We herein demonstrate that mast cells express all known members of the group II subfamily of secretory phospholipase A_2 ($sPLA_2$) isozymes, and those having heparin affinity markedly enhance the exocytotic response. Rat mastocytoma RBL-2H3 cells transfected with heparin-binding ($sPLA_2$-IIA, -V, and -IID), but not heparin-nonbinding ($sPLA_2$-IIC), enzymes released more granule-associated markers (β-hexosaminidase and histamine) than mock- or cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$)-transfected cells after stimulation with IgE and Ag. Site-directed mutagenesis of $sPLA_2$-IIA and -V revealed that both the catalytic and heparin-binding domains are essential for this function. Confocal laser and electron microscopic analyses revealed that $sPLA_2$-IIA, which was stored in secretory granules in unstimulated cells, accumulated on the membranous sites where fusion between the plasma membrane and granule membranes occurred in activated cells. These results suggest that the heparin-binding $sPLA_2$s bind to the perigranular membranes through their heparin-binding domain, and lysophospholipids produced in situ by their enzymatic action may facilitate the ongoing membrane fusion. In contrast to the redundant role of $sPLA_2$-IIA, -IID, and -V in the regulation of degranulation, only $sPLA_2$-V had the ability to markedly augment IgE/Ag-stimulated immediate PGD_2 production, which reached a level comparable to that elicited by $cPLA_2\alpha$. The latter observation reveals an unexplored functional segregation among the three related isozymes expressed in the same cell population. *The Journal of Immunology,* **2000, 165: 4007–4014.**

Cross-linking of the high affinity IgE receptor (FcεRI) on mast cells by IgE and multivalent Ag (IgE/Ag) or cytokine receptors by their cognate ligands elicits a biphasic mediator release response that is thought to promote allergic and chronic inflammatory diseases (1, 2). The immediate response, which occurs within a few minutes of cell activation, is accompanied by exocytosis of preformed mediators (such as histamine, serotonin, proteoglycans, and proteases) stored in secretory granules and generation of lipid mediators (such as PGD_2, leukotriene C_4, and platelet-activating factor). The immediate response is followed by induction of the immediate-early genes and various cytokines and prolonged production of PGD_2, which constitute the delayed phase of mast cell activation.

Phospholipase A_2 (PLA_2),[3] which liberates free fatty acids, including arachidonic acid (AA), from membrane phospholipids, represents a critical rate-limiting step for the biosynthesis of eicosanoids. To date, >10 PLA_2 gene products have been identified in mammals (3, 4). Of these, 85-kDa group IVA cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$) and several 14-kDa secretory PLA_2 ($sPLA_2$) enzymes have been shown to be involved in the regulation of eicosanoid biosynthesis (5–12). Analyses using bone marrow-derived mast cells (BMMC) obtained from $cPLA_2\alpha$-knockout mice have provided definitive evidence that $cPLA_2\alpha$ is essential for both the immediate and the delayed phases of eicosanoid biosynthetic responses (13). Of the $sPLA_2$ isozymes identified, the genes for groups IIA, V, IIC, IID, IIE, and IIF are clustered at the same chromosome locus and are classified as the group II subfamily because of their structural similarities (4–6, 9, 10, 14–17). Secretory PLA_2-IIA and V are two major $sPLA_2$s that have been implicated in PGD_2 generation in mast cells, although their expression and functional profiles before and after cell activation differ as a function of mast cell phenotype (18–21).

Several lines of evidence suggest the participation of $sPLA_2$ in the exocytosis of several endocrine cells, including mast cells (22–26). As the lysophospholipid, another product of the PLA_2 reaction, perturbs the structure of bilayer membranes (27, 28), its production has been postulated to promote membrane fusion, thereby facilitating the exocytotic process further. Some snake venom PLA_2s and rat $sPLA_2$-IIA added exogenously at very high concentrations directly elicit degranulation of rat serosal mast cells (25, 26). Several chemicals that inhibit the in vitro enzymatic activity of $sPLA_2$-IIA reduce histamine release from IgE/Ag-activated rat serosal mast cells (23, 24). Although these pharmacological studies have led to the hypothesis that $sPLA_2$-IIA stored in secretory granules may promote the exocytosis of mast cells, nonspecific effects of these agents cannot be ruled out. Moreover, recent advances in the $sPLA_2$ field have led to the identification of

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[3] Abbreviations used in this paper: PLA, phospholipase A_2; $sPLA_2$, secretory PLA_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; AA, arachidonic acid; COX, cyclo-oxygenase; BMMC, bone marrow-derived mast cells; β-HEX, β-hexosaminidase; TNP, trinitrophenyl; WT, wild type.

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several new members of this family (4, 14–17, 29), thus complicating our understanding of redundant and/or segregated functions among $sPLA_2$ enzymes.

To better understand the role of $sPLA_2$s in mast cell activation, we have conducted gain-of-function studies by transfecting rat mastocytoma cell line RBL-2H3 with various mammalian $sPLA_2$s. We show that, among $sPLA_2$s normally expressed in mast cells, the heparin-binding group II subfamily of $sPLA_2$s exhibits a redundant function in enhancing degranulation. In contrast, only $sPLA_2$-V has the ability to enhance immediate PGD_2 biosynthesis.

Materials and Methods

Cell culture

To obtain mouse BMMC, bone marrow cells from male BALB/cJ and C57BL/6J mice were cultured for up to 10 wk in 50% enriched medium (RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% FCS) and 50% WEHI-3 cell-conditioned medium as a source of IL-3. After 3 wk, >98% of the cells in the culture were BMMC (1, 2). Rat mastocytoma RBL-2H3 cells (Japanese Cancer Resources Bank) were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% FCS.

Materials and methods

The cDNAs for mouse $sPLA_2$-IIA (mIIA) and rat $sPLA_2$-V (rV) were subcloned into $pCXN_2$ (30); rat $sPLA_2$-IIA (rIIA), mouse $sPLA_2$-IIA mutants IIA-G30S and IIA-KE4 (31), rat $sPLA_2$-V mutants V-G30S and V-RS2 (5), and rat $sPLA_2$-IIC (rIIC) into pCR3.1 (Invitrogen, San Diego, CA); mouse $sPLA_2$-IID (mIID) into pCDNA3.1 (Invitrogen); human $sPLA_2$-IIA (hIIA) and human $sPLA_2$-V (hV) into pCI-neo (Pharmacia, Piscataway, NJ); and mouse $cPLA_2\alpha$ into pBK/CMV (Stratagene, La Jolla, CA). The cDNA probes for hamster cytosolic group VI Ca^{2+}-independent PLA_2 ($iPLA_2$) (5), human $sPLA_2$-X (8), mouse $sPLA_2$-IIE, and mouse $sPLA_2$-IIF (4) were described previously. Rabbit anti-rat $sPLA_2$-IIA Ab, which recognizes rat and mouse $sPLA_2$-IIAs but not other isozymes, was described previously (5, 7, 9). Rabbit anti-human $cPLA_2\alpha$ Ab was purchased from Cayman Chemicals (Ann Arbor, MI). Rabbit Abs against mouse cyclo-oxygenase-1 (COX-1) and COX-2 were described previously (9). Rabbit Ab against rat hemopoietic PGD_2 synthase was provided by Dr. Urade (Osaka Bioscience Institute, Osaka, Japan). An RIA kit for PGD_2 was purchased from Amersham (Arlington Heights, IL). The histamine ELISA kit was obtained from Immunotech (Marseille, France). Opti-MEM medium and TRIzol reagent were obtained from Life Technologies (Grand Island, NY). FITC-conjugated goat anti-rabbit IgG Ab was purchased from Zymed (South San Francisco, CA). IgE anti-trinitrophenyl (anti-TNP) and TNP-conjugated BSA were provided by Dr. H. Katz (Harvard Medical School, Boston, MA).

Establishment of transfectants

RBL-2H3 cells were seeded into 150-mm diameter dishes and cultured for 2–3 days to subconfluence. The cells (10^7 cells) were harvested, washed twice with Opti-MEM, and suspended in 400 μl of Opti-MEM. The cells were mixed with each cDNA (2–5 μg) and subjected to electroporation (BTX electroporator ECM600; Bio Medical Equipment, Tokyo, Japan) with a 200-V pulse amplitude and a 900-μF capacitance. After culture for 2 days, cells were resuspended in 10 ml of culture medium containing 800 μg/ml geneticin (Life Technologies) and seeded into 96-well plates (100 μl/well). After culture for 2 wk, single colonies were expanded into 12-well plates. After reaching confluence, the expression of each PLA_2 was assessed by RNA blotting or immunoblotting. Because mediator release by cells transfected with the empty vectors was comparable to that by parental cells (data not shown), we used a clone transfected with the empty pCDNA3.1 vector as a control for subsequent cell activation studies.

RNA blotting

All procedures were performed as described previously (9, 31). Briefly, equal amounts (5 μg) of total RNA, purified using TRIzol reagent, were applied to each lane of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore, Bedford, MA). The resulting blots were then sequentially probed with appropriate cDNA probes that had been labeled with [^{32}P]dCTP (NEN Life Science Products, Boston, MA) by random priming (Takara Biomedical, Ohtsu, Japan). All hybridizations were conducted at 42°C overnight in a solution comprising 50% (v/v) formamide, 0.75 M NaCl, 75 mM sodium citrate, 0.1% (w/v) SDS, 1 mM EDTA, 10 mM sodium phosphate (pH 6.8), 5× Denhardt's solution (Nacalai Tesque, Kyoto, Japan), 10% (w/v) dextran sulfate (Sigma, St. Louis, MO), and 100 μg/ml salmon sperm DNA (Sigma).

RT-PCR/Southern blotting

Synthesis of cDNA was performed using 0.5 μg of total RNA from BMMC and avian myeloblastosis virus reverse transcriptase, according to the manufacturer's instructions supplied with the RNA PCR kit (Takara Biomedical). Subsequent amplifications of the cDNA fragments for PLA_2 isozymes were performed using 1 μl of the reverse transcribed mixture as a template with specific oligonucleotide primers (Greiner Japan, Tokyo, Japan) as follows: $sPLA_2$-IB: sense, 5′-CCT CAC TCC TTC TGA AGA TG-3′; and antisense, 5′-CTG ACA GCA GGT ACT TTA TTA G-3′; $sPLA_2$-IIA: sense, 5′-TCA GCA TTT GGG CTT CTT-3′; and antisense, 5′-CCA TCC AAG AGA GCT GAC AGC-3′; $sPLA_2$-IIC: sense, 5′-ATG GAC CTC CTG GTC TCC TCA GG-3′; and antisense, 5′-CTA GCA ATG AGT TTG TCC CTG C-3′; $sPLA_2$-IID: sense, 5′-ATT TTT GAG ACT TGC CCT GCT GTG TG-3′; and antisense, 5′-TTA GCA TGC TGG AGT CTT GCC-3′; $sPLA_2$-IIE: sense, 5′-ATG AAA CCT CCC ATT GCC CTG-3′; and antisense, 5′-TCA GCA GGG TGG GGT GGG CCC AG-3′; $sPLA_2$-IIF: sense, 5′-ATG AAG AAA TTC TTT GCC ATC-3′; and antisense, 5′-CTA GCT TGA GAC AGG GGT CGC-3′; $sPLA_2$-V: sense, 5′-CAG GGG GCT TGC AAC TCA A-3′; and antisense, 5′-AAG AGG GTT GTA AGT CCA GAG G-3′; $sPLA_2$-X: sense, 5′-CTG GCA GGG ACC TTG GAT TGT G-3′; and antisense, 5′-GAG GTA TTT CAG GTG GTA CTC-3′; $cPLA_2\alpha$: sense, 5′-ATG TCA TTT ATA GAT CCT TAC C-3′; and antisense, 5′-TCA AAG TTC AAG AGA CAT TTC AG-3′; and $iPLA_2$: sense, 5′-TAC GTG AAG AAG CCT GC GG-3′; and antisense, 5′-GAA GCT GTT GTT TGC TGA TCT TGG A-3′. The PCR condition was 94°C for 30 s and then 33 cycles of amplification at 94°C for 5 s and 68°C for 4 min, using the Advantage cDNA polymerase mix (Clontech, Palo Alto, CA). The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide. The gels were further subjected to Southern blot hybridization using specific PLA_2 probes as described previously (20, 22).

SDS-PAGE/immunoblotting

Cell lysates (10^5 cell equivalents) or culture supernatants were subjected to SDS-PAGE using 15% (w/v) gels for $sPLA_2$s under nonreducing conditions, and 7.5% gels for $cPLA_2$ and 10% gels for COX-1, COX-2, and hemopoietic PGD_2 synthase under reducing conditions. The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using a semidry blotter (MilliBlot-SDE system, Millipore). The membranes were probed with the respective Abs and visualized using the ECL Western blot system (NEN Life Science Products) as described previously (9, 31).

Activation of RBL-2H3 cells

The cells (5×10^4 cells/ml) were seeded into 24-well plates and cultured for 2 days in 1 ml of culture medium. Then the cells were sensitized with 100 ng/ml IgE anti-TNP for 30 min, washed twice with culture medium, and activated for 10 min at 37°C with 10 ng/ml TNP-conjugated BSA in culture medium. This condition provided optimal activation of the cells. After harvesting the supernatants, the remaining cells were collected and disrupted by two freeze-thawing cycles. β-Hexosaminidase (β-HEX) release was assessed spectrophotometrically using *p*-nitrophenyl-β-D-2-acetamido-2-deoxyglucopyranoside (Sigma) as a substrate, as described previously (2). The release of histamine and PGD_2 was assessed by ELISA and RIA, respectively. The percent release of β-HEX and histamine was calculated by the formula $[S/(S + P)] \times 100$, where S and P are the amounts of β-HEX and histamine present in the supernatant and pellet, respectively.

Immunocytostaining (confocal laser microscopy)

Cells grown on collagen-coated coverglasses (Iwaki Glass, Tokyo, Japan) were fixed with 3% paraformaldehyde for 30 min in PBS. After three washes with PBS, the fixed cells were sequentially treated with 3% BSA (for blocking) and 1% saponin (for permeabilization) in PBS for 1 h, with an anti-$sPLA_2$-IIA Ab (1/500 dilution) for 1 h, and then with FITC-goat anti-rabbit IgG (1/100 dilution) for 1 h. After six washes with PBS, the cells were mounted on glass slips using Perma Fluor (Japan Tanner, Suita, Japan), and the $sPLA_2$-IIA signal was visualized using a laser scanning confocal microscope (IX70, Olympus, New Hyde Park, NY).

Immunocytostaining (electron microscopy)

The cells grown on chamber slide glasses (Iwaki Glass) were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 30 min in PBS. After three washes with PBS, the fixed cells were sequentially treated with 10% calf serum and 0.1% Triton X-100 in PBS for 30 min, with an anti-$sPLA_2$-IIA Ab (1/500 dilution) in PBS containing 10% calf serum overnight with biotinylated goat anti-rabbit IgG (1/100 dilution) for 1.5 h and then with HRP-conjugated streptavidin (1/500 dilution), for 1 h. After three washes with PBS, the cells were incubated with PBS containing 0.2% diaminobenzidine and 0.01% H_2O_2 for viewing. After three washes with PBS, the cells were treated for 30 min with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), washed three times, and incubated with 2% OsC_4 in 0.1 M cacodylate buffer. After six washes with cacodylate buffer, the cells were dehydrated with graded concentrations of ethanol and embedded in Epon epoxy resin. After incubating for 2 days at 60°C, ultrathin sections were prepared and examined with an electron microscope.

Statistical analysis

Data were analyzed by Student's *t* test. Results are expressed as the mean ± SD, with $p = 0.05$ as the limit of significance.

Results

Mast cells express all group II subfamily of $sPLA_2$s

Previous studies have demonstrated that mast cells express $sPLA_2$-IIA, -IIC, and -V, but not -IB, although $sPLA_2$ expression profiles differ in mast cells with different phenotypes and from different animal species (18–21, 32). To determine whether mast cells express recently discovered $sPLA_2$ enzymes, we conducted RT-PCR analysis using mouse BMMC RNA as a template and a set of primers specific for each mouse $sPLA_2$ and for mouse $cPLA_2\alpha$ and $iPLA_2$. As shown in Fig. 1, transcripts for $sPLA_2$-IIC, -IID, -IIE, -IIF, and –V; $cPLA_2\alpha$; and $iPLA_2$, but not for $sPLA_2$-IB and -X, were detected in BMMC prepared from BALB/cJ and C57BL/6J mice. Under the same conditions, $sPLA_2$-IB and -X were readily detected in rat stomach and lung (data not shown), the tissues where these enzymes are expressed (15–17). Secretory PLA_2-IIA was detected in BALB/cJ BMMC, but not in C57BL/6J BMMC (Fig. 1), consistent with the fact that the latter mouse strain has a natural disruption of the $sPLA_2$-IIA gene (33). Thus, mast cells express transcripts for all known group II $sPLA_2$ subfamily members. Direct comparison of the relative expression levels of the different PLA_2s was difficult, because they were varied among BMMC prepared from different mice.

The heparin-binding group II subfamily of $sPLA_2$s is a potent enhancer of degranulation

cDNAs for $sPLA_2$-IIA (mIIA, rIIA, and hIIA), $sPLA_2$-V (rV and hV), and mouse $cPLA_2\alpha$ were each subcloned into mammalian expression vectors and transfected into rat mastocytoma RBL-2H3 cells to establish drug-resistant stable transfectants. Expression of each PLA_2 in the established transfectants was assessed by RNA blotting ($sPLA_2$s) or immunoblotting ($cPLA_2\alpha$; Fig. 2, *inset*). Endogenous expression of $cPLA_2\alpha$ was detectable, whereas that of $sPLA_2$ was below detection, in control RBL-2H3 cells under the conditions employed here.

When these cells were sensitized with IgE and then activated with Ag for 10 min, the release of β-HEX, an exocytosis marker, was severalfold higher in the transfectants expressing mIIA (Fig. 2*A*), rIIA (Fig. 2*B*), hIIA (Fig. 2*C*), rV(Fig. 2*D*), and hV (Fig. 2*E*) than in replicate mock-transfected cells. The release of histamine, another granule-derived mediator, was also enhanced markedly in cells transfected with mIIA and rV (Fig. 2*G*). No significant augmentation of β-HEX release was observed in $cPLA_2\alpha$-transfected cells (Fig. 2*F*).

To determine the functional domains responsible for the degranulation-enhancing effect of $sPLA_2$-IIA and -V, we transfected the catalytically inactive mutants (mIIA-G30S and rV-G30S, in which Gly (31) essential for Ca^{2+} binding was replaced by Ser (5)) and those with significantly reduced heparanoid affinity (mIIA-KE4 and rV-RS2, in which Lys and Arg clusters in the C-terminal domain were replaced by Glu and Ser, respectively (5)) into RBL-2H3 cells. As shown in Fig. 3, neither the catalytically inactive mutants mIIA-G30S and rV-G30S nor the heparin-nonbinding mutants mIIA-KE4 and rV-RS2 enhanced IgE-dependent release of β-HEX, even though their expression levels were even higher than those in cells transfected with the respective wild-type (WT) enzymes (Fig. 3, *insets*). These results imply that both the catalytic and heparanoid-binding domains are essential for enhancing degranulation.

When cells expressing mIIA-KE4 and mIIA-WT were each stimulated with IgE/Ag, release of PLA_2 activities into the supernatants reached ~50 and 7% of the total $sPLA_2$ activity, respectively, with remaining activities being associated with the cells. Secretory PLA_2 activity was detected only minimally in the supernatants of unstimulated cells. These results suggest that mIIA-WT is retained on the outer surface of cells through its heparin-binding domain even after cell activation (see below) and that a large fraction of secreted mIIA-KE4 is released into the culture medium.

To further ascertain the importance of the heparanoid binding capacity in the degranulation-enhancing effect, we transfected RBL-2H3 cells with mIID, another heparin-binding $sPLA_2$ (15, 16), and rIIC, a heparin-nonbinding isozyme (5). Cells transfected with mIID (Fig. 4*B*), but not with rIIC (Fig. 4*A*), exhibited enhanced β-HEX release following IgE/Ag-directed activation. Thus, the three heparin-binding group II subfamily $sPLA_2$s, namely IIA, V, and IID, but not the heparin-nonbinding $sPLA_2$-IIC, play a redundant degranulation-enhancing role in mast cells.

Subcellular distribution of $sPLA_2$-IIA in RBL-2H3 transfectants

Confocal microscopic immunocytostaining analyses of permeabilized RBL-2H3 cells expressing mIIA-WT, using Ab specific for rodent $sPLA_2$-IIA, showed that the overexpressed enzyme accumulated in punctate domains, most likely vesicular compartments,

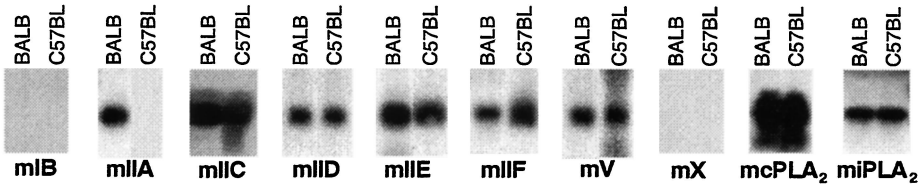

FIGURE 1. Expression of various PLA_2s in mouse BMMC. RNA prepared from BMMC obtained from BALB/cJ and C57BL/6J mice were subjected to RT-PCR using primers specific for mouse $sPLA_2$-IIA (mIIA), IIC (mIIC), IID (mIID), IIE (mIIE), IIF (mIIF), V (mV), and X (mX); $cPLA_2$ ($mcPLA_2$); and $iPLA_2$ ($miPLA_2$), followed by Southern blotting using their respective cDNA probes.

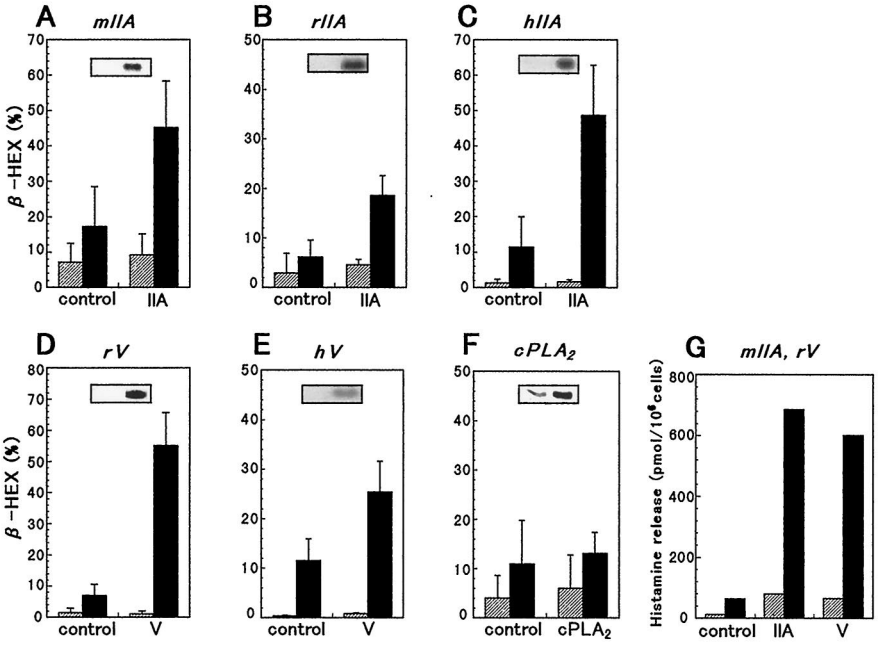

FIGURE 2. Degranulation of RBL-2H3 cells transfected with various PLA_2s. RBL-2H3 transfectants stably expressing mIIA (*A* and *G*), rIIA (*B*), hIIA (*C*), rV (*D* and *G*), hV (*E*), and mouse $cPLA_2$ (*F*) and mock-transfected cells (control, *A–G*) were sensitized for 30 min with IgE and then incubated for 10 min with (■) or without (▨) Ag. After collecting the culture supernatants, the release of β-HEX (*A–F*) and histamine (*G*) was assessed. Values shown in *A–F* are the mean ± SD of three to six experiments ($p < 0.05$ between unstimulated and stimulated cells in *A–G*, and $p < 0.05$ between $sPLA_2$-transfected and control cells after activation in *A–E* and *G*). A representative result of two reproducible experiments is shown in *G*. Expression of $sPLA_2$s (*A–E*, *inset*) and $cPLA_2$ (*F*, *inset*) was assessed by RNA blotting and immunoblotting, respectively.

that were distributed throughout the cytoplasm of unstimulated cells (Fig. 5). These signals for mIIA-WT moved in close proximity to the plasma membrane after IgE/Ag activation. Although cells expressing mIIA-KE4 gave a cytoplasmic punctate signal similar to that observed with mIIA-WT in the resting state, staining beneath the plasma membrane, which was obvious in activated mIIA-WT-expressing cells, was barely detected after IgE/Ag stimulation (Fig. 5). Moreover, the overall intensity of the signals for IIA-KE4 weakened after cell activation (Fig. 5), consistent with its secretion into the extracellular fluids.

Electron microscopic analyses of mIIA-WT-expressing cells demonstrated that the anti-$sPLA_2$-IIA Ab predominantly labeled the secretory granules, which are spherical bodies of varying density composed of vesicles and amorphous materials encased together in a limiting membrane (Fig. 6*A*). After $Fc_\epsilon RI$ cross-linking, mIIA-containing granules were fused with the plasma membrane, where positive mIIA signals were detected on the perigranular membranes, the area of fusion between the plasma and granule membranes in particular, and in the matrix components probably containing the granule proteoglycan serglycin (Fig. 6, *B* and *C*). The surface of the plasma membrane, apart from the opening granules, was not stained by the Ab.

$sPLA_2$-V, but not $sPLA_2$-IIA, promotes immediate PGD_2 biosynthesis

PGD_2 generation by the $sPLA_2$ and $cPLA_2\alpha$ transfectants 10 min after stimulation with IgE/Ag was examined next. Surprisingly,

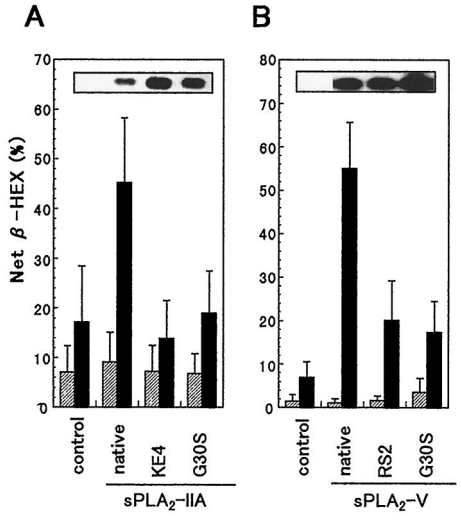

FIGURE 3. Both catalytic and heparin-binding domains are essential for the degranulation-enhancing effect of $sPLA_2$-IIA and V. RBL-2H3 transfectants stably expressing native and mutated mIIA (*A*) and rV (*B*) and mock-transfected cells were sensitized with IgE and then incubated for 10 min with (■) or without (▨) Ag, and β-HEX release was assessed (mean ± SD; $n = 4$; $p < 0.05$ between unstimulated and IgE/Ag-stimulated cells and between IgE/Ag-stimulated native $sPLA_2$-transfected and control cells). Expression of $sPLA_2$s was assessed by RNA blotting (*inset*).

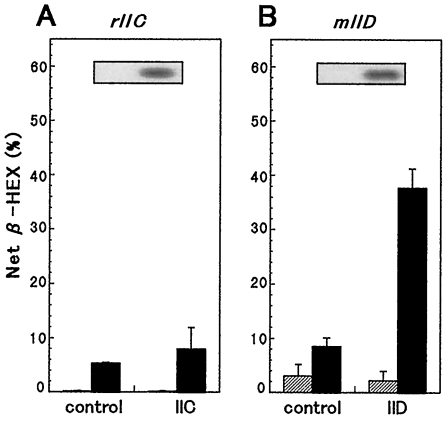

FIGURE 4. Degranulation of RBL-2H3 cells transfected with $sPLA_2$-IIC and -IID. RBL-2H3 cells stably transfected with rIIC (*A*) and mIID (*B*) and mock-transfected cells were sensitized with IgE and then incubated for 10 min with (■) or without (▨) Ag, and β-HEX release was examined (mean ± SD; $n = 3$; $p < 0.05$ between unstimulated and IgE/Ag-stimulated cells in *A* and *B*, and $p < 0.05$ between IgE/Ag-stimulated $sPLA_2$-IID-transfected and control cells in *B*). Expression of $sPLA_2$s was assessed by RNA blotting (*inset*).

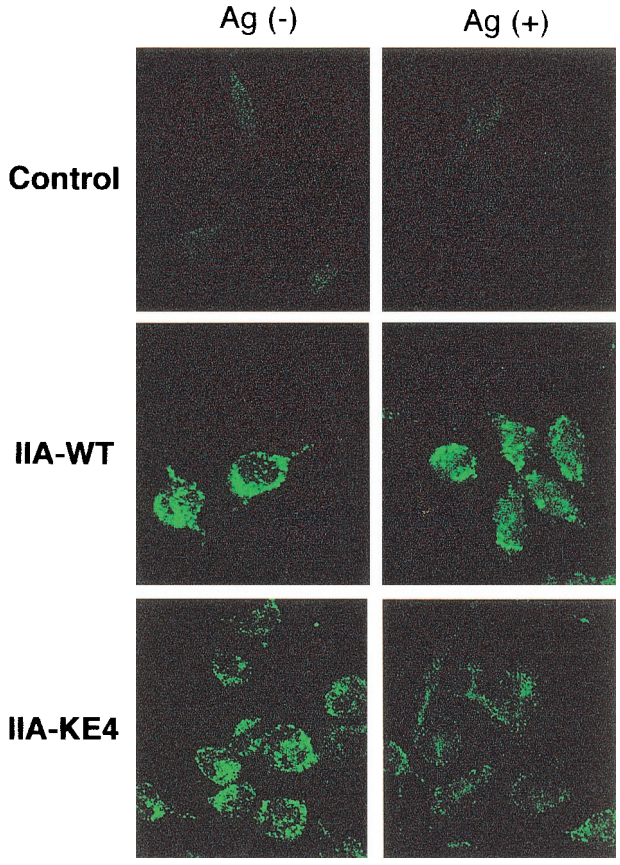

FIGURE 5. Subcellular distribution of $sPLA_2$-IIA as assessed by confocal microscopy. RBL-2H3 cells transfected with native mIIA (IIA-WT) and its heparin-nonbinding mutant (IIA-KE4), and control cells were sensitized with IgE and then incubated for 10 min in the presence (+) or the absence (−) of Ag. Cells were fixed, permeabilized, and sequentially incubated with anti-$sPLA_2$-IIA Ab and FITC-conjugated anti-rabbit IgG.

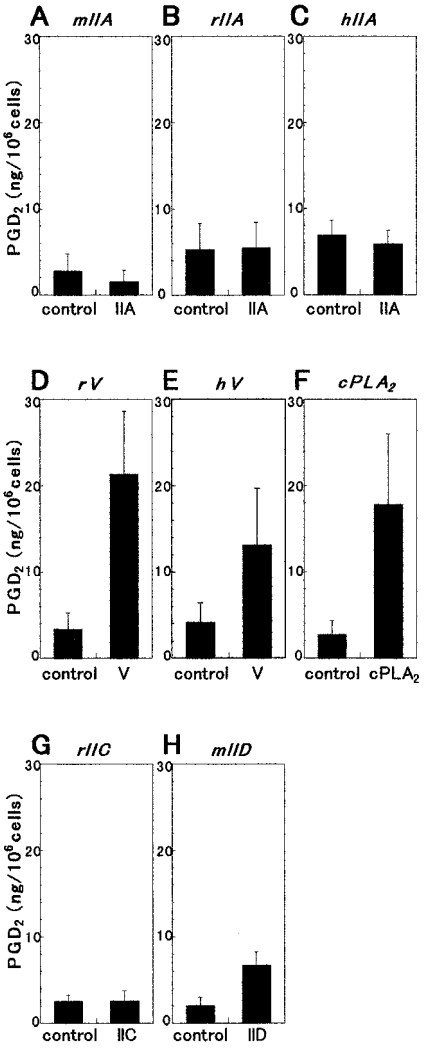

FIGURE 7. PGD_2 generation by RBL-2H3 cells transfected with various PLA_2s. RBL-2H3 transfectants stably expressing mIIA (*A*), rIIA (*B*), hIIA (*C*), rV (*D*), hV (*E*), mouse $cPLA_2$ (*F*), rIIC (*G*), and mIID (*H*) and mock-transfected cells (*A–H*) were sensitized for 30 min with IgE and then incubated for 10 min with Ag. PGD_2 released into the culture supernatants was quantified by RIA (mean ± SD; $n = 3–6$; $p < 0.05$ between $sPLA_2$-transfected and control cells in *D–F* and *H*). No production of PGD_2 was observed in the absence of Ag (not shown). The expression levels of each PLA_2 are shown in *insets* of Figs. 1 and 4.

PGD_2 generated by the transfectants expressing mIIA (Fig. 7*A*), rIIA (Fig. 7*B*), and hIIA (Fig. 7*C*) was almost equal to that by mock-transfected cells, whereas PGD_2 generation by cells expressing rV (Fig. 7*D*) and hV (Fig. 7*E*) was markedly augmented, reaching a level comparable to that produced by $cPLA_2\alpha$-transfected cells (Fig. 7*F*). The rIIC failed to affect PGD_2 generation (Fig. 7*G*), and mIID increased PGD_2 generation modestly (Fig. 7*H*). Thus, among the group II subfamily of $sPLA_2$s examined, only $sPLA_2$-V elicited an efficient PGD_2 biosynthetic response. The expression of endogenous COX-1 and hemopoietic PGD_2 synthase did not differ significantly, and that of endogenous COX-2 was undetectable among the transfectants used as assessed by immunoblotting (data not shown), indicating that the PGD_2 biosynthetic effect of $sPLA_2$-V was not due to alteration in the expression of downstream enzymes in the COX pathway as a result of forcible $sPLA_2$-V expression. As shown in Fig. 8, both rV-G30S and rV-RS2 failed to augment this PGD_2 generation, indicating that both the catalytic and heparanoid-binding domains are crucial for PGD_2 production, as in the case of the degranulation-enhancing effect (Fig. 3).

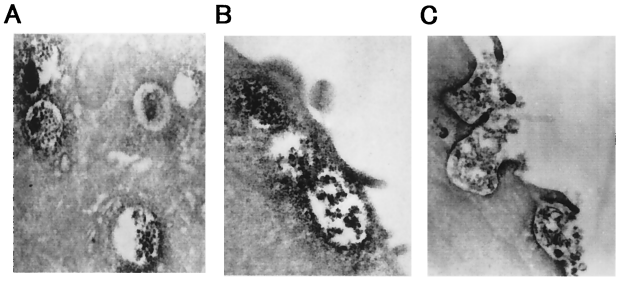

FIGURE 6. Subcellular distribution of $sPLA_2$-IIA as assessed by electron microscopy. RBL-2H3 cells transfected with mIIA were sensitized with IgE and then incubated for 0 min (*A*), 2 min (*B*), or 10 min (*C*) with Ag. After fixation, the cells were processed for immunoperoxidase staining using anti-$sPLA_2$-IIA Ab by electron microscopy as described in *Materials and Methods*. Reaction product was restricted to secretory granules and perigranular membranes, particularly the areas where the fusion between plasma and granule membranes take place.

Discussion

It has been reported that mast cells express $sPLA_2$-IIA, -IIC, and -V, but not $sPLA_2$-IB, the expression profiles of which differ among mast cell phenotypes (18–20). For instance, the mouse

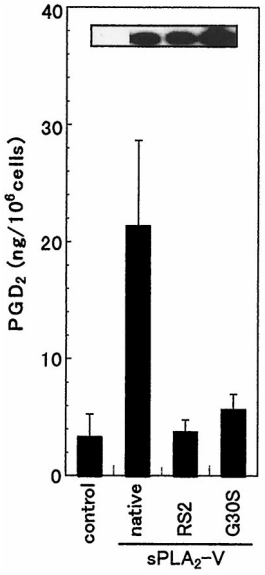

FIGURE 8. Both catalytic and heparin-binding domains are essential for the PGD_2-producing effect of $sPLA_2$-V. RBL-2H3 transfectants stably expressing native and mutated rV and mock-transfected cells were sensitized with IgE and then incubated for 10 min with Ag, and PGD_2 generation was examined (mean ± SD; $n = 3$; $p < 0.05$ between native $sPLA_2$-V-transfected and control cells). Expression of native and mutated rV was assessed by RNA blotting (*inset*).

mast cell line MMC-34 expresses $sPLA_2$-V, which participates in augmentation of COX-1-dependent immediate PGD_2 generation (18). Both $sPLA_2$-IIA and -V, but not $sPLA_2$-IB, are expressed in BALB/cJ mouse BMMC, and their expression increases after challenge with proinflammatory stimuli in accordance with COX-2-dependent delayed PGD_2 biosynthesis (19, 32). Rat serosal mast cells express $sPLA_2$-IIA but not $sPLA_2$-V, where concomitant induction of $sPLA_2$-IIA expression and delayed PGD_2 generation occur after stimulation with nerve growth factor (20). In the present study we have shown that BMMC from both BALB/cJ and C57BL/6J mice also express transcripts for $sPLA_2$-IID, -IIE, and -IIF, but not -X (Fig. 1). Although the expression of their functional proteins will need to be addressed in a future study using isozyme-specific Abs, our results nonetheless imply that mast cells have the capacity to express all known group II $sPLA_2$ subfamily members, the genes for which are clustered on mouse chromosome 4 (4, 14). This is reminiscent of the expression of chromosome 14-associated serine proteases (known as mouse mast cell protease MMCPs) in mouse BMMC (34, 35). Thus, it would be of particular importance to know the functional roles of each $sPLA_2$ in mast cells.

In an attempt to clarify the roles of the group II subfamily of $sPLA_2$s in mast cell activation, we have conducted transfection analyses using the rat mastocytoma cell line RBL-2H3 and demonstrated that those group II $sPLA_2$ subfamily members with heparin-binding capacity play a redundant role in enhancing the stimulus-induced exocytotic response. Cells transfected with the heparin-binding $sPLA_2$-IIA, -IID, and -V enzymes released more secretory granule-associated mediators, β-HEX and histamine, than control cells after $Fc_\epsilon RI$ cross-linking (Figs. 2–4). On the other hand, cells transfected with the heparin-nonbinding enzymes $sPLA_2$-IIC, $sPLA_2$-IIA-KE4, and $sPLA_2$-V-RS2 did not show enhanced degranulation. Catalytically inactive $sPLA_2$-IIA and -V mutants were without effect, implying that membrane phospholipid hydrolysis is an absolute requirement for augmenting degranulation. Even though overexpression studies do not always reflect the true functions of endogenous enzymes, our results are in line with previous pharmacological observations that $sPLA_2$-IIA inhibitors or a neutralizing Ab against $sPLA_2$-IIA blocked histamine release from rat serosal mast cells (23, 24). Although Fonteh et al. (36) have proposed that the action of $sPLA_2$ on mast cells depends on the M-type $sPLA_2$ receptor (37), our results argue against this hypothesis, because the rat $sPLA_2$ M-type receptor does not bind to rat and human $sPLA_2$-IIAs (38, 39).

Confocal laser and electron microscopic analyses revealed that $sPLA_2$-IIA, which resides in secretory granules in unstimulated cells, as has been observed by previous studies (32, 40), accumulates on the membranes where the granular membranes are fused with the plasma membrane (Figs. 4–6). This localization in a limited compartment was not observed with the heparin-nonbinding $sPLA_2$-IIA mutant KE4, which was largely released into the extracellular medium. Considering that lysophospholipids are fusogenic (27), an attractive model is that the heparin-binding $sPLA_2$s are associated with the particular membranous sites that are undergoing fusion through binding to certain heparan sulfate proteoglycans or other anionic components, and that this localization leads to spatially segregated lysophospholipid production, which enhances membrane fusion. This speculation is supported by the observation that the overexpression of $cPLA_2\alpha$, which acts on the perinuclear membrane in IgE/Ag-stimulated RBL-2H3 cells (41), did not affect degranulation (Fig. 2*G*). Chernomordik et al. (42) have reviewed the mechanisms by which lysophosphatidylcholine promotes fusion depending on which side of the membrane this lipid is produced. It is possible that lysophosphatidylcholine may be released by the heparin-binding $sPLA_2$s to promote degranulation on one side of the perigranular membranes. More recently, Schmidt et al. (26) have shown that the dynamic state between phosphatidic acid and lysophosphatidic acid alters membrane curvature and thereby influences granule invagination from the plasma membrane. This indicates that lysophospholipids are generally involved in both exocytosis and endocytosis processes. Moreover, hydrolysis of phosphatidylserine leads to the production of lysophosphatidylserine, a potent enhancer of mast cell degranulation (23, 24, 43). As heparin-binding group II $sPLA_2$s bind to anionic phosphatidic acid and phosphatidylserine vesicles in marked preference to zwitterionic phosphatidylcholine vesicles (44–46), these $sPLA_2$s may encounter these anionic phospholipids around the perigranular membranes of activated cells.

In contrast to the seemingly compensatory functions of the three heparin-binding $sPLA_2$s in degranulation, they displayed distinct roles in the regulation of immediate PGD_2 biosynthesis. Among them, $sPLA_2$-V exerted a potent enhancing effect on stimulus-dependent immediate production of PGD_2 (Fig. 7). This result is reminiscent of the previous observation that introduction of $sPLA_2$-V antisense into MMC-34 mast cells reduced immediate PGD_2 generation (18). Secretory PLA_2-IID also showed a modest effect, whereas $sPLA_2$-IIA was largely ineffective (Fig. 7). These observations are in marked contrast to PGE_2 generation observed in several adherent cells, where both $sPLA_2$-IIA and -V expression leads to AA release and PG formation (5–10). Site-directed mutagenesis strongly argues that the PGD_2-enhancing function of $sPLA_2$-V also depends on both catalytic and heparanoid-binding activities of this enzyme (Fig. 7).

In this context it is tempting to speculate that $sPLA_2$-V and, to a lesser extent, $sPLA_2$-IID cause membrane hydrolysis more efficiently than $sPLA_2$-IIA at the perigranular and plasma membrane fusion sites, where a large amount of AA, enough to reach the perinuclear COX-1, which is the dominant COX isozyme mediating the immediate phase of PGD_2 generation in mast cells (1, 2),

may be liberated. The amounts of AA released by $sPLA_2$-IIA may be below the threshold necessary to promote PGD_2 biosynthesis, even though lysophospholipids produced locally at the same time may be enough to accelerate the degranulation process. Another possibility is that $sPLA_2$-IIA would preferentially hydrolyze phospholipids that do not contain AA, whereas $sPLA_2$-V can act on those with AA, in the perigranular membranes. Alternatively, the ability of $sPLA_2$-V to elicit PGD_2 generation in RBL-2H3 cells may reflect the action of $sPLA_2$-V on phosphatidylcholine in the outer plasma membrane, because $sPLA_2$-V binds much more tightly to phosphatidylcholine-rich vesicles than does $sPLA_2$-IIA in vitro (47). In this model, $sPLA_2$-V may trigger degranulation at the fusion sites with the plasma membrane by locally producing lysophospholipids and is then secreted outside the cells and binds to the plasma membrane to produce AA from phosphatidylcholine for PGD_2 generation. In support of these speculations, $sPLA_2$-V is able to release AA far more efficiently than $sPLA_2$-IIA when added exogenously to human neutrophils (48) and to the mouse macrophage-like cell line $P388D_1$ (49). In fibroblastic cell lines HEK293 and 3Y1, expression of $sPLA_2$-IIA and -V leads to enhanced AA release and PGE_2 generation, probably not via action on the plasma membrane but on the caveolae-directed compartmentalized membranes (7). Nevertheless, additional studies are required to determine the mechanisms for different PGD_2 biosynthetic functions among $sPLA_2$s in mast cells.

Our present study has provided strong evidence that the heparin-binding group II $sPLA_2$ subfamily members, including IIA, IID, and V, function as enhancers of mast cell degranulation. This particular function of these $sPLA_2$s may not be limited to mast cells, as the involvement of an $sPLA_2$-IIA-like enzyme(s) in the exocytotic process has been reported in other cell types, such as neuroendocrine cells (22). In contrast to the redundant degranulation-enhancing role of these three $sPLA_2$s, $sPLA_2$-V (and, to a lesser extent, $sPLA_2$-IID) has the ability to elicit the PGD_2 biosynthetic response in mast cells. Although molecular mechanisms for the functional segregation of the related $sPLA_2$s in the PG biosynthetic response remain to be elucidated, these results together with our recent studies (5–9, 20, 31) imply that the actions of each $sPLA_2$ differ according to cell type and state of cell activation.

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