Secretery Phospholipase A2 Receptor-Mediated Activation of Cytosolic Phospholipase A2 in Murine Bone Marrow-Derived Mast Cells

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Secretory Phospholipase A$_2$ Receptor-Mediated Activation of Cytosolic Phospholipase A$_2$ in Murine Bone Marrow-Derived Mast Cells$^1$

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The current study examined the signal transduction steps involved in the selective release of arachidonic acid (AA) induced by the addition of secretory phospholipase A$_2$ (sPLA$_2$) isotypes to bone marrow-derived mast cells (BMMC). Overexpression of sPLA$_2$ receptors caused a marked increase in AA and PGD$_2$ release after stimulation of BMMC, implicating sPLA$_2$ receptors in this process. The hypothesis that the release of AA by sPLA$_2$ involved activation of cytosolic PLA$_2$ (cPLA$_2$) was next tested. Addition of group IB PLA$_2$ to BMMC caused a transient increase in cPLA$_2$ activity and translocation of this activity to membrane fractions. Western analyses revealed that these changes in cPLA$_2$ were accompanied by a time-dependent gel shift of cPLA$_2$ induced by phosphorylation of cPLA$_2$ at various sites. A noncatalytic ligand of the sPLA$_2$ receptor, p-amino-phenyl-$\alpha$-D-mannopyranoside BSA, also induced an increase in cPLA$_2$ activity in BMMC. sPLA$_2$ receptor ligands induced the phosphorylation of p44/p42 mitogen-activated protein kinase. Additionally, an inhibitor of p44/p42 mitogen-activated protein kinase (PD98059) significantly inhibited sPLA$_2$-induced cPLA$_2$ activation and AA release. sPLA$_2$ receptor ligands also increased Ras activation while an inhibitor of tyrosine phosphorylation (herbimycin) inhibited the increase in cPLA$_2$ activation and AA release. Addition of partially purified sPLA$_2$ from BMMC enhanced cPLA$_2$ activity and AA release. Similarly, overexpression of mouse groups IIA or V PLA$_2$ in BMMC induced an increase in AA release. These data suggest that sPLA$_2$ mediate the selective release of AA by binding to cell surface receptors and then inducing signal transduction events that lead to cPLA$_2$ activation. 


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hospholipase A$_2$s (PLA$_2$s)$^3$ are a family of enzymes that have the capacity to hydrolyze fatty acids from the sn-2 position of glycerophospholipids (for review, see Ref. 1). Groups I, IIa (IIA, IIC, IID, IE, IIF), III, V, and X PLA$_2$s are five sets of enzymes in a highly conserved family of secreted or extracellular PLA$_2$s (sPLA$_2$s) found in mammals (1–6). Other nonsecretory PLA$_2$s enzymes include group IV (IVA, IVB, IVC), cytosolic PLA$_2$s (cPLA$_2$s); group VI, calcium-independent PLA$_2$s; and group VII and group VIII, acetyl hydrolases (1, 7–9). The secretory family of enzymes has a number of features that distinguish them from other major PLA$_2$s families including a relatively low requirement for relatively high concentrations of calcium for catalysis. Many sPLA$_2$s are synthesized as proenzymes that contain signal peptide sequences that facilitate their release from cells. Although sPLA$_2$s have been studied extensively in mammals and in snake venoms, the physiological and pathophysiological roles of these enzymes are still not well known. Inspection of the numerous papers published in the past decade reveal that these sPLA$_2$s have the potential to mediate a wide range of biological activities including 1) potent antibacterial effects (10, 11); 2) a key component in phospholipid digestion; 3) production of lysophospholipids that contribute to electrophysiologic alterations that lead to arrhythmogenesis in the heart or altered airway permeability and surfactant properties in the lung (12–15); 4) serum markers and potential regulators of severe illnesses such as sepsis, shock, organ injury, and pancreatitis, all of which are linked to the development of adult respiratory distress syndrome and multiple organ failure (16–19); 5) regulators of platelet aggregation in hemorrhagic diseases (20); 6) proinflammatory components in diseases such as rheumatoid arthritis and asthma (21–24); 7) markers of cancer, initiators of cell proliferation in cancer cell lines, and a potent modifying locus in intestinal tumorigenesis in mice (25–30); and 8) enzymatic producers of arachidonic acid (AA) that contribute to eicosanoid generation (31–36). This daunting list of activities and diseases raises fundamental questions as to whether sPLA$_2$s cause or are merely associated with many of the aforementioned effects. It also raises the important question of how this family of enzymes could influence such a wide range of biological activities. To date, most of the biological activities of sPLA$_2$s have been attributed to its enzymatic capacity to hydrolyze membrane phospholipids. However, several findings have been difficult to reconcile merely based on this characteristic. For example, Nair and colleagues demonstrated that intradermal injection of inactivated sPLA$_2$ (no hydrolytic activity) causes similar phenotypic changes in skin to those observed with injection of fully active sPLA$_2$ (37).

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3 Abbreviations used in this paper: PLA$_2$, phospholipase A$_2$; sPLA$_2$, secretory PLA$_2$; cPLA$_2$, cytosolic PLA$_2$; LT, leukotriene; HSA, human serum albumin; MAPK, mitogen-activated protein kinase; NICI-GCMS, negative ion chemical ionization gas chromatography/mass spectrometry; [H$_2$], octadeuterated; [H$_3$], tritadeuterated; LA, linoleic acid; OA, oleic acid; AA, arachidonic acid; SA, stearic acid; APMP-BSA, p-amino-phenyl-$\alpha$-D-mannopyranoside BSA; BMMC, bone marrow-derived mast cells; DNP, dinitrophenol; PTX, pertussis toxin; PI3-K, phosphoinositide 3'-kinase; TX, thromboxane; PVDF, polyvinylidene difluoride; SEC, size-exclusion chromatography.

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Similarly, others have shown that the physiologic actions of sPLA₂ are not due to hydrolytic activity (38, 39). We have demonstrated that very low concentrations (low nanomolar) of certain sPLA₂ isotypes cause the selective release of AA (but not other more abundant fatty acids) from mast cells (bone marrow-derived mast cells (BMMC) and CFTL-15) and THP-1 cells (40). Several lines of evidence suggest that this response is not mediated by the capacity of sPLA₂ to hydrolyze membrane phospholipids but by specific binding of sPLA₂ to cell surface receptors.

In the last 10 years, different subtypes of membrane receptors for sPLA₂ have been identified in a variety of cell types by determining their affinities for various types of sPLA₂ (41, 42). Work by Arita and colleagues described the existence of a specific receptor family termed PLAP I that is abundant in brain and several other tissues and has high affinity for the binding of pancreatic-type sPLA₂ (41). More recently, receptors have been divided into two classes termed N-type receptors and M-type receptors. Lambeau and colleagues report that a major difference between N- and M-type receptors is their capacity to bind group III PLAP (beem venom) (42). For example, the N-type receptor associates very tightly with both group IB PLAP₂ and group III PLAP₂, while the rabbit muscle M-type receptor tightly binds groups IB and IIA PLAP₂ but does not associate with group III PLAP₂ (42). However, recent studies suggest that binding specificity may also depend on species or the glycosylation patterns of sPLA₂ isotypes or receptors (43–45).

M-type receptors have been cloned and sequenced and their structure shown to be homologous to the macrophage mannose receptor. Little is currently known about the signal transduction pathway that is initiated after occupancy of either the mannose or the sPLA₂ receptor. It is known that binding of group IB sPLA₂ to N-type receptor is calcium independent while the mannose receptor requires calcium for binding. Occupancy of the mannose receptor is also associated with tyrosine phosphorylation, while addition of group IBs sPLA₂ has been suggested to affect cell proliferation and AA release via its capacity to activate mitogen-activated protein kinase (MAPK) (46–48). The current study has focused on signal transduction events that are closely associated with AA mobilization induced by sPLA₂ in mast cells. These experiments reveal that addition of sPLA₂ isotypes to mast cells is associated with the activation and membrane translocation of group IV PLAP (sPLA₂).

Materials and Methods

Materials

Octadecanetetraol (5,6,8,9,11,12,14,15-²H) (²H₁₄) AA and trideuterated (²²H₁₄) stearic acid (SA) were purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Esssentially fatty acid-free human serum albumin (HSA), group IB PLA₂ (Naja naja) group III PLAP (beem venom), essential and nonessential amino acids, mouse IgE anti-dinitrophenol (IgE anti-DNP), heat-inactivated FBS, calcium ionophore A23187, DTT, pertussis toxin (PTX), HERG, and phosphoinositol 3-kinase (PI3-K) inhibitor, LY294002, were purchased from Sigma (St. Louis, MO). 1-Palmitoyl-2-[1-²H₈]-arachidonoyl-3-phosphocholine (55.6 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). Lyso-PC (3-8-²H₆) was purchased from Cambridge Isotope Laboratories (Ann Arbor, MI).

Mast cell culture and activation

BMMC were obtained from CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) and grown in RPMI 1640 culture medium (Life Technologies) containing 10% (v/v) FCS, 50 mM 2-ME, 2 mM L-glutamine, 1% (v/v) penicillin/streptomycin, and 0.1% gentamicin. The cell culture medium was supplemented twice a week with 50% WEHI supernatant fluid as a source of IL-3. After 3 wk in culture, BMMC (viability >98% determined by trypan blue exclusion) were used for activation and transfection studies.

BMMC were removed from culture media and washed (three times) using HBSS containing 0.25 mg/ml HSA. Cells were counted and resuspended in HBSS containing calcium at 5 × 10⁵ cells/assay. BMMC that had been sensitized with IgE anti-DNP (DNP-HSA), 50 μM p-a-phenylenediamine, 0.1 mM quercetin, 1 mM PMSF, 1 mM sodium orthovanadate, 0.1 mM NaF, 0.1 mM Na₂VO₃, 1 mM DTT, and 0.1% (v/v) fetal bovine serum (FBS) were obtained by transforming competent Escherichia coli (Valbonne, France). Mouse groups IIA and V PLA2 vectors were obtained from Dr. R. Lambeau (Centre National de la Recherche Scientifique-Institut de Pharmacologie Moleculaire et Cellulaire, Valbonne, France). Mouse groups IIA and V PLA₂ vectors were obtained from Dr. B. Kennedy (Merrck Frost, West Point, PA) and Dr. S. blonde (Harvard Medical School, Boston, MA), respectively. Enzyme immunoassay kit for detection of PGD₂ and thromboxane (TX) B₂ were purchased from Cayman Chemical (Ann Arbor, MI).

Overexpression of sPLA₂ receptor in mast cells

sPLA₂ M-type receptor cDNA was isolated from the pBluescript vector by digestion using XbaI followed by partial digestion using EcoRI. The 5.6-kb fragment was purifited using 1% agarose gel and asymmetrically subcloned into a mammalian expression vector (pCMV5 vector) provided by Dr. Zheng Cui (Wake Forest University School of Medicine, Winston-Salem, NC). Large quantities of the resulting plasmid (pCMV5/sPLA₂R) were obtained by transforming competent Escherichia coli followed by electroporation using the Wizard Plus plasmid purification system from Promega (Madison, WI). Before transfection studies were performed, the authenticity of pCMV5/sPLA₂ R was determined by sequencing using the dye terminator method. Before transfection, mast cells were initially placed in fresh culture media for 24 h. Subsequently, mast cells (4 × 10⁷ cells/ml) were transfected by electroporation. Briefly, 0.25 ml of cell suspension was incubated with nothing (control), with 15 μg pCMV5, or with pCMV5/sPLA₂ R and placed on ice for 10 min. Electroporation was performed using a Bio-Rad Gene Pulser (Richmond, CA) with voltage and capacitance set at 270 V and 960 μF, respectively. Cells were then dispersed in 10 ml of BMMC media supplemented with stem cell factor (100 ng/ml) or with IL-3 (10 ng/ml). A total of 65–75% of cells were recovered 48 h after transfection, and the viability of these cells was >90% as determined by trypan blue exclusion. The expression of sPLA₂ receptors was monitored at different time points after transfection by Western blot analysis as described below. Transfected and control cells were sensitized with 0.5 μg/ml IgE anti-DNP. Transfected BMMC were stimulated with 2 μg/ml Ag (DNP-HSA) or group IB PLAP₂ as described above, and mole quantities of fatty acids or prostanoids released into supernatant fluids were determined as described below.

Determination of sPLA₂ receptor expression by Western blot analysis

Amounts of sPLA₂ receptor expressed in mast cells were determined using cell lysates from 1 × 10⁷ cells. Lysates were obtained by incubating BMMC in lysis buffer (100 mM Tris-Cl, pH 7.5, containing 0.1% NaN₃, 2 mM EDTA, 1% Nonidet P-40, 1 mM NaVO₃, 50 mM NaF, 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone, 0.1 mM quercetin, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) for 10 min on ice. After
removal of nuclei by centrifugation, SDS-PAGE and Western blot analysis was performed. Briefly, extracts were mixed with an equal volume of 2× loading buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, and 10% mercaptoethanol, 0.05% bromophenol blue) and boiled for 5 min. Proteins were separated by SDS-PAGE on a 4–20% polyacrylamide gel. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, and the blots were incubated overnight with an anti-sPLA2 receptor Ab. Detection of the sPLA2 receptor was accomplished using HRP-conjugated goat IgG fraction to guinea pig IgG from Cappel and SuperSignal CL-HRP enhanced chemiluminescent substrate system from Pierce (Rockford, IL).

Quantitation of free fatty acids in supernatant fluids

After stimulation of BMMC, PGB2 (250 ng) was added to ethanol extracts of supernatant fluids as an internal standard, and the samples were concentrated under a stream of nitrogen. Mole quantities of PGB2 and TXB2 were determined using an enzyme immunoassay kit (Cayman Chemical) following the protocol provided by the manufacturer. Amounts of leukotrienes (LT) were determined by reversed-phase HPLC as previously described (31). Briefly, samples were suspended in 30% methanol in water and injected onto an Ultrasphere ODS column (2.1 × 250 mm; Supelco, Bellefonte, PA) that had been conditioned in a solvent that consisted of methanol/water/phosphoric acid (550:450:0.2 v/v, pH 5.7). The solvent was delivered at a flow rate of 0.4 ml/min, and products were monitored (270 and 206 nm) using an Hewlett-Packard diode array detection system. After 5 sPLA2 activity, reactions were eluted from the column by increasing the amount of methanol to 100% over 50 min. Mole quantities of LT were determined by UV spectroscopy.

Determination of cPLA2 activity in cytosol and membrane fractions

After stimulation of BMMC with sPLA2 receptor ligands, cells were washed (two times) with HBSS containing 0.25 mg/ml HSA and 5 mM EDTA. Cells were then suspended at 1 × 106 cells/ml in sonication buffer (10 mM HEPES, pH 7.4, containing 80 mM KCl, 1 mM EDTA, 40 mM leupeptin, 25 µM pepstatin, 10 mM NaF, 0.2 mM NaN3, and 4 mM DTT). Sonication (2 × 5 s) was performed using a probe sonicator (Heat System, Farmingdale, NY) at a power setting of 2 and 10% output. Cytosolic and membrane fractions were obtained after ultracentrifugation (100,000 × g for 1 h) and were maintained in sonication buffer containing 20% glycerol. Protein content of fractions was determined using the Coomassie Plus protein assay reagent (Pierce) and sPLA2 activity was measured using 400 pmol sonicated vesicles of 1-palmitoyl-2-[1-14C]-arachidonoyl-snglycero-3-phosphocholine as substrate and protein from cytosolic (50 µg) or membrane fractions (100 µg). sPLA2 activity was initiated by the addition of substrate to fractions that had been preincubated for 15 min at 37°C in an assay mixture that contained 5 mM EDTA. This preincubation was necessary for eliminating residual sPLA2 activity. Reactions were stopped by extracting lipids using the method of Bligh and Dyer (50). 1-Palmitoyl-2-[1-14C]-arachidonoyl-sn-glycero-3-phosphocholine and free fatty acid fractions were isolated by TLC on silica gel G developed in hexane/ethyl ether/formic acid (90:60:6 v/v/v) (31).

For size-exclusion chromatography (SEC), 300 µg of protein from cytosolic fractions of unstimulated and sPLA2-stimulated cells were loaded onto a Superose 12 column (Pharmacia, Piscataway, NJ), and proteins were eluted with 10 mM HEPES (pH 7.4) containing 80 mM KCl, 1 mM EDTA, 1 mM PMSF, and 1% glycerol at 0.4 ml/min. Fractions (1 min) were collected, and sPLA2 activity was determined using 100-µl aliquots of each fraction. Elution times for known protein standards (Bio-Rad) were obtained and used to calculate the molecular masses of the active fractions.

Western analysis of cPLA2 in cytosol and membrane fractions

Cytosolic and membrane fractions were prepared from BMMC that had been stimulated with sPLA2 receptor ligands as described above. Proteins in membrane fractions were concentrated by precipitation using 4 volumes of ethanol at −20°C. Equal amounts of cytosolic or membrane proteins were mixed with an equal volume of 2× SDS-PAGE loading buffer and then boiled for 5 min. Proteins were then separated on a 4–20% Tris/glycine polyacrylamide gel. Proteins were transferred onto PVDF membranes, and the blots were incubated using 400 pmol sonicated vesicles of cPLA2 with 1 µg/ml HRP-conjugated goat IgG fraction to guinea pig IgG from Cappel and SuperSignal CL-HRP enhanced chemiluminescent substrate system from Pierce (Rockford, IL).

Determination of MAPK activation

After BMMC stimulation with 100 nM group IB PLA2 for different periods of time, reactions were stopped using 4 volumes of ice-cold HBSS. Cell pellets were obtained after centrifugation and suspended in 50 µl lysis buffer. Protein amounts in lysates were determined using the Coomassie protein assay reagent (Pierce). Western analysis of cPLA2 in cytosol and membrane fractions were incubated with anti-cPLA2 Ab (1 µg/ml) in PBS-T containing 1% nonfat milk overnight. After washing with PBS-T (three times, 10 min), the blots were incubated with anti-rabbit IgG conjugated to HRP. Immunodetection was accomplished using the SuperSignal enhanced chemiluminescence reagents (Pierce).

Determination of Ras activation by affinity precipitation/immunoblotting

Ras activation was determined using the Ras activation kit (Upstate Biotechnology, Lake Placid, NY). Briefly, nonstimulated or group IB PLA2-stimulated BMMC were incubated in lysis/wash buffer (25 mM HEPES, pH 7.5, containing 0.15 M NaCl, 1% Igepal CA-630, 1 mM MgCl2, 1 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 25 mM NaF, 1 mM NaN3, and 2% glycerol). Ras-1 Ras Assay Reagent Agarose conjugate (10 µl) was added to each lysate and incubated at 4°C for 1 h, and the mixture was gently rocked at 4°C for 30 min. The agarose was then collected by microcentrifugation (14,000 × g, 5 s), washed (three times) using lysis buffer, and then resuspended in 2× SDS-PAGE buffer. Proteins were separated using a 4–20% Tris/glycine polyacrylamide gel and transferred to PVDF membranes. Immunodetection was accomplished using 1 µg/ml anti-Ras clone RAS10 Ab (overnight, 4°C) and goat anti-rabbit IgG conjugated IgG (Bio-Rad) at 1:5000 for 1.5 h at room temperature as primary and secondary Abs, respectively. Detection of the activated Ras was accomplished using SuperSignal CL-HRP enhanced chemiluminescent substrate system (Pierce).

Partial purification of endogenous sPLA2

sPLA2 was extracted from 1.5 × 107 BMMC using 0.18 M H2SO4 overnight at 4°C. The acid extract was then concentrated by ethanol precipitation and sPLA2 isolated by SEC using 0.1 M NaSO4, 0.1 M KH2PO4, pH 7.0, as the elution buffer at 0.5 ml/min. SEC was performed using an Altex Spherogel-TSK 300SW column (7.5 mm internal diameter × 30 cm, Beckman Coulter, Fullerton, CA). Protein elution was monitored by UV at 280 nm, fractions (0.5 ml) were collected, and sPLA2 activity was determined (31). The active fraction eluted from the SEC column with a molecular mass of ~14 kDa, and this procedure increased the sp. act. from 1.5 pmol/mg/min (acid extract) to 65 pmol/mg/min (active fraction from SEC). The active fraction was used to stimulate mast cells using protein concentrations ranging from 0 to 20 µg/ml.

Overexpression of mouse group IIA PLA2, or group V PLA2, in BMMC

Mouse group IIA PLA2 cDNA in a mammalian expression vector pSG5 or mouse group V PLA2 cDNA in a mammalian expression vector pSG5 was introduced into E. coli competent cells using the pUC18 cloning vector. E. coli competent cells were transformed and positive clones were selected with ampicillin. The authenticity of the construct was confirmed by restriction mapping using Eco RI (group IIA PLA2) or by PCR using the cDNA sequence as primer. Mouse group V PLA2 was amplified from mouse group V PLA2, 5′ primer, GATGAGGGCTCCTCAGACTG; 3′ primer, TAAGCAGAGAAGTTGGGGTAA from Gene Bank, accession no. AF162713.1). Transfection was performed by electroporation as described above for the sPLA2.
receptor. The expression of sPLA₂ was monitored at different time points after transfection by activity measurements in the culture medium. Mole quantities of fatty acids released into supernatant fluids were determined by NICI-GC/MS.

Data analysis

All data are expressed as means ± SEM of separate experiments. Statistics (p values) were obtained from Student’s t test for paired samples. Asterisks denote p < 0.05.

Results

sPLA₂ receptor expression is linked to the selective release of AA from stimulated BMMC

Our previous studies revealed that certain sPLA₂ isotypes selectively release AA from cells (BMMC, CFTL-15, and THP-1) that contain sPLA₂ receptors (40). To further examine the requirement of sPLA₂ receptors in the selective release of AA from mast cells, the sPLA₂ receptor was overexpressed in BMMC. BMMC transfected with a plasmid containing the cDNA of the M-type sPLA₂ receptor, but not control vector, expressed large quantities of a 180-kDa protein that was recognized by specific Abs for the sPLA₂ receptor (Fig. 1A).

To examine the relationship between sPLA₂ receptor expression and AA release, control, mock-transfected, and receptor-overexpressing BMMC were challenged with different concentrations of group IB PLA₂, and the release of AA was monitored by NICI-GC/MS. As shown in Fig. 1B, sPLA₂ induced the mobilization of AA from control and mock-transfected cells. However, more AA was mobilized from BMMC at all concentrations of sPLA₂ in cells overexpressing sPLA₂ receptor when compared with control or mock-transfected cells. It is important to note that the amount of AA released by receptor-overexpressing cells after incubation with low concentrations (5–20 nM) of sPLA₂ represents ~40% of the total cellular AA. This is likely the maximal amount of AA that can be released from the cell.

During mast cell stimulation with Ag, endogenous PLA₂ is rapidly released into supernatant fluid and competes with group IB PLA₂ for the same binding sites (31, 40). To determine whether this sPLA₂ induced AA release by binding to cell surface receptors, receptor-overexpressing cells were stimulated with Ag and AA release was monitored. Fig. 1C shows that Ag induced the release of AA in all three conditions (control, pCMV5, or pCMV5/sPLA₂R). However, Ag induced an ~8-fold higher release of AA from cells overexpressing the sPLA₂ receptor.

The role of sPLA₂ receptors in eicosanoid formation by stimulated mast cells was also examined. Mock-transfected cells formed PGD₂(16.5 pmol/5 × 10⁶ cell) and TXB₂ (15.8 pmol/5 × 10⁶) upon stimulation with 10 nM group IB PLA₂ for 5 min. Cells overexpressing sPLA₂ receptor formed more PGG₂ (51.3 pmol/5 × 10⁶ cells) and TXB₂ (25.7 pmol/5 × 10⁶ cells) than mock-transfected cells. Similarly, Ag stimulation induced more PGG₂ and TXB₂ formation in cells overexpressing sPLA₂ receptors than control or mock-transfected cells (data not shown). In contrast, levels of LTβ₄ were not altered when mock-transfected cells (5.8 ± 0.8 pmol/5 × 10⁶ BMMC, n = 3) or receptor-overexpressing cells (4.9 ± 1.4 pmol/5 × 10⁶ BMMC, n = 3) were stimulated with group IB PLA₂. In agreement with our previous studies, LTβ₄ was not detected by reversed-phase HPLC in the supernatant fluid when BMMC were stimulated with sPLA₂ for 5 min (31). Taken together, these data suggest that sPLA₂ receptor expression plays a role in the selective mobilization of AA and the formation of prostanoids when mast cells are stimulated with low nanomolar amounts of group IB PLA₂. The failure of sPLA₂ to increase LTβ₄ may be due to its incapacity to activate 5-LO in mast cells (31). Additionally, sPLA₂ mobilizes AA from mainly phosphatidylethanolamine, a pool that is not readily used for leukotriene biosynthesis in mast cells (51).

Activation of cPLA₂ by sPLA₂ receptor ligands

A potential mechanism by which sPLA₂ receptor ligands may induce the selective release of AA from mast cells is via cPLA₂ activation. To test this hypothesis, BMMC were treated with group...
IB PLA₂, and the activity of cPLA₂ was determined in cytosolic and membrane fractions. In control cells, >95% of cPLA₂ activity was located in the cytosol. After addition of different concentrations of group IB PLA₂, there was a significant increase (2-fold at 100 nM sPLA₂) in cPLA₂ activity in the cytosolic fraction (Fig. 2A). Additionally, incubation of BMMC with group IB PLA₂ resulted in a marked increase (5- to 15-fold) in membrane-associated cPLA₂ activity (Fig. 2B). To assure that the change in cytosolic activity was attributable to cPLA₂, cytosolic proteins were separated by gel filtration chromatography, and PLA₂ activities were assayed in 1-min fractions. SEC using a Pharmacia Superose 12 column (0.4 ml/min) indicated that the activity from cytosolic fractions obtained from untreated or group IB PLA₂-treated BMMC eluted at a time (36–38 min) that corresponded to a protein of ~100 kDa.

cPLA₂ activation is accompanied by a characteristic shift in the electrophoretic mobility that is associated with phosphorylation in several stimulated cells. To examine cPLA₂ phosphorylation after group IB PLA₂ stimulation, SDS-PAGE and immunodetection was performed on BMMC. Fig. 2C (upper panel) shows that group IB PLA₂ induces a time-dependent decrease in the mobility of cPLA₂ in the cytosol fraction. Furthermore, cPLA₂ is detected in the membrane fraction within 0.5 min after the addition of group IB PLA₂ to BMMC (Fig. 2C, lower panel). These data revealed that sPLA₂ receptor occupancy on BMMC is accompanied by the rapid activation and phosphorylation of cPLA₂ and translocation of the enzyme to membrane fractions.

We have previously shown that APMP-BSA, a noncatalytic ligand of the sPLA₂ receptor, induced the selective release of AA from BMMC (40). To determine whether cPLA₂ activation was required for the selective release of AA, we examined the effects of APMP-BSA on cPLA₂ activity in BMMC. Incubation of BMMC with APMP-BSA resulted in an increase cPLA₂ activity in homogenates from 12.5 ± 0.6 (basal activity) to 14.5 ± 0.4 (p < 0.05), 21.6 ± 4.8 (p < 0.05), and 20.4 ± 4.9 pmol/mg/min (n = 4), after 1, 2, and 5 min, respectively. These data revealed that sPLA₂ receptor occupancy alone and not catalytic activity is sufficient in inducing cPLA₂ activation in BMMC.

FIGURE 2. Activation of cPLA₂ by sPLA₂ receptor occupancy. A, BMMC were incubated without or with different concentrations of group IB PLA₂, and cytosolic fractions were prepared as described in Materials and Methods. cPLA₂ activity in these cytosolic fractions was determined, and the data were expressed as the amount of radiolabeled substrate hydrolyzed per milligram of protein. These data are the mean ± SEM of five separate experiments performed in duplicates (*, p < 0.05). B, cPLA₂ activity in membrane fractions (100 μg of protein) from untreated BMMC or from BMMC treated with different concentrations of group IB PLA₂ was determined as described above. These data are the mean ± SEM of four separate experiments (*, p < 0.05). C, Induction of cPLA₂ phosphorylation and translocation. BMMC were incubated with 100 nM group IB PLA₂ for different periods of time. After SDS-PAGE, the mobility of cPLA₂ obtained from the cytosolic fraction was determined by immunodetection and enhanced chemiluminescence (upper panel). Membrane fractions from the same experiment were concentrated, and proteins were separated by SDS-PAGE (lower panel). These data are representative of five separate experiments.

sPLA₂ receptor occupancy and MAPK activation

MAPK (p44/p42) has been shown to be responsible for cPLA₂ phosphorylation in some cells (52, 53). To determine whether the activation of cPLA₂ and subsequent selective release of AA by sPLA₂ may be mediated by MAPK pathways, we examined MAPK activation using a selective phospho p44/p42 Ab. As shown in Fig. 3A (upper panel), BMMC express both isoforms of MAPK (p44 and p42) and p44/p42 are constitutively phosphorylated in unstimulated BMMC (Fig. 3A, lane 1, middle panel). After incubation of BMMC with group IB PLA₂, there was transient phosphorylation 44/p42 MAPK (Fig. 3A, middle panel). Maximum phosphorylation (~2.5- and 1.5-fold for p44 and p42 MAPK, respectively) occurred within 0.5–2 min after stimulation with group IB PLA₂ (Fig. 3A, lower panel).

To determine whether catalytic activity is required to induce a signal through the sPLA₂ receptor, the effects of a noncatalytic ligand, APMP-BSA, were examined on p44/p42 MAPK phosphorylation. When equal amounts of protein were resolved by SDS-PAGE (Fig. 3B, upper panel), APMP-BSA induced a transient increase in p44/p42 MAPK phosphorylation (Fig. 3B, middle panel). Maximum phosphorylation (~2.5- and 1.4-fold for p44 and p42, respectively) occurred within 1–2 min after stimulation of BMMC with APMP-BSA (Fig. 3B, lower panel).
Decrease in cPLA₂ activation and AA release by MAPK inhibitor

Another set of experiments used a p44/p42 MAPK inhibitor (PD98059) to examine whether MAPK phosphorylation played a role in cPLA₂ activation and the selective release of AA from BMMC stimulated with group IB PLA₂. PD98059 significantly attenuated the increase in cPLA₂ activity induced by group IB PLA₂ (Fig. 4A). In addition, PD98059 inhibited AA release from BMMC stimulated with group IB PLA₂ (Fig. 4B). These data suggest that p44/p42 MAPK phosphorylation and cPLA₂ activation are involved in the selective release of AA when mast cells are incubated with group IB PLA₂.

Signal transduction and cPLA₂ activation

The activation of Ras by growth factor receptors or tyrosine kinases has been shown to result in the recruitment of cRAF, and activation of extracellular signal-regulated kinase/MAPK cascade (54, 55). To determine whether the activation of cPLA₂ by sPLA₂ is mediated via tyrosine kinase and Ras activation, we examined the effects of group IB PLA₂ on Ras activation after BMMC had been incubated with or without tyrosine kinase inhibitor. As shown in Fig. 5A, group IB sPLA₂ enhanced Ras activation in BMMC. Ras activation was attenuated by pretreatment of BMMC with tyrosine kinase inhibitor (herbimycin). Likewise, herbimycin inhibited cPLA₂ activation by group IB PLA₂-stimulated BMMC (Fig. 5B). Herbimycin also suppressed sPLA₂-induced AA release from 582.2 ± 57.8 to 426.2 ± 42.4 pmol/5 × 10⁶ BMMC (n = 4, p < 0.05). To examine other upstream signal transduction events that might link the sPLA₂ receptor to cPLA₂ activation, cPLA₂ activity and AA release were monitored in BMMC stimulated with group IB PLA₂ in the absence or presence of inhibitors of PI3-K or GTP-coupled receptors. cPLA₂ activity induced by sPLA₂ (68.1 ± 5.1 pmol/mg/min, n = 4) was not influenced by an inhibitor of PI3-K (LY294002, 58.1 ± 5.6 pmol/mg/min, n = 4) or PTX (59.2 ± 6.4 pmol/mg/min, n = 4) that uncouples membrane-bound receptors from GTP. Likewise, sPLA₂-induced AA release (1293.8 ± 84 pmol/5 × 10⁶ BMMC) was not influenced by pretreatment of BMMC with LY294002 (1345.6 ± 89.7 pmol/5 × 10⁶ BMMC) or by PTX (1464.3 ± 69.4 pmol/5 × 10⁶ BMMC). These data suggest that tyrosine phosphorylation is upstream of Ras activation and appears to be a major signal that links sPLA₂ receptors to cPLA₂ activation in mast cells.

Role of endogenous sPLA₂ in cPLA₂ activation and AA release

Recently, BMMC have been shown to contain primarily group IIA and group V sPLA₂ (56). To determine whether endogenous sPLA₂ might act in an autocrine fashion to activate cPLA₂ and induce AA release, BMMC were incubated with different concentrations of a sPLA₂-enriched extract. As shown in Table I, endogenous sPLA₂ induced an increase in cPLA₂ activity in BMMC. Furthermore, endogenous sPLA₂ released AA from BMMC (Table I). In contrast, levels of other unsaturated fatty acids (LA and OA) were not influenced when BMMC were incubated with partially purified endogenous sPLA₂ (Table I). To further test the hypothesis that group IIA or group V PLA₂ play key roles in AA release, these sPLA₂ isotypes were overexpressed in BMMC (Fig. 6A). BMMC expressing both isotypes release more AA into supernatant fluids than mock-transfected cells (Fig. 6B). Taken together, these data suggest that endogenous sPLA₂ can function in an autocrine fashion to activate cPLA₂ and enhance AA release from BMMC.

Discussion

The present study suggests that an important link between addition of sPLA₂ isotypes to cells and the selective release of AA is the receptor-mediated activation of cPLA₂. Furthermore, cPLA₂ activation and AA release appear to be mediated, in part, through tyrosine phosphorylation and p44/p42 MAPK activation but not through PI3-K activation or G protein-coupled receptors. Several lines of evidence support this sequence of molecular events: 1) mast cells overexpressing the sPLA₂ receptor release more AA and produce more prostanooids than mock-transfected cells; 2) sPLA₂ receptor ligands induce a dose- and time-dependent increase in cPLA₂ activity; 3) the increase in activity is accompanied by cPLA₂ phosphorylation and a shift of the activity from cytosolic to membrane fractions; 4) sPLA₂ receptor ligands induce the transient phosphorylation of p44/p42 MAPK; 5) an inhibitor of MAPK phosphorylation (PD98059) significantly attenuates sPLA₂-induced activation of cPLA₂ and the release of AA; 6) an inhibitor of tyrosine phosphorylation (herbimycin) attenuates cPLA₂ activation and AA release; and 7) addition of a sPLA₂-enriched extract from mast cells and the overexpression of
mouse groups IIA or V sPLA$_2$ in mast cells result in enhanced cPLA$_2$ activation and AA release.

Our previous results indicate that some sPLA$_2$ isotypes induce the selective release of AA from certain cells (BMMC, THP-1, and CFTL-15) but not from HL-60 cells. Interestingly, sPLA$_2$ isotypes can induce the selective release of AA from cells only if these cells express the sPLA2 receptor (40). The current study suggests that the selective release of AA from mast cells is initiated by the binding of sPLA$_2$ to its receptor. Over the past decade, sPLA$_2$ receptors have been described based on binding properties, tissue distribution, or species distribution (42, 43). A cloned sPLA$_2$ receptor termed M-type has homology with the macrophage mannose receptor and DEC-205 of dendrites, suggesting that these proteins may belong to a new class of receptors that possess Ag-recognition properties. Although several biological functions have been attributed to the occupancy of the sPLA$_2$ receptor, the molecular events responsible have not been elucidated.

The current study suggests that sPLA$_2$ receptor occupancy is linked to cPLA$_2$ activation, hence the selective release of AA and the formation of prostanoids by low nanomolar concentrations of sPLA$_2$. Our previous study showed that APMP-BSA, a sPLA$_2$ receptor agonist that has no hydrolytic activity, also induces the selective release of AA from cells expressing the sPLA$_2$ receptor (40). This observation raised the interesting possibility that binding alone (without hydrolytic activity) is sufficient to release AA from receptors.

Table I. Effects of endogenous sPLA$_2$ on cPLA$_2$ activity and fatty acid release$^a$

<table>
<thead>
<tr>
<th>sPLA$_2$ Extract (µg)</th>
<th>cPLA$_2$ Activity in Homogenate (pmol/mg/min)</th>
<th>AA (pmol/5 × 10$^6$ cells)</th>
<th>LA (pmol/5 × 10$^6$ cells)</th>
<th>OA (pmol/5 × 10$^6$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.5 ± 0.8</td>
<td>76.3 ± 16.8</td>
<td>29.6 ± 2.1</td>
<td>171.5 ± 14.7</td>
</tr>
<tr>
<td>5</td>
<td>15.0 ± 1.1$^*$</td>
<td>114.5 ± 37.7</td>
<td>31.5 ± 3.1</td>
<td>191.2 ± 15.3</td>
</tr>
<tr>
<td>10</td>
<td>16.9 ± 0.9$^*$</td>
<td>198.2 ± 24.7$^*$</td>
<td>29.7 ± 3.0</td>
<td>185.9 ± 22.2</td>
</tr>
<tr>
<td>20</td>
<td>15.2 ± 1.2$^*$</td>
<td>161.7 ± 23.7$^*$</td>
<td>29.3 ± 2.6</td>
<td>169.7 ± 13.1</td>
</tr>
</tbody>
</table>

$^a$ BMMC were challenged with different concentrations of partially purified sPLA$_2$ for 5 min at 37°C. The reactions were stopped by pelleting the cells and cPLA$_2$ activity in cell homogenates, and fatty acids (AA, LA, OA) released into supernatant fluids were determined as described in Materials and Methods. These data are the mean ± SEM of four separate experiments.

$^*, p < 0.05.$
cPLA₂ in AA metabolism has recently been demonstrated in a calcium binding (CALB) domain (57–59). An important role for calcium-dependent translocation of the enzyme from a cytosolic transfected with vector alone, group IIA, or group V PLA₂ cDNA were mast cells from cPLA₂ knockout mice do not display immediate or AA from a number of mammalian cells. cPLA₂ is activated by phospholipids. An ideal candidate for such an activity is group IV lytic activity within cells that hydrolyzes AA from membrane selective release of AA, there must be recruitment of another hydrolytic enzyme within mast cells can mobilize AA by activating cPLA₂. Our recent studies have identified groups IIA and V sPLA₂ as the major sPLA₂ isotypes found in mast cells (31, 35, 56). Consequently, it was important to determine whether endogenous sPLA₂ isotypes within mast cells can mobilize AA by activating cPLA₂. Our data suggest sPLA₂ extracted from mast cells induced cPLA₂ activation and the selective release of AA. Likewise, overexpression of groups IIA or V sPLA₂ in mast cells resulted in an increase in AA release. These data suggest that the selective release of AA from mast cells by sPLA₂ receptor ligands is due to the recruitment of cPLA₂.

It has been suggested that sPLA₂ can affect cell proliferation and AA release via its capacity to activate MAPK (46–48). The current study has focused on proximal signal transduction events that are closely associated with AA mobilization and metabolism. As mentioned above, AA release in many cells is due to the activation and translocation of cPLA₂ to a membrane location, and this process is often accompanied by phosphorylation of cPLA₂ by MAPK, p42/p44 MAPK initially appeared to be the major enzymes responsible for cPLA₂ phosphorylation (52). However, other kinase pathways have also been shown to activate cPLA₂ (61, 62). In fact, in stimulated platelets, inhibitor studies suggest that p38 kinase rather than extracellular signal-related kinases are responsible for cPLA₂ phosphorylation (63). In contrast, studies using these same inhibitors show that p44/p42 MAPK and not p38 kinase are involved in AA release from mast cells (64). The current study shows that during sPLA₂ receptor occupancy, there is transient phosphorylation of p44 and p42 MAPKs. Moreover, incubation of mast cells with a selective p44/p42 MAPK inhibitor (PD98059) significantly blocked cPLA₂ activation and AA release from BMMC in response to sPLA₂. Because PD98059 only partially reversed cPLA₂ activation, it is likely that other signal transduction pathways or communication between various pathways occur in activated mast cells (64).

Whereas cPLA₂ activation has been linked to various MAPKs in many cell types, there is paucity of information regarding upstream signals that link sPLA₂ receptor activation to MAPK and cPLA₂. In human astrocytoma cells, sPLA₂-induced cPLA₂ activation has been shown to be insensitive to PTX. In contrast, sPLA₂-induced calcium release is sensitive to PTX, caffeine, and herbimycin (49). In lung cancer cells, cPLA₂ has been recognized as a Ras-inducible regulator of eicosanoid biosynthesis (65). Therefore, there are several potential pathways that link receptor occupancy to cPLA₂ activity within cells. The current data suggest that tyrosine kinase plays a role in sPLA₂-induced cPLA₂ activation. Importantly, Ras activation was also implicated in the sPLA₂ receptor-mediated activation of cPLA₂. Further evidence for a role in tyrosine kinase/Ras activation is shown by the attenuation of sPLA₂-induced Ras activation by herbimycin. Together, these data provide evidence that binding of sPLA₂ to a receptor initiates several critical molecular events including the activation of tyrosine kinase, Ras, and p44/p42 MAPK. This sequence of molecular events is one potential mechanism by which sPLA₂ induces cPLA₂ activation and the selective release of AA.

Recent studies have identified groups IIA and V sPLA₂ as the major sPLA₂ isotypes found in mast cells (31, 35, 56). Consequently, it was important to determine whether endogenous sPLA₂ isotypes within mast cells can mobilize AA by activating cPLA₂. Our data suggest sPLA₂ extracted from mast cells induced cPLA₂ activation and the selective release of AA. Likewise, overexpression of groups IIA or V sPLA₂ in mast cells resulted in an increase in AA release. These data suggest that sPLA₂ isotypes in mast cells can act on sPLA₂ receptors in an autocrine fashion during mast cell activation. Overall, our data suggest that the physiologic roles of sPLA₂ may not only be mediated by the hydrolytic effects of this family of enzymes on phospholipids and cellular membranes, but also by

**FIGURE 6.** Overexpression of mouse groups IIA and V PLA₂ in BMMC and effects on cPLA₂ activity. A, BMMC transfected with vector alone or with vector containing groups IIA PLA₂ or V PLA₂ cDNA were placed in HBSS buffer for 10 min. sPLA₂ activity in supernatant fluid was determined as described in Materials and Methods. These data are expressed as a percentage of activity in vector-transfected BMMC and are the mean ± SEM of four separate experiments (*, p < 0.05). B, BMMC transfected with vector alone, group IIA, or group V PLA₂ cDNA were placed in buffer for 10 min at 37°C. Cell pellets were removed by centrifugation, and mole quantities of AA in supernatant fluids were determined by NICI-GC/MS. These data are expressed as a percentage of vector-transfected BMMC and are the mean ± SEM of four separate experiments.
their capacity to bind to cell surface receptors. Upon receptor binding, sPLA\(_2\) induces a signal transduction pathway that leads to cPLA\(_2\) activation and AA release. Further studies will be necessary to determine the signal transduction pathways that are associated with other sPLA\(_2\)-induced biological activities.

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References


