Presence of the M-type sPLA₂ receptor on neutrophils and its role in elastase release and adhesion

CHRISTOPHER C. SILLIMAN, ERNEST E. MOORE, GARRET ZALLEN, RICARDO GONZALEZ, JEFFREY L. JOHNSON, DAVID J. ELZI, XIANZHONG MENG, KOHJI HANASAKI, JUN ISHIZAKI, HITOSHI ARITA, LIHUA AO, KELLY M. ENGLAND, AND ANIRBAN BANERJEE

Bonfils Blood Center and Departments of Pediatrics and Surgery, University of Colorado School of Medicine, Denver 80262; Department of Surgery, Denver Health Medical Center, Denver, Colorado 80204; and Shionogi Research Laboratories, Shionogi & Company, Limited, Osaka 541, Japan

Received 20 December 2001; accepted in final form 24 May 2002

SECRETORY PHOSPHOLIPASE A₂ (sPLA₂) has been implicated in diverse inflammatory states including arthritis, pancreatitis, acute chest syndrome in patients with sickle cell anemia, and multiple organ failure (MOF) following traumatic injury (1, 4, 24, 37, 38, 56, 69). Moreover, circulating levels of sPLA₂ have been identified as a sensitive marker of mortality following pancreatitis, traumatic injury, acute chest syndrome, and sepsis (4, 62, 67, 70, 71, 74, 75, 79). Traditionally, the biological activity of these enzymes has been attributed to their ability to cleave lipids, causing release of arachidonate and resulting in eicosanoid generation (1, 4, 24, 37, 38, 56, 67, 69, 83). These lipid mediators then may precipitate cellular activation or even the systemic inflammatory response and predispose the patient to organ injury (55).

The presence of sPLA₂ receptors on a variety of cells, including smooth muscle, fibroblasts, Swiss 3T3 cells, and astrocytes, has been documented (2, 3, 27, 28, 32, 43–45, 48, 49, 59, 60, 85). Two distinct sPLA₂ receptors have been identified: the M-type, present on smooth muscle, and the N-type, found on cells of neural lineage (2, 3, 27, 28, 32, 43–45, 47–49, 59, 60, 85). These different receptors display selective affinities for the various groups of mammalian and reptile sPLA₂ (2, 3, 27, 28, 30, 32, 43–45, 47–49, 59, 60, 85). In addition, these receptors serve important physiological functions as ligand occupancy affects cell physiology, manifested by migration of vascular smooth muscle cells, inhibition of acetylcholine release, proliferation of Swiss 3T3 cells, and tumor invasion (6, 22, 34, 40). In concordance with their association with inflammation, a number of investigators have documented the proinflammatory effects of receptor occupancy (19, 20, 73, 82).

The identification of sPLA₂ receptors raises the possibility that ligand occupancy on leukocytes may produce inflammation through receptor activation. This study seeks to ascertain whether granulocytes, which are important cellular effectors of the systemic inflammatory response (10, 64, 80), possess sPLA₂ receptors. We hypothesize that sPLA₂ can directly affect polymorphonuclear neutrophil (PMN) function through occupancy of a membrane receptor. Previous studies from our laboratory demonstrated that sPLA₂-IB elicited...
the release of elastase in the presence of EGTA, but in the presence of EGTA, sPLA2-IA did not cause elastase release (84). Therefore, we focused our investigations to determine the presence of sPLA2 receptors on the PMN membrane, the type of receptor based on immunoreactivity, the changes in PMN function elicited by receptor ligation, and activation of mitogen-activated protein (MAP) kinases conferred by ligand occupancy.

**MATERIALS AND METHODS**

**Materials.** All chemical reagents, including a fluorescein isothiocyanate (FITC) protein labeling kit and sPLA2-IA (Naja naja) and sPLA2-IB (porcine, specific activity 600 U/mg), were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified. All reagents employed in these experiments were endotoxin free and were made from sterile water (USP) for intravenous administration, purchased along with sterile 0.9% saline for intravenous injection (USP), from Baxter Healthcare (Deerfield, NY). All buffers were made from injection-grade USP solutions obtained from the following manufacturers: 10% CaCl2, Fujisawa USA (Deerfield, IL); 23.4% NaCl, 20 Meq/ml KCl, and 50% MgSO4, American Regent Laboratories (Shirley, NY); and sodium phosphates (278 mg/ml monobasic and 142 mg/ml dibasic) and 50% dextrose, Abbott Laboratories (North Chicago, IL). In addition, all solutions were sterile-filtered before use by employing Nalgene MF75 series disposable sterilization filter units, purchased from Fischer Scientific (Pittsburgh, PA). Human sPLA2-IA was the kind gift of Dr. Hubertus Stockinger (Department of Biochemicals, Boehringer Mannheim, Mannheim, Germany), and the units of enzymatic activity were determined via a commercial assay done in Germany employing a specific sPLA2-IA phosphatidylserine substrate (results not shown). Cy3-labeled goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA); fluorescein-labeled wheat germ agglutinin (WGA) and mannose-BSA were procured from Molecular Probes (Eugene, OR); and Ficoll-Paque and protein A-Sepharose columns were purchased from Pharmacia Biotech (Uppsala, Sweden). Serum-free P1000 medium was obtained from Eiken Chemical (Tokyo, Japan). Polyacrylamide gel electrophoresis (PAGE) was performed on a Bio-Rad mini-gel system by using 4–20% Tris-HCl gels from Bio-Rad (Hercules, CA). Tris-SDS solution was obtained from Owl Scientific (Woburn, MA). Rabbit polyclonal antibodies to activated dual-phosphorylated (Thr180/Tyr182) p38 and activated dual-phosphorylated (Thr202/Tyr204) p42/44 MAPKs as well as a phosphatidylcholine from soybeans was prepared in a solution containing 150 mM NaCl and 5 mM CaCl2, pH 8.9 (10 mM NaOH). Ten milliliters of this emulsion were placed in a test tube, and the change in pH was monitored at room temperature. A solution containing 2.5 U/ml sPLA2 or buffer was added to the reaction medium and incubated for 10 min. During the reaction, 50-μl aliquots of 10 mM NaOH were added to maintain a pH of 8.9, and the total volume of NaOH required and the exact reaction time (min) were recorded. The dilution factor corresponds to the initial dilution of the sPLA2. sPLA2 activity was calculated from the equation

\[
sPLA2 (U/ml) = \frac{[NaOH] \times 1,000}{(\text{dilution factor}) \times 0.2} \quad (\text{reaction time})
\]

where [NaOH] is the volume of NaOH added to maintain the pH of the reaction mixture at pH 8.9 and NaOHbl is the volume of NaOH added to the “blank” to reach and maintain a pH of 8.9. This assay was used to assess the ability of 5 mM EGTA or 100 μM pBS to inhibit enzymatic activity (25). In these assays, 1 unit of enzyme activity was defined as the amount of enzyme needed to hydrolyze 1 μM 1-phosphatidylcholine into 1-α-lysophosphatidylcholine and fatty acid per minute. These assays were not employed to determine the units of activity for the respective sPLA2 enzymes, which were performed by the manufacturer and confirmed via electron mail.

**PMN isolation.** PMNs were isolated from heparinized whole blood of healthy volunteers after informed consent was obtained under a protocol approved by the Colorado Multiple Institutional Review Board. Briefly, the isolation procedure consisted of dextran sedimentation, Ficoll hypaque gradient centrifugation, and hypotonic lysis of contaminating red blood cells (63). The final cell population was >99% PMNs as determined by differential staining and was >99% viable as determined by trypan blue exclusion.

**FITC labeling of sPLA2.** The instructions for use of the FITC protein labeling kit (Sigma) were followed exactly. Briefly, types sPLA2-IA and sPLA2-IB were suspended in 0.1 M sodium carbonate (pH 9.0). FITC at ratios of 20:1, 10:1, and 5:1 (FITC:sPLA2) dissolved in dimethyl sulfoxide (DMSO) was added per 2 mg of sPLA2. The solution of sPLA2 and FITC was stirred in the dark for 2 h. Running the solution through a Sephadex 20 column separated the unbound FITC. The labeling index was determined for each ratio by calculating the FITC-to-protein molar ratio for optimal labeling by measuring the maximal absorbance of fractions at wavelengths of 280 and 495 nm, respectively. The optimum labeling indices were 38.5 and 37.8% for the 10:1 FITC: sPLA2 ratio, respectively, for two different kits.

**Cellular association of sPLA2 with PMNs.** To determine whether exogenously added sPLA2 was associated with PMNs, isolated PMNs were incubated with 10 μM sPLA2 and examined by digital microscopy. To examine the effects of enzymatic activity on the cellular association of exogenous sPLA2, the reaction mixture, which contained PMNs, was pretreated for 1 min with 5 mM EGTA or 100 μM pBS before the addition of type IA or type IB FITC-labeled sPLA2, which was dissolved in sterile saline. The associations of labeled, enzymatically inactive type IA and type IB sPLA2 with PMNs were examined by digital microscopy. Because digital microscopy can only examine a small number of cells per sample, we measured the number of sPLA2-labeled PMNs by using flow cytometry. PMNs (106) were warmed to 37°C or kept at 4°C, examined by digital microscopy. To examine the effects of enzymatic activity on the cellular association of exogenous sPLA2, the reaction mixture, which contained PMNs, was pretreated for 1 min with 5 mM EGTA or 100 μM pBS before the addition of type IA or type IB FITC-labeled sPLA2, which was dissolved in sterile saline. The associations of labeled, enzymatically inactive type IA and type IB sPLA2 with PMNs were examined by digital microscopy. Because digital microscopy can only examine a small number of cells per sample, we measured the number of sPLA2-labeled PMNs by using flow cytometry. PMNs (106) were warmed to 37°C or kept at 4°C,
incubated with EGTA for 30 s, and incubated with 10 μg/ml FITC-labeled sPLA2-IA or sPLA2-IB for 10 min at 37°C or for 30, 60, and 120 min of incubation at 4°C. The samples were assayed by flow cytometry and 5,000 events counted per sample. Controls for these experiments included unlabeled sPLA2-IA or sPLA2-IB and unbound FITC. These experiments were repeated by using PMNs from three different healthy donors.

**SPLA2 receptor saturation.** Saturation of the putative sPLA2 receptor was accomplished as a necessary preliminary experiment before we embarked upon formal Scatchard analysis. The reaction mixture, with PMNs, was incubated with EGTA for 1 min to bind Ca²⁺, and then increasing concentrations of labeled sPLA2-IB were added until the PMN-associated fluorescence was maximally intense as determined by digital microscopy. The quantification of brightness per PMN for all experiments was done by masking FITC signals, calculating the number of FITC pixels per 25 cells, and averaging the mean intensity in pixels/PMN for each sPLA2 concentration. The saturation concentrations were confirmed by flow cytometry.

**Scatchard analysis.** The existence of a putative sPLA2 receptor was investigated by using standard techniques for Scatchard analysis with fluorescently labeled ligands, except that digital microscopy rather than flow cytometry was employed (5, 26). Briefly, the reaction mixture, containing the isolated PMNs, was pretreated with 5 mM EGTA for 1 min to bind extracellular Ca²⁺ and was then incubated with different concentrations of FITC-labeled sPLA2-IB for 10 min at 37°C. The PMNs were fixed with 4% paraformaldehyde and examined by digital microscopy, and brightness was calculated by masking the individual PMNs as explained in sPLA2 receptor saturation. Analysis of the curves was accomplished by using standard techniques as previously reported (5, 7, 26, 35). These experiments were repeated three times for all concentrations of FITC-labeled sPLA2, employing two different lots of FITC-labeled sPLA2-IB. An estimate of total brightness was repeated for both aliquots of FITC-labeled sPLA2 by using the labeled protein alone at the highest concentration employed and serial dilutions of two orders of magnitude. Last, to determine whether unlabeled sPLA2 could competitively compete with the labeled compound, isolated PMNs, pretreated with EGTA, were exposed to various concentrations of unlabeled sPLA2 just before (5 s) the addition of a saturating concentration of labeled sPLA2, and the brightness was quantitated by digital microscopy. These competitive assays were performed by using methods previously published (46).

**Preparation of anti-sPLA2 receptor antibody.** A polyclonal antibody against the recombinant soluble form of the mouse M-type sPLA2 receptor was prepared as follows. cDNA encoding the signal peptide and the presumed extracellular domains (amino acids 1–1365) of the mouse sPLA2 receptor were placed in a mammalian expression vector that utilizes the SRα promoter for the recombinant transcription. This soluble sPLA2 receptor expression plasmid was introduced into CHO-K1 cells (American Type Culture Collection) with the calcium phosphate poration method, and cell clones were selected on the basis of acquired resistance to G418 (1 mg/ml). Selected cell lines were then cultured in serum-free P1000 medium, and the conditioned medium was collected. By using a sPLA2-IB-affinity column, soluble mouse M-type sPLA2 receptor was purified as a single 180-kDa band on SDS-PAGE as described previously (28). After immunization in rabbits, antisera was prepared, and the antibodies were purified by using a protein A-Sepharose column. Specificity of the antibody was demonstrated by immunohistochemistry detection of antibody signal in the glomerular region of mouse kidney that was abolished by the addition of soluble sPLA2 receptor and was not detected in kidneys of sPLA2 receptor-deficient mice. In addition, Western blot detected a single band in mouse lung, uterus, and spleen that completely disappeared in the tissues of the sPLA2 receptor-deficient mice (results not shown). Furthermore, the antibody did display immunoreactivity in the membranes of a human smooth muscle cell line, cells that express the M-type sPLA2 receptor on their membranes (results not shown).

**Separation of proteins from neutrophil lysates and immunoblotting for the sPLA2 receptor.** The proteins from PMN lysates (1 × 10⁶ cell equivalents) were separated by PAGE, transferred to polyyvinylidene difluoride (PVDF) membrane, and incubated with a rabbit polyclonal antibody to the M-type sPLA2 receptor. A secondary incubation was performed with a goat anti-rabbit HRP polyclonal antibody, and the immunoreactivity was visualized by using an enhanced chemiluminescence system with exposure of X-ray film.

**sPLA2 receptor staining.** Immunofluorescence staining was applied to detect and localize M-type sPLA2 receptors on PMNs. PMNs were isolated and suspended in Krebs-Ringer phosphate (pH 7.35) with 2% dextrose (KRPD). A rabbit polyclonal antibody (see above) against the M-type sPLA2 receptor was added to the PMN suspension in a 1:10 volume dilution and incubated at 4°C for 30 min. After two washes with KRPD, the PMNs were suspended and treated with Cy3-labeled goat anti-rabbit IgG (1:400 dilution) at 4°C for 45 min. Cells were washed and then fixed in 2% paraformaldehyde for 20 min. After being thoroughly washed with PBS, the Cy3-labeled PMNs were smeared onto glass slides, counterstained with fluorescein-labeled WGA (5 μg/ml, for cell surface staining) and bis-benzimide (1 μg/ml, for nuclear staining), and then mounted with aqueous antiquenching medium. To assess the specificity of the immunostaining, an aliquot of cells were suspended in KRPD at 4°C without antibody and treated with Cy3-labeled goat anti-rabbit IgG (1:400 dilution) for 45 min at 4°C. Fixation and counterstaining were processed under identical conditions. The PMNs were analyzed by digital microscopy.

**Separation of proteins from neutrophil lysates and immunoblotting for MAPK activation.** Isolated PMNs (1.25 × 10⁶) were incubated at 37°C over a time course of 0.5–5 min with sPLA2. The cells were then pipetted into sample buffer (Tris-SDS-Eagle’s basal medium) and fresh inhibitor mix (40 mM sodium orthovanadate, 1 M nitrophenylphosphate, 100 mM PMSF, and 1 mg/ml leupeptin) and boiled for 5 min. The proteins were then separated by 4–20% gradient PAGE and transferred to PVDF membranes. The membranes were blocked with 5% BSA and then incubated with either a rabbit anti-dual-phosphorylated (Tyr185/Thr186) p38 MAPK antibody or a rabbit anti-dual-phosphorylated (Tyr202/Thr204) ERK1/2 antibody. The membranes were then washed with Trizma-buffered saline (TBS) plus 0.1% Tween and incubated with an HRP-conjugated goat anti-rabbit antibody. Immunoreactivity was visualized by enhanced chemiluminescence and subsequent exposure to X-ray film. Density of the bands was measured by using a Hewlett Packard model 6201C scanner and Scion 4.02 software downloaded from the National Institutes of Health web site.

**PMN elastase release assay.** PMNs (1.5 × 10⁶) were warmed in 1.5-ml tubes to 37°C in a shaking water bath, preincubated with saline or 5 mM EGTA, and then stimulated with sPLA2-IA (10 and 100 U/ml), sPLA2-IB (10 and 100 U/ml), and sPLA2-IIA (1–10 U/ml) for 5 min at 37°C. PMNs were also incubated with mannose-BSA (1–10 μM) for 5 min at 37°C, and in selected experiments these PMNs were

*AJP-Cell Physiol* • VOL 283 • OCTOBER 2002 • www.ajpcell.org
RESULTS

Inhibition of sPLA₂ activity. As a necessary preliminary, an in vitro assay based on the change in pH was employed to determine the effects of EGTA Ca²⁺ chelation and BPB incubation on sPLA₂ activity. Chelation of Ca²⁺ with EGTA (5 mM) inhibited the activity of sPLA₂-IA, sPLA₂-IB, and sPLA₂-IIA by 98 ± 3, 98 ± 4, and 99 ± 2%, respectively, supporting previous reports that Ca²⁺ is required for sPLA₂ enzymatic activity (25). In addition, pretreatment with 100 μM BPB also inhibited sPLA₂-IA, sPLA₂-IB, and sPLA₂-IIA activity by 98.5 ± 4 to 99 ± 3%. Lesser concentrations of EGTA and BPB did not completely inhibit sPLA₂ activity for any of the types tested (results not shown). Thus EGTA preincubation is an effective inhibitor of sPLA₂ activity, which was confirmed by the use of a second antagonist. These assays were not employed to ascertain the units of sPLA₂ activity but to determine whether Ca²⁺ chelation and BPB treatment were efficacious methods to inhibit sPLA₂ activity (25).

Membrane binding of sPLA₂. Because sPLA₂ may act as a ligand to provoke elastase release, we examined the cellular association of sPLA₂ with PMNs (69). These studies used FITC-labeled sPLA₂-IA and sPLA₂-IB and both flow cytometry and digital microscopy. Digital microscopy revealed strong cellular association of sPLA₂-IB but not sPLA₂-IA (Fig. 1). Because enzymatically inactive sPLA₂-IB was avidly associated with the PMN membrane, and digital microscopy is only able to examine a limited number of cells from a given sample, the number of PMNs labeled with inactive sPLA₂ was measured by flow cytometry (Fig. 2). The incubations with labeled sPLA₂ were either identical to those of the digital microscopy, 10 min at 37°C, or the PMNs were incubated for 30, 60, and 120 min at 4°C. Controls for these experiments included unlabeled sPLA₂ and unattached “free” FITC. Compared with PMNs treated with unlabeled sPLA₂-IB (Fig. 2, peak A), PMNs incubated with FITC-linked sPLA₂-IB for 30 min at 4°C demonstrated almost a 2-log shift in mean fluorescence intensity at 30 min (Fig. 2, peak B) without marked increases with longer incubation times (results not shown), and ~98% of the PMNs evidenced cellular association of labeled-sPLA₂. In addition, incubation of PMNs with FITC-labeled sPLA₂ for 10 min at 37°C yielded a 10-fold shift in mean fluorescence intensity (unlabeled sPLA₂: 8.4 ± 1.2 vs. labeled sPLA₂: 127 ± 25) and a 24.4 ± 1.0 shift compared with buffer with free FITC-treated PMNs with labeling of 70% of the cells (results not shown). In addition, FITC-
labeled sPLA₂-IA did demonstrate minimal cell association of this sPLA₂ when EGTA was deleted, implying that the enzymatic activity of type IA sPLA₂ may occur in the PMN plasma membrane (results not shown). These findings provide supportive evidence that a receptor for sPLA₂ resides on the PMN membrane.

Scatchard analysis of the sPLA₂ receptor on PMNs. Before Scatchard analysis was initiated, it was important to determine saturation of the putative receptor. FITC-labeled sPLA₂-IB was added in increasing concentrations of isolated PMNs, and the fluorescent intensity was calculated. Saturation of the putative sPLA₂ receptor was found to be 2.77 fM (Fig. 3A, n = 3). After the receptor saturation was determined, Scatchard analysis was completed (37°C with a 10-min incubation time) and the graphical relationship was revealed (Fig. 3B). From the shape of the curve, the receptor is heterogeneous and there is negative cooperativity. From standard calculation, the dissociation constant (Kᵋ) is 167 pM, and the calculated number of receptors per cell is ~1,672 (5, 7, 35). To ensure that the labeled sPLA₂ affinity for the PMN membrane was specific for the protein itself and not due to the labeling of sPLA₂ to FITC, isolated PMNs, pretreated with 5 mM EGTA, were incubated with FITC-labeled sPLA₂-IB in the presence of unlabeled sPLA₂-IB (Fig. 3C). As the concentration of unlabeled sPLA₂ was increased, the amount of cell-associated FITC-tagged sPLA₂ decreased, demonstrating that the unlabeled sPLA₂ was able to compete for the sPLA₂ receptor sites on the PMN.

Presence and location of the M-type sPLA₂ receptor. To determine the presence of the M-type sPLA₂ receptor on PMNs, the proteins from whole cell lysates were separated, transferred to nitrocellulose, and probed with a polyclonal antibody to the M-type sPLA₂ receptor. A single band of immunoreactivity was identified at ~185 kDa (Fig. 4). To confirm the membrane location of this receptor, we employed a triple stain technique and digital microscopy. Isolated PMNs were incubated with the polyclonal antibody, fixed with paraformaldehyde, and smeared onto slides. After appropriate washing, the membranes were labeled with fluorescein-labeled WGA (green), the nuclei were labeled with bis-benzimide (blue), and the receptor im-

---

**Fig. 2.** Cellular association of FITC-labeled sPLA₂ by flow cytometry. Flow cytometric analysis was performed on PMNs incubated with unlabeled (peak A) and FITC-labeled sPLA₂-IB (peak B) for 30 min at 4°C. The number of events (counts) is depicted as a function of the FITC intensity. Data are representative of PMNs from 4 separate donors and 2 different preparations of FITC-labeled sPLA₂.

**Fig. 3.** Saturation of PMN sPLA₂ receptors with labeled sPLA₂-IB (A), Scatchard analysis of sPLA₂-IB binding to PMNs (B), and inhibition of cell-associated labeled sPLA₂-IB with unlabeled sPLA₂-IB (C). PMNs (1 × 10⁶) were incubated with differing concentrations of FITC-labeled sPLA₂-IB (porcine) for 5 min at 37°C, immediately fixed with 4% paraformaldehyde for 5 min at 4°C, smeared onto charged slides, and mounted with antifluorescent medium. The amount of cellular fluorescence was determined by digital microscopy. A: determination of receptor saturation employing increasing concentrations of labeled sPLA₂-IB. Data consist of PMNs from 3 separate donors and 2 different lots of FITC-labeled sPLA₂-IB. B: Scatchard analysis of differing concentrations of FITC-labeled sPLA₂ with a set number of PMNs (1 × 10⁶). Data are from 3 separate donors and 2 different lots of FITC-labeled sPLA₂-IB. C: competitive inhibition of cellular fluorescence by incubation of PMNs with a saturating concentration of FITC-labeled sPLA₂-IB and increasing concentrations of unlabeled sPLA₂-IB. Data are from 3 separate donors and 2 different lots of both FITC-labeled sPLA₂-IB and unlabeled sPLA₂-IB.
200 kD

Fig. 4. Immunoblot of PMN lysates for the M-type sPLA2 receptor. PMNs (5 \times 10^6) were lysed with Laemmli sample buffer in the presence of 1 mM PMSF and 10 μg/ml leupeptin. The proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The blot was probed with a polyclonal antibody to the human M-type sPLA2 receptor, and bands of immunoreactivity were visualized by employing an enhanced chemiluminescence detection system and X-ray film. The blot is representative of 4 identical experiments.

Immunoreactivity was labeled with a Cy3-labeled secondary antibody (red) (Fig. 5). The bright red signal shown in Fig. 5C (anti-sPLA2 receptor antibody + Cy3-labeled secondary antibody) demonstrates the presence of the sPLA2 receptor on the PMNs. The lack of red signal in secondary antibody (Cy3-only stained cells) (Fig. 5). The bright red signal shown in Fig. 5C is not due to nonspecific binding of the Cy3 secondary antibody. The receptor labeling correlated directly with the membrane labeling. The sPLA2 receptor plus Cy3 secondary antibody-produced area of red staining overlapped the green membrane staining as shown by the yellow color (green overlapping red) in Fig. 5D. The Cy3-only stained cells had no evidence of any yellow intensity. Thus the receptor localizes to the PMN membrane, because the intact, live PMNs were incubated with the polyclonal antibody to the M-type receptor before fixation.

sPLA2-mediated elastase release. To confirm that sPLA2 was able to affect degranulation, PMNs were treated with sPLA2-IA, sPLA2-IB, and sPLA2-IIA, and elastase release was measured (Fig. 6). To inhibit sPLA2 activity, the reaction mixture, which includes the PMNs, was treated with 5 mM EGTA for 1 min before the addition of sPLA2. All three types of sPLA2 caused the release of significant amounts of elastase from PMNs compared with buffer-treated control cells [sPLA2-IA: 100 U/ml = 16.0 ± 0.2%, 10 U/ml = 13.5 ± 3.0%; sPLA2-IB: 100 U/ml = 32.0 ± 6.0%, 10 U/ml = 12.5 ± 2.0%; and sPLA2-IIA: 10 U/ml = 30.0 ± 4.0%, 1 U/ml = 15.0 ± 3.0% vs. control: 5.1 ± 0.1% (n = 8, P < 0.05)]. Type IB sPLA2 had a response at 100 U/ml that was equivalent to that of PAF (200 nM)-primed and fMLP (1 μM)-activated elastase release (46.1 ± 4.5%).

Having confirmed that “active” sPLA2 directly stimulated PMNs to release elastase, we tested the effects of enzymatically inactive sPLA2 on PMN elastase release. The reaction mixture, which included the PMNs, was pretreated with 5 mM EGTA for 1 min before the addition of sPLA2. In the presence of EGTA, sPLA2-IA did not cause release of elastase, indicating that sPLA2-IA requires enzymatic function to facilitate elastase release (Fig. 7). In contrast, EGTA preincubation of sPLA2-IB did not affect its ability to cause elastase release from PMNs (Fig. 8). Finally, sPLA2-IIA was able to stimulate elastase release in the presence of EGTA, but its activity was attenuated by 39.7 ± 0.3% (Fig. 6). This attenuation was statistically significant at 10 U/ml (P < 0.05, n = 8). These data indicated that sPLA2-IA, from N. naja venom, caused PMN elastase release through its enzymatic function but not secondary to ligand occupancy. Type IB sPLA2 demonstrated significant PMN elastase release regardless of its enzymatic activity, implying stimulation of a membrane receptor. Type IIA sPLA2 induced PMN elastase.

Fig. 5. Membrane localization of the sPLA2 receptor on PMNs. PMNs (1 \times 10^6) were incubated with a rabbit polyclonal antibody against the M-type sPLA2 receptor for 30 min at 4°C, washed twice, and incubated with Cy3-labeled goat anti-rabbit antibody (red; C and D). PMNs were fixed with 2% paraformaldehyde (20 min), smeared onto glass slides, and counterstained with 1 μg/ml bis-benzimide nuclear stain (blue; A–D) and FITC-labeled wheat germ agglutinin (WGA) membrane stain (green; B and D). The receptor immunofluorescence (red) colocalized with the membrane stain (green), producing a yellow color (D). Controls stained with the Cy3-linked rabbit IgG alone did not demonstrate any red color (results not shown). Results are representative of the results of the PMNs from 2 separate, healthy donors.
release that appeared to be the result of both enzymatic activity and receptor activation. Inclusion of sPLA2-IIA was important because of its reported role in systemic inflammation in humans (23, 67, 71, 76, 77) As controls, we determined the role of extracellular Ca\(^{2+}\) in fMLP-mediated elastase release in both buffer-pretreated and PAF-primed PMNs. Chelation of extracellular Ca\(^{2+}\) moderately decreased fMLP-elicted elastase (fMLP: 10.3 ± 0.5% vs. fMLP + EGTA: 8.3 ± 0.7%); however, chelation of extracellular Ca\(^{2+}\) did significantly decrease PAF priming of fMLP-mediated elastase release (PAF/fMLP: 48 ± 3.2% vs. PAF/fMLP + EGTA: 32 ± 6.8%), though not nearly to the levels of fMLP alone (10.3 ± 0.5%) or the buffer-treated controls (4 ± 0.5%). These results were concordant with previous data that did not find a strict requirement for extracellular Ca\(^{2+}\) in PMN exocytosis (50, 51, 61, 68).

Because it has been postulated that the sPLA2 receptor may represent the mannose receptor on PMNs and other leukocytes, we employed mannose bound to BSA to determine its effects on PMN elastase release in the presence or absence of enzymatically inactive sPLA2-IIA (73). As demonstrated in Table 1, mannose-BSA did not cause a significant release of elastase from PMNs, nor did it affect the sPLA2-IIA-mediated elastase release in the presence of EGTA. Furthermore, BSA alone did not affect elastase release from control or sPLA2-IIA stimulated PMNs (Table 1, results not shown). In addition, mannose-BSA primed fMLP-mediated elastase release 5.1 ± 1.0-fold, whereas BSA alone did not affect PMN elastase release in response to fMLP (results not shown).

sPLA2-mediated PMN adhesion. To ensure that sPLA2 affected other PMN functions in a nonenzymatic manner, we assayed the ability of enzymatically inactive (EGTA preincubation) sPLA2-IB and sPLA2-IIA to affect PMN adhesion to fibrinogen-coated plates. It is important to note that β2-mediated integrin adhesion of PMNs to RGD ligands is not dependent on extracellular Ca\(^{2+}\); rather, PMNs require extracellular Mg\(^{2+}\) for firm adhesion (11, 15, 33). S PLA2-IB and sPLA2-IIA were employed because they both elicited PMN elastase independent of their enzymatic activity. Both S PLA2-IB and S PLA2-IIA caused PMN adhesion to fibrinogen-coated plates in a concentration-dependent

![Graph](https://via.placeholder.com/150)

**Fig. 6.** S PLA2 causes PMN elastase release independent of its enzymatic activity. Isolated PMNs were pretreated with buffer or 5 mM EGTA for 1 min at 37°C to abrogate the enzymatic activity of S PLA2. The PMNs were then incubated with buffer control, 10 or 100 U/ml S PLA2-IA, 10 or 100 U/ml S PLA2-IB, or 1 or 10 U/ml S PLA2-IIA for 5 min at 37°C. Elastase release into the supernatant was measured as described. Open bar (C) represents the elastase release by buffer-treated control PMNs (P < 0.05).

**Fig. 7.** S PLA2 elicits PMN adhesion to fibrinogen-coated plates. PMNs were loaded with \(^{31}C\)Cr, washed twice, warmed to 37°C, and added to fibrinogen-coated plates. After settling, the PMNs were incubated with 5 mM EGTA to inhibit S PLA2 activity and were then treated with saline (C), the positive controls (2 μM platelet-activating factor (PAF), 1 μM N-formyl-methionyl-leucyl-phenylalanine (fMLP), or 200 ng/ml PMA, 1–100 U/ml S PLA2-IB, or S PLA2-IIA for 30 min at 37°C. The plates were covered and centrifuged inverted at 200 g for 5 min. The remaining adherent PMNs were lysed with 0.1% SDS and placed into scintillation cocktail, and the amount of radioactivity was counted for each sample. Data represent the results of PMNs from 9 healthy donors. *Statistical significance compared with control PMNs (P < 0.05).
Data are representative of 3 experiments. The sPLA2 are members of a large family of enzymes that are capable of producing biologically active compounds and signaling molecules from membrane lipids (13, 14, 25). Such phospholipase activity has been delineated in the production of eicosanoids, the remodeling pathway of PAF biosynthesis, and the generation of other intracellular signaling molecules (13, 14, 25, 67). Both eicosanoids and PAF have been implicated in a number of PMN-mediated syndromes of organ injury.

**DisCUSSION**

p38 MAPK with diminution of the signal at 5 min. Neither BPB nor EGTA caused p38 MAPK activation (data not shown). In addition, because p42/44 MAPK is another important signaling molecule in PMNs, we tested the ability of sPLA2 to activate this MAPK. Incubation of PMNs with sPLA2 did not activate (dual phosphorylation) p42/44 MAPK (data not shown) (16, 53).

**MAPK inhibition.** To demonstrate that sPLA2-mediated elastase release was dependent on activation of p38 MAPK, PMNs were preincubated with the specific p38 MAPK inhibitor SB-203580 or the selective MAP kinase kinase (MEK)1/2 inhibitor PD-98059 (12). In these experiments, elastase release was measured after the addition of BPB-inactivated sPLA2. Inhibition of p38 MAPK decreased elastase release in a concentration-dependent fashion with maximal inhibition (36 ± 3.4%) at 10⁻⁵ M SB-203580 (P < 0.05, n = 8; Fig. 9). In contrast, the specific MEK1 inhibitor PD-98059, which obviates p42/44 MAPK activity, did not inhibit elastase release.

**Study design.** The sPLA2 are members of a large family of enzymes that are capable of producing biologically active compounds and signaling molecules from membrane lipids (13, 14, 25). Such phospholipase activity has been delineated in the production of eicosanoids, the remodeling pathway of PAF biosynthesis, and the generation of other intracellular signaling molecules (13, 14, 25, 67). Both eicosanoids and PAF have been implicated in a number of PMN-mediated syndromes of organ injury.
Increased circulating levels of sPLA2 have been demonstrated in a number of PMN-mediated syndromes, including acute lung injury, postinjury MOF, acute pancreatitis, and arthritis (4, 62, 67, 70, 71, 74, 75, 79). Moreover, the levels of sPLA2 and/or sPLA2 activity in these maladies correlate with patient outcome (56, 70, 71, 74, 75, 84). For example, the level of sPLA2 activity in sickle cell patients accurately predicts the patients who will develop acute chest syndrome, a life-threatening variant of acute lung injury that is particular to patients with sickle cell anemia (71). Few data with respect to the sPLA2 receptor in PMN-mediated organ injury exist; however, sPLA2-IB receptor-deficient mice are resistant to endotoxic shock (30). Although the precise mechanisms of protection against endotoxic shock is not known, these studies provide provocative evidence that sPLA2 receptors may have relevance in inflammatory diseases (30). Collectively, these clinical data and animal models strongly suggest that the activity of sPLA2 is associated with inflammation and the development of acute, multiple organ injury, a syndrome that is PMN mediated (55).

The current study has demonstrated that the M-type sPLA2 receptor was present on the PMN membrane in numbers similar to the PAF receptor, ~1,600 for sPLA2 vs. 1,100 for PAF (45). Receptor occupancy caused activation of p38 MAPK that was directly linked to PMN elastase release. The cellular association of inactive sPLA2-IB and sPLA2-IIA with PMNs and the inability of inactive sPLA2-IA to associate with the PMN membrane are entirely consistent with previous reports of the smooth muscle M-type receptor affinity for specific sPLA2 isoenzymes (40, 43–45, 49). Furthermore, PMN receptor occupancy by both sPLA2-IB and sPLA2-IIA caused PMN adhesion to RGD ligands, elastase release, and activation of p38 MAPK, all in the absence of phospholipase activity. Furthermore, pretreatment of PMNs with mannose-BSA had little effect on sPLA2-IIA-mediated elastase release and provides evidence that the M-type sPLA2 receptors and the mannose receptor on PMNs comprise distinct entities (73). sPLA2-IIA activation of p38 MAPK was rapid, within 30–60 s, and this time course was consistent with both elastase release, which begins at 3 min and is maximal at 5 min (results not shown and Fig. 8), and activation of p38 MAPK by other PMN agonists, including fMLP or PAF (18, 39, 57, 58). To demonstrate that p38 MAPK activation leads to the release of elastase, we used the p38 inhibitor SB-203580 to block activated p38 MAPK. SB-203580 attenuated the release of elastase but did not eliminate it completely, similar to SB-203580 inhibition of fMLP activation of PMN oxidase (58). Although previous reports have documented activation of p42/44 MAPKs as the result of sPLA2 receptor occupancy in astrocytes (31, 41, 54), we were unable to demonstrate p42/44 MAPK activation in PMNs following incubation with sPLA2. Moreover, specific MEK1 inhibition with PD-98059, which abrogates p42/44 MAPK activation, did not affect sPLA2 receptor mediated elastase release. The reason for the incomplete inhibition with SB-203580 may lie in parallel signaling pathways. There are multiple signaling pathways for elastase release in the PMN that involve the activation of a number of other kinases, including both protein kinase C and phosphatidylinositol 3-kinase (17, 65, 78). It is likely that there are redundant signaling pathways involved in sPLA2 receptors that merit further investigation.

The ability of active sPLA2-IA and sPLA2-IIA to affect PMN elastase release implies cleavage of membrane lipids. Previous studies have demonstrated that sPLA2 induces the surface expression of β2-integrins on the PMN, an activity ascribed to its phospholipase activity; moreover, sPLA2-IB can also cause the release of β-glucuronidase from macrophages (72, 73, 82). Lipid extraction of the supernatant of isolated PMNs does not contain a detectable amount of chloroform-soluble compounds as assayed by diode array spectroscopy at 235 nm or by gas chromatography mass spectroscopy (results not shown). The activation of PMNs by exogenous secretory phospholipase activity was not expected and merits further investigation.

To date, most of the sPLA2 binding data of the M-type receptor employed transfection of COS, 293, or CHO cells, with one transfection yielding only transient expression of the human M-type receptor (3, 32). Such transfection models are attractive but may have little correlation with native receptor ligand interactions due to differences in cellular processing of the receptor protein or the requirements of accessory proteins in the binding of ligand. Two reports have suggested the possibility of such accessory proteins for binding sPLA2 to the M-type receptor; moreover, a recent review stressed the need for sPLA2 binding studies employing cells with “native” receptors (21, 29, 36).

To our knowledge, the existence of sPLA2 receptors on PMNs has not been previously reported, and their presence may further explain the role of sPLA2 in inflammatory processes that implicate the release of inflammatory mediators. Recently, sPLA2-IA and sPLA2-IIA were shown to activate human pulmonary macrophages, causing both release of β-glucuronidase and production of IL-6; moreover, inhibition of sPLA2 activity did not affect β-glucuronidase release; however, treatment of these macrophages with p-aminophenyl-mannopyranoside-BSA (mp-BSA), a mannose receptor agonist, caused similar release of β-glucuronidase (73). In addition, pretreatment with mp-BSA did
not affect β-glucuronidase release by sPLA₂-IIA but augmented release by sPLA₂-I A (73). Taken together, these findings indicate that both the sPLA₂-II A and sPLA₂-IIA appear to activate human macrophages in lieu of their enzymatic activity and through either the mannone receptor or an sPLA₂-specific receptor (73). In contrast, the current study characterizes the function of sPLA₂ receptors on PMNs and provides evidence that these receptors could be involved in PMN-mediated inflammation, a role in which sPLA₂ has historically been placed (1, 4, 22, 24, 34, 37, 38, 40, 56, 67, 69, 74–77, 79). This proinflammatory effect from sPLA₂ receptor stimulation may be clinically important in diverse arenas, including PMN-mediated organ dysfunction. Both acute lung injury and postinjury MOF have been associated with increased levels of sPLA₂; however, the methods used to measured sPLA₂ include ELISAs, or activity assays that employed varied substrates, making comparisons among these studies difficult (56, 62, 67, 74–77). Further work employing a uniform measurement of sPLA₂ activity is required to identify clinically relevant levels of these enzymes. Thus modulation of sPLA₂ receptor occupancy as well as its inherent phospholipase activity has the potential to lead to novel therapeutic strategies that could attenuate the inflammatory response seen in these clinical scenarios.

REFERENCES


60. Seilhamer JJ, Pruzanski W, Vadas P, Plant S, Miller JA, Kloss J, and Johnson LK. Cloning and recombinant expres-


