Groups IV, V and X Phospholipases A2 in Human Neutrophils

ROLE IN EICOSANOID PRODUCTION AND GRAM NEGATIVE BACTERIAL PHOSPHOLIPID HYDROLASES

Norbert Degousee‡, Farideh Ghomashchi¶, Eva Stefanski‡, Alan Singer¶, Brian P. Smart¶, Niels Borregaard†, Reinhardt Reithmeier†‡, Thomas F. Lindsay‡, Cornelia Lichtenberger§, Walter Reinisch§, Gerard Lambeau††, Jonathan Arm‡‡, Jay Tischfield¶¶, Michael H. Gelb¶ ** and Barry B. Rubin‡ §§ **

From ‡‡The Division of Vascular Surgery, Max Bell Research Center, Toronto General Hospital, University Health Network, ¶¶Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195, USA, †Granulocyte Research Laboratory, Department of Hematology, Copenhagen University Hospital, Copenhagen, DK-2100, ††Medical Research Council Group in Membrane Biology, Departments of Medicine and Biochemistry, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8, Ontario, §Clinic of Internal Medicine IV, Department of Gastroenterology and Hepatology, University of Vienna, Austria, ††Institut de Pharmacologie Moleculaire et Cellulaire, CNRS-UPR 411, 660 route des Lecioles, Sophia Antipolis, 06560 Valbonne, France, ‡‡Department of Allergy and Immunology, Harvard Medical School, Boston, MA and ¶¶The Department of Genetics, Rutgers, The State University of New Jersey, 604 Allison Road, Piscataway, NJ 08854-8082.

** Address correspondence to: Michael H. Gelb, Depts. of Chemistry and Biochemistry, University of Washington, Seattle, WA 98195. Tel.: 206-543-7142; Fax: 206-685-8665; E-
Manuscript M1:09083.

mail: gelb@chem.washington.edu, or Barry B. Rubin, Division of Vascular Surgery, 200 Elizabeth Street, EC5-302a, Toronto General Hospital, Toronto, Ontario, Canada M5G-2C4. Tel.: 416-340-3645; fax: 416-340-5029; E-mail: barry.rubin@uhn.on.ca.

Running title: Group IV, V and X sPLA₂s in human neutrophils.

Keywords: secretory phospholipase A₂, cytosolic phospholipase A₂, neutrophil, leukotriene B₄.

E. coli.
SUMMARY. Exposure to the bacterial tri-peptide formyl-Met-Leu-Phe (fMLP) induces the secretion of enzyme(s) with phospholipase A$_2$ (PLA$_2$) activity from human neutrophils. Nine human secreted PLA$_2$ (sPLA$_2$) are known. We show that a cell sorted, homogenous preparation of circulating human neutrophils express groups V and X sPLA$_2$ (GV and GX sPLA$_2$) mRNA and contain GV and GX sPLA$_2$ proteins, whereas GIB, GIIA, GIID, GIIE, GIIF, GIII and GXII sPLA$_2$s are undetectable. GV sPLA$_2$ is a component of both azurophilic and specific granules, while GX sPLA$_2$ is confined to azurophilic granules. Exposure to fMLP or opsonized zymosan (OZ) results in the release of GV but not GX sPLA$_2$. By using the sPLA$_2$ inhibitor Indoxam and characterizing its specificity, we show that most, if not all, of the PLA$_2$ activity in the extracellular fluid of fMLP-stimulated neutrophils is due to GV sPLA$_2$. GV sPLA$_2$ does not contribute to fMLP-stimulated LTB$_4$ production, but may support the anti-bacterial properties of the neutrophil, since we show that 10 to 100 ng per ml concentrations of this enzyme leads to gram-negative bacterial membrane phospholipid hydrolysis in the presence of human serum. By use of a recently-described and specific inhibitor of cytosolic PLA$_2$-α (Group IV PLA$_2$α), we show that this enzyme produces virtually all of the arachidonic acid used for the biosynthesis of LTB$_4$ in fMLF- and OZ-stimulated neutrophils, the major eicosanoid produced by these pro-inflammatory cells.
INTRODUCTION

Neutrophils (polymorphonuclear leukocytes) are one of the principal effector cells in the inflammatory response. Following activation, neutrophils release a barrage of cytotoxic products, such as reactive oxygen species, degradative enzymes and phospholipases, including phospholipase A2 (PLA2) (1-3). PLA2 enzymes catalyze the hydrolysis of phospholipids, yielding free fatty acids and lysophospholipids. The identity of the PLA2 enzyme(s) that are contained in and secreted by human neutrophils has not been defined.

A family of 10 secreted PLA2 (sPLA2) enzymes has been described in mammals that currently includes Group (G) IB, IIA, IIC, IID, IIE, IIF, III, V, X and XII sPLA2 (4,5). sPLA2 enzymes exert biological effects through multiple mechanisms, including the release of arachidonic acid, which may be metabolized to leukotrienes and prostaglandins (6,7), bactericidal activity via hydrolysis of the outer membrane of gram-positive bacteria (8,9) and through binding to specific sPLA2 receptors (10-12). The different sPLA2's are not close isoforms of each other since their amino acid sequences are approximately 30-50% identical among the paralogs. This plus the fact that the sPLA2 enzymes have distinct tissue distributions argues for different physiological functions for each enzyme (5,13). Thus, GIB sPLA2 has been identified in the pancreas and functions in phospholipid digestion (14), but is also found in non-digestive tissues, where its functions remain unknown (5). GIIA sPLA2 is expressed at high levels during inflammatory reactions (15,16) and, until recently (4,5,13), was thought to be the principal sPLA2 isoform in the immune system. Human GIIA sPLA2 mRNA has been identified in myocardium, skeletal muscle, lung, liver, placenta, prostate
and the small and large intestine, but was not detected in peripheral blood leukocytes (17). GIIC sPLA₂ is present in the mouse but appears as a pseudo gene in humans (4). GIID sPLA₂ mRNA was detected in human spleen, thymus, small intestine, colon, lung, pancreas and placenta (17,18), while GIIE sPLA₂ expression in humans is restricted to the brain, heart, lung, and placenta (19). mRNA coding for GIIF sPLA₂ has been identified in many human tissues, with the highest levels detected in the placenta, thymus, prostate, testes, kidney, liver, and thyroid (17). GII sPLA₂, which is homologous to bee venom PLA₂, is expressed in the pancreas, kidney, liver, lung, skeletal muscle and myocardium (17,20). GV sPLA₂ has been identified in myocardium, placenta, mast cells and macrophages (21-24). While GV sPLA₂ was shown to contribute to PAF-mediated PGE₂ production by a murine macrophage-like cell line (24), GV sPLA₂ mRNA was not identified in human peripheral blood leukocytes (17). GX sPLA₂ is expressed in organs associated with the immune response (25), and induces cyclooxygenase-2 dependent PGE₂ synthesis by adherent mammalian cells (26-28). One study identified GX sPLA₂ mRNA in peripheral blood leukocytes (25), while two others did not (17,29). Transcripts for GXII sPLA₂ were identified in multiple tissues, including myocardium, skeletal muscle, kidney, pancreas and type 2 helper T cells (30,31).

sPLA₂ enzymes share a number of structural characteristics, including several intramolecular disulfide bridges, a Ca²⁺-binding loop and the requirement for sub-millimolar to millimolar concentrations of Ca²⁺ for catalytic activity (32-34). In addition, some of the sPLA₂ enzymes have N-terminal prepropeptide sequences of varying lengths (5,13). The mechanism by which prepropeptide
sPLA₂ enzymes mature into secreted proteins has not been fully defined. Some sPLA₂ enzymes have dibasic motifs at the C-terminus of their signal sequence, such as the arginine doublet in GX sPLA₂ (25), that may be efficiently cleaved by subtilisin-like protein convertases in the Golgi apparatus (35). Some sPLA₂ enzymes, including GIB and GX sPLA₂, are secreted as proenzymes and then cleaved to yield mature, catalytically active proteins, while others, like GIIA and GV sPLA₂, do not have an extra N-terminal peptide. GX sPLA₂ also contains a single consensus sequence for post-translational modification, the N-glycosylation acceptor site (Asn-X-Ser/Thr) at Asn113 (25), and N-glycosylation of GX sPLA₂ was demonstrated when this enzyme was expressed in HEK293 cells (36).

PLA₂ enzymatic activity has been detected in intracellular granules in human neutrophils (37-39). Subcellular fractionation experiments have shown that neutrophils have a heterogenous population of granules that have distinct intra-granular and membrane-bound proteins (40). Thus, neutrophils contain azurophilic, specific and gelatinase granules, as well as secretory vesicles, that function as regulated storage organelles. When neutrophils are stimulated, the granules may release their protein contents into the extracellular environment, or may fuse with phagosomes to form phagolysosomes, where the contents of the granules cooperate in the killing of microbes (41). Fusion of granule membranes, which are important reservoirs of membrane-bound proteins, with the plasma membrane and phagolysosomes may also participate in the eradication of microbes (40). The identity of the granules that contain sPLA₂ enzymes in human neutrophils has not been established.

A large body of experimental evidence supports the hypothesis that agonist-stimulated
release of arachidonic acid from phospholipids is mediated, at least in part, by cPLA_2 (reviewed in (42)). First, cPLA_2 selectively hydrolyzes phospholipids with arachidonic acid in the sn-2 position (43). Second, exposing neutrophils to fMLP results in a decrease in the electrophoretic mobility of cPLA_2, a finding consistent with cPLA_2 phosphorylation, and stimulates the translocation of cPLA_2 from cytosolic to microsomal and nuclear compartments (44,45). Third, co-incubating neutrophils with the cPLA_2 inhibitor methyl arachidonyl fluorophosphonate (MAFP) decreases fMLP-stimulated arachidonic acid mass release (44,45). Fourth, peritoneal macrophages from mice subjected to targeted disruption of the cPLA_2 gene (cPLA_2^{-/-}) produce less PGE_2, LTB_4 and PAF following exposure to inflammatory stimuli than macrophages from wild type mice (cPLA_2^{+/+}) (46,47). Three isoforms of cPLA_2 have been identified; cPLA_2\alpha, cPLA_2\beta and cPLA_2\gamma (43,48,49). While cPLA_2\alpha is thought to mediate arachidonic acid release by a variety of cells (42), the physiologic roles of cPLA_2\beta and cPLA_2\gamma have not been defined.

The purpose of this study was to identify the sPLA_2 enzymes that are expressed by neutrophils, define the subcellular localization of these enzymes, determine which sPLA_2 enzymes are released by neutrophils, and evaluate the role of the extracellular sPLA_2 enzymes in neutrophil LTB_4 biosynthesis and gram-negative bacterial phospholipid hydrolysis. We identified GV and GX sPLA_2 mRNA in a homogenous population of human neutrophils, showed that these cells contain GV and GX sPLA_2 protein, demonstrated that GV and GX sPLA_2 are both present in azurophilic granules, and found that GV sPLA_2 is also a component of
specific granules. Furthermore, we showed that GV sPLA$_2$ was released into the extracellular environment following exposure to formyl-Met-Leu-Phe (fMLP) or opsonized zymosan (OZ), while mature GX sPLA$_2$ was not. Inhibition of extracellular sPLA$_2$ activity with the active-site directed, tight-binding inhibitor Indoxam (50), which was found to inhibit GV sPLA$_2$ activity 125-fold more efficiently than GX sPLA$_2$ activity, had no effect on fMLP stimulated neutrophil LTB$_4$ synthesis. In contrast, pre-treatment of neutrophils with Pyrrolidine-1 (51), a highly specific inhibitor of cPLA$_2$$\alpha$ activity that has no effect on the catalytic activity of recombinant GIIA, V or X sPLA$_2$ (52) abrogated fMLP- and OZ-induced neutrophil LTB$_4$ biosynthesis. Finally, we showed that recombinant GIIA or GV sPLA$_2$, but not GX sPLA$_2$, efficiently hydrolyzed $[^{3}H]$oleic acid from $[^{3}H]$oleic acid labeled live E. coli in the presence of serum.
EXPERIMENTAL PROCEDURES

Materials.

Ficoll-Paque was from Amersham Pharmacia Biotech (Baie D’Urfé, Quebec), and was used for neutrophil isolation. DNase I RNase-free was from Qiagen (Mississauga, ON). Thermus aquaticus (Taq) polymerase was from MBI Fermentas (Burlington, ON). Rhodamine-phycoerythrin (R-PE)-conjugated anti-CD16 MoAb (clone 3G8; Monosan, Uden, Netherlands), Fluorescein isothiocyanate (FITC)-conjugated anti-CD19 (Immunotech, Luminy, France), FITC- conjugated anti-CD3 (Beckman Coulter Inc., Fullerton, CA) and FITC-conjugated anti-HLA-DR (Immunotech, Luminy, France) were used for cell sorting procedures. [9,10-3H]oleic acid (NFT 289, 5 Ci/mmol) was from New England Nuclear (Mississauga, ON). Myocardial RNA was from Ambion (Austin, TX). DNA sequence analysis was performed at the “DNA Sequencing Facility” of The Hospital for Sick Children (Toronto, ON). Isolation of the IgG fraction from serum was done with the ImmunoPure Immobilized Protein G Plus Orientation Kit (Pierce, Rockford, IL). The E. coli strain pldA was kindly provided by Dr. Peter Elsbach (New York University School of Medicine, NY). Zymosan was obtained from Sigma (St. Louis, MI), and OZ was prepared as previously described (53). Indoxam and Pyrrolidine-l were synthesized as described elsewhere (50,52).

Neutrophil isolation.

After obtaining informed consent, 100 ml of blood was obtained from healthy donors that were not taking any medications and anticoagulated in citrate. Neutrophils were then isolated by dextran sedimentation and Ficoll-Paque density gradient centrifugation, exactly as described (44).
Neutrophils (1 × 10^7) enriched by Ficoll centrifugation were incubated for 30 min at 4°C with 20% heat inactivated AB-serum prepared in KRPD buffer, washed twice with KRPD, and simultaneously labeled for 30 min at 4°C with anti-CD16 R-PE, anti-CD19 FITC, anti-CD3 FITC and anti-HLA-DR FITC, according to the manufacturer’s instructions. After two washes with cold KRPD buffer, cells were subjected to dual channel FACS analysis on a MoFlo cytometer (Cytomation Inc., Fort Collins, CO) using a 150-MW Coherent Innova Enterprise II ion laser (Coherent Inc., Santa Clara, CA) tuned at 488 nm and equipped with CyCLOPS Summit software. Fluorescence was measured using 570/40 (R-PE) and 530/40 (FITC) band pass filters. CD16^+, CD19^−, CD3^−, HLA-DR^− cells were separated by sorting in the “sort purify” mode setting with a flow rate of 10,000 cells/sec. Gates were set to exclude debris and nonviable cells on the basis of light scatter properties. Neutrophils were defined by a combination of forward, pulse width and side scatter characteristics, as well as the fluorescence intensity of anti-CD16 R-PE. Aliquots of the Ficoll-enriched, CD16^+ sorted cells were reanalyzed on a FACscan using the CellQuest program (Becton Dickinson, San Jose, CA), and were routinely > 99.9% pure. Flow cytometric and FACS analysis were performed at the “Flow Cytometry Facility” (Princess Margaret Hospital, Toronto, ON). Cell viability was determined by trypan blue exclusion and was always more than 90%. Sorted cells were placed on ice and immediately processed for RNA isolation.

RNA extraction and reverse transcription.

Ficoll-enriched or FACS-sorted CD16^+ cells (1 × 10^6) were directly lysed in 1 ml of TRIzol
Reagent for 5 min at room temperature. After addition of 0.2 ml of CHCl₃ and vigorous shaking, tubes were centrifuged at 12,000×g for 15 min at 4C. The aqueous phase was aspirated, supplemented with glycogen (10 µg/ml) and total RNA was precipitated by mixing with 0.5 ml of isopropyl alcohol. After 10 min at room temperature, samples were centrifuged at 12,000×g for 10 min at 4C. RNA pellets were washed with 75% ethanol, briefly air-dried, dissolved in 55 µl of RNAse-free water and incubated with 5 µl of DNAse I RNAse-free (10 U/ml) at 37C for 30 min. DNAse I was then inactivated by heat treatment for 5 minutes at 70C. The absence of genomic DNA in the RNA preparations was confirmed by performing PCR analysis for “minus-RT controls” (using RNA that was not reverse transcribed as the template).

cDNA mixtures were prepared in a 20 µl reaction using a first strand cDNA synthesis kit (MBI Fermentas, Burlington, ON) according to manufacturer’s instructions. Briefly, 5 µl of the DNA-free RNA preparations from Ficoll-enriched or FACS-sorted CD16+ human neutrophils, or 2 µg DNA-free RNA extracted from myocardial tissue (Ambion, Austin, TX) was reverse transcribed using 40 units of Moloney murine leukemia virus reverse transcriptase (M-MuLV RT) in the presence of 0.5 µg oligo (dT₁₈) primers, 50 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 4 mmol/L MgCl₂, 10 mmol/L DTT, deoxynucleotide (dNTP) mix (1 mmol/L each), and 20 units ribonuclease (RNase) inhibitor. The reaction mixture was incubated for 60 min at 39C (transcription) and 10 minutes at 70C (inactivation of RT). The cDNA mixture was then diluted with RNAse-free water to a final volume of 100 µl.

Polymerase Chain Reaction (PCR).
A total of 5 µl of the diluted first strand cDNA was amplified in a PCR reaction that included the cDNA from 4166 cells in the case of Ficoll-enriched or FACS-sorted CD16+ neutrophils, or 100 ng of total RNA from human myocardium. PCR was performed in 50 µl reactions containing 20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, dNTP mix (0.2 mmol/L each), 2 U Taq polymerase and 0.5 µmol/L of the specific primers. After 4 min at 95°C, 40 cycles of amplification with a PCR processor (PTC-100 Thermal Cycler, MJ Research, Waltham, MA) was carried out as follows: 30 sec at 95°C, 45 sec at 60°C and 50 sec at 72°C, followed by 10 min at 72°C to ensure a complete extension of the amplified DNA. Hot start PCR was employed to increase the specificity of the amplification.

PCR primers.

Primers were selected that showed insignificant homology to other genes in the EMBL DNA sequence database. When gene sequence data was available, primer pairs were chosen to span introns in their genomic sequences, thus ensuring mRNA-specific amplification. In addition, primers were selected to have a G + C content between 45% to 65%, a size between 18 to 23 mer and to exclude primer-dimer structures. The sequences of the sPLA₂ primers and predicted MW of the PCR products for GIB, GIIA, GIID, GIIF, GIII, GV and GX sPLA₂, and for CD52, c-fms, HLA-DRα and β-actin are listed in Table 1.

Negative controls were performed by omitting reverse transcriptase from cDNA synthesis or by omitting cDNA from the PCR amplifications. PCR products were analyzed by electrophoresis.
through 3% agarose gels and viewed under UV light after ethidium bromide staining. PCR product specificities were confirmed by DNA sequence analysis using an ABI Prism 377 DNA Sequencer (Applied Biosystems, Forster City, CA).

**Generation of recombinant sPLA$_2$ proteins.**

Recombinant human GIIA, GIIF, GX, and GXII sPLA$_2$ were produced by refolding the inclusion body protein obtained from expression in *E. coli*, as described previously (17,26,30,54). The preparation of recombinant human GIB, GIIE and GV sPLA$_2$ will be reported elsewhere. All recombinant sPLA$_2$s were judged to be pure by Laemmli gel electrophoresis. Electrospray mass spectrometry analysis of recombinant human sPLA$_2$s was carried out on a Bruker/Hewlett-Packard Esquire LC ion trap HPLC/mass spectrometer. The observed mass of all sPLA$_2$s was within 0.5 amu of the theoretical mass, indicating that all disulfides were formed.

**Generation of anti-sPLA$_2$ antisera.**

Antiserum to each recombinant human sPLA$_2$ was prepared by Cocalico Biologicals (Reamstown, PA). Rabbits were immunized with 100 µg of antigen with Complete Freund’s Adjuvant by multiple s.c. and i.m. injections. On days 14 and 21, rabbits received a booster injection with 50 µg of antigen with Incomplete Freund’s Adjuvant. A test bleed was collected at day 35, and a third boost with 50 µg of antigen in Incomplete Freund’s adjuvant on day 49 was carried out. The second test bleed was on day 56, and after a final boost with 50 µg of antigen in Incomplete Freund’s adjuvant on day 60, exanguination bleeds were obtained on day 67.
All anti-sPLA₂ antisera were tested for specificity toward the various human sPLA₂s (hGIB, hGIIA, hGIIE, hGIIF, hGV, hGX, and hGXII), as described below. While each anti-serum readily detected 1 ng of recombinant sPLA₂, no signal was detected when 50 ng of each of the other human sPLA₂s were analyzed (ECL detection, Pharmacia-Amersham). Thus, the individual antisera were highly specific for each of the human sPLA₂ enzymes. The anti-GV sPLA₂ and anti-GX sPLA₂ antisera were further purified by passage through a protein-G agarose column, as described by the manufacturer (Pierce).

**Preparation of soluble and microsomal fractions for western blot analysis.**

Cells were washed twice with PBS and centrifuged. The pellet was suspended in 66 mM HEPES buffer, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 25 mM NaF, 1 mM DFP, and 10 µg/ml leupeptin and aprotinin, sonicated on ice with 3 bursts (20 % maximum power) of 15 s each and centrifuged for 5 min at 14,000×g to remove nuclei. The supernatant was then centrifuged at 150,000×g for 20 min at 4°C to resolve the soluble and microsomal fractions, which were resuspended in Laemmli loading buffer.

**Preparation of neutrophil granule fractions.**

Neutrophils (1.1×10⁹) were resuspended in 18 ml of KRPD + 5 mM glucose, pelleted by centrifugation, resuspended in 18 ml of KRPD + 5 mM glucose (relaxation buffer) and incubated with 5 mM DFP for 15 min on ice. Cells were then pelleted by centrifugation, resuspended in 13 ml of relaxation buffer and subjected to nitrogen cavitation, as described (55). 10 ml of the post-nuclear supernatant was then put on a 3-layer Percoll gradient (generated in relaxation buffer
containing 1 mM PMSF) and centrifuged (55). Samples were collected in fractions of 1 ml. Fractions 1-6 were pooled and designated as the $\alpha$-band, fractions 7-12 the $\beta_1$-band, fractions 13-18 the $\beta_2$-band and fractions 19-24 the $\gamma$-band. Analysis for the granule markers MPO, NGAL, LF, gelatinase, HSA and HLA was carried out exactly as described (56), and showed that less than 0.5 % of the MPO, LF and gelatinase was identified in the soluble fraction (not shown), thereby confirming that nitrogen cavitation left the granules largely intact (55).

**Western blot analysis.**

Cell lysates, extracellular fluid, granules fractions and soluble or microsomal fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 16.5% gels at a constant voltage of 100V in 100 mM Tris, 100 mM Tricine and 0.1 % SDS as running buffer. Proteins were transferred to a PVDF membrane (NEN) in 25 mM TrisHCl, 192 mM glycine, 20% methanol (0.1% SDS for GIIA sPLA$_2$, 0.01 % SDS for GV sPLA$_2$ and GX sPLA$_2$, no SDS for the other sPLA$_2$ enzymes), pH 8.3, ± 0.1 at 100 V for 1 hour, followed by overnight blocking in 5 % milk and 1 % goat serum. The blots were then incubated with primary antibody (see legends under figures for specific antibodies) for 2 hrs at room temperature, washed and incubated with horseradish peroxidase conjugated secondary antibody for 1 hour. Detection of immunoreactive bands was carried out using enhanced chemiluminescence.

**Measurement of PLA$_2$ activity.**

Neutrophils (2×10$^7$) were resuspended in KRPD with 1 mM CaCl$_2$ and 0.25% BSA, warmed to 37C for 10 minutes and treated with 5 $\mu$M cytochalasin B for 2 min and either 0.1 % DMSO
or 1 µM fMLP for 10 min. Stimulations were terminated by centrifugation at 14,000×g for 1 min. The PLA2 activity in 50 µl of extracellular fluid was determined by measuring the amount of free \[^{3}\text{H}]\text{oleic acid} released from \[^{3}\text{H}]\text{oleic labeled, autoclaved} \text{E. coli membranes}, according to the protocol developed by Elsbach (57). The reaction was carried out in a total volume of 1.5 ml of 0.1 M Tris buffer, pH 7.5, containing 7 mM CaCl2, 10 mg fatty acid free BSA and \(2.8 \times 10^8\) radiolabelled, autoclaved \textit{E. coli} (corresponding to 5.6 nmole of phospholipid). After a 30 minute incubation at 37C, the reaction was terminated by filtration through a 0.45 µm Millipore filter, and the released \[^{3}\text{H}]\text{oleic acid} bound to the BSA carrier was measured by liquid scintillation counting (58). All cpm measurements were corrected for nonenzymatic hydrolysis. One unit of PLA2 activity is defined as the amount of enzyme that hydrolyzes 56 pmoles of phospholipid substrate in 30 min at 37C, which corresponds to 1% of the total \textit{E. coli} substrate.

\textit{LTB}_4 \textit{production}.

In some experiments, neutrophils were pre-incubated with increasing concentrations of Indoxam or Pyrridoline-1 for 10 min at 37C, as indicated in the Figure legends. Following exposure to vehicle, cytochalasin B and fMLP, or OZ (5 mg/ml), cells were centrifuged at 1000×g for 5 min at 4C and \textit{LTB}_4 release was subsequently determined by ELISA, as described by the manufacturer (Cayman).

\textit{Radiolabeling of live} \textit{E. coli}.

An inoculum of \textit{E coli} pldA\(^{-}\) (lacking the principal envelope phospholipase) was diluted 1:10 in fresh LB medium and grown for 2 h at 37C to mid-log phase in a shaking water bath.
Bacteria were harvested at 3000 rpm for 10 min and resuspended in 0.2% lactalbumin medium supplemented with 3 μCi/ml of [9,10-3H]oleic acid (NEN # NFT 289, 5 Ci/mmol) complexed with 0.02% bovine serum albumin (fatty acid free) and incubated for 2 h while shaking. After harvesting, bacteria were suspended in fresh 0.2% lactalbumin medium containing 1% bovine serum albumin and incubated for 30 min at 37°C. After centrifugation, cells were washed in 150 mM NaCl containing 1% BSA three times to remove unincorporated [9,10-3H]oleic acid. The labeled bacteria were then resuspended in 150 mM NaCl, adjusted to a concentration of 1×10^9/ml by measuring the absorbance at 550 nm and kept on ice until ready for use.

Measurement of bacterial phospholipid degradation in live radiolabeled E. coli by recombinant GIIA, GV and GX sPLA2.

Two ml of serum from the blood of healthy donors was pooled and passed over a 1 ml heparin column (Pharmacia), resulting in the removal of detectable PLA2 activity (data not shown). For the assay, [9,10-3H]oleic acid labeled live E. coli (2.5 × 10^7) were resuspended in 250 μl of 40% (v/v) Hanks’ balanced salts solution, 1.5% (w/v) BSA, 120 mM HEPES pH 7.4 and 2% heparin column-purified normal human serum. After a 15 min pre-incubation at 37°C, human recombinant GIIA, V or X sPLA2 was added to final concentration ranging from 0 to 500 ng/ml and incubated for 60 min at 37°C. The reaction was terminated by adding 250 μl of ice cold 150 mM NaCl and centrifugation at 14,000×g for 4 min. Radioactivity in the recovered supernatants was measured by liquid scintillation counting, and results were corrected for the non-specific hydrolysis of labeled E. coli.
Statistical analysis.

Results are expressed as the mean ± SD of triplicate determinations. Comparisons between groups were made by repeated measures analysis of variance, followed by post-hoc analysis with paired t-tests, where indicated. A p value < 0.05 was considered significant. When multiple comparisons were made, a Bonferroni correction factor was applied.
RESULTS

Identification of GV and GX sPLA2 mRNA in human neutrophils.

In order to determine which of the sPLA2 mRNA species were expressed by neutrophils, sets of primers for GIB, GIIA, GIID, GIIF, GIII, GV and GX sPLA2 were designed (Table 1.). The ability of each of these primer sets to amplify their respective sPLA2 mRNA by RT-PCR was confirmed with mRNA that had been isolated from human myocardium, as shown in Fig. 1A. Multiple primer sets were also generated for GIIE sPLA2. However, we were unable to detect GIIE sPLA2 mRNA in myocardium. Hanasaki and co-workers reported that GIIE sPLA2 mRNA was detected only after multiple rounds of PCR amplification, indicating that this protein is expressed at very low levels (59), and we have not been able to detect GIIE sPLA2 using a variety of human tissue cDNAs (Invitrogen multiple tissue panel). RT-PCR analysis of neutrophils that had been prepared by centrifugation on a discontinuous Ficoll-Paque density gradient identified GIIA, GIID, GV and GX sPLA2 mRNA species (Fig. 1B), but failed to identify GIB, GIIF or GIII sPLA2 mRNA.

Caution must be exercised in the interpretation of these results, as neutrophils isolated on a Ficoll-Paque density gradient are contaminated by macrophages, eosinophils and lymphocytes (Fig. 2A), cells that could be a source of sPLA2 mRNA (60,61). Indeed, mRNA from the Ficoll-Paque neutrophil preparation contained c-fms, a monocyte specific antigen (62), HLA-DR, which is expressed by lymphoid cells, and CD52, which is expressed by monocytes, eosinophils and lymphoid cells, but not by neutrophils (Fig. 2B) (63,64). Neutrophils express exceptionally high levels of CD16 on their surface in comparison with other leukocytes (65), and do not express CD3 or
CD19. In order to generate a preparation of highly purified neutrophils, cells isolated on a Ficoll-Paque density gradient were simultaneously incubated with anti-CD3, anti-CD19 and anti-HLA-DR antibodies conjugated to FITC, as well as a PE-conjugated anti-CD16 antibody. Dual label fluorescence activated cell sorting (FACS) was then carried out to separate CD16 positive cells from cells that express CD3, CD19 or HLA-DR (66). With this protocol, a homogenous population of cells (> 99.9 % neutrophils, Fig. 2C) was generated. Following extraction of the RNA from this purified population of neutrophils, RT-PCR failed to identify c-fms, HLA-DR or CD52 mRNA, thereby demonstrating that the neutrophil population generated by cell sorting was free of contaminating macrophages, eosinophils and lymphocytes (Fig. 2D). When RNA from neutrophils that had been purified by cell sorting was subjected to RT-PCR analysis, GV and GX sPLA₂ mRNA species were identified, while GIIA and GIID mRNA species were not detected (Fig. 2E).

Sequence analysis of the GV and GX sPLA₂ RT-PCR products.

RT-PCR analysis with a primer set designed to amplify GV sPLA₂ mRNA yielded 2 distinct products when human myocardium (Fig. 1A) or FACS purified human neutrophils (Fig. 2E) were used as the source of RNA. Following amplification of the putative GV sPLA₂ PCR products, nucleotide sequence analysis demonstrated that the 358 bp PCR product corresponds to nucleotides 24 to 381 of human GV sPLA₂ mRNA. Sequence analysis of the 251 bp PCR product also confirmed a 100 % match with GV sPLA₂ mRNA from nucleotides 24 to 381, in which nucleotides 186 to 292, which correspond to Exon 4 of the hGV sPLA₂ gene, have been deleted. Nucleotide sequence analysis also demonstrated that the 370 bp PCR product generated
with the primer set for GX sPLA₂ mRNA corresponded to nucleotides 569-938 of hGX sPLA₂ mRNA. No sequence data was obtained from the lower MW product that was generated with the primer set for GX sPLA₂ mRNA. These results provide direct evidence that circulating neutrophils express GV and GX sPLA₂ mRNA species.

Identification of GV and GX sPLA₂ enzymes in circulating human neutrophils.

To determine which sPLA₂ enzymes are present in circulating human neutrophils, western blot analysis was carried out for GIB, IIA, IIE, IIF, V, X and XII sPLA₂. The antisera used in these studies were specific for each of the sPLA₂ enzymes, and did not cross react with any other known human sPLA₂’s (Fig. 3). To evaluate neutrophils for the presence of individual sPLA₂ enzymes, and to define the subcellular location of these enzymes, neutrophils were sonicated in disruption buffer, resolved into soluble and microsomal fractions, incubated with vehicle or recombinant sPLA₂ enzyme (as a positive control) and evaluated by western blotting. The corresponding sPLA₂ enzymes were also run alone, in separate lanes, as additional positive controls. Therefore, we were able to determine if proteins identified by individual anti-sPLA₂ antisera migrated at the same apparent MW as recombinant sPLA₂ proteins following SDS-PAGE. With this approach, we identified proteins that were detected with the anti-GV sPLA₂ and anti-GX sPLA₂ antisera, in whole cell lysates and in the soluble fraction of neutrophils, that co-migrated with the respective recombinant enzymes (Fig. 4E and 4F). When the soluble fraction of neutrophils was spiked with recombinant GV sPLA₂ and evaluated by western
blotting, a single band was identified that co-migrated with recombinant GV sPLA₂ (Fig. 4E). Similarly, when the soluble fraction of neutrophils was spiked with recombinant GX sPLA₂, western blotting studies identified a single band that co-migrated with recombinant GX sPLA₂ (Fig. 4F). In contrast, proteins that co-migrated with recombinant GIB, GIIA, GIIE, GIIF or GXII sPLA₂ were not detected by western blot analyses using highly specific antisera (Fig. 4). For each of these later sPLA₂’s, we confirmed that the addition of the respective recombinant sPLA₂ protein to the soluble or microsomal fraction of neutrophils resulted in the appearance of a band that co-migrated with the recombinant sPLA₂ protein. These positive controls indicate that GIB, GIIA, GIIE, GIIF or GXII sPLA₂ are not being degraded in neutrophil lysates. Furthermore, the detection limit for sPLA₂ proteins using these antisera in western blots was in the range of 0.05–0.5 ng with enhanced chemiluminescence detection (data not shown). Taken together, these results provide direct evidence that only GV and GX sPLA₂ mRNAs and proteins are present in circulating human neutrophils.

*Distribution of GV and GX sPLA₂ in the granules of circulating human neutrophils.*

To determine if GV and GX sPLA₂ were present in neutrophil granules, cells were disrupted by nitrogen cavitation and resolved on a 3 layer Percoll density gradient into fractions that contain azurophil, specific and gelatinase granules, and secretory vesicles (55). Analysis of granule marker enzyme content in the α, β₁, β₂ and γ fractions demonstrated that this protocol resulted in the separation of the azurophil, specific and gelatinase granules and secretory vesicles (Table
2). As shown in Fig. 5A, we identified a protein in the α, β1 and β2 fractions that co-migrated with recombinant GV sPLA2. Slightly less GV sPLA2 was detected in the β1 fraction than in the α fraction, with minimal GV sPLA2 in the β2 fraction. No proteins that co-migrated with recombinant GV sPLA2 were identified in the γ fraction, which contains both secretory vesicles and plasma membranes (67). Similarly, we identified a protein in the α, β1 and β2 fractions that co-migrated with recombinant GX sPLA2 (Fig. 5B). The majority of the GX sPLA2 was identified in the α fraction, with comparatively little GX sPLA2 in the β1 or β2 fractions. No proteins that co-migrated with recombinant GX sPLA2 were identified in the γ fraction. The distribution of GX sPLA2 follows the distribution of the azurophil granule marker, MPO (Table 2). In contrast, the distribution of GV sPLA2 follows the distribution of the azurophil granule marker MPO and the specific granule markers lactoferrin and NGAL. This indicates that GX sPLA2 is localized to azurophil granules solely, while GV sPLA2 is localized to both azurophil and specific granules.

*Neutrophils release GV sPLA2 following exposure to fMLP or OZ.*

sPLA2 enzymes may be released by cells into their extracellular environment (37). Thus, when neutrophils were exposed to the bacterial tri-peptide fMLP, sPLA2 activity (as measured with radiolabeled bacterial membranes) in the extracellular fluid increased by 3-4 fold (Fig. 6A), a finding consistent with previous results (44). To determine if the increase in extracellular PLA2 activity was associated with the release of GV or GX sPLA2, cells were treated with fMLP, and the extracellular fluid was evaluated by western blot analysis. As shown in Fig. 6B, extracellular
GV sPLA$_2$ was detected that co-migrated with recombinant GV sPLA$_2$, and addition of recombinant GV sPLA$_2$ to the supernatant of fMLP stimulated cells resulted in the appearance of a single, more intense band on western blots. A protein with an apparent MW of approximately 18-19 kDa that cross-reacted with the anti-GX sPLA$_2$ antiserum was also identified in the supernatant of vehicle (DMSO) and fMLP stimulated cells, but this protein did not co-migrate with recombinant GX sPLA$_2$ (mature form, lacking an N-terminal extension) (Fig. 6C). Similarly, exposure to OZ resulted in the release of GV sPLA$_2$ by neutrophils, but did not result in the release of any proteins that were identified by the anti-GX sPLA$_2$ antiserum (Figs. 6D and 6E). No sPLA$_2$ enzymatic activity was detected in the extracellular fluid of OZ-stimulated cells (not shown). The failure to detect sPLA$_2$ activity in the extracellular fluid of OZ-stimulated cells appears to be due to inhibition of sPLA$_2$ enzymatic activity by OZ, as co-incubation of recombinant GV sPLA$_2$ or the supernatant of fMLP-stimulated cells with OZ decreased sPLA$_2$ enzymatic activity by $>95\%$ (not shown). These results demonstrate that circulating neutrophils release GV sPLA$_2$, but release little or no mature GX sPLA$_2$ into their extracellular environment following exposure to fMLP or OZ ($<0.2$ ng per $10^6$ neutrophils).

*Indoxam exhibits selectivity for the inhibition of recombinant GIIA and GV sPLA$_2$.*

LY311727 and Indoxam are potent active site-directed inhibitors of GIIA sPLA$_2$ (50,68). Prior to evaluating the ability of LY311727 or Indoxam to inhibit the PLA$_2$ activity released by fMLP stimulated cells, we evaluated the ability of these compounds to inhibit recombinant GV and
recombinant GX sPLA₂. The effect of LY311727 or Indoxam on recombinant GIIA sPLA₂ activity was also assessed for comparison. Assays were carried out under conditions where sPLA₂ activity was linear with respect to the amount of enzyme used (Fig. 7A) and time (Fig. 7B). Under these conditions, the specific activity of recombinant GIIA sPLA₂ (dotted line) was approximately 4-fold higher than recombinant GV sPLA₂ (hatched line), and 14-fold higher than recombinant GX sPLA₂ (solid line). As shown in Fig. 7C, co-incubation with LY311727 inhibited recombinant GIIA sPLA₂ activity (IC₅₀ ≈ 50 nM) more potently than recombinant GV sPLA₂ activity (IC₅₀ ≈ 2 μM) or recombinant GX sPLA₂ activity (IC₅₀ ≈ 0.75 μM). In contrast, co-incubation with Indoxam (Fig. 7D) inhibited recombinant GIIA sPLA₂ activity (IC₅₀ ≈ 10 nM) and recombinant GV sPLA₂ activity (IC₅₀ ≈ 40 nM) to a similar degree, but had a much less potent effect on recombinant GX sPLA₂ activity (IC₅₀ ≈ 5 μM). Therefore, Indoxam exhibited a 125-fold selectivity for inhibition of recombinant GV sPLA₂ in comparison with recombinant GX sPLA₂ in vitro. The IC₅₀ value for inhibition of human GX sPLA₂ by Indoxam is similar to that measured for mouse GX sPLA₂ (28).

\textit{Co-incubation with Indoxam inhibits extracellular sPLA₂ activity but does not attenuate LTB₄ production by neutrophils treated with fMLP.}

Following exposure of neutrophils (2 × 10⁷) to 1 μM fMLP for 10 min, PLA₂ activity in the extracellular medium was 202 ± 4 U/ml (Fig. 8A). Co-incubation of the extracellular fluid from
fMLP-treated neutrophils with Indoxam decreased PLA₂ activity in a dose-dependent manner (Fig. 8A). As fMLP-stimulated neutrophils released a protein that cross-reacted with the anti-GV sPLA₂ antiserum (c.f. Figs. 6B), these results are consistent with the notion that circulating neutrophils release catalytically active GV sPLA₂ following exposure to fMLP. The concentration of Indoxam required to inhibit the major portion of PLA₂ activity secreted from neutrophils (IC₅₀ ~ 50 nM) is similar to the IC₅₀ of Indoxam measured with recombinant GV sPLA₂ using the assay with radiolabelled E. coli membranes (Fig. 7D). The difference between the PLA₂ activity released by untreated neutrophils (40 ± 3 U / ml) and the PLA₂ activity in the supernatant of fMLP-treated neutrophils following incubation with 10 µM Indoxam (45 ± 3 U / ml) was negligible. This indicates that Indoxam inhibited essentially all of the PLA₂ activity that was released from neutrophils in response to fMLP. The effect of Indoxam on OZ-induced PLA₂ release by neutrophils was not studied, as OZ inhibited the activity of the PLA₂ released by these cells. Since the concentrations of Indoxam used in these studies do not inhibit GX sPLA₂, these results are consistent with the western blotting data indicating that fMLP-stimulated neutrophils do not release catalytically-active GX sPLA₂.

To evaluate the role of extracellular GV sPLA₂ in neutrophil LTB₄ biosynthesis, cells were pre-treated with Indoxam and stimulated with fMLP. As shown in Fig. 8B, inhibition of extracellular GV sPLA₂ activity with Indoxam did not inhibit fMLP-stimulated LTB₄ biosynthesis by human neutrophils. Therefore, GV sPLA₂ released from neutrophils in response to fMLP does not participate in neutrophil LTB₄ biosynthesis. In contrast, pre-incubating
neutrophils with the selective cPLA2α inhibitor, Pyrrolidine-1 (51,52), resulted in a dose-dependent inhibition of both fMLP- and OZ-induced neutrophils LTB4 biosynthesis (IC50 ≈ 0.1 – 0.5 µM, Figs. 9A and 9B). These findings indicate that cPLA2α participates in fMLP- and OZ-induced LTB4 synthesis by neutrophils. Pre-incubation with Pyrrolidine-1 had no effect on the fMLP-induced release of GV sPLA2, as assessed by western blotting, or on PLA2 activity released by fMLP stimulated cells (data not shown).

Recombinant GIIA and GV sPLA2, but not recombinant GX sPLA2, catalyze the hydrolysis of phospholipids in live E. coli.

A clear link between E. coli envelope phospholipid degradation and overall bacterial destruction by rabbit neutrophils has been established (69). As we have demonstrated that human neutrophils contain GV sPLA2 and release this enzyme upon exposure to fMLP or OZ, we evaluated the ability of recombinant GV sPLA2 to hydrolyze bacterial phospholipids. Studies with GIIA and GX sPLA2s were also carried out for comparison. Live E. coli were labeled with [3H]oleic acid during the log growth phase, washed extensively in buffer with 1% BSA and co-incubated with increasing concentrations of recombinant GIIA, GV or GX sPLA2. For these studies, human serum that had been eluted from a heparin column (to remove all PLA2 activity) was added at a final concentration of 2 % serum. Co-incubation of serum purified over the heparin column with live [3H]oleic acid labeled E. coli did not result in measurable phospholipid hydrolysis (data not shown). Co-incubation of live [3H]oleic acid labeled E. coli with 10 ng
Manuscript M1:09083.

recombinant GIIA sPLA₂ for 60 min resulted in the hydrolysis of approximately 25% of the labeled bacterial phospholipids, and this value did not change significantly as the amount of recombinant GIIA sPLA₂ was increased to 500 ng (Fig. 10). Co-incubation of live [³H]oleic acid labeled *E. coli* with recombinant GV sPLA₂ resulted in a dose-dependent increase in bacterial phospholipid hydrolysis that reached a plateau at 100 ng recombinant GV sPLA₂, while co-incubation of up to 500 ng of recombinant GX sPLA₂ with live [³H]oleic acid labeled *E. coli* resulted in virtually no hydrolysis (Fig. 10). When recombinant GIIA sPLA₂ or recombinant GV sPLA₂ were co-incubated with live [³H]oleic acid labeled *E. coli* in the absence of serum, no bacterial phospholipid hydrolysis was detected (data not shown). These results provide direct evidence that recombinant GIIA and recombinant GV sPLA₂ can hydrolyze phospholipids present in the outer membrane of live *E. coli*, and that this phospholipid hydrolysis is dependent on the presence of serum.
DISCUSSION

GIIA sPLA₂ has been purified from rabbit inflammatory exudates (70,71), and immunohistochemical studies identified GIIA sPLA₂ in human neutrophils (37). These seminal findings led to the notion that human neutrophils express and release GIIA sPLA₂ during an inflammatory response (72). However, recent studies indicate that multiple sPLA₂ enzymes, in addition to GIIA sPLA₂, may play important roles in the generation of an inflammatory response (4). Thus, some myeloid cells express more than one sPLA₂ enzyme (73), and sPLA₂ enzymes other than GIIA sPLA₂, including GV and GX sPLA₂, have been shown to participate in the generation of pro-inflammatory mediators (5,24). Furthermore, nine human sPLA₂ enzymes have now been described (13). The existence of multiple sPLA₂ enzymes raises the possibility that the anti-GIIA antisera used in earlier studies could have cross-reacted with other sPLA₂ enzymes (37). Based on these considerations, we decided to systematically evaluate which sPLA₂ mRNA species and sPLA₂ proteins exist in circulating human neutrophils, and to define the subcellular localization of these enzymes. Such information is necessary as a prelude for studying the biological functions of sPLA₂ enzymes in these cells. In this study, we also focused on the identification of the enzyme(s) that are released by neutrophils that were stimulated with two distinct agonists, fMLP and OZ, and on the role that these sPLA₂ enzymes play in neutrophil LTB₄ biosynthesis and gram-negative bacterial phospholipid hydrolysis. We also studied the role of cPLA₂α in fMLP- and OZ-stimulated LTB₄ biosynthesis by incubating cells with Pyrrolidine-1 (52), since this inhibitor is more specific than previously used inhibitors of cPLA₂α.
Identification of GV and GX sPLA2 mRNAs and proteins in neutrophils.

Extensive experimental evidence has documented the existence of sPLA2 enzyme(s) in human neutrophils. Thus, neutrophils contain an acid-stable enzyme with PLA2 activity (74), sPLA2 has been localized to the granules of resting human neutrophils and shown to translocate to phagolysosomes following exposure to agonists (37), and neutrophils stimulated with fMLP release sPLA2 activity (44). In the present study we have convincingly shown by the combined use of RT-PCR and western blot analysis with highly specific anti-sPLA2 antisera that circulating and non-stimulated human neutrophils contain GV and GX sPLA2, while GIB, GIIA, GIID, GIIE, GIIF, GIII, and GXII sPLA2 were undetectable in these cells. Our results underscore the importance of using highly-purified neutrophils for RT-PCR analysis, as GIIA and GIID sPLA2 mRNA species were detected in neutrophils isolated on a Ficoll-Paque density gradient but were not identified in FACS purified cells that were free of contaminating macrophages, eosinophils and lymphocytes. For GIID and GIII sPLA2, only RT-PCR was used for detection, with successful execution of positive controls, since antisera for these proteins are not yet available. Although GIIE sPLA2 mRNA could not be detected by RT-PCR (Hanasaki and co-workers reported that GIIE sPLA2 cDNA could be detected by PCR only after multiple rounds of extensive amplification (59)), GIIE sPLA2 protein was not detected by western blot analysis even though spiking the neutrophil lysate with 1.5 ng of recombinant GIIE sPLA2 gave a readily detected band. In contrast to our results, a previous study reported that GV sPLA2 is not present in human neutrophils (75). This discrepancy may be due to the fact that we used a high affinity
antiserum that could detect < 1 ng of recombinant GV sPLA₂, while the earlier study made use of a lower affinity monoclonal antibody that was only able to detect approximately 30 ng of this antigen (76). Despite earlier reports that circulating human neutrophils contain GIIA sPLA₂ based on immunohistochemical analysis (37), we could not detect GIIA sPLA₂ in these cells either by RT-PCR or western blot. For the latter analyses, the GIIA sPLA₂ western blot band was easily seen when the neutrophil lysate was spiked with 1 ng of recombinant GIIA sPLA₂, thus the amount of GIIA sPLA₂ is << 1 ng per 10⁶ neutrophils. Our findings underscore the cell type specificity of sPLA₂ expression; for example, GIIA, GIIC, GIID, GIIE, GIIF and GV sPLA₂ were detected in bone marrow derived mast cells from BALB/cJ mice, while transcripts for GIB and GX sPLA₂ were not identified in these cells (73). As neutrophils express some mRNA species during myelopoiesis that are not expressed in mature cells (77), we could not exclude the possibility that other sPLA₂ mRNA species besides GV and GX sPLA₂ are expressed at an earlier phase of neutrophil maturation.

Sequence analysis demonstrated that the 358 bp GV sPLA₂ RT-PCR product was identical to the corresponding sequence in GV sPLA₂, while the 251 bp GV sPLA₂ product had a 107 bp deletion. Deletion of this 107 bp sequence, which corresponds to exon 4 of the human GV sPLA₂ gene, would result in a frame shift that would introduce a premature TGA stop codon. Therefore, translation of this alternatively spliced GV sPLA₂ mRNA would be predicted to give rise to a catalytically inactive, truncated GV sPLA₂ protein.
We identified proteins in neutrophils that were recognized by anti-GV and anti-GX sPLA₂ antisera, and that co-migrated exactly with recombinant GV sPLA₂ (Fig. 4E) and recombinant GX sPLA₂ (Fig. 4F). These recombinant protein were generated in *E. coli*, and lack N-terminal signal sequences. Therefore, our findings are consistent with the notion that the N-terminal signal sequence of GV and GX sPLA₂ in neutrophils had been cleaved.

The identification of multiple sPLA₂ enzymes in neutrophils is consistent with the results of studies of other types of cells, in which multiple sPLA₂ enzymes were expressed. Thus, GIIA and GV sPLA₂ were identified in mouse bone marrow mast cells (23), cultured rat astrocytes (78) and rat neonatal cardiomyocytes,³ while GIB and GIIA sPLA₂ were both identified in epidermis (79) and rat splenic macrophages (80,81). In mast cells, GIIA sPLA₂ was recognized in secretory granules (82), consistent with the putative role of sPLA₂ in facilitating secretory granule exocytosis (83), while GV sPLA₂ was identified in the Golgi apparatus, nuclear envelope and plasma membrane (23). To our knowledge, other than human neutrophils, no myeloid cells have been identified that express both GV and GX sPLA₂.

*Subcellular localization of GV and GX sPLA₂ in human neutrophils.*

Neutrophils contain four types of regulated storage organelles, namely azurophilic, specific and gelatinase granules, and secretory vesicles (40). In this study, we identified GV sPLA₂ in azurophilic and specific granules, while GX PLA₂ was confined to azurophilic granules. In human neutrophils, most of the bactericidal proteins are localized in the azurophil granules (84),

Manuscript M1:09083.
including bactericidal/permeability increasing protein (BPI) (85), CAP37 (azurocidin) (86) and defensins (87). Therefore, the intracellular fusion of azurophilic granules with phagocytosed bacteria, which results in the formation of a phagolysosome (41), would be predicted to generate a high local concentration of GV sPLA₂, GX sPLA₂ and multiple bactericidal proteins. Indeed, phagocytosis of OZ results in the fusion of the phagosome with both azurophilic and specific granules, and in the release of membrane-bound BPI into the phagolysosome (88). As BPI enhances the ability of sPLA₂ enzymes to hydrolyze bacterial phospholipids, it is likely that the combined action of multiple bactericidal proteins, working simultaneously, effects bacterial destruction inside the phagolysosome. The precise roles of GV and GX sPLA₂ in bacterial eradication following phagocytosis remains to be defined. However, the recent demonstration that human GV and GX sPLA₂ can both kill gram positive bacteria in vitro (89) supports a role for these proteins in bacterial eradication following phagocytosis.

**Identification of GV sPLA₂ in the extracellular fluid of fMLP- and OZ-stimulated neutrophils.**

Both GV and GX sPLA₂s are both expected to be secreted enzymes. SignalP analysis (90) of the amino acid sequences of GV and GX sPLA₂ for the presence of potential cleaved signal sequences predicted that both GV sPLA₂ (signal peptide probability = 1.0) and GX sPLA₂ (signal peptide probability = 0.997) have a cleaved signal sequence. The predicted cleavage site for GV sPLA₂ is between Gly20 and Gly21 (maximum cleavage site probability = 0.864), while
the predicted cleavage site for GX sPLA₂ is between Gly31 and Glu32 (maximum cleavage site probability = 0.502).

Stimulation of human neutrophils with fMLP or OZ leads to the release of GV sPLA₂ into the extracellular fluid (c.f. Fig. 6). By comparing the density of the band on the western blot that corresponds to released GV sPLA₂ with the density of the band corresponding to 1.5 ng of recombinant GV sPLA₂ (Fig. 6B), we estimate than 10⁶ fMLP-stimulated neutrophils release approximately 0.9 ng of GV sPLA₂. As shown in Fig. 7A, 1 ng of recombinant GV sPLA₂ corresponds to approximately 10 Units of PLA₂ activity. As 20 million PMN release approximately 100 Units of PLA₂ activity / ml after exposure to fMLP (or 0.5 ng / million neutrophils, c.f. Fig. 6A), the amount of GV sPLA₂ release by neutrophils, estimated by western blotting and activity analysis, are in general agreement.

In contrast to the results of the western blot analysis for GV sPLA₂, proteins that co-migrated with mature GX sPLA₂ were not detected in the extracellular fluid of fMLP- or OZ-stimulated neutrophils, despite the fact that the positive control with 1 ng of added recombinant GX sPLA₂ gave a readily detectable band. Furthermore, as little as 0.2 ng of recombinant GX sPLA₂ could be detected by western blot analysis, even when added to the crude neutrophil lysate (not shown). Therefore, if neutrophils release mature GX sPLA₂ following exposure to fMLP or OZ, the amount released is less than 0.2 ng / 10⁶ cells. Control and fMLP-stimulated neutrophils both released a protein into the extracellular fluid with a MW of approximately 19
kDa that cross-reacts with the anti-GX sPLA2 antiserum. This cross-reacting protein, which was also identified in the soluble fraction of neutrophils (Fig. 4F), could represent pro-GX sPLA2 (i.e. GX sPLA2 prior to cleavage of the N-terminal pro-peptide) or glycosylated GX sPLA2 (36).

As pro-GX sPLA2 has relatively low enzymatic activity compared to that of the mature protein (36), the presence of pro-GX sPLA2 in the extracellular fluid of neutrophils would not be predicted to contribute to the PLA2 activity released by neutrophils. In any case, our results showing that concentrations of Indoxam that inhibit GV but not GX sPLA2 block virtually all of the PLA2 enzymatic activity released by neutrophils after fMLP stimulation (Fig. 8) also strongly argues that enzymatically active GX sPLA2 is not present in the extracellular fluid of fMLP-stimulated cells.

The appearance of relatively small amounts of GV PLA2 in the extracellular fluid of neutrophils exposed to fMLP is consistent with the fact that azurophilic and specific granule contents are poorly mobilized in response to this bacterial tri-peptide (55). However, as GV and GX sPLA2 were both identified in azurophilic granules, we anticipated that GV sPLA2 release by fMLP or OZ stimulated cells would be associated with some GX sPLA2 release. The reason for the failure to identify mature GX PLA2 in the extracellular fluid of fMLP- or OZ-stimulated cells is not readily apparent, but may have be due to proteolytic degradation of this protein following degranulation. Alternatively, it is possible that GX PLA2, like BPI (88), is a membrane-associated protein in azurophilic granules, and is not released into the extracellular fluid after exposure to fMLP or OZ. It may be noted that recent studies with rat mesangial cells show that
~80% of the GIIA sPLA2 in these cells is secreted after agonist stimulation, while most of the GV sPLA2 remains in an intracellular compartment (91). Thus, it would appear that not all sPLA2s are secreted to the extracellular space following agonist-induced cell activation.

*Extracellular GV sPLA2 does not participate in neutrophil LTB4 synthesis.*

A model of plasma membrane phospholipid hydrolysis by sPLA2 enzymes has been proposed in which endogenous sPLA2 enzymes are released and bind to the plasma membrane in a paracrine fashion (92). In this model, hydrolysis of plasma membrane phospholipids by the bound sPLA2 enzyme leads to the production of free fatty acids, including arachidonic acid, that may be utilized for subsequent eicosanoid production (24,24,93). Endogenous GV sPLA2 released by neutrophils could theoretically hydrolyze neutrophil plasma membrane phospholipids via a similar mechanism, as exogenously added recombinant human GV sPLA2 promotes free fatty acid release and LTB4 synthesis from these cells (94). Catalytically active recombinant GV sPLA2 was required to induce LTB4 synthesis by neutrophils, as the H48A GV sPLA2 mutant, which lacks catalytic activity, failed to induce LTB4 release by these cells (95). Taken together, these studies are consistent with a role for extracellular GV sPLA2 in neutrophil LTB4 synthesis. In support of this hypothesis, we found that fMLP-stimulated neutrophils release GV sPLA2, which accounts for most, if not all, of the PLA2 activity in the extracellular medium. However, we found that co-incubation with Indoxam, which showed strong potency and specificity for the inhibition of GV sPLA2, completely blocked extracellular PLA2 activity, but had no effect on
LTB₄ synthesis by fMLP-stimulated neutrophils. The reason for the discrepancy between our results and others (75,94) is not established, but may be due to the fact that much higher concentrations of GV sPLA₂ were added to neutrophils (approximately 1,400 ng/ml) (95) than are generated endogenously (≈ 1 ng/10⁶ neutrophils, or 20 ng/ml in this study) following exposure to fMLP (c.f. Fig. 6B). The failure of the relatively low levels of endogenous GV sPLA₂ that were released by fMLP-stimulated neutrophils to participate in ex vivo LTB₄ synthesis does not exclude the possibility that GV sPLA₂ plays a role in neutrophil LTB₄ synthesis during an inflammatory response in vivo, as GV sPLA₂ released by other myeloid cells, such as macrophages, could induce this response if present in the extracellular fluid at relatively high concentrations. Alternately, it is possible that higher local concentrations of GV sPLA₂ are generated by neutrophils during an inflammatory response in vivo than by neutrophils treated with fMLP in vitro.

It is possible that the 19 kDa band detected by western blot analysis in the extracellular medium of control and fMLP-stimulated neutrophils, using the anti-GX sPLA₂ antiserum (c.f. Fig. 6C), is pro-GX sPLA₂. If this is the case, GX sPLA₂ released by neutrophils could participate in LTB₄ biosynthesis, if pro-GX sPLA₂ could be converted to mature GX sPLA₂ by extracellular proteases during an inflammatory response in vivo. For this to occur, neutrophils would have to generate at least 10 ng GX sPLA₂/ml, as this is the concentration of GX sPLA₂ that is required to produce detectable eicosanoid formation in a variety of mammalian cells treated with exogenous GX sPLA₂ (26,27). In general, it is very difficult to assess the role of
extracellular sPLA$_2$s in eicosanoid generation from in vitro studies alone. This is because it is difficult to relate the concentrations of sPLA$_2$s in the extracellular medium of neutrophils in vitro to the concentrations of these enzymes in an inflammatory exudate in vivo. Whole animal studies with sPLA$_2$-deficient mice or highly specific sPLA$_2$ inhibitors are warranted to evaluate the role of sPLA$_2$ enzymes in eicosanoid biosynthesis in vivo. The failure to identify GIIA PLA$_2$ in neutrophils (c.f. Figs. 2E and 4B) does not preclude a role for this enzyme in the generation of eicosanoids during an inflammatory response in vivo, as GIIA sPLA$_2$ could be released by other pro-inflammatory cells, such as macrophages or eosinophils (61,96). In fact, the identification of high levels of GIIA sPLA$_2$ in inflammatory exudates strongly supports a role for GIIA sPLA$_2$ in inflammation (97-100).

cPLA$_2$$\alpha$ regulates fMLP-induced neutrophil LTB$_4$ biosynthesis.

In a previous study, we showed that the potent cPLA$_2$ inhibitor MAFP blocked virtually all of the arachidonate release by neutrophils stimulated with fMLP (44). Similarly, Bauldry et al. showed that cPLA$_2$ activation could provide arachidonic acid for LTB$_4$ biosynthesis by permeabilized human neutrophils (101). In addition, co-incubation with DTT, which would inactivate endogenous sPLA$_2$ activity, had no effect on LTB$_4$ biosynthesis by permeabilized neutrophils (101). These results support the hypothesis that cPLA$_2$, rather than a sPLA$_2$, catalyzes the release of arachidonic acid that is the precursor of LTB$_4$ in neutrophils. To directly evaluate the role of cPLA$_2$ in neutrophil LTB$_4$ biosynthesis, cells were incubated with the selective cPLA$_2$$\alpha$ inhibitor, Pyrrolidine-1 (51,52). This compound offers a number of advantages over MAFP. It is highly
potent against cPLA2α, leading to 50% inhibition when present at a mole fraction of approximately 0.002 in a number of in vitro assays, is a poor inhibitor of cPLA2γ and calcium-independent group VI PLA2 (MAFP inhibits this enzyme), and does not cause detectable inhibition of GIIA, V or X sPLA2 activity (52). In addition, Pyrrolidine-1 blocks arachidonic acid release by Ca2+ ionophore-stimulated CHO cells that were stably transfected with cPLA2α (52). We found that pre-incubation with Pyrrolidine-1 results in a dose-dependent and complete inhibition of fMLP- or OZ-induced LTB4 release by neutrophils. The IC50 for Pyrrolidine-1 inhibition of LTB4 release was between 0.1 to 1.0 µM, which is similar to the IC50 for Pyrrolidine-1 inhibition of cPLA2α activity in multiple cell lines (52). Therefore, it appears that cPLA2α, and not extracellular GV sPLA2, is responsible for the rapid liberation of arachidonate that is metabolized to LTB4 following neutrophil activation by fMLP or OZ.

*Possible role of GV sPLA2 in neutrophil mediated gram-negative bacterial phospholipid hydrolysis.*

Gram-negative bacteria have a plasma membrane, an anionic peptidoglycan cell wall and an outer layer of lipopolysaccharide (LPS) (72). The highly dense and anionic cell wall and LPS layers limit the ability of extracellular sPLA2 enzymes to access and hydrolyze the plasma membrane. However, following disruption of the cell wall by polymixin antibiotics or BPI (48,72) cationic enzymes such as GIIA sPLA2 may gain access to the plasma membrane and kill the bacteria (102,103), and a clear link has been established between envelope phospholipid degradation and overall gram-negative
bacterial destruction (104). We found that recombinant GIIA sPLA\textsubscript{2}, in the presence of serum, hydrolyzed phospholipids from \textit{E. coli} that lack PLA\textsubscript{2} activity more efficiently than recombinant GV sPLA\textsubscript{2}, while recombinant GX sPLA\textsubscript{2} did not catalyze detectable bacterial phospholipid hydrolysis. This data is consistent with the fact that GIIA sPLA\textsubscript{2} has a high pI (> 10.5) (72), is well designed to hydrolyze anionic bacterial phospholipids, and has been shown to hydrolyze \textit{E. coli} \textit{in vivo} (105). GV sPLA\textsubscript{2} has a lower pI but is still > 7, and so it would be predicted to have intermediate activity on anionic cell wall-bound \textit{E. coli} membranes, while GX sPLA\textsubscript{2} has an acidic pI and does not hydrolyze \textit{E. coli} phospholipids. The permissive effect of serum on GIIA and GV sPLA\textsubscript{2} mediated \textit{E. coli} phospholipid hydrolysis was likely due to the presence of BPI and (sublethal) assemblies of the membrane-attack complex of complement, which promote GIIA PLA\textsubscript{2} action against Gram-negative bacteria by producing sublethal alterations of the outer envelope of these bacteria (48,106). As GV sPLA\textsubscript{2} was the only sPLA\textsubscript{2} enzyme released by neutrophils, these results point to a potential role for extracellular GV sPLA\textsubscript{2} in neutrophil-mediated \textit{E. coli} phospholipid hydrolysis \textit{in vivo}. It is also possible that GV sPLA\textsubscript{2} bound to \textit{E. coli} may augment bacterial phospholipid hydrolysis after phagocytosis of the bacteria by neutrophils, as described for GIIA sPLA\textsubscript{2} (69,107). Extracellular GV sPLA\textsubscript{2} released by neutrophils may also participate in the eradication of gram-positive bacteria, including \textit{L. monocytogenes}, \textit{E. faecium}, \textit{E. faecalis} and methicillin sensitive- and resistant-\textit{S. aureus}, as co-incubation of these bacteria with rat, human, and mouse GV sPLA\textsubscript{2s} resulted in significant bactericidal activity \textit{in vitro} (89,108). However, the
in vivo bactericidal activity GV sPLA₂, which is less potent than GIIA sPLA₂, remains to be established.

In summary, we have shown that human neutrophils express GV and GX sPLA₂ mRNA and contain GV and GX sPLA₂ protein. GV sPLA₂ is present in both azurophil and specific granules, while GX sPLA₂ is confined to azurophil granules. Stimulation with fMLP or OZ results in the selective release of GV sPLA₂ by neutrophils. However, inhibition of extracellular GV sPLA₂ activity has no effect on neutrophil LTB₄ synthesis, which was shown to be fully dependent on cPLA₂α activity. In contrast, 10-100 ng/ml amounts of GV sPLA₂ were able to hydrolyze E. coli phospholipids in the presence of human serum, and may therefore participate in neutrophil mediated E. coli degradation in vivo. The possibility that granule bound GV and/or GX sPLA₂ is released into phagolysosomes and participates in the eradication of ingested bacteria or other pathogens remains to be evaluated.
REFERENCES


Manuscript M1:09083.


Manuscript M1:09083.

1925-1935


46


Manuscript M1:09083.


FOOTNOTES

* This work was supported by grants from The Heart and Stroke Foundation of Canada (NA-4151, BBR), The Canadian Institutes of Health Research (RR), CNRS and the Association pour le Recherche sur le Cancer (GL), The Danish Medical Research Council (52-00-0887, NB) and The National Institutes of Health (HL36235 and HL50040, MG; PO1 HL36110, JA and DK38185, JT).

§§ Is the recipient of the Wylie Scholar Award in Academic Vascular Surgery from The Pacific Vascular Research Foundation, San Francisco, CA and a Fellowship from the Bickel Foundation.

The abbreviations used are: GIIA PLA₂, Group IIA phospholipase A₂; sPLA₂, secretory phospholipase A₂; cPLA₂, Ca²⁺-dependent cytosolic phospholipase A₂ (= Group IV PLA₂); cPLA₂α, cPLA₂β and cPLA₂γ, α, β and γ isoforms of cPLA₂; iPLA₂, Ca²⁺-independent cytosolic phospholipase A₂; PMN, polymorphonuclear leukocyte (neutrophil); MAFP, methyl arachidonyl fluorophosphonate; PAF, platelet activating factor; PGE₂, prostaglandin E₂; PCR, polymerase chain reaction; dNTP, deoxynucleotide; FACS, fluorescence activated cell sorter; M-MuLV RT, Moloney murine leukemia virus reverse transcriptase; R-PE, Rhodamine-phycoerythrin; FBS, fetal bovine serum; KRPD, Krebs Ringers Phosphate Dextrose; LTB₄, leukotriene B₄; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; fMLF, formyl-Met-Leu-Phe; BPI, bactericidal/permeability-increasing protein; ECL, enhanced chemiluminescence; MPO, myeloperoxidase; NGAL, neutrophil gelatinase-associated lipocalin; LF, lactoferrin; HSA,
human serum albumin; OZ, serum opsonized zymosan; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis, HPLC, high performance liquid chromatography.

2 M. Gelb, manuscripts in preparation.

3 N. Degousee, E. Stefanski and B. Rubin, submitted for publication.
Table 1. Characteristics of the primers used for RT-PCR analysis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sense and antisense PCR primers</th>
<th>Corresponding nucleotides in human cDNA</th>
<th>Predicted mw of DNA product (bp)</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIB sPLA₂</td>
<td>5′-TCCTTGTGCTAGCTGTGCTG, 5′-TGAAGGCCTACACTCTTTG</td>
<td>45–64, 366–385</td>
<td>341</td>
<td>NM_000928</td>
</tr>
<tr>
<td>GIIA sPLA₂</td>
<td>5′-ATGAAGACCCCTCCTACTGTT, 5′-TCAGCAACGAGGGTGCTCC</td>
<td>136-155, 551–570</td>
<td>434</td>
<td>NM_000300</td>
</tr>
<tr>
<td>GIID sPLA₂</td>
<td>5′-GAAAATGCCCATCCTCTCCT, 5′-AAACGCAGTCGCTTCTGTA</td>
<td>138-157, 412–431</td>
<td>294</td>
<td>NM_012400</td>
</tr>
<tr>
<td>GIIF sPLA₂</td>
<td>5′-ATGAAGAAGTTCTTCACCGTGGC, 5′-CAGTGTTGTCTCGATGGTGTGA</td>
<td>1-23, 261–283</td>
<td>283</td>
<td>NM_022819</td>
</tr>
<tr>
<td>GIIII sPLA₂</td>
<td>5′-TGCCCTACAGAATCAGCAGGA, 5′-TTGAGCAGCTGGAACTCGAT</td>
<td>911-930, 1391–1410</td>
<td>500</td>
<td>AF_220490</td>
</tr>
<tr>
<td>GV sPLA₂</td>
<td>5′-TTGGTTCTGGCTTGTAGTGTG, 5′-TTGGGTGTAGCTCCGTAGGTG</td>
<td>156-177, 492–513</td>
<td>358</td>
<td>NM_000929</td>
</tr>
<tr>
<td>GX sPLA₂</td>
<td>5′-GATCCTGGAACTGGCAGGAA, 5′-TCAGTCACACTTGGCAGAT</td>
<td>569-588, 919–938</td>
<td>370</td>
<td>NM_0003561</td>
</tr>
<tr>
<td>CD52</td>
<td>5′-GCGCTTCTTCCTCTCCTACT, 5′-GAGTGTGATGGTGACGCTGTTT</td>
<td>39-60, 254–275</td>
<td>235</td>
<td>X62466</td>
</tr>
<tr>
<td>c-fms</td>
<td>5′-AGGAGAGAGCGGGACTATACC, 5′-GAAGTGATGCTGCGATAGCTC</td>
<td>3052-3072, 3375–3395</td>
<td>344</td>
<td>X03663</td>
</tr>
<tr>
<td>HLA-DRα</td>
<td>5′-TGGGACCACATTCTCATCTAAGG, 5′-GGGCATTCAGCAGCACGACAGAC</td>
<td>713-735, 1069–1092</td>
<td>380</td>
<td>K01171</td>
</tr>
<tr>
<td>β-actin</td>
<td>5′-GCTATCCGTAGCGCTGTG, 5′-CTCCTTGTGCATCTGTCGG</td>
<td>458-477, 970–989</td>
<td>532</td>
<td>X00351</td>
</tr>
</tbody>
</table>
Table 2. Analysis of marker enzymes in the neutrophil granule fractions.

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$</th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO$^a$</td>
<td>1651</td>
<td>334</td>
<td>131</td>
<td>94</td>
</tr>
<tr>
<td>NGAL$^a$</td>
<td>2.98</td>
<td>36.90</td>
<td>28.22</td>
<td>2.55</td>
</tr>
<tr>
<td>LF$^a$</td>
<td>41.3</td>
<td>527.8</td>
<td>314.5</td>
<td>32.9</td>
</tr>
<tr>
<td>Gelatinase$^a$</td>
<td>1.6</td>
<td>33.7</td>
<td>115.5</td>
<td>20.9</td>
</tr>
<tr>
<td>HSA$^b$</td>
<td>117</td>
<td>1471</td>
<td>3439</td>
<td>18870</td>
</tr>
<tr>
<td>HLA$^c$</td>
<td>1.3</td>
<td>2.3</td>
<td>3.8</td>
<td>52.8</td>
</tr>
<tr>
<td>Protein$^d$</td>
<td>3.1</td>
<td>3.6</td>
<td>1.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

MPO was used as a marker for azurophilic granules, lactoferrin and NGAL were used as specific granule markers, gelatinase was used as a marker for gelatinase granules, and HSA and HLA were used as markers for secretory vesicles. $^a$µg/ml, $^b$ng/ml, $^c$arbitrary units, $^d$mg/ml.
Figure 1. **RT-PCR analysis of sPLA2 mRNA species expressed in neutrophils isolated by Ficoll density gradient centrifugation.** A, DNA-free RNA extracted from human cardiac myocytes was reverse transcribed and subjected to PCR analysis with primer sets designed to amplify specific regions of GIB, GIIA, GIID, GIIF, GIII, GV and GX sPLA2 cDNA. For each sPLA2 enzyme a PCR product corresponding to the predicted size of the segment of cDNA to be amplified was detected. For GV sPLA2, a second PCR product with a MW of approximately 250 bp was also detected. B, Neutrophils were isolated by dextran sedimentation and Ficoll density gradient centrifugation. Following RNA extraction, RT-PCR analysis was carried out with each set of sPLA2 primers, as described in “Materials and Methods”. The blot shows that GIIA, GIID, GV and GX sPLA2 mRNA was detected in this population of neutrophils. - RT, negative control in which PCR was carried out without RT; act, actin; MW, molecular weight standards. Blots are representative of 4 studies.

Figure 2. **Identification of GV and GX sPLA2 mRNA in neutrophils purified by four-label FACS analysis.** A, Neutrophils were isolated by dextran sedimentation and Ficoll density gradient centrifugation. Cells were then incubated with a PE-conjugated anti-CD16 antibody and FITC conjugated anti-CD3, anti-CD19 and anti-HLA-DR antibodies, and analyzed by dual channel fluorescence. The scatter-gram shows that a readily detectable fraction of the cells in the preparation expressed CD3, CD19 and/or HLA-DR on their cell surface, and that some cells had very low levels of CD16 surface expression. B, RNA extracted from neutrophils was treated with
DNase I, reverse transcribed and analyzed by PCR with primer sets designed to amplify specific regions of c-fms, HLA-DR and CD52, as described in “Materials and Methods”. The detection of c-fms, HLA-DR and CD52 mRNA confirmed that this cell preparation contained macrophages, eosinophils and/or lymphocytes. C, Neutrophils were labeled as in A and subjected to FACS analysis in which PE-labeled cells (i.e. CD16 positive) were separated from FITC labeled cells (i.e. CD3, CD19 or HLA-DR positive cells). In addition, only cells with the forward and side light scattering characteristics of neutrophils were retained. With this protocol, a population of cells that was > 99.9% neutrophils was generated. D, RNA was extracted from FACS purified neutrophils, treated with DNase I, reverse transcribed and analyzed by PCR. No c-fms, HLA-DR and CD52 mRNA was detected in this cell preparation. E, RNA was isolated from neutrophils purified by FACS, treated with DNase I and reverse transcribed. PCR analysis identified GV and GX sPLA₂ mRNA, but failed to identify GIIA or GIID sPLA₂ mRNA. Blots are representative of 3 studies.

Figure 3. Specificity of individual anti-sPLA₂ antisera. Recombinant GIB, GIIA, GIIE, GIIF, GV, GX or GXII sPLA₂ (1 ng each) were applied to a PVDF membrane, washed and probed with anti-GIB, GIIA, GIIE, GIIF, GV, GX or GXII sPLA₂ antisera, as described in “Materials and Methods”. The blot demonstrates that each of the anti-sPLA₂ antisera identified the respective sPLA₂ protein, and did not cross-react with other sPLA₂ proteins. The blot is representative of 3 studies.
Figure 4. Neutrophils contain GV and GX sPLA₂. Neutrophils (10⁶) were disrupted by sonication and resolved into soluble (S) and membrane fractions (MF). The soluble or membrane fractions were then incubated in the presence (+) or absence (-) of 1 ng of recombinant GIB or GIIA sPLA₂, or 1.5 ng of recombinant GIIE, GIIF, GV, GX or GXII sPLA₂ on ice for 10 min. Intact neutrophils (PMN) and the soluble (50 µg) or membrane fraction (8 µg) of these cells, plus or minus the spiked recombinant sPLA₂ proteins, were then resolved by SDS-PAGE, transferred to PVDF membrane and probed with: A, anti-GIB; B, anti-GIIA; C, anti-GIIE; D, anti-GIIF; E, anti-GV; F, anti-GX; or G, anti-GXII sPLA₂ antisera, as described in “Materials and Methods”. For each blot, the recombinant sPLA₂ enzyme was run alone as a control. Representative results for 4-8 separate experiments are shown.

Figure 5. Distribution of GV and GX sPLA₂ in neutrophil granules. Cells were disrupted by N₂ cavitation. The post-nuclear supernatant was loaded on a three-layer Percoll gradient, centrifuged and collected in 1 ml aliquots. The α, β₁, β₂ and γ fractions were formed by pooling fractions 1-6, 7-12, 13-18 and 19-24, respectively, as described in “Materials and Methods”. Aliquots of individual granule fractions (corresponding to 10⁶ neutrophils) were then incubated with vehicle or recombinant GV or GX sPLA₂, as indicated, resolved by SDS-PAGE, transferred to PVDF membrane and probed with A, anti-GV sPLA₂ or B, anti-GX sPLA₂ antisera. For each blot, the recombinant sPLA₂ enzyme was run alone as a control. Representative results for granule preparations from 2 different neutrophil donors are shown.
Figure 6. fMLP and OZ both stimulate the release of GV sPLA_2 from neutrophils. A, 1 ml of cells (2 × 10^7/ml) were resuspended in KRPD with 1 mM CaCl_2 and 0.25% BSA, treated with cytochalasin B and DMSO (open bars) or fMLP (filled bars), centrifuged and the supernatant was assayed for PLA_2 activity, as described in “Materials and Methods”. Results are the mean ± S.D. of 4 experiments. B, Following an identical experimental protocol, 25 µl of the supernatant of DMSO or fMLP treated cells (corresponding to 5 × 10^5 cells) was incubated in the presence (rV) or absence (control) of 1.5 ng of recombinant GV sPLA_2 on ice for 10 min, resolved by SDS-PAGE, transferred to PVDF membrane and probed with the anti-GV sPLA_2 antisera. C, The supernatant of DMSO or fMLP treated cells was incubated in the presence (rX) or absence (control) of 1 ng of recombinant GX sPLA_2 on ice for 10 min, resolved by SDS-PAGE, transferred to PVDF membrane and probed with the anti-GX sPLA_2 antisera, as described in “Materials and Methods”. D, Cells (2 × 10^7) were resuspended in KRPD with 1 mM CaCl_2 and 0.25% BSA, treated with vehicle or OZ for 10 min. The supernatant (25 µl, 5 × 10^5 cells) was then incubated in the presence (rV) or absence (control) of 0.5 ng of recombinant GV sPLA_2 on ice for 10 min, resolved by SDS-PAGE, transferred to PVDF membrane and probed with the anti-GV sPLA_2 antisera. E, The supernatant of vehicle or OZ treated cells was incubated in the presence (rX) or absence (control) of 1 ng of recombinant GX sPLA_2 on ice for 10 min, resolved by SDS-PAGE, transferred to PVDF membrane and probed with the anti-GX sPLA_2 antisera. Results are representative of 8 separate experiments.
Figure 7. **Effect of LY311727 or Indoxam on the catalytic activity of recombinant GIIA, GV and GX sPLA₂.** A, Increasing quantities of recombinant GIIA, GV or GX sPLA₂ were incubated with [³H]oleate labeled *E. coli* membranes for 30 min, and PLA₂ activity was measured, as described in “Materials and Methods”. B, 0.25 ng of GIIA sPLA₂, 2.5 ng of GV sPLA₂ or 5 ng of GX sPLA₂ were incubated with [³H]oleate labeled *E. coli* membranes for 15, 30 or 45 min, and PLA₂ activity was measured, as described in “Materials and Methods”. The mean specific activity for recombinant GIIA, GV and GX sPLA₂ was 45.6 ± 3.8, 10.8 ± 0.3 and 3.2 ± 0.5 U/ng, respectively. C, 0.25 ng of GIIA sPLA₂, 2.5 ng of GV sPLA₂ or 5 ng of GX sPLA₂ was incubated with increasing concentrations of LY311727 or D, Indoxam for 10 min. Following addition of [³H]oleate labeled *E. coli* membranes for 30 min, and PLA₂ activity was measured. All results are the mean ± S.D. of at least 4 separate experiments.

Figure 8. **Effect of inhibition of extracellular GV sPLA₂ activity on LTB₄ production by fMLP stimulated neutrophils.** A, Cells (2×10⁷/ml) were resuspended in KRPD with 1 mM CaCl₂ and 0.25% BSA, treated sequentially with cytochalasin B and fMLP and centrifuged, as described in “Materials and Methods”. The supernatant from these cells was then incubated with increasing concentrations of Indoxam (0 to 10 μM), and PLA₂ activity was measured. B, Cells (20×10⁶/ml) were resuspended in KRPD with 1 mM CaCl₂ and 0.25% BSA, incubated with increasing concentrations of Indoxam (0 to 10 μM) and treated sequentially with cytochalasin B and...
DMSO (open bars) or fMLP (filled bars). Following centrifugation, the supernatant was assayed for LTB₄, as described in “Materials and Methods”. Results are the mean ± S.D. of 4 experiments.

Figure 9. Effect of inhibition of cPLA₂α on LTB₄ production by fMLP or OZ stimulated neutrophils. Cells (2×10⁷/ml) were resuspended in KRPD with 1 mM CaCl₂ and 0.25% BSA, pre-incubated with increasing concentrations of Pyrolidine-1 for 10 min and treated with A, cytochalasin B and DMSO (open bars) or fMLP (filled bars) or B, vehicle (open bars) or OZ (filled bars). Following centrifugation, the supernatant was assayed for LTB₄, as described in “Materials and Methods”. Results are the mean ± S.D. of 3 independent experiments.

Figure 10. RecombinantGIIaandGVsPLA₂, but not recombinantGXsPLA₂, hydrolyze gram-negative bacterial phospholipids. E. coli was metabolically labeled with [³H]oleic acid, washed extensively with 150 mM NaCl and 1% BSA and co-incubated with 0-500 ng of recombinant GIIA (open bars), GV (filled bars) or GX sPLA₂ (hatched bars). Bacterial phospholipid hydrolysis was estimated by measuring the release of [³H]oleic acid into the supernatant, as described in “Materials and Methods”. Results are the mean ± S.D. of 4 experiments.
Figure 3
A  PMN  S  MF  S  MF
  24 -  19 -
  -  -  -  +  +  +  rGIB PLA₂

B  PMN  S  MF  S  MF
  24 -  19 -
  -  -  -  +  +  +  rGIIA PLA₂

C  PMN  S  MF  S  MF
  24 -  19 -
  -  -  -  +  +  +  rGIIE PLA₂

D  PMN  S  MF  S  MF
  24 -  19 -
  -  -  -  +  +  +  rGIIIF PLA₂

Figure 4
Figure 4
Figure 5
Figure 6
Figure 7
Figure 9