THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 7, Issue of February 15, pp. 5061–5073, 2002

Printed in U.S.A.

Groups IV, V, and X Phospholipases A_2s in Human Neutrophils

ROLE IN EICOSANOID PRODUCTION AND GRAM-NEGATIVE BACTERIAL PHOSPHOLIPID HYDROLYSIS*

Received for publication, September 20, 2001, and in revised form, December 5, 2001
Published, JBC Papers in Press, December 6, 2001, DOI 10.1074/jbc.M109083200

Norbert Degousee,[a] Farideh Ghomashchi,[b] Eva Stefanski,[a] Alan Singer,[b] Brian P. Smart,[b] Niels Borregaard,[c] Reinhardt Reithmeier,[d] Thomas F. Lindsay,[a] Cornelia Lichtenberger,[e] Walter Reinisch,[e] Gerard Lambeau,[f] Jonathan Arm,[g] Jay Tischfield,[h] Michael H. Gelb,[b,i] and Barry B. Rubin[a,j]

From the [a]Division of Vascular Surgery, Max Bell Research Center, Toronto General Hospital, University Health Network, Toronto M5G 2C4, Canada, the [b]Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195, [c]Granulocyte Research Laboratory, Department of Hematology, Copenhagen University Hospital, Copenhagen DK-2100, Denmark, [d]Medical Research Council Group in Membrane Biology, Departments of Medicine and Biochemistry, Medical Sciences Building, University of Toronto, Toronto, Ontario M5S 1A8, Canada, [e]Clinic of Internal Medicine IV, Department of Gastroenterology and Hepatology, University of Vienna, Vienna A-1090, Austria, the [f]Institut de Pharmacologie Moleculaire et Cellulaire, CNRS-UPR 411, 660 Route des Lucioles, Sophia Antipolis, 06560 Valbonne, France, [g]Department of Allergy and Immunology, Harvard Medical School, Boston, Massachusetts 20115, and the [h]Department of Genetics, Rutgers, the State University of New Jersey, Piscataway, New Jersey 08854-8082

The bacterial tripeptide formyl-Met-Leu-Phe (fMLP) induces the secretion of enzyme(s) with phospholipase A_2 (PLA_2) activity from human neutrophils. We show that 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, whereas GX $sPLA_2$ is confined to azurophilic granules. Exposure to fMLP or opsonized zymosan results in the release of GV but not GX $sPLA_2$ and 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 leukotriene B_4 production but may support the anti-bacterial properties of the neutrophil, because 10–100 ng per ml concentrations of this enzyme lead 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 $PLA_2\alpha$), we show that this enzyme produces virtually all of the arachidonic acid used for the biosynthesis of leukotriene B_4 in fMLP- and opsonized zymosan-stimulated neutrophils, the major eicosanoid produced by these pro-inflammatory cells.

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 A_2 (PLA_2)[1] (1–3). PLA_2 enzymes catalyze the hydrolysis of phospholipids, yielding free fatty acids and lysophospholipids. The identity of the PLA_2 enzyme(s) that are contained in and secreted by human neutrophils has not been defined.

A family of 10 secreted PLA_2 ($sPLA_2$) enzymes has been described in mammals that currently includes group (G) IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII $sPLA_2$ (4, 5). $sPLA_2$ 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 $sPLA_2$ receptors (10–12). The different $sPLA_2$s are not close isoforms of each other because their amino acid sequences are ~30–50% identical among the paralogs. This plus the fact that the $sPLA_2$ enzymes have distinct tissue distributions argue for different physiological functions for each enzyme (5, 13). Thus, GIB $sPLA_2$ 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 $sPLA_2$ is expressed at high levels during inflammatory reactions (15, 16) and, until recently (4, 5, 13), was thought to be the principal $sPLA_2$ isoform in the immune system. Human GIIA $sPLA_2$ mRNA has been identified in myocardium, skeletal muscle, lung, liver, placenta, prostate, and the small and large intes-

* This work was supported in part by Heart and Stroke Foundation of Canada Grant NA-4151 and the Physician Service Inc. Foundation of Ontario (to B. B. R.), by the Canadian Institutes of Health Research (to R. R.), by CNRS and the Association pour le Recherche sur le Cancer (to G. L.), by Danish Medical Research Council Grant 52-00-0887 (to N. B.), and by National Institutes of Health Grants HL36235 and HL50040 (to M. G.), PO1 HL36110 (to J. A.), and DK38185 (to J. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[i] To whom correspondence may be addressed: Depts. of Chemistry and Biochemistry, Campus Box 351700, University of Washington, Seattle, WA 98195. Tel.: 206-543-7142; Fax: 206-685-8665; E-mail: gelb@chem.washington.edu.

[j] 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. To whom correspondence may be addressed: Division of Vascular Surgery, 200 Elizabeth St., EC5-302a, Toronto General Hospital, Toronto, Ontario *M5G-2C4, Canada. Tel.: 416-340-3645; Fax: 416-340-5029; E-mail: barry.rubin@uhn.on.ca.

[1] The abbreviations used are: PLA_2, phospholipase A_2; GIIA PLA_2, group IIA phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; $cPLA_2$, Ca^{2+}-dependent cytosolic phospholipase A_2 (= group IV PLA_2); $cPLA_2\alpha$, $cPLA_2\beta$ and $cPLA_2\gamma$, α, β and γ isoforms of $cPLA_2$; PMN, polymorphonuclear leukocyte (neutrophil); MAFP, methylarachidonyl fluorophosphonate; PGE_2, prostaglandin E_2; FACS, fluorescence-activated cell sorter; RT, reverse transcriptase; R-PE, rhodamine-phycoerythrin; LTB_4, leukotriene B_4; FITC, fluorescein isothiocyanate; BPI, bactericidal/permeability-increasing protein; MPO, myeloperoxidase; NGAL, neutrophil gelatinase-associated lipocalin; OZ, serum opsonized zymosan; BSA, bovine serum albumin; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PVDF, polyvinylidene difluoride; h, human.

tine but was not detected in peripheral blood leukocytes (17). GIIC sPLA$_2$ is present in the mouse but appears as a pseudo gene in humans (4). GIID sPLA$_2$ mRNA was detected in human spleen, thymus, small intestine, colon, lung, pancreas, and placenta (17, 18), whereas GIIE sPLA$_2$ expression in humans is restricted to the brain, heart, lung, and placenta (19). mRNA coding for GIIF sPLA$_2$ has been identified in many human tissues, with the highest levels detected in the placenta, thymus, prostate, testes, kidney, liver, and thyroid (17). GIII sPLA$_2$, which is homologous to bee venom PLA$_2$, is expressed in the pancreas, kidney, liver, lung, skeletal muscle, and myocardium (17, 20). GV sPLA$_2$ has been identified in myocardium, placenta, mast cells, and macrophages (21–24). Although GV sPLA$_2$ was shown to contribute to platelet-activating factor-mediated PGE$_2$ production by a murine macrophage-like cell line (24), GV sPLA$_2$ mRNA was not identified in human peripheral blood leukocytes (17). GX sPLA$_2$ is expressed in organs associated with the immune response (25) and induces cyclooxygenase-2-dependent PGE$_2$ synthesis by adherent mammalian cells (26–28). One study identified GX sPLA$_2$ mRNA in peripheral blood leukocytes (25), whereas two others did not (17, 29). Transcripts for GXII sPLA$_2$ were identified in multiple tissues, including myocardium, skeletal muscle, kidney, pancreas, and type 2 helper T cells (30, 31).

sPLA$_2$ enzymes share a number of structural characteristics, including several intramolecular disulfide bridges, a Ca^{2+}-binding loop, and the requirement for sub-millimolar to millimolar concentrations of Ca^{2+} for catalytic activity (32–34). In addition, some of the sPLA$_2$ enzymes have N-terminal prepropeptide sequences of varying lengths (5, 13). The mechanism by which prepropeptide sPLA$_2$ enzymes mature into secreted proteins has not been fully defined. Some sPLA$_2$ enzymes have dibasic motifs at the C terminus of their signal sequence, such as the arginine doublet in GX sPLA$_2$ (25), that may be efficiently cleaved by subtilisin-like protein convertases in the Golgi apparatus (35). Some sPLA$_2$ enzymes, including GIB and GX sPLA$_2$, are secreted as proenzymes and then cleaved to yield mature, catalytically active proteins, whereas others, like GIIA and GV sPLA$_2$, do not have an extra N-terminal peptide. GX sPLA$_2$ also contains a single consensus sequence for post-translational modification, the *N*-glycosylation acceptor site (Asn-X-Ser/Thr) at Asn-113 (25), and *N*-glycosylation of GX sPLA$_2$ was demonstrated when this enzyme was expressed in HEK293 cells (36).

PLA$_2$ 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 sPLA$_2$ 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 Ref. 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 methylarachidonyl 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 platelet-activating factor following exposure to inflammatory stimuli than macrophages from wild type mice (cPLA$_2^{+/+}$) (46, 47). Three isoforms of cPLA$_2$ have been identified as follows: cPLA$_2\alpha$, cPLA$_2\beta$, and cPLA$_2\gamma$ (43, 48, 49). Although 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 homogeneous 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), whereas 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, pretreatment 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 *Escherichia coli* in the presence of serum.

EXPERIMENTAL PROCEDURES

Materials—Ficoll-Paque was from Amersham Biosciences and was used for neutrophil isolation. DNase I RNase-free was from Qiagen (Mississauga, Ontario, Canada). *Thermus aquaticus* (*Taq*) polymerase was from MBI Fermentas (Burlington, Ontario, Canada). Rhodamine-phycoerythrin (R-PE)-conjugated anti-CD16 monoclonal antibody (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-^{3}H]Oleic acid (NFT 289, 5 Ci/mmol) was from PerkinElmer Life Sciences. Myocardial RNA was from Ambion (Austin, TX). DNA sequence analysis was performed at the "DNA Sequencing Facility" of The Hospital for Sick Children (Toronto, Ontario, Canada). Isolation of the IgG fraction from serum was done with the ImmunoPure Immobilized Protein G Plus Orientation Kit (Pierce). The *E. coli* strain pldA$^-$ was kindly provided by Dr. Peter Elsbach (New York University School of Medicine, New York). Zymosan was obtained from Sigma, and OZ was prepared as described previously (53). Indoxam and pyrrolidine-1 were synthesized as described elsewhere (50, 52).

Neutrophil Isolation—After obtaining informed consent from healthy donors that were not taking any medications, 100 ml of blood was obtained and anticoagulated in citrate. Neutrophils were then isolated by dextran sedimentation and Ficoll-Paque density gradient centrifugation, exactly as described (44).

Neutrophil Purification by Fluorescence-activated Cell Sorting (FACS) Analysis—Neutrophils (1×10^7) enriched by Ficoll centrifuga-

tion were incubated for 30 min at 4 °C with 20% heat-inactivated AB serum prepared in KRPD buffer (where KRPD is Krebs-Ringers phosphate dextrose), 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-megawatt 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$^-$, and HLA-DR$^-$ cells were separated by sorting in the "sort purify" mode setting with a flow rate of 10,000 cells/s. 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, Ontario, Canada). 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 $CHCl_3$ and vigorous shaking, tubes were centrifuged at 12,000 × *g* for 15 min at 4 °C. The aqueous phase was aspirated and 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 4 °C. 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 units/ml) at 37 °C for 30 min. DNase I was then inactivated by heat treatment for 5 min at 70 °C. 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 the manufacturer's instructions. Briefly, 5 μl of the DNA-free RNA preparations from Ficoll-enriched or FACS-sorted CD16$^+$ human neutrophils or 2 μg of DNA-free RNA extracted from myocardial tissue (Ambion, Austin, TX) was reverse-transcribed using 40 units of Moloney murine leukemia virus-reverse transcriptase in the presence of 0.5 μg of oligo(dT_{18}) primers, 50 mmol/liter Tris-HCl, pH 8.3, 50 mmol/liter KCl, 4 mmol/liter $MgCl_2$, 10 mmol/liter dithiothreitol, deoxynucleotide (dNTP) mix (1 mmol/liter each), and 20 units of RNase inhibitor. The reaction mixture was incubated for 60 min at 39 °C (transcription) and 10 min at 70 °C (inactivation of RT). The cDNA mixture was then diluted with RNase-free water to a final volume of 100 μl.

PCR—A total of 5 μl of the diluted first strand cDNA was amplified in a PCR 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/liter Tris-HCl, pH 8.4, 50 mmol/liter KCl, 1.5 mmol/liter $MgCl_2$, dNTP mix (0.2 mmol/liter each), 2 units of *Taq* polymerase, and 0.5 μmol/liter 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 s at 95 °C, 45 s at 60 °C, and 50 s 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 data base. When gene sequence data were 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 and 65%, a size between 18- and 23-mer, and to exclude primer-dimer structures. The sequences of the sPLA$_2$ primers and predicted molecular weight of the PCR products for GIB, GIIA, GIID, GIIF, GIII, GV, and GX sPLA$_2$ and for CD52, c-Fms, HLA-DRα, and β-actin are listed in Table I.

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, Foster 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.[2] 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 high performance liquid chromatography/mass spectrometer. The observed mass of all sPLA$_2$s was within 0.5 atomic mass units 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 subcutaneous and intramuscular 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-sPLA$_2$ antisera were tested for specificity toward the various human sPLA$_2$s (hGIB, hGIIA, hGIIE, hGIIF, hGV, hGX, and hGXII), as described below. Whereas each antiserum readily detected 1 ng of recombinant sPLA$_2$, no signal was detected when 50 ng of each of the other human sPLA$_2$s were analyzed (ECL detection, Amersham Biosciences). Thus, the individual antisera were highly specific for each of the human sPLA$_2$ enzymes. The anti-GV sPLA$_2$ and anti-GX sPLA$_2$ 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 phosphate-buffered saline and centrifuged. The pellet was suspended in 66 mM HEPES buffer, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 25 mM NaF, 1 mM diisopropylfluorophate, 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 (1.1×10^9) 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 diisopropylfluorophate 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 phenylmethylsulfonyl fluoride) and centrifuged (55). Samples were collected in fractions of 1 ml. Fractions 1–6 were pooled and designated as the α-band, fractions 7–12 the β_1-band, fractions 13–18 the β_2-band, and fractions 19–24 the γ-band. Analysis for the granule markers MPO, NGAL, lactoferrin, gelatinase, human serum albumin, and HLA was carried out exactly as described (56) and showed that less than 0.5% of the MPO, lactoferrin, 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, granule fractions, and soluble or microsomal fractions were analyzed by SDS-PAGE using 16.5% gels at a constant 100 V in 100 mM Tris, 100 mM Tricine, and 0.1% SDS as running buffer. Proteins were transferred to a PVDF membrane (PerkinElmer Life Sciences) in 25 mM Tris-HCl, 192 mM glycine, 20% methanol (0.1% SDS for GIIA sPLA$_2$, 0.01% SDS for GV sPLA$_2$ and GX sPLA$_2$, and no SDS for the other sPLA$_2$ enzymes), pH 8.3 ±0.1 at 100 V for 1 h, 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 h at room temperature, washed, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. 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 37 °C for

[2] M. Gelb, manuscript in preparation.

TABLE I
Characteristics of the primers used for RT-PCR analysis

Protein	Sense and antisense PCR primers	Corresponding nucleotides in human cDNA	Predicted M_r of DNA product	GenBank™ accession number
			bp	
GIB sPLA$_2$	5′-TCCTTGTGCTAGCTGTGCTG, 5′-TGAAGGCCTCACACTCTTTG	45–64, 366–385	341	NM_000928
GIIA sPLA$_2$	5′-ATGAAGACCCTCCTACTGTT, 5′-TCAGCAACGAGGGGTGCTCC	136–155, 551 –570	434	NM_000300
GIID sPLA$_2$	5′-GAAAATGCCCATCCTCTCCT, 5′-AAACGCAGTCGCTTCTGGTA	138–157, 412 –431	294	NM_012400
GIIF sPLA$_2$	5′-ATGAAGAAGTTCTTCACCGTGGC, 5′-CAGTGTTGTTCTCGATGGTGTGA	1–23, 261–283	283	NM_022819
GIII sPLA$_2$	5′-TGCCTACAGAATCAGCACGA, 5′-TTGAGCAGCTGGAACTCGAT	911–930, 1391–1410	500	AF_220490
GV sPLA$_2$	5′-TTGGTTCCTGGCTTGTAGTGTG, 5′-TGGGTTGTAGCTCCGTAGGTTT	156–177, 492–513	358	NM_000929
GX sPLA$_2$	5′-GATCCTGGAACTGGCAGGAA, 5′-TCAGTCACACTTGGGCGAGT	569–588, 919–938	370	NM_0003561
CD52	5′-GCGCTTCCTCTTCCTCCTACTC, 5′-GAGTGATGGTGGCAGCTGTTTC	39–60, 254–275	235	X62466
c-Fms	5′-AGGAGAGAGCGGGACTATACC, 5′-GAAGTGGGATCCTCTGACCTC	3052–3072, 3375–3395	344	X03663
HLA-DRα	5′-TGGGACCATCTTCATCATCAAGG, 5′-GGGCATTCCATAGCAGAGACAGAC	713–735, 1069–1092	380	K01171
β-Actin	5′-GCTATCCCTGTACGCCTCTG, 5′-CTCCTTCTGCATCCTGTCGG	458–477, 970–989	532	X00351

10 min, and treated with 5 μM cytochalasin B for 2 min and either 0.1% Me_2SO or 1 μM fMLP for 10 min. Stimulations were terminated by centrifugation at 14,000 × *g* for 1 min. The PLA$_2$ activity in 50 μl of extracellular fluid was determined by measuring the amount of free [^{3}H]oleic acid released from [^{3}H]oleic acid-labeled autoclaved *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 $CaCl_2$, 10 mg of fatty acid-free BSA, and 2.8 × 10^8 radiolabeled, autoclaved *E. coli* (corresponding to 5.6 nmol of phospholipid). After a 30-min incubation at 37 °C, the reaction was terminated by filtration through a 0.45-μm Millipore filter, and the released [^{3}H]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 PLA$_2$ activity is defined as the amount of enzyme that hydrolyzes 56 pmol of phospholipid substrate in 30 min at 37 °C, which corresponds to 1% of the total *E. coli* substrate.

LTB$_4$ Production—In some experiments, neutrophils were preincubated with increasing concentrations of indoxam or pyrridoline-1 for 10 min at 37 °C, 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 4 °C, and LTB$_4$ release was subsequently determined by enzyme-linked immunosorbent assay, as described by the manufacturer (Cayman).

Radiolabeling of Live E. coli—An inoculum of *E. coli* pldA$^-$ (lacking the principal envelope phospholipase) was diluted 1:10 in fresh LB medium and grown for 2 h at 37 °C to mid-log phase in a shaking water bath. Bacteria were harvested at 3000 rpm for 10 min, resuspended in 0.2% lactalbumin medium supplemented with 3 μCi/ml [9,10-^{3}H]oleic acid (PerkinElmer Life Sciences catalog number NFT 289, 5 Ci/mmol) complexed with 0.02% bovine serum albumin (fatty acid-free), and incubated for 2 h while being shaken. 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 three times in 150 mM NaCl containing 1% BSA to remove unincorporated [9,10-^{3}H]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 sPLA$_2$—Two ml of serum from the blood of healthy donors was pooled and passed over a 1-ml heparin column (Amersham Biosciences), resulting in the removal of detectable PLA$_2$ activity (data not shown). For the assay, [9,10-^{3}H]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 preincubation at 37 °C, human recombinant GIIA, V, or X sPLA$_2$ was added to a 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 nonspecific hydrolysis of labeled *E. coli*.

Statistical Analysis—Results are expressed as the mean ± S.D. 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 sPLA$_2$ mRNA in Human Neutrophils—To determine which of the sPLA$_2$ mRNA species were expressed by neutrophils, sets of primers for GIB, GIIA, GIID, GIIF, GIII, GV, and GX sPLA$_2$ were designed (Table I). The ability of each of these primer sets to amplify their respective sPLA$_2$ mRNA by RT-PCR was confirmed with mRNA that had been isolated from human myocardium, as shown in Fig. 1*A*. Multiple primer sets were also generated for GIIE sPLA$_2$. However, we were unable to detect GIIE sPLA$_2$ mRNA in myocardium. Hanasaki and co-workers (19) reported that GIIE sPLA$_2$ mRNA was detected only after multiple rounds of PCR amplification, indicating that this protein is expressed at very low levels, and we have not been able to detect GIIE sPLA$_2$ 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 sPLA$_2$ mRNA species (Fig. 1*B*) but failed to identify GIB, GIIF, or GIII sPLA$_2$ 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. 2*A*), cells that could be a source of sPLA$_2$ mRNA

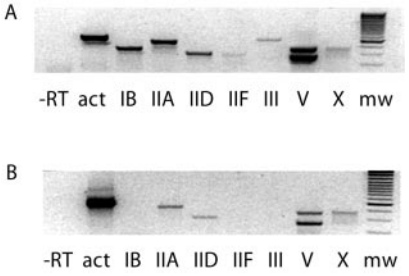

FIG. 1. **RT-PCR analysis of sPLA$_2$ mRNA species expressed in neutrophils isolated by Ficoll density gradient centrifugation.** *A,* DNA-free RNA extracted from human cardiac myocytes was reverse-transcribed and subjected to PCR analysis with primer sets designed to amplify specific regions of GIB, GIIA, GIID, GIIF, GIII, GV, and GX sPLA$_2$ cDNA. For each sPLA$_2$ enzyme a PCR product corresponding to the predicted size of the segment of cDNA to be amplified was detected. For GV sPLA$_2$, a second PCR product with a molecular weight of ~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 sPLA$_2$ primers, as described under "Materials and Methods." The blot shows that GIIA, GIID, GV, and GX sPLA$_2$ mRNA were 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 four studies.

(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. 2*B*) (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. 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 homogeneous population of cells (>99.9% neutrophils, Fig. 2*C*) 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. 2*D*). When RNA from neutrophils that had been purified by cell sorting was subjected to RT-PCR analysis, GV and GX sPLA$_2$ mRNA species were identified, whereas GIIA and GIID mRNA species were not detected (Fig. 2*E*).

Sequence Analysis of the GV and GX sPLA$_2$ RT-PCR Products—RT-PCR analysis with a primer set designed to amplify GV sPLA$_2$ mRNA yielded two distinct products when human myocardium (Fig. 1*A*) or FACS-purified human neutrophils (Fig. 2*E*) were used as the source of RNA. Following amplification of the putative GV sPLA$_2$ PCR products, nucleotide sequence analysis demonstrated that the 358-bp PCR product corresponds to nucleotides 24–381 of human GV sPLA$_2$ mRNA. Sequence analysis of the 251-bp PCR product also confirmed a 100% match with GV sPLA$_2$ mRNA from nucleotides 24–381, in which nucleotides 186–292, which correspond to exon 4 of the hGV sPLA$_2$ gene, have been deleted. Nucleotide sequence analysis also demonstrated that the 370-bp PCR product generated with the primer set for GX sPLA$_2$ mRNA corresponded to nucleotides 569–938 of hGX sPLA$_2$ mRNA. No sequence data were obtained from the lower molecular weight product that was generated with the primer set for GX sPLA$_2$ mRNA.

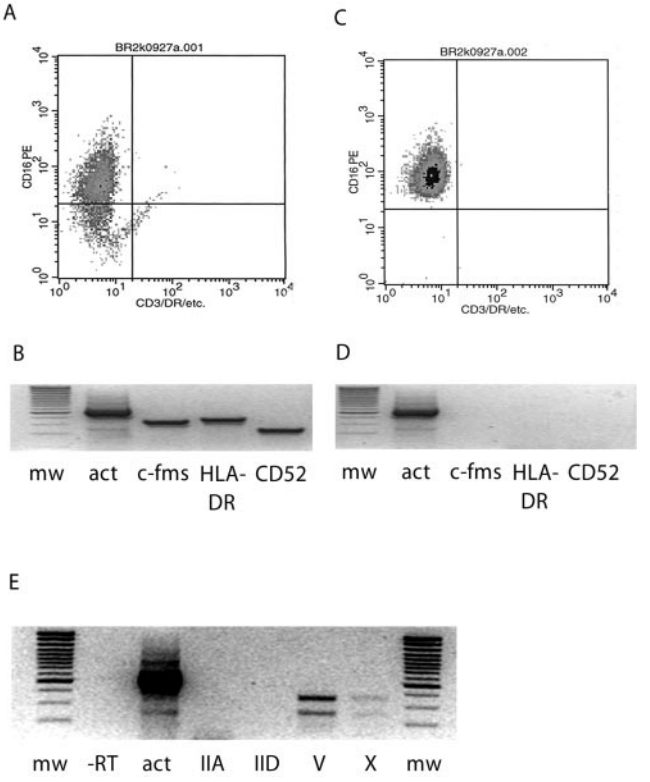

FIG. 2. **Identification of GV and GX sPLA$_2$ mRNA in neutrophils purified by four-label FACS analysis.** *A,* neutrophils were isolated by dextran sedimentation and Ficoll density gradient centrifugation. Cells were then incubated with a PE-conjugated anti-CD16 antibody and FITC-conjugated anti-CD3, anti-CD19, and anti-HLA-DR antibodies and analyzed by dual channel fluorescence. The scattergram 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 under "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 cells) 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 sPLA$_2$ mRNA but failed to identify GIIA or GIID sPLA$_2$ mRNA. Blots are representative of three studies. *act,* actin; *mw*, molecular weight standards.

These results provide direct evidence that circulating neutrophils express GV and GX sPLA$_2$ mRNA species.

Identification of GV and GX sPLA$_2$ Enzymes in Circulating Human Neutrophils—To determine which sPLA$_2$ enzymes are present in circulating human neutrophils, Western blot analysis was carried out for GIB, IIA, IIE, IIF, V, X, and XII sPLA$_2$. The antisera used in these studies were specific for each of the sPLA$_2$ enzymes and did not cross-react with any other known human sPLA$_2$s (Fig. 3). To evaluate neutrophils for the presence of individual sPLA$_2$ 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 sPLA$_2$ enzyme (as a positive control), and evaluated by Western blotting. The corresponding sPLA$_2$ enzymes were also run alone, in separate

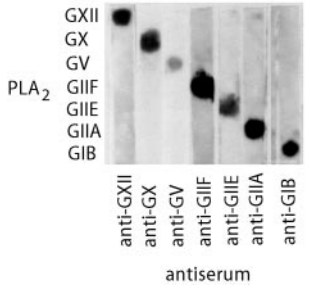

FIG. 3. **Specificity of individual anti-sPLA$_2$ antisera.** Recombinant GIB, GIIA, GIIE, GIIF, GV, GX, or GXII sPLA$_2$ (1 ng each) were applied to a PVDF membrane, washed, and probed with anti-GIB, GIIA, GIIE, GIIF, GV, GX, or GXII sPLA$_2$ antisera, as described under "Materials and Methods." The blot demonstrates that each of the anti-sPLA$_2$ antisera identified the respective sPLA$_2$ protein and did not cross-react with other sPLA$_2$ proteins. The blot is representative of three studies.

lanes, as additional positive controls. Therefore, we were able to determine whether proteins identified by individual anti-sPLA$_2$ antisera migrated at the same apparent molecular weight as recombinant sPLA$_2$ proteins following SDS-PAGE. With this approach, we identified proteins that were detected with the anti-GV sPLA$_2$ and anti-GX sPLA$_2$ antisera, in whole cell lysates and in the soluble fraction of neutrophils, that co-migrated with the respective recombinant enzymes (Fig. 4, *E* and *F*). When the soluble fraction of neutrophils was spiked with recombinant GV sPLA$_2$ and evaluated by Western blotting, a single band was identified that co-migrated with recombinant GV sPLA$_2$ (Fig. 4*E*). Similarly, when the soluble fraction of neutrophils was spiked with recombinant GX sPLA$_2$, Western blotting studies identified a single band that co-migrated with recombinant GX sPLA$_2$ (Fig. 4*F*). In contrast, proteins that co-migrated with recombinant GIB, GIIA, GIIE, GIIF, or GXII sPLA$_2$ were not detected by Western blot analyses using highly specific antisera (Fig. 4). For each of these later sPLA$_2$s, we confirmed that the addition of the respective recombinant sPLA$_2$ protein to the soluble or microsomal fraction of neutrophils resulted in the appearance of a band that co-migrated with the recombinant sPLA$_2$ protein. These positive controls indicate that GIB, GIIA, GIIE, GIIF, or GXII sPLA$_2$ are not being degraded in neutrophil lysates. Furthermore, the detection limit for sPLA$_2$ 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 sPLA$_2$ mRNAs and proteins are present in circulating human neutrophils.

Distribution of GV and GX sPLA$_2$ in the Granules of Circulating Human Neutrophils—To determine whether GV and GX sPLA$_2$ 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, β_1, β_2, and γ fractions demonstrated that this protocol resulted in the separation of the azurophil, specific and gelatinase granules, and secretory vesicles (Table II). As shown in Fig. 5*A*, we identified a protein in the α, β_1, and β_2 fractions that co-migrated with recombinant GV sPLA$_2$. Slightly less GV sPLA$_2$ was detected in the β_1 fraction than in the α fraction, with minimal GV sPLA$_2$ in the β_2 fraction. No proteins that co-migrated with recombinant GV sPLA$_2$ were identified in the γ fraction, which contains both secretory vesicles and plasma membranes (67). Similarly, we identified a protein in the α, β_1, and β_2 fractions that co-migrated with recombinant GX sPLA$_2$ (Fig. 5*B*). The majority

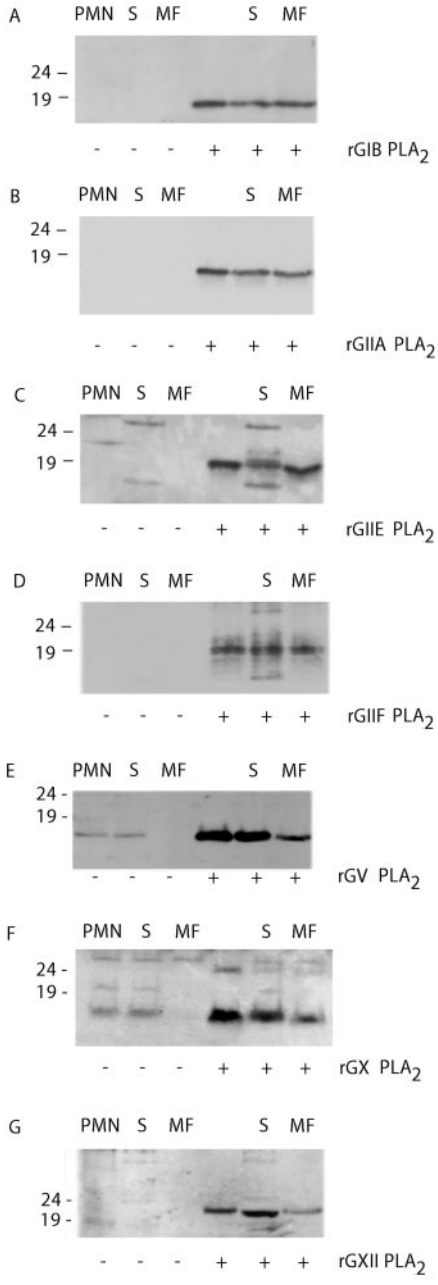

FIG. 4. **Neutrophils contain GV and GX sPLA$_2$.** Neutrophils (10^6) were disrupted by sonication and resolved into soluble (*S*) and membrane fractions (*MF*). The soluble or microsomal fractions were then incubated in the presence (+) or absence (−) of 1 ng of recombinant GIB or GIIA sPLA$_2$ or 1.5 ng of recombinant GIIE, GIIF, GV, GX, or GXII sPLA$_2$ on ice for 10 min. Intact neutrophils (*PMN*) and the soluble (50 μg) or microsomal fraction (8 μg) of these cells, plus or minus the spiked recombinant sPLA$_2$ proteins, were then resolved by SDS-PAGE, transferred to PVDF membrane, and probed with the following: *A,* anti-GIB; *B,* anti-GIIA; *C,* anti-GIIE; *D,* anti-GIIF; *E,* anti-GV; *F,* anti-GX; or *G,* anti-GXII sPLA$_2$ antisera as described under "Materials and Methods." For each blot, the recombinant sPLA$_2$ enzyme was run alone as a control. Representative results for 4–8 separate experiments are shown.

of the GX sPLA$_2$ was identified in the α fraction, with comparatively little GX sPLA$_2$ in the β_1 or β_2 fractions. No proteins that co-migrated with recombinant GX sPLA$_2$ were identified in the γ fraction. The distribution of GX sPLA$_2$ follows the distribution of the azurophil granule marker, MPO (Table II). In contrast, the distribution of GV sPLA$_2$ follows the distribution of the azurophil granule marker MPO and the specific granule markers lactoferrin and NGAL. This indicates that GX

TABLE II

Analysis of marker enzymes in the neutrophil granule fractions

MPO was used as a marker for azurophilic granules; lactoferrin (LF) and NGAL were used as specific granule markers; gelatinase was used as a marker for gelatinase granules; and human serum albumin (HSA) and HLA were used as markers for secretory vesicles.

	α	β_1	β_2	γ
MPO[a]	1651	334	131	94
NGAL[a]	2.98	36.90	28.22	2.55
LF[a]	41.3	527.8	314.5	32.9
Gelatinase[a]	1.6	33.7	115.5	20.9
HSA[b]	117	1471	3439	18,870
HLA[c]	1.3	2.3	3.8	52.8
Protein[d]	3.1	3.6	1.6	1.5

[a] Values are shown as μg/ml.
[b] Values are shown as ng/ml.
[c] Values are shown as arbitrary units.
[d] Values are shown as mg/ml.

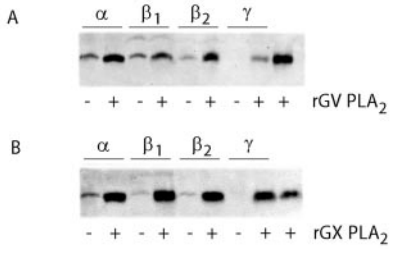

FIG. 5. **Distribution of GV and GX sPLA$_2$ in neutrophil granules.** Cells were disrupted by N_2 cavitation. The post-nuclear supernatant was loaded on a three-layer Percoll gradient, centrifuged, and collected in 1-ml aliquots. The α, β_1, β_2, and γ fractions were formed by pooling fractions 1–6, 7–12, 13–18, and 19–24, respectively, as described under "Materials and Methods." Aliquots of individual granule fractions (corresponding to 10^6 neutrophils) were then incubated with vehicle or recombinant GV or GX sPLA$_2$, as indicated, resolved by SDS-PAGE, transferred to PVDF membrane, and probed with anti-GV sPLA$_2$ (*A*) or anti-GX sPLA$_2$ (*B*) antisera. For each blot, the recombinant sPLA$_2$ enzyme was run alone as a control. Representative results for granule preparations from two different neutrophil donors are shown.

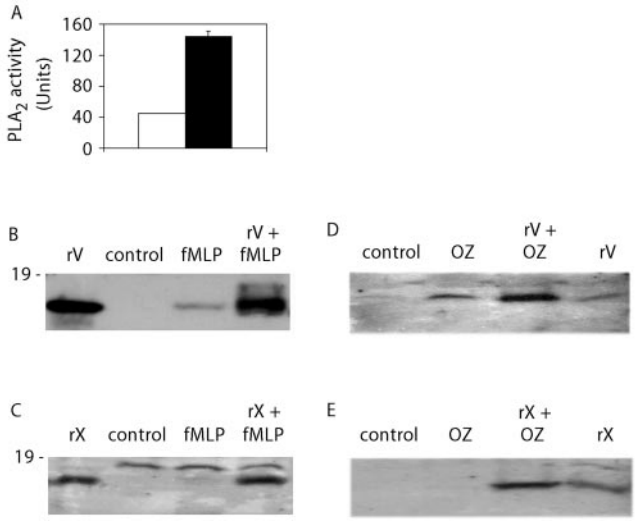

FIG. 6. **fMLP and OZ both stimulate the release of GV sPLA$_2$ from neutrophils.** *A,* 1 ml of cells (2×10^7/ml) was resuspended in KRPD with 1 mM $CaCl_2$ and 0.25% BSA, treated with cytochalasin B and Me_2SO (*open bars*) or fMLP (*filled bars*), and centrifuged, and the supernatant was assayed for PLA$_2$ activity as described under "Materials and Methods." Results are the mean ± S.D. of four experiments. *B,* following an identical experimental protocol, 25 μl of the supernatant of Me_2SO- 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 Me_2SO- 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 under "Materials and Methods." *D,* cells (2×10^7) were resuspended in KRPD with 1 mM $CaCl_2$ and 0.25% BSA and 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 eight separate experiments.

sPLA$_2$ is localized to azurophil granules solely, whereas GV sPLA$_2$ is localized to both azurophil and specific granules.

Neutrophils Release GV sPLA$_2$ following Exposure to fMLP or OZ—sPLA$_2$ enzymes may be released by cells into their extracellular environment (37). Thus, when neutrophils were exposed to the bacterial tripeptide fMLP, sPLA$_2$ activity (as measured with radiolabeled bacterial membranes) in the extracellular fluid increased by 3–4-fold (Fig. 6*A*), a finding consistent with previous results (44). To determine whether the increase in extracellular PLA$_2$ activity was associated with the release of GV or GX sPLA$_2$, cells were treated with fMLP, and the extracellular fluid was evaluated by Western blot analysis. As shown in Fig. 6*B*, 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 molecular mass of ~18–19 kDa that cross-reacted with the anti-GX sPLA$_2$ antiserum was also identified in the supernatant of vehicle (Me_2SO) and fMLP-stimulated cells, but this protein did not co-migrate with recombinant GX sPLA$_2$ (mature form, lacking an N-terminal extension) (Fig. 6*C*). 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 (Fig. 6, *D* and *E*). 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 and 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 sPLA$_2$. The effect of LY311727 or indoxam on recombinant GIIA sPLA$_2$ activity was also assessed for comparison. Assays were carried out under conditions where sPLA$_2$ activity was linear with respect to the amount of enzyme used (Fig. 7*A*) and time (Fig. 7*B*). Under these conditions, the specific activity of recombinant GIIA sPLA$_2$ (*dotted line*) was ~4-fold higher than recombinant GV sPLA$_2$ (*dashed line*) and 14-fold higher than recombinant GX sPLA$_2$ (*solid line*). As shown in Fig. 7*C*, co-incubation with LY311727 inhibited recombinant GIIA sPLA$_2$ activity (IC_{50} ≈50 nM) more potently than recombinant GV sPLA$_2$ activity (IC_{50} ≈2 μM) or recombinant GX sPLA$_2$ activity (IC_{50} ≈0.75 μM). In contrast, co-incu-

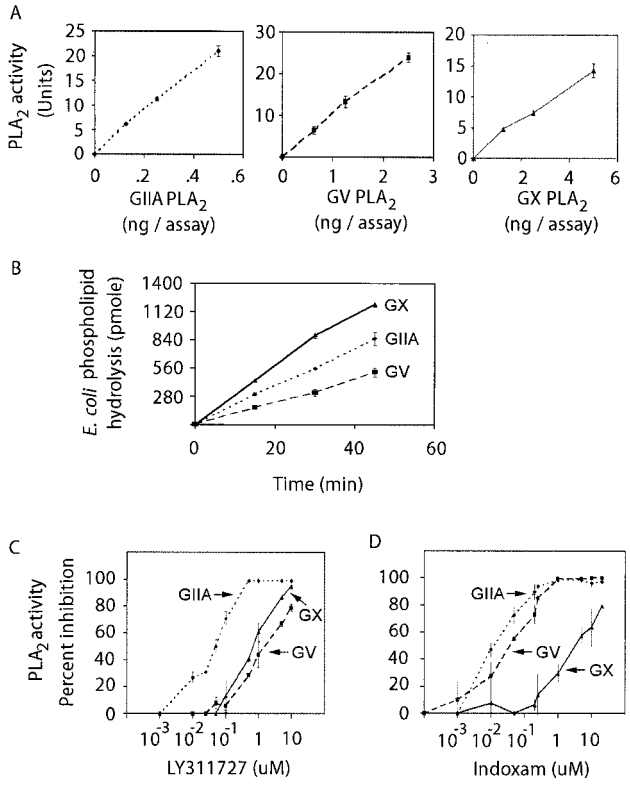

FIG. 7. **Effect of LY311727 or indoxam on the catalytic activity of recombinant GIIA, GV, and GX sPLA$_2$s.** *A,* increasing quantities of recombinant GIIA, GV, or GX sPLA$_2$ were incubated with [^{3}H]oleate-labeled *E. coli* membranes for 30 min, and PLA$_2$ activity was measured as described under "Materials and Methods." *B,* 0.25 ng of GIIA sPLA$_2$, 2.5 ng of GV sPLA$_2$, or 5 ng of GX sPLA$_2$ were incubated with [^{3}H]oleate-labeled *E. coli* membranes for 15, 30, or 45 min, and PLA$_2$ activity was measured as described under "Materials and Methods." The mean specific activity for recombinant GIIA, GV, and GX sPLA$_2$ was 45.6 ± 3.8, 10.8 ± 0.3, and 3.2 ± 0.5 units/ng, respectively. 0.25 ng of GIIA sPLA$_2$, 2.5 ng of GV sPLA$_2$, or 5 ng of GX sPLA$_2$ was incubated with increasing concentrations of LY311727 (*C*) or indoxam (*D*) for 10 min. Following addition of [^{3}H]oleate-labeled *E. coli* membranes for 30 min, PLA$_2$ activity was measured. All results are the mean ± S.D. of at least four separate experiments.

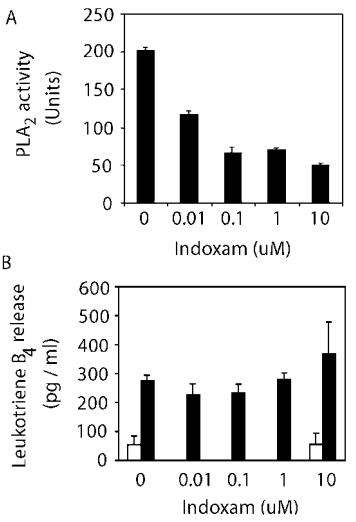

FIG. 8. **Effect of inhibition of extracellular GV sPLA$_2$ activity on LTB$_4$ production by fMLP-stimulated neutrophils.** *A,* cells (2×10^7/ml) were resuspended in KRPD with 1 mM $CaCl_2$ and 0.25% BSA, treated sequentially with cytochalasin B and fMLP, and centrifuged as described under "Materials and Methods." The supernatant from these cells was then incubated with increasing concentrations of indoxam (0–10 μM), and PLA$_2$ activity was measured. *B,* cells (20×10^6/ml) were resuspended in KRPD with 1 mM $CaCl_2$ and 0.25% BSA, incubated with increasing concentrations of indoxam (0–10 μM), and treated sequentially with cytochalasin B and Me_2SO (*open bars*) or fMLP (*filled bars*). Following centrifugation, the supernatant was assayed for LTB$_4$ as described under "Materials and Methods." Results are the mean ± S.D. of four experiments.

bation with indoxam (Fig. 7*D*) inhibited recombinant GIIA sPLA$_2$ activity (IC_{50} ≈10 nM) and recombinant GV sPLA$_2$ activity (IC_{50} ≈40 nM) to a similar degree but had a much less potent effect on recombinant GX sPLA$_2$ activity (IC_{50} ≈5 μM). Therefore, indoxam exhibited a 125-fold selectivity for inhibition of recombinant GV sPLA$_2$ in comparison with recombinant GX sPLA$_2$ *in vitro*. The IC_{50} value for inhibition of human GX sPLA$_2$ by indoxam is similar to that measured for mouse GX sPLA$_2$ (28).

Co-incubation with Indoxam Inhibits Extracellular sPLA$_2$ Activity but Does Not Attenuate LTB$_4$ Production by Neutrophils Treated with fMLP—Following exposure of neutrophils (2×10^7) to 1 μM fMLP for 10 min, PLA$_2$ activity in the extracellular medium was 202 ± 4 units/ml (Fig. 8*A*). Co-incubation of the extracellular fluid from fMLP-treated neutrophils with indoxam decreased PLA$_2$ activity in a dose-dependent manner (Fig. 8*A*). Because fMLP-stimulated neutrophils released a protein that cross-reacted with the anti-GV sPLA$_2$ antiserum (*cf.* Fig. 6*B*), these results are consistent with the notion that circulating neutrophils release catalytically active GV sPLA$_2$ following exposure to fMLP. The concentration of indoxam required to inhibit the major portion of PLA$_2$ activity secreted from neutrophils (IC_{50} ~50 nM) is similar to the IC_{50} of indoxam measured with recombinant GV sPLA$_2$ using the assay with radiolabeled *E. coli* membranes (Fig. 7*D*). The difference between the PLA$_2$ activity released by untreated neutrophils (40 ± 3 units/ml) and the PLA$_2$ activity in the supernatant of fMLP-treated neutrophils following incubation with 10 μM indoxam (45 ± 3 units/ml) was negligible. This indicates that indoxam inhibited essentially all of the PLA$_2$ activity that was released from neutrophils in response to fMLP. The effect of indoxam on OZ-induced PLA$_2$ release by neutrophils was not studied, because OZ inhibited the activity of the PLA$_2$ released by these cells. Because the concentrations of indoxam used in these studies do not inhibit GX sPLA$_2$, these results are consistent with the Western blotting data indicating that fMLP-stimulated neutrophils do not release catalytically active GX sPLA$_2$.

To evaluate the role of extracellular GV sPLA$_2$ in neutrophil LTB$_4$ biosynthesis, cells were pretreated with indoxam and stimulated with fMLP. As shown in Fig. 8*B*, inhibition of extracellular GV sPLA$_2$ activity with indoxam did not inhibit fMLP-stimulated LTB$_4$ biosynthesis by human neutrophils. Therefore, GV sPLA$_2$ released from neutrophils in response to fMLP does not participate in neutrophil LTB$_4$ biosynthesis. In contrast, preincubating neutrophils with the selective cPLA$_2\alpha$ inhibitor, pyrrolidine-1 (51, 52), resulted in a dose-dependent inhibition of both fMLP- and OZ-induced neutrophils LTB$_4$ biosynthesis (IC_{50} ≈0.1–0.5 μM, Fig. 9, *A* and *B*). These findings indicate that cPLA$_2\alpha$ participates in fMLP- and OZ-induced LTB$_4$ synthesis by neutrophils. Preincubation with pyrrolidine-1 had no effect on the fMLP-induced release of GV sPLA$_2$, as assessed by Western blotting, or on PLA$_2$ activity released by fMLP-stimulated cells (data not shown).

Recombinant GIIA and GV sPLA$_2$, but Not Recombinant GX sPLA$_2$, 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). We have demonstrated that human neutrophils contain GV sPLA$_2$ and release this enzyme upon exposure to fMLP or OZ, so we evaluated the ability of recom-

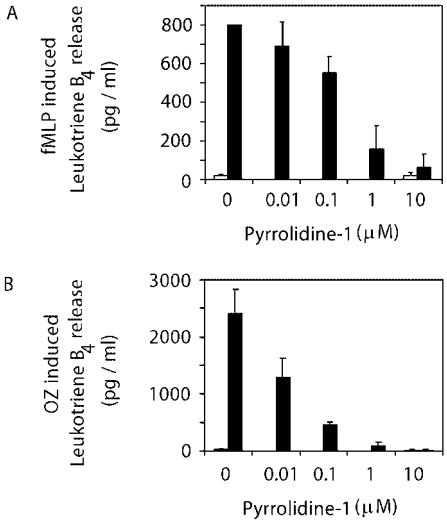

FIG. 9. **Effect of inhibition of cPLA$_2\alpha$ on LTB$_4$ production by fMLP- or OZ-stimulated neutrophils.** Cells (2×10^7/ml) were resuspended in KRPD with 1 mM $CaCl_2$ and 0.25% BSA, preincubated with increasing concentrations of pyrrolidine-1 for 10 min, and treated with cytochalasin B and Me_2SO (*open bars*) or fMLP (*filled bars*) (*A*) or vehicle (*open bars*) or OZ (*filled bars*) (*B*). Following centrifugation, the supernatant was assayed for LTB$_4$ as described under "Materials and Methods." Results are the mean ± S.D. of three independent experiments.

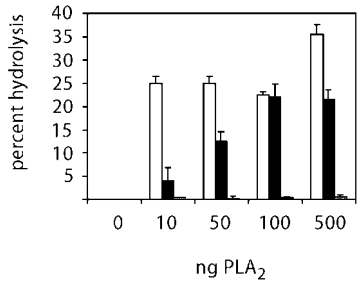

FIG. 10. **Recombinant GIIa and GV sPLA$_2$, but not recombinant GX sPLA$_2$, hydrolyze Gram-negative bacterial phospholipids.** *E. coli* was metabolically labeled with [^{3}H]oleic acid, washed extensively with 150 mM NaCl and 1% BSA, and co-incubated with 0–500 ng of recombinant GIIA (*open bars*), GV (*filled bars*), or GX sPLA$_2$ (*dashed bars*). Bacterial phospholipid hydrolysis was estimated by measuring the release of [^{3}H]oleic acid into the supernatant as described under "Materials and Methods." Results are the mean ± S.D. of four experiments.

binant GV sPLA$_2$ to hydrolyze bacterial phospholipids. Studies with GIIA and GX sPLA$_2$s were also carried out for comparison. Live *E. coli* were labeled with [^{3}H]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 sPLA$_2$. For these studies, human serum that had been eluted from a heparin column (to remove all PLA$_2$ activity) was added at a final concentration of 2% serum. Co-incubation of serum purified over the heparin column with live [^{3}H]oleic acid-labeled *E. coli* did not result in measurable phospholipid hydrolysis (data not shown). Co-incubation of live [^{3}H]oleic acid-labeled *E. coli* with 10 ng of recombinant GIIA sPLA$_2$ for 60 min resulted in the hydrolysis of ~25% of the labeled bacterial phospholipids, and this value did not change significantly as the amount of recombinant GIIA sPLA$_2$ was increased to 500 ng (Fig. 10). Co-incubation of live [^{3}H]oleic acid-labeled *E. coli* with recombinant GV sPLA$_2$ resulted in a dose-dependent increase in bacterial phospholipid hydrolysis that reached a plateau at 100 ng of recombinant GV sPLA$_2$, whereas co-incubation of up to 500 ng of recombinant GX sPLA$_2$ with live [^{3}H]oleic acid-labeled *E. coli* resulted in virtually no hydrolysis (Fig. 10). When recombinant GIIA sPLA$_2$ or recombinant GV sPLA$_2$ was co-incubated with live [^{3}H]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 sPLA$_2$ 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 sPLA$_2$ has been purified from rabbit inflammatory exudates (2, 71), and immunohistochemical studies identified GIIA sPLA$_2$ in human neutrophils (37). These seminal findings led to the notion that human neutrophils express and release GIIA sPLA$_2$ during an inflammatory response (72). However, recent studies (4) indicate that multiple sPLA$_2$ enzymes, in addition to GIIA sPLA$_2$, may play important roles in the generation of an inflammatory response. Thus, some myeloid cells express more than one sPLA$_2$ enzyme (73), and sPLA$_2$ enzymes other than GIIA sPLA$_2$, including GV and GX sPLA$_2$, have been shown to participate in the generation of pro-inflammatory mediators (5, 24). Furthermore, nine human sPLA$_2$ enzymes have now been described (5). The existence of multiple sPLA$_2$ enzymes raises the possibility that the anti-GIIA antisera used in earlier studies (37) could have cross-reacted with other sPLA$_2$ enzymes. Based on these considerations, we decided to evaluate systematically which sPLA$_2$ mRNA species and sPLA$_2$ 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 sPLA$_2$ 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 sPLA$_2$ enzymes play in neutrophil LTB$_4$ biosynthesis and Gram-negative bacterial phospholipid hydrolysis. We also studied the role of cPLA$_2\alpha$ in fMLP- and OZ-stimulated LTB$_4$ biosynthesis by incubating cells with pyrrolidine-1 (52), because this inhibitor is more specific than previously used inhibitors of cPLA$_2\alpha$.

Identification of GV and GX sPLA$_2$ mRNAs and Proteins in Neutrophils—Extensive experimental evidence has documented the existence of sPLA$_2$ enzyme(s) in human neutrophils. Thus, neutrophils contain an acid-stable enzyme with PLA$_2$ activity (74); sPLA$_2$ 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 sPLA$_2$ 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-sPLA$_2$ antisera that circulating and non-stimulated human neutrophils contain GV and GX sPLA$_2$, whereas GIB, GIIA, GIID, GIIE, GIIF, GIII, and GXII sPLA$_2$ were undetectable in these cells. Our results underscore the importance of using highly purified neutrophils for RT-PCR analysis, as GIIA and GIID sPLA$_2$ 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 sPLA$_2$, only RT-PCR was used for detection, with successful execution of positive controls, because antisera for these proteins are not yet available. Although GIIE sPLA$_2$ mRNA could not be detected by RT-PCR (Hanasaki and co-workers (19) reported that GIIE sPLA$_2$ cDNA could be detected by PCR only after multiple rounds of extensive amplification), GIIE sPLA$_2$ protein was not detected by Western blot analysis even though spiking the neutrophil lysate with 1.5 ng of recombinant GIIE

sPLA$_2$ gave a readily detected band. In contrast to our results, a previous study (75) reported that GV sPLA$_2$ is not present in human neutrophils. This discrepancy may be due to the fact that we used a high affinity antiserum that could detect <1 ng of recombinant GV sPLA$_2$, while the earlier study made use of a lower affinity monoclonal antibody that was only able to detect ~30 ng of this antigen (76). Despite earlier reports that circulating human neutrophils contain GIIA sPLA$_2$ based on immunohistochemical analysis (37), we could not detect GIIA sPLA$_2$ in these cells either by RT-PCR or Western blot. For the latter analyses, the GIIA sPLA$_2$ Western blot band was easily seen when the neutrophil lysate was spiked with 1 ng of recombinant GIIA sPLA$_2$, thus the amount of GIIA sPLA$_2$ is $\ll$1 ng per 10^6 neutrophils. Our findings underscore the cell type specificity of sPLA$_2$ expression; for example, GIIA, GIIC, GIID, GIIE, GIIF, and GV sPLA$_2$ were detected in bone marrow-derived mast cells from BALB/cJ mice, whereas transcripts for GIB and GX sPLA$_2$ 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 sPLA$_2$ mRNA species besides GV and GX sPLA$_2$ are expressed at an earlier phase of neutrophil maturation.

Sequence analysis demonstrated that the 358-bp GV sPLA$_2$ RT-PCR product was identical to the corresponding sequence in GV sPLA$_2$, whereas the 251-bp GV sPLA$_2$ product had a 107-bp deletion. Deletion of this 107-bp sequence, which corresponds to exon 4 of the human GV sPLA$_2$ gene, would result in a frameshift that would introduce a premature TGA stop codon. Therefore, translation of this alternatively spliced GV sPLA$_2$ mRNA would be predicted to give rise to a catalytically inactive, truncated GV sPLA$_2$ protein.

We identified proteins in neutrophils that were recognized by anti-GV and anti-GX sPLA$_2$ antisera and that co-migrated exactly with recombinant GV sPLA$_2$ (Fig. 4*E*) and recombinant GX sPLA$_2$ (Fig. 4*F*). These recombinant proteins 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 sPLA$_2$ in neutrophils had been cleaved.

The identification of multiple sPLA$_2$ enzymes in neutrophils is consistent with the results of studies of other types of cells, in which multiple sPLA$_2$ enzymes were expressed. Thus, GIIA and GV sPLA$_2$ were identified in mouse bone marrow mast cells (23), cultured rat astrocytes (78), and rat neonatal cardiomyocytes,[3] whereas GIB and GIIA sPLA$_2$ were both identified in epidermis (79) and rat splenic macrophages (80, 81). In mast cells, GIIA sPLA$_2$ was recognized in secretory granules (82), consistent with the putative role of sPLA$_2$ in facilitating secretory granule exocytosis (83), whereas GV sPLA$_2$ 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 sPLA$_2$.

Subcellular Localization of GV and GX sPLA$_2$ 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 sPLA$_2$ in azurophilic and specific granules, whereas GX sPLA$_2$ was confined to azurophilic granules. In human neutrophils, most of the bactericidal proteins are localized in the azurophil granules (84), 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 sPLA$_2$, GX sPLA$_2$, 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 sPLA$_2$ 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 sPLA$_2$ in bacterial eradication following phagocytosis remains to be defined. However, the recent demonstration that human GV and GX sPLA$_2$ can both kill Gram-positive bacteria *in vitro* (89) supports a role for these proteins in bacterial eradication following phagocytosis.

Identification of GV sPLA$_2$ in the Extracellular Fluid of fMLP- and OZ-stimulated Neutrophils—Both GV and GX sPLA$_2$s are both expected to be secreted enzymes. SignalP analysis (90) of the amino acid sequences of GV and GX sPLA$_2$ for the presence of potential cleaved signal sequences predicted that both GV sPLA$_2$ (signal peptide probability = 1.0) and GX sPLA$_2$ (signal peptide probability = 0.997) have a cleaved signal sequence. The predicted cleavage site for GV sPLA$_2$ is between Gly-20 and Gly-21 (maximum cleavage site probability = 0.864), whereas the predicted cleavage site for GX sPLA$_2$ is between Gly-31 and Glu-32 (maximum cleavage site probability = 0.502).

Stimulation of human neutrophils with fMLP or OZ leads to the release of GV sPLA$_2$ into the extracellular fluid (*cf.* Fig. 6). By comparing the density of the band on the Western blot that corresponds to released GV sPLA$_2$ with the density of the band corresponding to 1.5 ng of recombinant GV sPLA$_2$ (Fig. 6*B*), we estimate than 10^6 fMLP-stimulated neutrophils release ~0.9 ng of GV sPLA$_2$. As shown in Fig. 7*A*, 1 ng of recombinant GV sPLA$_2$ corresponds to ~10 units of PLA$_2$ activity. As 20 million PMNs release ~100 units of PLA$_2$ activity/ml after exposure to fMLP (or 0.5 ng/million neutrophils, *cf.* Fig. 6*A*), the amount of GV sPLA$_2$ release by neutrophils, estimated by Western blotting and activity analysis, is in general agreement.

In contrast to the results of the Western blot analysis for GV sPLA$_2$, proteins that co-migrated with mature GX sPLA$_2$ 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 sPLA$_2$ gave a readily detectable band. Furthermore, as little as 0.2 ng of recombinant GX sPLA$_2$ could be detected by Western blot analysis, even when added to the crude neutrophil lysate (not shown). Therefore, if neutrophils release mature GX sPLA$_2$ following exposure to fMLP or OZ, the amount released is less than 0.2 ng/10^6 cells. Control and fMLP-stimulated neutrophils both released a protein into the extracellular fluid with a mass of ~19 kDa that cross-reacts with the anti-GX sPLA$_2$ antiserum. This cross-reacting protein, which was also identified in the soluble fraction of neutrophils (Fig. 4*F*), could represent pro-GX sPLA$_2$ (*i.e.* GX sPLA$_2$ prior to cleavage of the N-terminal propeptide) or glycosylated GX sPLA$_2$ (36). As pro-GX sPLA$_2$ has relatively low enzymatic activity compared with that of the mature protein (36), the presence of pro-GX sPLA$_2$ in the extracellular fluid of neutrophils would not be predicted to contribute to the PLA$_2$ activity released by neutrophils. In any case, our results showing that concentrations of indoxam that inhibit GV but not GX sPLA$_2$ block virtually all of the PLA$_2$ enzymatic activity released by neutrophils after fMLP stimulation (Fig. 8) also strongly argue that enzymatically active GX sPLA$_2$ is not present in the extracellular fluid of fMLP-stimulated cells.

[3] N. Degousee, E. Stefanski, and B. Rubin, submitted for publication.

The appearance of relatively small amounts of GV sPLA$_2$ 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 tripeptide (55). However, as GV and GX sPLA$_2$ were both identified in azurophilic granules, we anticipated that GV sPLA$_2$ release by fMLP- or OZ-stimulated cells would be associated with some GX sPLA$_2$ release. The reason for the failure to identify mature GX PLA$_2$ 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 sPLA$_2$, 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 sPLA$_2$ in these cells is secreted after agonist stimulation, whereas most of the GV sPLA$_2$ remains in an intracellular compartment (91). Thus, it would appear that not all sPLA$_2$s are secreted to the extracellular space following agonist-induced cell activation.

Extracellular GV sPLA$_2$ Does Not Participate in Neutrophil LTB$_4$ Synthesis—A model of plasma membrane phospholipid hydrolysis by sPLA$_2$ enzymes has been proposed in which endogenous sPLA$_2$ enzymes are released and bind to the plasma membrane in a paracrine fashion (22). In this model, hydrolysis of plasma membrane phospholipids by the bound sPLA$_2$ 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 sPLA$_2$ released by neutrophils could theoretically hydrolyze neutrophil plasma membrane phospholipids via a similar mechanism, as exogenously added recombinant human GV sPLA$_2$ promotes free fatty acid release and LTB$_4$ synthesis from these cells (94). Catalytically active recombinant GV sPLA$_2$ was required to induce LTB$_4$ synthesis by neutrophils, because the H48A GV sPLA$_2$ mutant, which lacks catalytic activity, failed to induce LTB$_4$ release by these cells (95). Taken together, these studies are consistent with a role for extracellular GV sPLA$_2$ in neutrophil LTB$_4$ synthesis. In support of this hypothesis, we found that fMLP-stimulated neutrophils release GV sPLA$_2$, which accounts for most, if not all, of the PLA$_2$ activity in the extracellular medium. However, we found that co-incubation with indoxam, which showed strong potency and specificity for the inhibition of GV sPLA$_2$, completely blocked extracellular PLA$_2$ activity, but had no effect on LTB$_4$ 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 sPLA$_2$ were added to neutrophils (~1400 ng/ml) (95) than are generated endogenously (≈1 ng/10^6 neutrophils or 20 ng/ml in this study) following exposure to fMLP (*cf.* Fig. 6*B*). The failure of the relatively low levels of endogenous GV sPLA$_2$ that were released by fMLP-stimulated neutrophils to participate in *ex vivo* LTB$_4$ synthesis does not exclude the possibility that GV sPLA$_2$ plays a role in neutrophil LTB$_4$ synthesis during an inflammatory response *in vivo*, because GV sPLA$_2$ 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 sPLA$_2$ 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 sPLA$_2$ antiserum (*cf.* Fig. 6*C*), is pro-GX sPLA$_2$. If this is the case, GX sPLA$_2$ released by neutrophils could participate in LTB$_4$ biosynthesis, if pro-GX sPLA$_2$ could be converted to mature GX sPLA$_2$ by extracellular proteases during an inflammatory response *in vivo*. For this to occur, neutrophils would have to generate at least 10 ng of GX sPLA$_2$/ml, as this is the concentration of GX sPLA$_2$ that is required to produce detectable eicosanoid formation in a variety of mammalian cells treated with exogenous GX sPLA$_2$ (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 (*cf.* Fig. 2*E* and Fig. 4*B*) 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 (15, 97, 99, 100).

cPLA$_2\alpha$ Regulates fMLP-induced Neutrophil LTB$_4$ Biosynthesis—In a previous study (44), we showed that the potent cPLA$_2$ inhibitor MAFP blocked virtually all of the arachidonate release by neutrophils stimulated with fMLP. Similarly, Bauldry and Wooten (101) showed that cPLA$_2$ activation could provide arachidonic acid for LTB$_4$ biosynthesis by permeabilized human neutrophils. In addition, co-incubation with dithiothreitol, 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 evaluate directly 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 cPLA$_2\alpha$, leading to 50% inhibition when present at a mole fraction of ~0.002 in a number of *in vitro* assays, is a poor inhibitor of cPLA$_2\gamma$ and calcium-independent group VI PLA$_2$ (MAFP inhibits this enzyme), and does not cause detectable inhibition of GIIA, V, or X sPLA$_2$ activity (52). In addition, pyrrolidine-1 blocks arachidonic acid release by Ca^{2+} ionophore-stimulated Chinese hamster ovary cells that were stably transfected with cPLA$_2\alpha$ (52). We found that preincubation with pyrrolidine-1 results in a dose-dependent and complete inhibition of fMLP- or OZ-induced LTB$_4$ release by neutrophils. The IC$_{50}$ value for pyrrolidine-1 inhibition of LTB$_4$ release was between 0.1 and 1.0 μM, which is similar to the IC$_{50}$ value for pyrrolidine-1 inhibition of cPLA$_2\alpha$ activity in multiple cell lines (52). Therefore, it appears that cPLA$_2\alpha$, and not extracellular GV sPLA$_2$, is responsible for the rapid liberation of arachidonate that is metabolized to LTB$_4$ following neutrophil activation by fMLP or OZ.

Possible Role of GV sPLA$_2$ 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 (72). The highly dense and anionic cell wall and lipopolysaccharide layers limit the ability of extracellular sPLA$_2$ enzymes to access and hydrolyze the plasma membrane. However, following disruption of the cell wall by polymyxin antibiotics or BPI (48, 72), cationic enzymes such as GIIA sPLA$_2$ may gain access to the plasma membrane and kill the bacteria (59, 102), and a clear link has been established between envelope phospholipid deg-

radation and overall Gram-negative bacterial destruction (69). We found that recombinant GIIA sPLA$_2$, in the presence of serum, hydrolyzed phospholipids from *E. coli* that lack PLA$_2$ activity more efficiently than recombinant GV sPLA$_2$, whereas recombinant GX sPLA$_2$ did not catalyze detectable bacterial phospholipid hydrolysis. These data are consistent with the fact that GIIA sPLA$_2$ has a high pI (>10.5) (72), is well designed to hydrolyze anionic bacterial phospholipids, and has been shown to hydrolyze *E. coli in vivo* (70). GV sPLA$_2$ has a lower pI but is still >7, and so it would be predicted to have intermediate activity on anionic cell wall-bound *E. coli* membranes, whereas GX sPLA$_2$ has an acidic pI and does not hydrolyze *E. coli* phospholipids. The permissive effect of serum on GIIA and GV sPLA$_2$-mediated *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$_2$ action against Gram-negative bacteria by producing sublethal alterations of the outer envelope of these bacteria (48, 92). As GV sPLA$_2$ was the only sPLA$_2$ enzyme released by neutrophils, these results point to a potential role for extracellular GV sPLA$_2$ in neutrophil-mediated *E. coli* phospholipid hydrolysis *in vivo*. It is also possible that GV sPLA$_2$ bound to *E. coli* may augment bacterial phospholipid hydrolysis after phagocytosis of the bacteria by neutrophils, as described for GIIA sPLA$_2$ (3, 69). Extracellular GV sPLA$_2$ released by neutrophils may also participate in the eradication of Gram-positive bacteria, including *Listeria monocytogenes*, *Enterobacter faecium*, *Enterococcus faecalis,* and methicillin-sensitive and -resistant *Staphylococcus aureus*, because co-incubation of these bacteria with rat, human, and mouse GV sPLA$_2$s resulted in significant bactericidal activity *in vitro* (69, 89). However, the *in vivo* bactericidal activity GV sPLA$_2$, which is less potent than GIIA sPLA$_2$, remains to be established.

In summary, we have shown that human neutrophils express GV and GX sPLA$_2$ mRNA and contain GV and GX sPLA$_2$ protein. GV sPLA$_2$ is present in both azurophil and specific granules, whereas GX sPLA$_2$ is confined to azurophil granules. Stimulation with fMLP or OZ results in the selective release of GV sPLA$_2$ by neutrophils. However, inhibition of extracellular GV sPLA$_2$ activity has no effect on neutrophil LTB$_4$ synthesis, which was shown to be fully dependent on cPLA$_2\alpha$ activity. In contrast, 10–100 ng/ml amounts of GV sPLA$_2$ 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 sPLA$_2$ is released into phagolysosomes and participates in the eradication of ingested bacteria or other pathogens remains to be evaluated.

REFERENCES

1. Weiss, S. J. (1989) *N. Engl. J. Med.* **320,** 365–376
2. Wright, G. W., Ooi, C. E., Weiss, J., and Elsbach, P. (1990) *J. Biol. Chem.* **265,** 6675–6681
3. Weiss, J., Inada, M., Elsbach, P., and Crowl, R. M. (1994) *J. Biol. Chem.* **269,** 26331–26337
4. Tischfield, J. A. (1997) *J. Biol. Chem.* **272,** 17247–17250
5. Valentin, E., and Lambeau, G. (2000) *Biochim. Biophys. Acta* **1488,** 59–70
6. Ancian, P., Lambeau, G., Mattei, M. G., and Lazdunski, M. (1995) *J. Biol. Chem.* **270,** 8963–8970
7. Murakami, M., Koduri, R. S., Enomoto, A., Shimbara, S., Seki, M., Yoshihara, K., Singer, A., Valentin, E., Ghomashchi, F., Lambeau, G., Gelb, M. H., and Kudo, I. (2001) *J. Biol. Chem.* **276,** 10083–10096
8. Foreman-Wykert, A. K., Weiss, J., and Elsbach, P. (2000) *Infect. Immun.* **68,** 1259–1264
9. Qu, X. D., and Lehrer, R. I. (1998) *Infect. Immun.* **66,** 2791–2797
10. Lambeau, G., and Lazdunski, M. (1999) *Trends Pharmacol. Sci.* **20,** 162–170
11. Ancian, P., Lambeau, G., and Lazdunski, M. (1995) *Biochemistry* **34,** 13146–13151
12. Fonteh, A. N., Atsumi, G., LaPorte, T., and Chilton, F. H. (2000) *J. Immunol.* **165,** 2773–2782
13. Six, D. A., and Dennis, E. A. (2000) *Biochim. Biophys. Acta* **1488,** 1–19
14. Dennis, E. A. (1994) *J. Biol. Chem.* **269,** 13057–13060
15. Vadas, P., Browning, J., Edelson, J., and Pruzanski, W. (1993) *J. Lipid Mediators* **8,** 1–30
16. Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K., and Kudo, I. (1997) *Crit. Rev. Immunol.* **17,** 225–283
17. Valentin, E., Singer, A. G., Ghomashchi, F., Lazdunski, M., Gelb, M. H., and Lambeau, G. (2000) *Biochem. Biophys. Res. Commun.* **279,** 223–228
18. Ishizaki, J., Suzuki, N., Higashino, K., Yokota, Y., Ono, T., Kawamoto, K., Fujii, N., Arita, H., and Hanasaki, K. (1999) *J. Biol. Chem.* **274,** 24973–24979
19. Suzuki, N., Ishizaki, J., Yokota, Y., Higashino, K., Ono, T., Ikeda, M., Fujii, N., Kawamoto, K., and Hanasaki, K. (2000) *J. Biol. Chem.* **275,** 5785–5793
20. Valentin, E., Ghomashchi, F., Gelb, M. H., Lazdunski, M., and Lambeau, G. (2000) *J. Biol. Chem.* **275,** 7492–7496
21. Chen, J., Engle, S. J., Seilhamer, J. J., and Tischfield, J. A. (1994) *J. Biol. Chem.* **269,** 2365–2368
22. Reddy, S. T., Winstead, M. V., Tischfield, J. A., and Herschman, H. R. (1997) *J. Biol. Chem.* **272,** 13591–13596
23. Bingham, C. O., III, Fijneman, R. J., Friend, D. S., Goddeau, R. P., Rogers, R. A., Austen, K. F., and Arm, J. P. (1999) *J. Biol. Chem.* **274,** 31476–31484
24. Balboa, M. A., Balsinde, J., Winstead, M. V., Tischfield, J. A., and Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 32381–32384
25. Cupillard, L., Koumanov, K., Mattei, M. G., Lazdunski, M., and Lambeau, G. (1997) *J. Biol. Chem.* **272,** 15745–15752
26. Bezzine, S., Koduri, R. S., Valentin, E., Murakami, M., Kudo, I., Ghomashchi, F., Sadilek, M., Lambeau, G., and Gelb, M. H. (2000) *J. Biol. Chem.* **275,** 3179–3191
27. Morioka, Y., Ikeda, M., Saiga, A., Fujii, N., Ishimoto, Y., Arita, H., and Hanasaki, K. (2000) *FEBS Lett.* **487,** 262–266
28. Morioka, Y., Saiga, A., Yokota, Y., Suzuki, N., Ikeda, M., Ono, T., Nakano, K., Fujii, N., Ishizaki, J., Arita, H., and Hanasaki, K. (2000) *Arch. Biochem. Biophys.* **381,** 31–42
29. Raeder, E. M., Mansfield, P. J., Hinkovska-Galcheva, V., Kjeldsen, L., Shayman, J. A., and Boxer, L. A. (1999) *Blood* **93,** 686–693
30. Gelb, M. H., Valentin, E., Ghomashchi, F., Lazdunski, M., and Lambeau, G. (2000) *J. Biol. Chem.* **275,** 39823–39826
31. Ho, I. C., Arm, J. P., Bingham, C. O., III, Choi, A., Austen, K. F., and Glimcher, L. H. (2001) *J. Biol. Chem.* **276,** 18321–18326
32. Scott, D. L., White, S. P., Browning, J. L., Rosa, J. J., Gelb, M. H., and Sigler, P. B. (1991) *Science* **254,** 1007–1010
33. Verheij, H. M., Slotboom, A. J., and De Haas, G. H. (1981) *Rev. Physiol. Biochem. Pharmacol.* **91,** 91–203
34. Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., and Sigler, P. B. (1990) *Science* **250,** 1541–1546
35. Halban, P. A., and Irminger, J. C. (1994) *Biochem. J.* **299,** 1–18
36. Hanasaki, K., Ono, T., Saiga, A., Morioka, Y., Ikeda, M., Kawamoto, K., Higashino, K., Nakano, K., Yamada, K., Ishizaki, J., and Arita, H. (1999) *J. Biol. Chem.* **274,** 34203–34211
37. Rosenthal, M. D., Gordon, M. N., Buescher, E. S., Slusser, J. H., Harris, L. K., Franson, and RC. (1995) *Biochem. Biophys. Res. Commun.* **208,** 650–656
38. Balsinde, J., Diez, E., Schuller, A., and Mollinedo, F. (1988) *J. Biol. Chem.* **263,** 1929–1936
39. Diez, E., Balsinde, J., and Mollinedo, F. (1990) *Biochim. Biophys. Acta* **1047,** 83–89
40. Borregaard, N., and Cowland, J. B. (1997) *Blood* **89,** 3503–3521
41. Sorensen, O. E., Follin, P., Johnsen, A. H., Calafat, J., Tjabringa, G. S., Hiemstra, P. S., and Borregaard, N. (2001) *Blood* **97,** 3951–3959
42. Leslie, C. C. (1997) *J. Biol. Chem.* **272,** 16709–16712
43. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) *Cell* **65,** 1043–1051
44. Marshall, J., Krump, E., Lindsay, T., Downey, G., Ford, D. A., Zhu, P., Walker, P., and Rubin, B. (2000) *J. Immunol.* **164,** 2084–2091
45. Surette, M. E., Dallaire, N., Jean, N., Picard, S., and Borgeat, P. (1998) *FASEB J.* **12,** 1521–1531
46. Bonventre, J. V., Huang, Z., Taheri, M. R., O'Leary, E., Li, E., Moskowitz, M. A., and Sapirstein, A. (1997) *Nature* **390,** 622–625
47. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., and Tashiro, F. (1997) *Nature* **390,** 618–622
48. Elsbach, P., Weiss, J., Franson, R. C., Beckerdite-Quagliata, S., Schneider, A., and Harris, L. (1979) *J. Biol. Chem.* **254,** 11000–11009
49. Underwood, K. W., Song, C., Kriz, R. W., Chang, X. J., Knopf, J. L., and Lin, L. L. (1998) *J. Biol. Chem.* **273,** 21926–21932
50. Yokota, Y., Hanasaki, K., Ono, T., Nakazato, H., Kobayashi, T., and Arita, H. (1999) *Biochim. Biophys. Acta* **1438,** 213–222
51. Seno, K., Okuno, T., Nishi, K., Murakami, Y., Watanabe, F., Matsuura, T., Wada, M., Fujii, Y., Yamada, M., Ogawa, T., Okada, T., Hashizume, H., Kii, M., Hara, S., Hagishita, S., Nakamoto, S., Yamada, K., Chikazawa, Y., Ueno, M., Teshirogi, I., Ono, T., and Ohtani, M. (2000) *J. Med. Chem.* **43,** 1041–1044
52. Ghomashchi, F., Stewart, A., Hefner, Y., Ramanadham, S., Turk, J., Leslie, C. C., and Gelb, M. H. (2001) *Biochim. Biophys. Acta* **1513,** 160–166
53. Wheelan, P., and Murphy, R. C. (1997) *Anal. Biochem.* **244,** 110–115
54. Koduri, R. S., Baker, S. F., Snitko, Y., Han, S. K., Cho, W., Wilton, D. C., and Gelb, M. H. (1998) *J. Biol. Chem.* **273,** 32142–32153
55. Kjeldsen, L., Sengelov, H., and Borregaard, N. (1999) *J. Immunol. Methods* **232,** 131–143
56. Sorensen, O., and Borregaard, N. (1999) *J. Immunol. Methods* **232,** 179–190
57. Elsbach, P., and Weiss, J. (1991) *Methods Enzymol.* **197,** 24–31
58. Pruzanski, W., Stefanski, E., Vadas, P., Kennedy, B. P., and van den Bosch, H. (1998) *Biochim. Biophys. Acta* **1403,** 47–56
59. Wiese, A., Brandenburg, K., Lindner, B., Schromm, A. B., Carroll, S. F., Rietschel, E. T., and Seydel, U. (1997) *Biochemistry* **36,** 10301–10310
60. Cifone, M. G., Cironi, L., Roncaioli, P., Martinotti, S., Toniato, E., Cilenti, L., Botti, D., Solito, R., Parente, L., and Santoni, A. (1996) *Cell. Immunol.* **170,** 274–282
61. Blom, M., Tool, A. T., Wever, P. C., Wolbink, G. J., Brouwer, M. C., Calafat, J.,

Egesten, A., Knol, E. F., Hack, C. E., Roos, D., and Verhoeven, A. J. (1998) *Blood* **91,** 3037–3043
62. Paietta, E., Racevskis, J., Stanley, E. R., Andreeff, M., Papenhausen, P., and Wiernik, P. H. (1990) *Cancer Res.* **50,** 2049–2055
63. Hale, G., Bright, S., Chumbley, G., Hoang, T., Metcalf, D., Munro, A. J., and Waldmann, H. (1983) *Blood* **62,** 873–882
64. Elsner, J., Hochstetter, R., Spiekermann, K., and Kapp, A. (1996) *Blood* **88,** 4684–4693
65. Huizinga, T. W., van der Schoot, C. E., Jost, C., Klaassen, R., Kleijer, M., dem Borne, A. E., Roos, D., and Tetteroo, P. A. (1988) *Nature* **333,** 667–669
66. Lichtenberger, C., Zakeri, S., Baier, K., Willheim, M., Holub, M., and Reinisch, W. (1999) *J. Immunol. Methods* **227,** 75–84
67. Sengelov, H., and Borregaard, N. (1999) *J. Immunol. Methods* **232,** 145–152
68. Schevitz, R. W., Bach, N. J., Carlson, D. G., Chirgadze, N. Y., Clawson, D. K., Dillard, R. D., Draheim, S. E., Hartley, L. W., Jones, N. D., and Mihelich, E. D. (1995) *Nat. Struct. Biol.* **2,** 458–465
69. Wright, G. C., Weiss, J., Kim, K. S., Verheij, H., and Elsbach, P. (1990) *J. Clin. Invest.* **85,** 1925–1935
70. Weinrauch, Y., Abad, C., Liang, N. S., Lowry, S. F., and Weiss, J. (1998) *J. Clin. Invest.* **102,** 633–638
71. Forst, S., Weiss, J., Elsbach, P., Maraganore, J. M., Reardon, I., and Heinrikson, R. L. (1986) *Biochemistry* **25,** 8381–8385
72. Buckland, A. G., and Wilton, D. C. (2000) *Biochim. Biophys. Acta* **1488,** 71–82
73. Enomoto, A., Murakami, M., Valentin, E., Lambeau, G., Gelb, M. H., and Kudo, I. (2000) *J. Immunol.* **165,** 4007–4014
74. Marki, F., and Franson, R. (1986) *Biochim. Biophys. Acta* **879,** 149–156
75. Cho, W. (2000) *Biochim. Biophys. Acta* **1488,** 48–58
76. Munoz, N. M., Kim, K. P., Han, S. K., Boetticher, E., Sperling, A. I., Sano, H., Zhu, X., Cho, W., and Leff, A. R. (2000) *Hybridoma* **19,** 171–176
77. Cowland, J. B., and Borregaard, N. (1999) *J. Leukocyte Biol.* **66,** 989–995
78. Thomas, G., Bertrand, F., and Saunier, B. (2000) *J. Biol. Chem.* **275,** 10876–10886
79. Maury, E., Prevost, M. C., Simon, M. F., Redoules, D., Ceruti, I., Tarroux, R., Charveron, M., and Chap, H. (2000) *J. Invest. Dermatol.* **114,** 960–966
80. Inada, M., Tojo, H., Kawata, S., Tarui, S., and Okamoto, M. (1991) *Eur. J. Biochem.* **197,** 323–329
81. Tojo, H., Ono, T., Kuramitsu, S., Kagamiyama, H., and Okamoto, M. (1988) *J. Biol. Chem.* **263,** 5724–5731
82. Chock, S. P., Schmauder-Chock, E. A., Cordella-Miele, E., Miele, L., and Mukherjee, A. B. (1994) *Biochem. J.* **300,** 619–622
83. Blackwood, R. A., Transue, A. T., Harsh, D. M., Brower, R. C., Zacharek, S. J., Smolen, J. E., and Hessler, R. J. (1996) *J. Leukocyte Biol.* **59,** 663–670
84. Gabay, J. E., Heiple, J. M., Cohn, Z. A., and Nathan, C. F. (1986) *J. Exp. Med.* **164,** 1407–1421
85. Weiss, J., and Olsson, I. (1987) *Blood* **69,** 652–659
86. Campanelli, D., Detmers, P. A., Nathan, C. F., and Gabay, J. E. (1990) *J. Clin. Invest.* **85,** 904–915
87. Ganz, T., Selsted, M. E., Szklarek, D., Harwig, S. S., Daher, K., Bainton, D. F., and Lehrer, R. I. (1985) *J. Clin. Invest.* **76,** 1427–1435
88. Calafat, J., Janssen, H., Knol, E. F., Malm, J., and Egesten, A. (2000) *Acta Pathol. Microbiol. Immunnol. Scand.* **108,** 201–208
89. Koduri, R. S., Gronroos, J. O., Laine, V. J., Le Calvez, C., Lambeau, G., Nevalainen, T. J., and Gelb, M. H. (2001) *J. Biol. Chem.* **276,** in press
90. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) *Protein Eng.* **10,** 1–6
91. van der Helm, H. A., Buijtenhuijs, P., and Van den, B. H. (2001) *Biochim. Biophys. Acta* **1530,** 86–96
92. Madsen, L. M., Inada, M., and Weiss, J. (1996) *Infect. Immun.* **64,** 2425–2430
93. Shinohara, H., Balboa, M. A., Johnson, C. A., Balsinde, J., and Dennis, E. A. (1999) *J. Biol. Chem.* **274,** 12263–12268
94. Han, S. K., Kim, K. P., Koduri, R. S., Bittova, L., Munoz, N. M., Leff, A. R., Wilton, D. C., Gelb, M. H., and Cho, W. (1999) *J. Biol. Chem.* **274,** 11881–11888
95. Kim, K. P., Rafter, J. D., Bittova, L., Han, S. K., Snitko, Y., Munoz, N. M., Leff, A. R., and Cho, W. (2001) *J. Biol. Chem.* **276,** 11126–11134
96. Arbibe, L., Vial, D., Rosinski-Chupin, I., Havet, N., Huerre, M., Vargaftig, B. B., and Touqui, L. (1997) *J. Immunol.* **159,** 391–400
97. Seilhamer, J. J., Pruzanski, W., Vadas, P., Plant, S., Miller, J. A., Kloss, J., and Johnson, L. K. (1989) *J. Biol. Chem.* **264,** 5335–5338
98. Gronroos, J. O., Laine, V. J., Janssen, M. J., Egmond, M. R., and Nevalainen, T. J. (2001) *J. Immunol.* **166,** 4029–4034
99. Pruzanski, W., Albin-Cook, K., Laxer, R. M., MacMillan, J., Stefanski, E., Vadas, P., and Silverman, E. D. (1994) *J. Rheumatol.* **21,** 1951–1954
100. Kortekangas, P., Aro, H. T., and Nevalainen, T. J. (1994) *Scand. J. Rheumatol.* **23,** 68–72
101. Bauldry, S. A., and Wooten, R. E. (1996) *Biochim. Biophys. Acta* **1303,** 63–73
102. Wiese, A., Brandenburg, K., Carroll, S. F., Rietschel, E. T., and Seydel, U. (1997) *Biochemistry* **36,** 10311–10319