THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 50, Issue of December 13, pp. 48535–48549, 2002
Printed in U.S.A.

Interfacial Kinetic and Binding Properties of the Complete Set of Human and Mouse Groups I, II, V, X, and XII Secreted Phospholipases A_2*

Received for publication, June 12, 2002, and in revised form, September 27, 2002
Published, JBC Papers in Press, September 30, 2002, DOI 10.1074/jbc.M205855200

Alan G. Singer‡, Farideh Ghomashchi‡, Catherine Le Calvez§, James Bollinger‡, Sofiane Bezzine‡¶, Morgane Rouault§‖, Martin Sadilek‡, Eric Nguyen§, Michel Lazdunski§, Gérard Lambeau§§§, and Michael H. Gelb‡ ‡‡**

From the ‡Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195 and the §Institut de Pharmacologie Moléculaire et Cellulaire, CNRS-UPR 411, 660 route des Lucioles, Sophia Antipolis, Valbonne 06560, France

Expression of the full set of human and mouse groups I, II, V, X, and XII secreted phospholipases A_2 ($sPLA_2$s) in *Escherichia coli* and insect cells has provided pure recombinant enzymes for detailed comparative interfacial kinetic and binding studies. The set of mammalian $sPLA_2$s display dramatically different sensitivity to dithiothreitol. The specific activity for the hydrolysis of vesicles of differing phospholipid composition by these enzymes varies by up to 4 orders of magnitude, and yet all enzymes display similar catalytic site specificity toward phospholipids with different polar head groups. Discrimination between *sn*-2 polyunsaturated *versus* saturated fatty acyl chains is <6-fold. These enzymes display apparent dissociation constants for activation by calcium in the 1–225 μM range, depending on the phospholipid substrate. Analysis of the inhibition by a set of 12 active site-directed, competitive inhibitors reveals a large variation in the potency among the mammalian $sPLA_2$s, with Me-Indoxam being the most generally potent $sPLA_2$ inhibitor. A dramatic correlation exists between the ability of the $sPLA_2$s to hydrolyze phosphatidylcholine-rich vesicles efficiently *in vitro* and the ability to release arachidonic acid when added exogenously to mammalian cells; the group V and X $sPLA_2$s are uniquely efficient in this regard.

The mammalian family of phospholipases A_2 includes the secreted forms ($sPLA_2$s),[1] which are 14–19-kDa (with some exceptions), Ca^{2+}-dependent, disulfide-rich enzymes that catalyze the hydrolysis of phospholipids at the *sn*-2 position to release fatty acids and lysophospholipids (1–4). 10 $sPLA_2$s have been identified in mice (groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII), and humans contain all of these except IIC, which occurs as a pseudogene (5, 6). Many of these $sPLA_2$s were identified in the past few years, and studies are under way to determine their physiological functions. A catalytically inactive $sPLA_2$-like protein called otoconin-95 was also identified in mice and humans (7, 8). The different $sPLA_2$ paralogs are not closely related isoforms because the amino acid identity between any two is in the range of <15–50%. This plus the fact that each $sPLA_2$ displays a distinct tissue distribution pattern argue for distinct physiological functions of these enzymes. Furthermore, the tissue distributions of the human $sPLA_2$s are often different from those of the mouse orthologs, suggesting that the function of a particular $sPLA_2$ in the mouse may be distinct from that of its human ortholog.

Well established physiological functions for mammalian $sPLA_2$s include the following. Pancreatic $sPLA_2$ (group IB) has a well known role in the digestion of dietary phospholipids, but other $sPLA_2$s are probably also involved in phospholipid degradation in the gastrointestinal track (9). The first nonpancreatic mammalian $sPLA_2$ to be identified was the group IIA enzyme, which is expressed at high levels during inflammation (10) and is the principal bactericidal agent against Gram-positive bacteria in human tears (11) and also works in concert with neutrophils as a bactericidal agent (12). Groups IIA and V $sPLA_2$s are involved in liberation of arachidonic acid from phospholipids, for example in endothelial cells and macrophages, for the biosynthesis of eicosanoids (13, 14). One or more

* This work was supported in part by National Science Foundation Grant 9807748 for the Esquire mass spectrometer. 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.

¶ Recipient of a postdoctoral fellowship from the Association de la Recherche contre le Cancer.

‖ Recipient of a doctoral ingenior fellowship from the CNRS.

** Supported by the CNRS, the Association pour la Recherche sur le Cancer, and the Fonds de Recherche Hoechst Marion Roussel.

‡‡ Supported by National Institutes of Health Grant HL36236. To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, University of Washington, Box 351700, Seattle, WA 98195. Tel.: 206-543-7142; Fax: 206-685-8665; E-mail: gelb@chem.washington.edu.

§§ To whom correspondence should be addressed. Tel.: 33-4-93-95-77-31; Fax: 33-4-93-95-77-04; E-mail: lambeau@ipmc.cnrs.fr.

[1] The abbreviations used are: $sPLA_2$(s), secreted phospholipase A_2(s); $DO_{et}PC$ and $DO_{et}PS$, 1,2-dioleyl-*sn*-glycero-3-phosphocholine and -phosphoserine, respectively; [^{3}H]DPPA and [^{3}H]DPPC, 1-palmitoyl-2-[9,10-^{3}H]palmitoyl phosphatidic acid and phosphatidylcholine, respectively; DTT, dithiothreitol; HEK, human embryonic kidney; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MOPS, 4-morpholinepropanesulfonic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate; POPC/G/M/S, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/glycerol/methanol/serine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; PS, phosphatidylserine; [^{14}C]SAPC, 1-stearoyl-2-[1-^{14}C]arachidonylphosphatidylcholine; TPCK, tosylphenylalanyl chloromethyl ketone. A comprehensive abbreviation system for the various mammalian $sPLA_2$s is used: each $sPLA_2$ is abbreviated with a lowercase letter indicating the $sPLA_2$ species (m and h for mouse and human, respectively), followed by uppercase letters identifying the $sPLA_2$ group (GIB, GIIA, GIIC, GIID, GIIE, GIIF, GIII, GV, GIX, GX, GXI, and GXII). hGIIFΔC is the mutant of hGIIF lacking the C-terminal 23 amino acids. $hGIID_{ng}$ is the nonglycosylated form of hGIID. LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LIP, lysophosphatidylinositol; LPM, lysophosphatidylmethanol; LPS, lysophosphatidylserine.

keratinocyte sPLA$_2$ is involved in the generation of free fatty acids, which are one of the main constituents of the permeability barrier of the outermost layer of skin (stratum corneum) (15, 16). Physiological functions for groups IIC, IID, IIE, IIF, III, and XII sPLA$_2$s have not yet been reported, although overexpression of groups IID, IIE, and IIF in HEK293 cells results in arachidonic acid release, which can be converted into prostaglandins (17–19). It is also clear that mammals contain a collection of proteins that bind sPLA$_2$s tightly. Two types of sPLA$_2$ receptor (M- and N-type), the cell surface proteoglycan glypican, and soluble sPLA$_2$-binding proteins have been identified and are likely to play a role in the physiological functions of mammalian sPLA$_2$s and in the toxicity of a wide variety of myotoxic and neurotoxic sPLA$_2$s found in reptile and invertebrate venoms (17, 20, 21).

sPLA$_2$s are water-soluble enzymes that must bind to the membrane interface to gain access to their highly water-insoluble phospholipid substrates (interfacial enzymes) (3, 4). Kinetic and x-ray structural studies of sPLA$_2$s have established that they contain an interfacial recognition site that allows attachment of enzyme to the interface, which is distinct from the catalytic site where the esterolysis of a single phospholipid molecule occurs. Thus, the substrate specificity of sPLA$_2$s is dictated by the type of membrane interface to which the enzyme prefers to bind (interfacial specificity) and by the type of phospholipid that is accommodated in the catalytic site (catalytic site specificity). These features can be studied separately using suitable methods (22). Interfacial binding specificity of sPLA$_2$s has important physiological consequences. For example, human group IIA sPLA$_2$ binds several orders of magnitude more tightly to anionic phospholipid membranes than to an interface composed mainly of charge-neutral phosphatidylcholine (PC) (23). This may explain why the extracellular face of the plasma membrane of mammalian cells, which is rich in PC and sphingomyelin, is normally resistant to degradation by group IIA sPLA$_2$, and yet the phosphatidylglycerol-rich membrane of Gram-positive bacteria is readily degraded by this enzyme (24–26).

Special consideration is also required to analyze reversible sPLA$_2$ inhibitors properly because nonspecific effects often result from a decrease in the amount of interface-bound enzyme caused by the presence of the inhibitor candidate in the interface (3). Highly specific sPLA$_2$ inhibitors have been reported for some of the group members (27–29).

In the present study, we have characterized the interfacial kinetic and binding properties of the full set of mouse and human groups I, II, V, X, and XII sPLA$_2$s (mGIB, hGIB, mGIIA, hGIIA, mGIIC, mGIID, hGIID, mGIIE, hGIIE, mGIIF, hGIIF, mGV, hGV, mGX, hGX, and hGXII). The group III sPLA$_2$ gene predicts a protein consisting of a central sPLA$_2$ domain flanked by large N- and C-terminal extensions. This sPLA$_2$ was not included in the present studied because the structure of the mature protein remains to be elucidated. Mouse group XII was not studied because its sequence is 94% identical to that of hGXII. The interfacial kinetic properties (turnover numbers on various different phospholipid vesicles, catalytic site specificity with regard to phospholipid head group and *sn*-2 acyl chain, and calcium affinity) were studied systematically, allowing the comparison of the enzymatic properties of mammalian sPLA$_2$s measured with a common set of conditions. The interfacial binding affinities of the mammalian sPLA$_2$s for vesicles composed of zwitterionic phospholipids containing various amounts of anionic phospholipids were quantified as a prelude to interpreting the ability of these enzymes to hydrolyze the outer plasma membrane of mammalian cells, which is also reported. Given the importance of sPLA$_2$ inhibitors as medicinal agents and for unraveling the physiological functions of these enzymes, we also report the full set of inhibition data using several previously described compounds that have been established to act by a catalytic site-directed mechanism. All of these studies rely on a source of pure recombinant sPLA$_2$s, and refolding protocols are described which permit native enzymes to be obtained in relatively high yields from *Escherichia coli*-generated insoluble inclusion bodies except for two sPLA$_2$s that were obtained by expression in insect cells.

EXPERIMENTAL PROCEDURES

Materials—All phospholipids are from Avanti Polar Lipids Inc. unless specified otherwise. PI is from soybeans and is mainly 1-palmitoyl-2-linoleoylphosphatidylinositol. sPLA$_2$ inhibitors were obtained as follows: DDC-1 (30); Me-Indoxam (31) (previously designated Indoxam), LY311727 (gift from Dr. E. Mihelich, Lilly Laboratories) (32); MJ33, MJ50, and triterpenoid-1 and -2 (33, 34) (gift from Prof. M. K. Jain, University of Delaware); SB203347 (gift from Dr. L. A. Marshall, Smith Kline Beechem Pharmaceuticals) (35); phosphonate-6b, -10b, and -12b (36); Pyr-1 (37). All inhibitor structures are given in Fig. 4. mGIID was prepared by expression in *E. coli* (38). Detailed procedures for the preparation of mGIB, mGIIA, mGIIE, mGIIF, mGV, and mGX by expression in *E. coli* and of mGIIC by expression in *Drosophila* S2 cells will be reported elsewhere. Cobra venom sPLA$_2$ was purified from *Naja naja naja* venom (37).

Preparation of Recombinant Human sPLA$_2$s—Except as noted below, sPLA$_2$s were produced by *in vitro* refolding of inclusion body protein produced by expression in *E. coli*. All sPLA$_2$s were analyzed by mass spectrometry using electrospray ionization on a Bruker/Hewlett-Packard Esquire LC Ion Trap machine. For some sPLA$_2$s, MALDI-TOF was used (Applied Biosystems Voyager DE-Pro), and the mass was measured in linear mode using sinapinic acid as a matrix. As necessary, sPLA$_2$s were purified on a C18 ZipTip (Millipore) to remove traces of salts prior to electrospray mass spectrometry. The tip was rinsed twice with 50% CH_3CN and 0.1% trifluoroacetic acid, then three times with water and 0.1% trifluoroacetic acid. sPLA$_2$ solution (10 μl) was loaded onto the tip, which was washed twice with water and 0.1% trifluoroacetic acid and then with 80% CH_3CN to elute the protein.

hGIIA, and hGX were produced in *E. coli* as described previously (23, 39–41). *E. coli* expression plasmids for hGIB and hGIIF were prepared from the respective cDNAs as described for hGXII (41). The expression plasmids are based on the pAB$_3$ plasmid encoding the first 8.4 kDa of glutathione *S*-transferase followed by a factor Xa protease recognition site fused to the N terminus of mature hGIB and hGIIF (*i.e.* without signal peptides or prepropeptides) (38). Because cDNA for hGIIE could not be PCR amplified from a number of commercial human tissue cDNAs (Clontech), a synthetic gene was prepared as described previously for hGX (37) and used to construct a pAB$_3$ plasmid as for hGIB (sequence available from the authors upon request). For hGIIFΔC, a PCR fragment coding for a factor Xa cleavage site (Ile-Glu-Gly-Arg) followed by the hGIIFΔC mature protein ending with the peptide sequence PTPNC (42) was amplified with *Pwo* DNA polymerase and subcloned in-frame in pAB$_3$ (43). For hGV expression, the cDNA coding for the mature enzyme was PCR amplified and then cloned in-frame to the initiator Met codon encoded by the *Nde*I site present in the pET21a expression plasmid (Novagen Inc.).

hGIB, hGIIE, hGIIF, hGIIFΔC, and hGXII were expressed in *E. coli* BL21, and hGV was expressed in BL21 (DE3). Inclusion body protein was isolated and sulfonated as described (41). Specific refolding procedures for each human sPLA$_2$ are described below. Enzymatic activity was followed using a fluorometric assay with 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphomethanol (44).

Sulfonated hGIB fusion protein (40 mg) was dissolved in 200 ml of 6 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, at room temperature by stirring for 30–60 min. The solution was dialyzed against 6.5 liters of prechilled refolding buffer (0.9 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, 5 mM freshly added cysteine, 5 mM EDTA) for 5–7 h at 4 °C and then against a second portion of buffer overnight. A small aliquot of dialyzed protein solution was assayed for enzymatic activity using the fluorometric assay (44) until it reached a maximum (typically 1–2 days after initiation of dialysis). The dialysis bag was transferred to 6.5 liters of prechilled protease buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$). The buffer was changed twice for a total of 20 liters. The contents of the dialysis bag were centrifuged at 8,000 $\times$ *g* for 20 min at 4 °C to remove protein precipitate. The protein concentration in the supernatant was measured with the Bradford dye binding assay (Bio-

Rad, using bovine serum albumin as a standard). TPCK-treated trypsin was added (0.025 μg/μg of hGIB fusion protein), and digestion was allowed to proceed with stirring at room temperature until the enzymatic activity reached a maximum (typically ~2 h). The protein solution was centrifuged to remove any particulate and then pumped at 3 ml/min directly onto the HPLC column (60 ml/run, Vydac 218 TP1010 C18 reverse phase) that had been equilibrated previously with solvent A (0.06% trifluoroacetic acid in water). The column was developed with a program of 0–30% solvent B (0.06% trifluoroacetic acid in acetonitrile) over 40 min, then to 60% B over 110 min, then to 100% B over 20 min. hGIB elutes at 43 min. The peak fractions were combined, 2 volumes of water were added, and the sample was lyophilized. The overall yield of pure, refolded hGIB is ~1.7 mg/liter of bacterial culture. The protein was judged to be >98% pure on a 15% SDS-polyacrylamide gel. The observed molecular weight is 14,125.8, and the calculated weight is 14,125.0.

Sulfonated hGIIE fusion protein was refolded by the dialysis method described above for hGIB. Although sPLA$_2$ activity was detected after dialysis against refolding buffer, no activity was detected in the supernatant following centrifugation of the protein solution that was dialyzed against protease buffer. The protein pellet, obtained after submitting 150 mg of sulfonated fusion protein to refolding, was dissolved in 150 ml of protease buffer containing 10 mM lauryl sulfobetaine (Calbiochem) by stirring for 10–20 min at room temperature. After centrifugation (8,000 × *g* at 4 °C for 15 min), TPCK-treated trypsin was added (0.02 μg/μg of fusion protein) to the supernatant. The sPLA$_2$ activity was followed until it reached a maximum (~1.5 h). The sample was centrifuged (8,000 × *g* at 4 °C for 15 min). One-fourth of the supernatant was pumped directly onto the HPLC column (as for hGIB) that had been equilibrated previously with 10% solvent B. The column was developed with 10–30% B over 20 min then to 45% B over 110 min, and hGIIE elutes at 45 min. The protein solution was concentrated ~2-fold in a SpeedVac (Savant Instruments) and dialyzed against 10 mM Tris, pH 8.0, at 4 °C. The dialyzed hGIIE solution was stored at −20 °C. The overall yield of pure, refolded hGIIE is ~1.7 mg/liter of bacterial culture. The protein was judged to be >98% pure on a 15% SDS-polyacrylamide gel. The observed molecular weight (electrospray mass spectrometry) is 13,951.5, and the calculated is 13,952.1.

Sulfonated hGIIF fusion protein was dissolved to 10 mg/ml in 6 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0 (this and all subsequently used buffers and HPLC solvents also contained 1 mM methionine to prevent oxidation of protein methionines), by stirring for 2 h at room temperature or overnight at 4 °C. The sample was centrifuged at 4 °C at 12,000 rpm for 20 min to remove undissolved protein. Four ml of protein solution was added dropwise (~1 drop/s) to 2 liters of hGIIF-refolding buffer (50 mM Tris-HCl, pH 8.0, 0.9 M guanidine-HCl, 10 mM $CaCl_2$, 5 mM freshly added cysteine, 30% acetonitrile (by volume), acetonitrile added last to buffer preadjusted to pH 8.0) with stirring in an Erlenmeyer flask at room temperature. Stirring was continued for a few min, and the solution was kept at room temperature without stirring until the sPLA$_2$ activity reached a maximum (~2–3 days). The volume of the solution was reduced to 1,400 ml by rotary evaporation at 30 °C. Lauryl sulfobetaine was added to a concentration of 5 mM followed by the addition of 14 ml of 100 mM methionine (previously filtered through a 0.45-μm syringe filter). The solution was concentrated to 40–50 ml in an ultrafiltration cell (Amicon, YM-10 membrane) with stirring under 30–40 p.s.i. of N_2 pressure. The solution was dialyzed against protease buffer at 4 °C (three cycles, each with 40 volumes of buffer). Trypsin was added to a final concentration of 0.2 μg/ml, and the sample was left at room temperature overnight. Phenylmethylsulfonyl fluoride was added to a final concentration of 0.5 mM, and the solution was filtered through a 0.45-μm nylon HPLC solvent filter. An aliquot (12.5 ml) was pumped onto the reverse phase HPLC column (previously equilibrated with 20% acetonitrile, 0.1% trifluoroacetic acid, solvent A) at 3 ml/min. The column was washed with 15 ml of solvent A followed by a gradient of 0–6.3% B (100% acetonitrile, 0.1% trifluoroacetic acid) in 2 min, then to 27.5% B in 42 min, then to 100% B in 4 min. hGIIF elutes at 36 min. The purified protein solution was neutralized by adding 2 M Tris (10 μl/ml of HPLC fraction), and lauryl sulfobetaine was added to a concentration of 5 mM. The sample was concentrated in a Centriprep-10 (Amicon) and then dialyzed against 40 volumes of 10 mM Tris, 0.1 mM DTT, pH 8.0, at 4 °C for one cycle and then 40 volumes of 10 mM Tris-HCl, pH 8.0, for two cycles. The protein solution was stored at −20 °C. The overall yield of pure, refolded hGIIF is ~3.7 mg/liter of bacterial culture. The protein was judged to be >98% pure on a 15% SDS-polyacrylamide gel. To ensure complete removal of buffer cysteine that may be disulfide-linked to the hGIIF cysteine residue in its C-terminal extension, 0.64 mg of hGIIF in 1 ml of 20 mM Tris, pH 8.0, was treated with 1 mM DTT for 1 h at room temperature. The sample was diluted to 1.5 ml with 20% CH_3CN and 0.1% trifluoroacetic acid and loaded onto an analytical HPLC column (Supelco Discovery BIO wide pore C5, 5 μm, 4.6 × 250 mm). The column was developed for 5 min in 20% CH_3CN and 0.1% trifluoroacetic acid followed by a gradient to 50% CH_3CN and 0.1% trifluoroacetic acid over 120 min. hGIIF eluted at 69.4 min, and the fraction was diluted with water and lyophilized. The protein was resuspended in 31.5% CH_3CN and 0.1% trifluoroacetic acid, and the solution was loaded onto the same HPLC column. After 5 min at 31.5% CH_3CN and 0.1% trifluoroacetic acid, a gradient to 36.5% CH_3CN and 0.1% trifluoroacetic acid was started. hGIIF eluted at 46.3 min and was diluted with water and lyophilized. The observed molecular weight (electrospray mass spectrometry) is 16,583.4, and the calculated is 16,583.7.

hGIIFΔC was expressed in *E. coli* as follows. A PCR fragment coding for a factor Xa cleavage site (Ile-Glu-Gly-Arg) followed by the hGIIFΔC mature protein ending with the peptide sequence PTPNC (43) was amplified with *Pwo* DNA polymerase and subcloned in-frame in the pAB3 vector. Bacterial induction, preparation of inclusion bodies, sulfonation of the fusion protein, and dialysis against 1% acetic acid were performed as described (38). Sulfonated hGIIFΔC protein was dissolved at 0.2 mg of protein/ml in 500 ml of 6 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, and refolded by dialysis against 8 liters of 0.7 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM L-methionine, and 5 mM L-cysteine for 48 h at 4 °C. The refolded protein was dialyzed twice against 8 liters of 2 M urea, 0.1 M NH_4Cl, 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0. The protein solution was filtered through Sephadex G-50 and subjected to digestion with trypsin (0.025 μg of trypsin/μg of protein) at room temperature. The cleavage of the fusion protein was followed by measuring the increase of sPLA$_2$ activity using *E. coli* membranes as substrate (45). After about 2 h of incubation with trypsin, the reaction was stopped by acidification with 0.1% trifluoroacetic acid, and the mixture was concentrated to 40 ml using an Amicon stirred cell concentrator with a YM-10 membrane. The solution was filtered and loaded directly onto a Beckman C18 reverse phase HPLC column (10 × 250 mm, 19.6 ml, 100 Å, 5 μm). Elution was performed at 4 ml/min using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (20–35% acetonitrile over 150 min). Fractions containing sPLA$_2$ activity were lyophilized, dissolved in 23% acetonitrile and 0.1% trifluoroacetic acid, and loaded on a Waters Symmetry Shield C8 column (4.6 × 250, 4.6 ml, 100 Å, 5 μm) equilibrated with the same buffer. hGIIFΔC protein was eluted with a shallow gradient of acetonitrile in 0.1% trifluoroacetic acid (23–35% over 120 min). The hGIIFΔC protein was eluted at 92.7 min. The observed molecular weight (MALDI-TOF) is 14,171.2, and the calculated weight is 14,171.0. The protein appeared as a single band (>98% pure) on a 14% SDS-polyacrylamide gel (not shown).

Sulfonated hGV was dissolved to 10 mg/ml in 6 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0 (this and all subsequently used buffers and HPLC solvents also contained 1 mM methionine), by stirring for 2 h at room temperature or overnight at 4 °C. The sample was centrifuged at 4 °C at 12,000 rpm for 20 min to remove undissolved protein. Protein solution (4 ml) was added to 2 liters of room temperature refolding buffer (50 mM Tris-HCl, pH 8.0, 0.9 M guanidine-HCl, 10 mM $CaCl_2$, 5 mM freshly added cysteine, 30% acetonitrile (by volume), acetonitrile added last to buffer preadjusted to pH 8.0) as described for hGIIF. The protein was allowed to refold as for hGIIF. The volume was reduced to 70% by rotary evaporation at 30 °C. Lauryl sulfobetaine was added to a final concentration of 5 mM, and the protein solution was concentrated to a final volume of 40–50 ml in an Amicon stirred cell with a YM-10 membrane at room temperature. The concentrated protein solution was dialyzed against 20% acetonitrile, 0.1% trifluoroacetic acid at 4 °C (three cycles, each cycle with 40 volumes of buffer). The dialyzed solution was filtered and pumped directly onto the HPLC column that was used for hGIIF except that the column was preequilibrated with 20% solvent B in solvent A (same solvents as for hGIIF). Up to 25 ml of protein solution was injected per run. After injection, a solvent gradient was started: 0% B to 12.5% B in 2 min, then to 22.5% B in 34 min, then to 100% B in 4 min (3 ml/min). hGV elutes at 23.6 min. HPLC fractions were combined and stored at −20 °C. The active fraction was concentrated to about 70% of its initial volume by rotary evaporation and then lyophilized. The overall yield of pure, refolded hGV is 7 mg/liter of bacterial culture. The protein was judged to be >98% pure on a 15% SDS-polyacrylamide gel. The observed molecular weight (MALDI mass spectrometry) is 13,577.70, and the calculated weight is 13,578.60.

hGXII was produced by a modification of the original procedure (41), resulting in improved yields. Sulfonated hGXII fusion protein from a 4.5-liter bacterial culture was dissolved in 200 ml of 6 M guanidine-HCl,

50 mM Tris, pH 8.0, by stirring at room temperature for ~1 h. The sample was dialyzed for 5 h against 6.5 liters of 0.9 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 5 mM freshly added cysteine at 4 °C. The buffer was replaced and dialysis continued for an additional 48 h (enzymatic activity followed with the fluorometic assay). Solid in the dialysis bag was removed by centrifugation, and the sample was dialyzed against 6.5 liters of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$ at 4 °C for 4–5 h, and the dialysis was continued for an additional ~12 h with fresh buffer that also contained 5 mM lauryl sulfobetaine. Solid was removed by centrifugation, the supernatant was saved at 4 °C, and the pellet was stirred with 100 ml of dialysis buffer containing 5 mM lauryl sulfobetaine for 2 h at room temperature. After centrifugation, the supernatant was combined with the original supernatant. The sample was concentrated to ~250 ml with an ultrafiltration cell equipped with a YM-10 membrane. To the sample was added 400 units of factor Xa protease (Amersham Biosciences), and the digestion was allowed to proceed overnight at room temperature. Solid was removed by centrifugation, and the supernatant was pumped directly onto the HPLC column (Vydac 218TP1010 C18 reverse phase) (in four equal portions) that had been previously equilibrated with 10% solvent B in solvent A at 1 ml/min. The column was developed by washing with 10% solvent B in solvent A for 3 min, then increasing to 30% solvent B in 20 min, then to 35% solvent B in 25 min, then to 60% solvent B in 30 min, and finally to 100% solvent B in 10 min. hGXII elutes at ~55 min, and the remaining fusion protein elutes ~1.5 min later (the factor Xa digestion time can be increased if large amounts of fusion protein remain). hGXII-containing fractions are combined, adjusted to pH ~5, and half of the sample is applied to a cation exchange HPLC column (Toso Haas SP-5PW, 0.75 × 7.5 cm) previously equilibrated with 20 mM sodium acetate, pH 5.0, 30% acetonitrile (solvent C) (pH adjusted with acetonitrile present) at 1 ml/min. After a 15-min wash at 100% solvent C, a gradient over 30 min to 100% solvent D (solvent C + 0.5 M NaCl) was used to elute the protein (elution time ~37 min). To desalt the protein, it was diluted with water to give 10% acetonitrile and reapplied to the C18 HPLC column. After developing the column as above, the hGXII-containing fraction was diluted with water and lyophilized to obtain 8–15 mg of pure hGXII. Alternatively, the HPLC fraction was diluted with water and concentrated in a Centriprep (Y-10, Amicon, prewashed with water), and buffer was exchanged to 20 mM sodium acetate, pH 4, by repeated dilution with buffer and ultrafiltration. The final protein concentration was 7.3 mg/ml (stored at −20 °C). The protein was judged to be >99% pure on a 15% SDS-polyacrylamide gel. The observed molecular weight (electrospray) is 18,701.4, and the calculated weight is 18701.15.

hGIID was obtained by expression in *Drosophila* S2 cells as several attempts to refold hGIID protein in high yield from *E. coli*-produced inclusion bodies failed. The cDNA coding for the full-length hGIID protein (GenBank AF112982) was amplified from human thymus cDNA by reverse transcription-PCR with *Pwo* DNA polymerase (Roche Molecular Biochemicals), cloned into pGEM-T easy (Promega), and sequenced. This resulted in the amplification of a polymorphic variant of hGIID which differs from AF112982 by the presence of a serine instead of a glycine at position 60. The amplified cDNA was then subcloned into the *Drosophila* expression vector pMT/V5-His (Invitrogen) to allow for the recombinant expression of hGIID in S2 cells according to the Invitrogen *Drosophila* expression system manual. S2 cells were grown at 24–27 °C in *Drosophila*-SFM medium (Invitrogen) supplemented with 1% fetal bovine serum and antibiotics (Invitrogen). After sequencing, the expression plasmid hGIID-pMT/V5-His and a plasmid carrying a G418-resistance selection cassette were cotransfected into *Drosophila* S2 cells using the calcium-phosphate method. The calcium-phosphate-DNA precipitate was incubated with cells for 4 h, after which the cells were washed by centrifugation and incubated in fresh medium. Two days after transfection, 2 mg/ml G418 sulfate was added to the cell culture medium to select for stably transfected cells. Fresh selection medium was added every 4–5 days. Five days after transfection and 4 weeks after selection, hGIID expression was examined by measuring membrane-bound and soluble sPLA$_2$ activity present in cell medium, as described previously for hGIIA produced in *Sf9* insect cells (45). sPLA$_2$ activity was measured using labeled *E. coli* membranes as substrate (45). For these assays, cells were plated at 3×10^6 cells/ml in 24-well plates and induced the day after with 500 μM $CuSO_4$ for 3–7 days. For large scale production of hGIID, stably transfected cells were seeded into 500 ml of complete medium at a density of $2–3 \times 10^6$ cells/ml in 1-liter Spinner flasks (Integra Biosciences). Upon reaching a density of $5–6 \times 10^6$ cells/ml, cells were induced with 500 μM $CuSO_4$. Three days after induction, the cells were pelleted, and the medium was collected. Membrane-bound hGIID activity was extracted for 30 min at 4 °C with 50 ml/Spinner of phosphate-buffered saline containing 1 M KCl. The cells were again pelleted, and the KCl extract was combined with the cell-free medium.

The purification of hGIID was routinely performed from batches of 2 liters of induced cells. To prevent extensive oxidation of hGIID, 1 mM L-methionine was added to all of the purification buffers. The cell medium and KCl extract from 2 liters of induced cells were combined, diluted to 4 liters with water, and applied under vacuum to 150 ml of heparin-Sepharose CL-6B (Amersham Biosciences), poured into a 500-ml glass-sintered funnel. The gel was subsequently washed with 1 liter of 20 mM Tris, pH 7.4, containing 0.3 M NaCl and 1 liter of the same buffer containing 0.4 M NaCl. sPLA$_2$ activity was eluted with 2 liters of 20 mM Tris, pH 7.4, containing 1 M NaCl, 8 M urea, and 0.1 M NH_4Cl. Fractions containing sPLA$_2$ activity were diluted twice with water and acidified to pH 3.0 with 0.1% trifluoroacetic acid. Acetonitrile was added to 5% by volume, and the sample was loaded onto 100 ml of C18 silica gel 40–63 μm (Merck), poured into a 500 ml glass-sintered funnel. The gel was washed with 1 liter of acetonitrile/water/trifluoroacetic acid (5/95/0.1) and 1 liter of acetonitrile/water/trifluoroacetic acid (12/88/0.1). Bound sPLA$_2$ was eluted with 2 liters of acetonitrile/water/trifluoroacetic acid (60/40/0.1). Fractions containing sPLA$_2$ activity were pooled, and the sample evaporated to a small volume (typically 2 liters to about 400 ml) with a rotary evaporator to remove acetonitrile, diluted 2-fold with water, and then lyophilized. The sPLA$_2$ material was dissolved in 1% acetic acid containing 10% acetonitrile, filtered, and applied to a Tosohaas Spherogel TSK SP-5PW HPLC column (10 μm, 21 × 150 mm, 55 ml), equilibrated with the same buffer. The column was eluted at 8 ml/min using a linear gradient of ammonium acetate (0–2 M, pH 6.8 over 100 min) in 10% acetonitrile. The main fractions containing sPLA$_2$ activity were pooled, lyophilized, and applied to a C18 reverse phase HPLC column (Beckman 10 × 250 mm, 19.6 ml, 5 μm, 100 Å). Elution was performed at 4 ml/min using a gradient of acetonitrile in 0.1% trifluoroacetic acid (10–25% acetonitrile over 15 min, followed by 25–45% over 60 min). This purification step led to two major peaks containing oxidized and nonoxidized hGIID that eluted at 38.4 and 44.6 min, respectively. These two peaks were lyophilized separately, dissolved in 23% acetonitrile and 0.1% trifluoroacetic acid, and loaded onto a C4 develosil reverse phase HPLC column (Nomura Chemicals, 4.6 × 250 mm, 4.6 ml, 5 μm, 300 Å) equilibrated in the same solvent. Elution was performed at 1 ml/min using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (23–28% acetonitrile over 100 min). Oxidized glycosylated hGIID, oxidized nonglycosylated hGIID, nonoxidized glycosylated hGIID, and nonoxidized nonglycosylated hGIID eluted from the C4 column at 23.7, 31.4, 41.6, and 52.5 min, respectively. The different protein fractions were judged to be more than 99% pure on a 14% SDS-polyacrylamide gel (not shown) and were all analyzed by MALDI-TOF mass spectrometry using internal calibration. The observed molecular mass determined for nonoxidized nonglycosylated hGIID (14522.9) is consistent with the calculated value of the hGIID polypeptide with a serine at position 60 (14,522.6). The glycosylated hGIID fraction appears to be glycosylated heterogeneously with several molecular masses of 15,412.2, 15,558.4, 15,737.9, and 15,945.2, corresponding to sugar masses of 891.8, 1,037.9, 1,217.5, and 1,424.8. The heterogeneous glycosylation pattern of hGIID appears to be similar to those observed for bee venom sPLA$_2$ or other proteins endogenously or heterogeneously expressed in insect cells (46–48). Treatment of glycosylated hGIID with *N*-glycopeptidase (Roche Molecular Biochemicals) followed by mass spectrometry analysis confirmed that hGIID produced in S2 cells is *N*-glycosylated (not shown), in good agreement with the presence of an *N*-glycosylation site in its sequence (49). On the other hand, treatment with *O*-glycosidase and neuraminidase (Roche Molecular Biochemicals) does not affect the molecular mass of the glycosylated hGIID protein, suggesting the absence of sialic acids and *O*-glycosylation (not shown). The oxidized forms of glycosylated and nonglycosylated hGIID eluted earlier during reverse phase HPLC and appear to be monooxidized, as indicated by delta masses of about 16 mass units. Fractions containing pure hGIID sPLA$_2$s were lyophilized and stored in aliquots at −20 °C. The final yield of purified, nonoxidized glycosylated hGIID sPLA$_2$ was about 50 μg/liter of cell medium.

Interfacial Kinetics with Phospholipid Vesicles—The initial rate of hydrolysis of phospholipid vesicles by sPLA$_2$s was carried out with the fatty acid-binding protein assay (37). Assays were carried out in Hanks' balanced salt solution with 1.27 mM Ca^{2+} and 0.90 mM Mg^{2+}. Assays also contained 9.7 μg of fatty acid-binding protein (37), 1 μM of 11-dansylundecanoic acid (Molecular Probes Inc.), and 30 μM phospholipid (POPG, POPS, POPC) extruded vesicles (37). The final assay volume was 1.3 ml, present in a fluorescence cuvette with a magnetic stir bar at 37 °C. Excitation was at 350 nm and emission at 500 nm with both slits

at 10 nm. Assays were calibrated by adding a known amount of oleic acid and measuring the decrease in fluorescence. Reactions were started by adding 2–10 μl of sPLA$_2$. When the concentration of sPLA$_2$ in the stock solution was less than 100 μg/ml, stock solutions were prepared in buffer containing 1 mg/ml bovine serum albumin to prevent absorption of enzyme on the walls of the tube.

sPLA$_2$ Sensitivity to DTT—sPLA$_2$ (0.5–10 μg) was added to 100 μl of 100 mM Tris, pH 7.4. The sample was split in half, and DTT was added to one tube to give a final concentration of 10 mM. Samples were incubated at room temperature or 50 °C for the indicated times, and aliquots were assayed using a fluorometric assay with 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphomethanol (44). The activity in the sample with DTT was compared with that in the sample without DTT to obtain the percent remaining activity at each time point.

Enzyme Catalytic Site Phospholipid Headgroup Substrate Specificity—The concentrations of phospholipids in $CHCl_3$/CH_3OH stock solutions were determined by inorganic phosphate assay. An equimol mixture of POPA, POPC, POPE, POPG, POPM, POPS, and PI in $CHCl_3$/CH_3OH was dried down *in vacuo*, and small vesicles were made by sonication (50) in water (0.86 mM phospholipid). Reaction mixtures contained 3 ml of 1 mM NaCl, 0.6 mM $CaCl_2$, and a total phospholipid concentration of 0.25 mM at 21 °C. A sufficient quantity of sPLA$_2$ was added until ~3–5% of total phospholipid was hydrolyzed. Reactions were quenched by the addition of EGTA to give a concentration in the assay mixture of 2 mM. The internal standard d_{31}-LPG (37), 5.5 nmol, was added to the quenched reaction.

To a disposable column containing 1 ml of C18 reverse phase silica packing (Bakerbond spe™, J. T. Baker) was added 3 ml of $CHCl_3$, and the column was hung on the rim of a glass tube and centrifuged in a clinical table top centrifuge to elute all of the solvent. In this way the column was then washed with 2 ml of methanol and then twice with 3-ml portions of purified water (Milli-Q, Millipore). After spin elution of the last water wash, the quenched pH-stat reaction was loaded onto the column. After centrifugation, the column was washed twice with 3-ml portions of purified water. The column was transferred to a new glass tube, and phospholipids were eluted by washing with two 1-ml portions of $CHCl_3$/methanol (2/1). The solvent in the glass tube was removed with a stream of N_2 at room temperature, and remaining water was removed in a SpeedVac. A control pH-stat reaction containing all components except sPLA$_2$ was worked up as described above. The residue was dissolved in 50 μl of 70% methanol in water, and 2 μl was injected onto the HPLC column for electrospray ionization mass spectrometry using a modification of the earlier procedure (37) as described below.

HPLC was carried out on a reverse phase column (Vydac) using solvent A (water and 0.5% concentrated NH_4OH), solvent B (methanol and 0.5% concentrated NH_4OH), and solvent C (33% hexane in methanol and 0.5% concentrated NH_4OH). The program is 30% A in B, then to 100% B over 10 min, then holding at 100% B for 6 min, then to 40% B in C over 10 min, and finally holding at 40% B in C for 2 min, all at a flow rate of 0.2 ml/min. Mass spectrometry was carried out as described (37), switching from negative ion mode to positive ion mode at 16 min into the solvent program. Ion intensities for each LPX species (LPA, 409–410; LPC, 496–497, LPE, 452–453; LPG, 483–484; d_{31}-LPG, 514–515; LPI, 599–600; LPM, 423–424; LPS, 496–497) were extracted from the data, and each peak was integrated. The data set obtained with the sPLA$_2$ and with the minus enzyme control were scaled to each other by using the ion intensity of the common d_{31}-LPG internal standard, and the scaled integrals for each LPX species in the minus enzyme control were subtracted from the corresponding LPX ion intensities in the run that included the sPLA$_2$. The background corrected ion intensities were normalized to the ion intensity for LPG, and the normalized ion intensities were multiplied by the mass spectrometer response factors for each LPX species relative to LPG (obtained by HPLC/mass spectrometry analysis of a standard sample containing equal mol of each LPX) to give the moles of each LPX species generated by action of the sPLA$_2$ relative to the moles of LPG formed. Because the LPX species were generated only after a small amount of total phospholipid was hydrolyzed, the relative LPX mol ratios approximate the relative initial velocities for the hydrolysis of each phospholipid species. Because each phospholipid species is present in the vesicle at an equal mol fraction, the relative LPX mol ratio gives the specificity constant k_{cat}*/K_m* (concentration normalized initial velocity (22)) for the hydrolysis of each LPX species relative to that for LPG. These relative k_{cat}*/K_m* values are plotted in Fig. 2, *A* and *B*.

The quantification of LPA was also checked by an independent method as LPA eluted early from the HPLC column in a region just after salt and other impurities, agents that can potentially interfere with mass spectrometry detection. Also, the variation in the amount of d_{31}-LPA internal standard in different samples was large, suggesting differential loss of LPA in independent experiments. Reaction mixtures (100 μl) contained 20 μM total phospholipid (vesicles composed of equal mol amounts of POPA, POPC, POPE, POPG, POPM, POPS, and PI, as for the mass spectrometry studies, and also containing 20,000 dpm of [^{3}H]DPPA (400 Ci/mol) and 20,000 dpm of [^{14}C]DPPC (50 Ci/mol, American Radiochemicals Inc.)) in 50 mM Tris-HCl, pH 8.0, 0.6 mM $CaCl_2$ at room temperature. [^{3}H]DPPA was prepared as for the radiolabeled phosphatidylglycerol (38) except that glycerol was omitted from the reaction mixture. Sufficient sPLA$_2$ was added to hydrolyze less than 20% of the total radiolabeled phospholipid in a reaction time of 30 min. Reactions were quenched with organic solvent, and fatty acids were separated from phospholipids as described (51). Double channel scintillation counting was used to determine the ratio of ^{3}H- to ^{14}C-fatty acid produced (these data were corrected for cpm of fatty acids in a minus sPLA$_2$ control reaction). An aliquot of the reaction mixture prior to the addition of enzyme was submitted to scintillation counting to obtain the ratio of ^{3}H to ^{14}C phospholipid substrates.

sn-2 Fatty Acyl Chain Specificity—Chloroform solutions of POPG, [^{3}H]DPPC (92 Ci/mmol, American Radiochemicals Inc.), and [^{14}C]SAPC (53 Ci/mol, American Radiochemicals Inc.) were mixed, solvent was removed *in vacuo*, and vesicles were prepared by sonication (50) in water. Assay mixtures contained 5 μM total phospholipid and 40,000–50,000 cpm of each radiolabeled phospholipid in 100 μl of 100 mM Tris, pH 8.0, 2.5 mM $CaCl_2$, and an appropriate amount of sPLA$_2$ to allow ~10–20% of the radiolabeled phospholipid to be hydrolyzed at the end of the 20-min reaction period to ensure that the initial velocity was being measured. Reaction workup, product, substrate ratio determination, and correction for the minus enzyme control were as described above for the studies with [^{3}H]DPPA and [^{14}C]DPPC.

Calcium Affinity Studies—The dependence of the initial velocity for the hydrolysis of phospholipid vesicles on the concentration of Ca^{2+} was carried out as described above for the kinetic studies using fatty acid-binding protein and 30 μM POPG, POPS, or POPC extruded vesicles as substrates. Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} was used. Various concentrations of Ca^{2+} in the assay mixture were established as described (52). The initial velocity *versus* the concentration of free Ca^{2+} was fitted to the simple hyperbolic equation to obtain values of $K_{Ca(app)}$ (see "Results").

Inhibition Analysis—Inhibition of sPLA$_2$s was analyzed using a radiometric assay consisting of POPG vesicles containing a trace amount of [^{3}H]DPPC. Phospholipids were mixed in chloroform to give a final specific activity of 50 Ci/mol. After removal of solvent *in vacuo*, assay buffer (0.1 M Tris-HCl, pH 8.0, 10 mM $CaCl_2$) was added to give a lipid concentration of 5 μM, and small unilamellar vesicles were prepared by sonication (50). Assays (100 μl) were carried out in polypropylene microfuge tubes. After addition of inhibitor, reactions were started by adding sPLA$_2$. After incubation at 37 °C, reactions were quenched with organic solvent, and liberated [^{3}H]palmitic assay was determined as described (51). Sufficient sPLA$_2$ was added to give ~10,000 dpm of released product (10–20% of the total) after a 10–20-min incubation.

Inhibition of sPLA$_2$s by Me-Indoxam was analyzed using a microtiter plate fluorescence assay using 1-palmitoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol (Molecular Probes Inc.) at a concentration in the assay of 1 μM. The assay is similar to that described previously using the analogous phosphomethanol substrate (37) (full details to be published separately). Inhibition by Me-Indoxam was also analyzed using radiolabeled *E. coli* membranes as substrate as described (53) except that the incubation buffer was 100 mM Tris, pH 8.0, 10 mM $CaCl_2$, 0.1% bovine serum albumin. In these assays, Me-Indoxam was preincubated with sPLA$_2$s for 10 min before the addition of substrate.

Interfacial Binding to Vesicles—Preparation and size analysis of 0.1-μm diameter, sucrose-loaded, unilamellar vesicles of diether vesicles $DO_{et}PC$/$DO_{et}PS$ was carried out as described (23). sPLA$_2$ binding studies were carried out in 5 mM MOPS, pH 7.4, 0.1 M KCl, 2 mM $CaCl_2$ at room temperature using the centrifugation method in which the amount of enzyme remaining in the supernatant above pelleted vesicles is quantified by enzymatic assay (23) (pH of the binding mixture was checked after the addition of all components). Because the specific activity of the different sPLA$_2$s varies considerably, the amount of supernatant taken and the extent of its dilution with 3% bovine serum albumin in water were chosen such that the reaction velocity measured in the assay was at least 5-fold higher than the minus-sPLA$_2$ background rate (23). In the case of hGIB, the buffer for binding studies also contained 1 mg/ml bovine serum albumin to prevent loss of enzyme to the walls of the centrifuge tube.

Cellular Arachidonate Release—RBL-2H3.1 cells (obtained from Prof. B. Helm, University of Sheffield, UK) were cultured at 37 °C in a

humidified atmosphere of 5% CO_2 in minimal essential medium (S-MEM; Invitrogen) with 12% heat-inactivated fetal calf serum, 2 mM glutamine and penicillin/streptomycin/Fungizone. For arachidonic acid release studies, 5×10^5 cells were plated in each well of a 24-well plate in 1 ml of medium. After incubation for 24 h at 37 °C, cells were labeled with [^{3}H]arachidonic acid (~100 Ci/mmol, 0.1 μCi/well) for 24 h. The monolayer of cells was washed twice with complete medium, and 1 ml of complete medium was added to each well followed by the indicated amount of sPLA$_2$. Cells were incubated for 6 h at 37 °C, and the supernatant was collected and centrifuged briefly to pellet any dislodged cells. A 0.5-ml aliquot of the supernatant was analyzed by scintillation counting. To the cell pellet was added 1 ml of $CHCl_3$/CH_3OH (2/1), and the solution was transferred to a scintillation vial. After removal of solvent with a stream of nitrogen, the residue was submitted to scintillation counting. the percent of arachidonate release is expressed as 100 × (supernatant cpm)/(total cpm).

HEK293 cells (American Type Culture Collection) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G, and 10 μg/ml streptomycin sulfate at 37 °C in a humidified atmosphere of 5% CO_2. Cells were labeled with [^{3}H]arachidonic acid (or in some experiments with 0.5 μCi/well [9,10-^{3}H]oleic acid (15 Ci/mol), American Radiochemicals Inc.) and analyzed for released [^{3}H]arachidonic acid as described above for RBL-2H3.1 cells.

RESULTS

Production of Recombinant Mouse and Human sPLA$_2$s—Expressing large amounts of functional sPLA$_2$s is made difficult by the fact that these enzymes are generally toxic to cells when overexpressed in native form. Furthermore, the N terminus of most sPLA$_2$s is part of a catalytically important hydrogen bond network (54), thus N-terminal fusion peptides, which are useful for driving high expression, must be removed by selective proteolysis. If efficient *in vitro* refolding conditions can be obtained for these disulfide-rich proteins, *E. coli* expression can provide tens of milligrams of sPLA$_2$s, and it is this approach that we have taken to express most of the mammalian sPLA$_2$s. In this case, the insoluble inclusion body fraction of *E. coli* was isolated, protein was solubilized in denaturant, all disulfides were reduced, and free SH groups were sulfonated with Thanhausser reagent as described under "Experimental Procedures." Refolding was initiated by removal of denaturant in the presence of free cysteine to remove protein-bound sulfonates. This method was used previously by us and others to generate native sPLA$_2$s (43, 44, 55–58), but the yields are low (typically less than a few percent) because of protein precipitation in refolding buffer. By evaluating two different denaturant removal procedures (slow dialysis or rapid dilution) and systematically exploring the use of protein-solubilizing agents including water-miscible organic solvents and nonionic detergents (59), we have been able to improve dramatically the refolding yields for most of the mouse and human sPLA$_2$s.

For those sPLA$_2$s that did not express well when they lack an N-terminal fusion peptide, the pAB_3 expression plasmid (38) was used to generate the sPLA$_2$ fused to the N-terminal ~10-kDa portion of glutathione *S*-transferase. A factor Xa protease site is present in the fusion peptide just preceding the N-terminal residue of the mature sPLA$_2$, and factor Xa can be used to cleave all of the fusion proteins in high yield. However, in all cases, except for hGXII, we have found that treatment with trypsin provides a much less expensive alternative to the use of factor Xa, and with careful monitoring of the increase in sPLA$_2$ enzymatic activity, the desired proteins are obtained in high yield. For hGXII, trypsin treatment lead to multisite cleavage. The improvement in overall yields of native enzymes compared with previously reported procedures is considerable. For example, 7 mg of hGV was obtained per liter of bacterial culture, which is more than an order of magnitude better than yields reported previously (58, 60). Several attempts to refold hGIID fusion protein in high yield from pAB_3 plasmid-produced *E. coli* inclusion bodies failed, and only small amounts could be provided using a pET vector similar to that used for hGV (not shown). hGIID was thus produced in *Drosophila* S2 cells in both glycosylated and nonglycosylated forms (for details, see "Experimental Procedures"). All of the mouse sPLA$_2$s (except mGIIC, which was produced in S2 cells) could be obtained by *E. coli* expression and *in vitro* refolding (the methods will be published elsewhere).

hGIIF and mGIIF are unique among mammalian sPLA$_2$s in that they have a 23-amino acid C-terminal extension containing a cysteine residue. We found that solutions of mGIIF and hGIIF stored in the absence of 0.1 mM DTT exist as a mixture of monomer and homodimer proteins (separated by reverse phase HPLC). The homodimer could be converted quantitatively to the monomer by addition of 0.1 mM DTT without the reduction of disulfides present in the mGIIF and hGIIF monomer (established by mass spectrometry). All subsequent studies of mGIIF and hGIIF were carried out with the monomeric proteins. It remains to be established *in vivo* whether these monomers are linked together by a disulfide bridge or whether they are disulfide-linked to a different cellular protein. We also prepared hGIIFΔC (hGIIF lacking the C-terminal 23-amino acid extension) by bacterial expression.

All human and mouse recombinant sPLA$_2$s were shown to be >98% pure by SDS-PAGE (not shown). Analysis of the sPLA$_2$ molecular weights was carried out with electrospray ionization or MALDI-TOF mass spectrometry. In all cases, the observed mass of the $M+H^+$ ion agreed with the calculated mass to within 1 atomic mass unit. These data establish that all disulfide bonds are formed in the refolded sPLA$_2$s and that trypsin did not cleave any of the proteins internally.

Sensitivity of Mouse and Human sPLA$_2$s to DTT—There are numerous reports in the literature in which loss of PLA$_2$ activity upon treatment of the cellular extract with DTT is taken as firm evidence that the activity is caused by an sPLA$_2$. The sensitivity of the full set of mouse and human groups I, II, V, X, and XII sPLA$_2$s to DTT is summarized in Table I (numerical values in Table II). The most DTT-sensitive sPLA$_2$s are hGIIA and mGIIA, mGIIC, hGIID, and mGIID, and hGV and mGV. For these, complete loss of activity occurs in less than 30–60 min of exposure to 10 mM DTT at room temperature. The activity of hGIB and mGIB, hGIIF and mGIIF, mGX, and hGXII falls to zero but only after a 30–60-min DTT treatment at elevated temperature (50 °C). Remarkably, hGIIE and hGX retain measurable activity, 9 and 16%, respectively, even after treatment with 10 mM DTT for 60 min at 50 °C, and mGIIE retains 28% activity after treatment with DTT for 30 min at 50 °C. In general the DTT sensitivity of each human sPLA$_2$ is similar to that for the mouse ortholog. Importantly, the data show that caution must be exercised to use thiol reduction data to establish whether a PLA$_2$ enzymatic activity in a cell or tissue extract is caused by an sPLA$_2$. In addition, DTT sensitivity data provided in Table I may be useful to provide information on the type of sPLA$_2$ being detected in a cell lysate.

Activity of Mouse and Human sPLA$_2$s on Phospholipid Vesicles—We determined the specific activities for each sPLA$_2$ on phospholipid vesicles composed of the single phospholipids POPG, POPS, or POPC (summarized in Table I, numerical values in Table III) using the real time fluorometric assay employing fatty acid-binding protein (see "Experimental Procedures"). With the anionic vesicles POPG and POPS, the reaction started immediately after the addition of all sPLA$_2$s with no discernible lag, and the specific activities in Table III were calculated from the initial velocities. With charge-neutral POPC vesicles, a lag in the onset of hydrolysis was seen for some of the sPLA$_2$s (Table III). The initial velocities were close

TABLE I
Interfacial kinetic and binding properties of human and mouse sPLA$_2$s

sPLA$_2$	DTT sensitivity[a]	Catalytic efficiency[b]	Vesicle preference (single phospholipid vesicles)[c]	Catalytic site phospholipid preference[d]	*sn*-2 Fatty acyl chain specificity 20:4/16:0[e]	$K_{Ca(app)}$[f]	Most potent inhibitor[g]	Interfacial binding K_d[h]	Exogenous action on mammalian cells[i]
						μM		$m M$	
hGIB	++	+++	POPG ≫ POPS ≫ POPC	PG ~ PC ~ PA > PS > PE > PI	1.8	8 ± 3, POPG	Phosphonate-6b	~3 (30% PS/PC)	−
hGIIA	+++	+++	POPG > POPS ≫ POPC	PG ~ PC ~ PA > PS > PE > PI	0.8	13 ± 3, POPG	Me-Indoxam	No binding at 2 mM (10% PS/PC) ~0.02 (30% PS/PC)	−
hGIID	+++	+	POPG > POPS ≈ POPC	PG ~ PC ~ PE > PS > PA > PI	2.1	11 ± 3, POPG	Phosphonate-10b	0.12 (10% PS/PC) 100% bound at 0.14 mM (30% PS/PC)	−
hGIIE	+	+	POPG ≈ POPS ≈ POPC	PC > PA > PG > PE > PS > PI	0.7	112 ± 39, POPG	Me-Indoxam	No binding at 2 mM (10% PS/PC) No binding at 2 mM (30% PS/PC)	−
hGIIF	++	++	POPG ≫ POPS ≈ POPC	PG ~ PC > PE ~ PA > PS > PI	2.0	27 ± 14, POPG	MJ33 and MJ50	0.35 (10% PS/PC) 0.16 (30% PS/PC)	−
hGV	+++	++	POPG > POPS > POPC	PG ~ PC > PA > PS ~ PE > PI	0.3	1 ± 0.5, POPG 18 ± 4, POPS 225 ± 42, POPC	Me-Indoxam	0.05 (10% PS/PC) 90% bound at 0.1 mM (30% PS/PC)	++
hGX	+	++	POPC > POPG > POPS	PC > PG > PA > PE > PS > PI	2.3	1.5 ± 0.4, POPG 16 ± 4, POPS 44 ± 11, POPC	Me-Indoxam	0.13 (10% PS/PC) 0.036 (30% PS/PC)	+++
hGXII	++	+	POPG ≈ POPC > POPS	PC > PG ~ PA > PE > PS > PI	1.9	45 ± 13, POPG	Me-Indoxam	1.1 (10% PS/PC) 0.20 (30% PS/PC)	−
mGIB	++	+++	POPG > POPS ≫ POPC	PG ~ PC ~ PA > PS ~ PE > PI	1.3	8 ± 3, POPG	Phosphonate-12b	2.0 (10% PS/PC) 0.18 (30% PS/PC)	−
mGIIA	+++	+++	POPG > POPS ≫ POPC	PG > PC ~ PA > PE > PS > PI	0.4	4 ± 1, POPG	Me-Indoxam	No data (10% PS/PC) 100% bound at 0.1 mM (30% PS/PC)	−
mGIIC	+++	++	POPG > POPS > POPC	PG > PC ~ PA > PS > PE > PI	1.9	14 ± 4, POPG	Me-Indoxam	0.03 (10% PS/PC) 100% bound at 0.1 mM (30% PS/PC)	−
mGIID	+++	+	POPG ≈ POPS > POPC	PG ~ PC ~ PS > PE ~ PA > PI	0.4	46 ± 15, POPG	DDC-1 and Me-Indoxam	<10% bound at 2 mM (10% PS/PC) 0.04 (30% PS/PC)	−
mGIIE	+	++	POPG ≈ POPC > POPS	PC > PG > PA > PE > PS > PI	1.0	100 ± 40, POPG	LY311727 and Me-Indoxam	No binding at 2 mM (10% PS/PC) 3 (30% PS/PC)	−
mGIIF	++	++	POPG > POPS ≈ POPC	PG ~ PC > PS ~ PA > PE > PI	1.7	35 ± 10, POPG	Me-Indoxam	0.04 (10% PS/PC) ~0.02 (30% PS/PC)	−
mGV	+++	++	POPG > POPS > POPC	PC > PG ~ PA > PE ~ PS > PI	0.3	1 ± 0.5, POPG 200 ± 35, POPC	Me-Indoxam	~0.02 (10% PS/PC) ~0.01 (30% PS/PC)	++
mGX	++	++	POPG ≈ POPS > POPC	PC > PG ~ PS ~ PA > PE > PI	2.2	48 ± 15, POPG	Me-Indoxam	0.07 (10% PS/PC) ~0.02 (30% PS/PC)	+++

[a] Actual values are in Table II.
[b] Specific activity on the most preferred of the three pure phospholipid vesicles (POPG, POPS, POPC) is ranked as +++ (140–1,030 μmol/min/mg), ++ (4–24 μmol/min/mg), and + (0.1–0.9 μmol/min/mg). Actual values are given in Table III.
[c] Ranking of specific activities on pure phospholipid vesicles.
[d] Ranked according to relative $k_{cat}*/K_m*$ values for the hydrolysis of mixed phospholipid vesicles. Actual values are given in Fig. 2, *A* and *B*.
[e] $k_{cat}*/K_m*$ for the hydrolysis of [^{14}C]SAPC divided by $k_{cat}*/K_m*$ for the hydrolysis of [^{3}H]DPPC in POPG vesicles. Estimated errors are <20% based on duplicate or triplicate analyses.
[f] Values of $K_{Ca(app)}$ were determined from fitting the initial velocity for the hydrolysis of the indicated pure phospholipid vesicle as a function of the free calcium concentration to the hyperbolic equation (shown in Fig. 3 for two examples).
[g] Most potent of the 11 inhibitors shown in Fig. 3 for each sPLA$_2$ is listed. Actual potencies are shown in Table IV.
[h] Determined by the centrifugation method using sucrose-loaded vesicles (for example, see Fig. 5).
[i] For detailed values, see Fig. 6, *A–C*.

TABLE II
Remaining enzymatic activity of human and mouse sPLA$_2$s after treatment with 10 mM DTT

sPLA$_2$	Room temperature			50 °C		
	5 min	30 min	60 min	5 min	30 min	60 min
	%	%	%	%	%	%
hGIB	100[a]	100	100	73	0	ND[b]
hGIIA	36	0	ND	ND	ND	ND
hGIID	3	0	ND	ND	ND	ND
hGIIE	100	100	100	100	55	9
hGIIF	100	98	85	40	0	ND
hGV	31	0	ND	0	ND	ND
hGX	100	100	100	100	56	16
hGXII	84	63	46	23	0	ND
mGIB	100	100	100	61	0	ND
mGIIA	13	0	ND	ND	ND	ND
mGIIC	13	0	ND	ND	ND	ND
mGIID	23	0	ND	ND	ND	ND
mGIIE	100	100	100	100	28	0
mGIIF	100	100	100	40	13	0
mGV	46	10	0	ND	ND	ND
mGX	100	97	37	89	11	0

[a] Percent remaining enzymatic activity after exposure to DTT under the indicated conditions.
[b] Not determined.

TABLE III
Action of human and mouse sPLA$_2$ on phospholipid vesicles

Specific activities were derived either from the initial velocity, when no lag was observed, or from the velocity measured after the lag, when a lag was observed. Assays were carried out with 30 μM extruded vesicles in Hanks' balanced salt solution with 1.27 mM Ca^{2+} and 0.90 mM Mg^{2+} at 37 °C.

sPLA$_2$	Specific activity		
	On POPG	On POPS	On POPC
	μmol/(min × mg)	*μmol/(min × mg)*	*μmol/(min × mg)*
hGIB	1,030 ± 490, NL[a]	55 ± 30, NL	0.2 ± 0.07, NL
hGIIA	220 ± 90, NL	40 ± 18, NL	0.7 ± 0.2, L
hGIID	0.9 ± 0.2, NL	0.1 ± 0.03, NL	0.05 ± 0.02, NL
hGIID$_{ng}$[b]	0.7 ± 0.2, NL	No data[c]	No data
hGIIE	0.50 ± 0.2, NL	0.26 ± 0.05, NL	0.33 ± 0.1, L
hGIIF	18 ± 1, NL	0.04 ± 0.01, NL	0.03 ± 0.01, NL
hGIIFΔC	15 ± 1, NL	0.05 ± 0.01, NL	0.09 ± 0.02, NL
hGV	24 ± 5, NL	9 ± 3, NL	5 ± 1, NL
hGX	14 ± 0.8, NL	4 ± 2, NL	30 ± 2, NL
hGXII	0.32 ± 0.02, NL	0.022 ± 0.005, NL	0.23 ± 0.06, L
mGIB	720 ± 150, NL	270 ± 40, NL	0.4 ± 0.2, L
mGIIA	140 ± 60, NL	24 ± 7, NL	0.12 ± 0.03, L
mGIIC	12 ± 5, NL	2.5 ± .5, NL	0.1 ± 0.03, NL
mGIID	0.9 ± 0.2, NL	0.7 ± 0.1, NL	No activity with 3 μg
mGIIE	0.1 ± 0.04, NL	0.034 ± 0.013, NL	0.12 ± 0.05, L
mGIIF	4 ± 1.6, NL	0.35 ± 0.15, NL	0.4 ± 0.2, SL
mGV	40 ± 5, NL	9 ± 3, NL	5.6 ± 1.3, NL
mGX	30 ± 15, NL	20 ± 8, NL	7.3 ± 2, NL

[a] NL, L, and SL designate no lag, lag, and short lag, respectively, seen in the reaction progress curve.
[b] hGIID$_{ng}$ is the nonglycosylated fraction of hGIID produced in *Drosophila* S2 cells.
[c] No data means that a reliable K_d value could not be obtained because of loss of enzyme to the wall of the microfuge tube.

to zero, and the reaction accelerated giving way to a linear, steady-state reaction progress (Fig. 1). In these cases, the specific activities in Table III were derived from the post-lag reaction velocity. The lag was either long (lasting 3–8 min for hGIIA, hGIIE, hGXII, mGIB, mGIIA, and mGIIE), short (lasting <1 min for mGIIF), or not observed (hGIB, hGIID, hGIIF, hGIIFΔC, hGV, hGX, mGIIC, mGV, and mGX) (examples shown in Fig. 1).

POPG was the greatly preferred substrate for most of the sPLA$_2$s. The exceptions are that hGIIE displays similar, albeit low, specific activity on POPG, POPS, and POPC vesicles (mGIIE shows similar behavior but with a slight preference for POPG and POPC over POPS), mGIID and mGX show similar specific activity on POPG and POPS, hGX shows highest activity on POPC, and hGXII shows comparable activity on POPG and POPC. Despite these exceptions, POPG may be considered to be the mostly generally useful sPLA$_2$ substrate. In general, each human sPLA$_2$ behaved similarly to its mouse ortholog with regard to specific activity and lag behavior. The exceptions are that mGIB hydrolyzed POPC vesicles with a lag, whereas the reaction started immediately after the addition of hGIB, as well as slight differences in specificity of hGIID *versus* mGIID and of hGX *versus* mGX. It is also apparent that the absolute magnitude of the specific activity of the most preferred substrate for each enzyme vary dramatically among the various sPLA$_2$s with a maximum span of ~30,000-fold. The highest values are seen for hGIB and mGIB acting on POPG, and the lowest values are found for hGIID and mGIID, hGIIE and mGIIE, and hGXII (summarized in Table I). In general, these specific activities are a function of the amount of enzyme bound to the interface (interfacial specificity), and the interfacial turnover kinetic constants for the action of the membrane-bound sPLA$_2$ (k_{cat}*/K_m*) (catalytic site specificity) (22). These features were studied separately as described below.

The specific activities of glycosylated and nonglycosylated hGIID, both produced in *Drosophila* S2 cells, are similar to the value obtained for hGIID obtained from *in vitro* refolding of inclusion bodies produced in *E. coli* (data not shown). Because

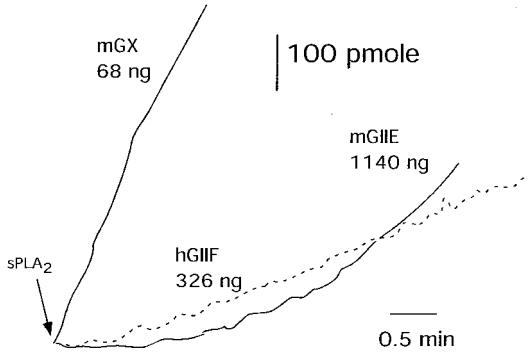

FIG. 1. **Reaction progress curves for the action of sPLA$_2$s on POPC vesicles.** The hydrolysis of POPC vesicles was measured using the fatty acid-binding protein assay. Negative fluorescence is plotted *versus* the reaction time (*vertical bar* designates 100 pmol of liberated oleic acid). The indicated amounts of the sPLA$_2$s were added at the *arrow*. Other conditions are detailed under "Experimental Procedures." Data for all enzymes are summarized in Tables I and III. At least three independent progress curves were obtained for each sPLA$_2$.

mGIID contains a putative *N*-glycosylation site (38) and because hGIID was found to be glycosylated when produced in S2 cells, we have also produced mGIID in S2 cells (not shown but prepared in a manner similar to that used for hGIID). We found that the mGIID protein produced in S2 cells is not glycosylated and is indistinguishable from the mGIID protein first produced in *E. coli* (38). Both proteins have identical molecular masses as checked by SDS-PAGE and MALDI-TOF mass spectrometry. Their specific activities differ by <5% when assayed with radiolabeled *E. coli* membranes (see "Experimental Procedures"). These results with hGIID and mGIID strongly argue that the low specific activity of these sPLA$_2$s is an intrinsic property of these proteins and is not caused by misfolding. Finally, it appears from the data in Table III that the C-terminal, 23-amino acid, extension of hGIIF is not required for its activity on POPG, POPS, and POPC vesicles *in vitro* because hGIIFΔC behaves similarly to hGIIF toward these vesicle substrates.

Catalytic Site Phospholipid Specificity—The catalytic site specificity for an interfacial enzyme is defined as the relative values of the specificity constant, $k_{cat}*/K_m*$, (constants with an asterisk are for interfacial enzyme action) for the hydrolysis of different substrates present in the vesicle to which the sPLA$_2$ is bound (22). This specificity is analogous to classical substrate specificity for the action of noninterfacial enzymes in the aqueous phase and is, to a first approximation, independent of the specificity for binding of sPLA$_2$ to the vesicle interface. Catalytic site specificity of the human and mouse sPLA$_2$s toward phospholipids with different polar headgroups was obtained by measuring the ratio of lysophospholipids formed from a vesicle containing a mixture of phospholipids. The product ratio was determined under conditions of <10% of the total phospholipid hydrolyzed so that the product ratios are approximately equal to the ratio of velocities for the action of enzyme on each phospholipid species. For these studies, each sPLA$_2$ was added to a solution of mixed phospholipid vesicles containing an equal mol ratio of POPA, POPC, POPE, POPG, PI, and POPS. After phospholipid extraction and desalting, the mol amount of each lysophospholipid species was determined with the aid of deuterated lysophospholipid internal standards using HPLC/electrospray ionization mass spectrometry as described under "Experimental Procedures."

Values of the specificity constant $k_{cat}*/K_m*$ relative to that for POPG are shown in Fig. 2, *A* and *B*, and a qualitative summary is provided in Table I. Mass spectrometry was used to quantify all LPX species except LPA; the latter was quantified

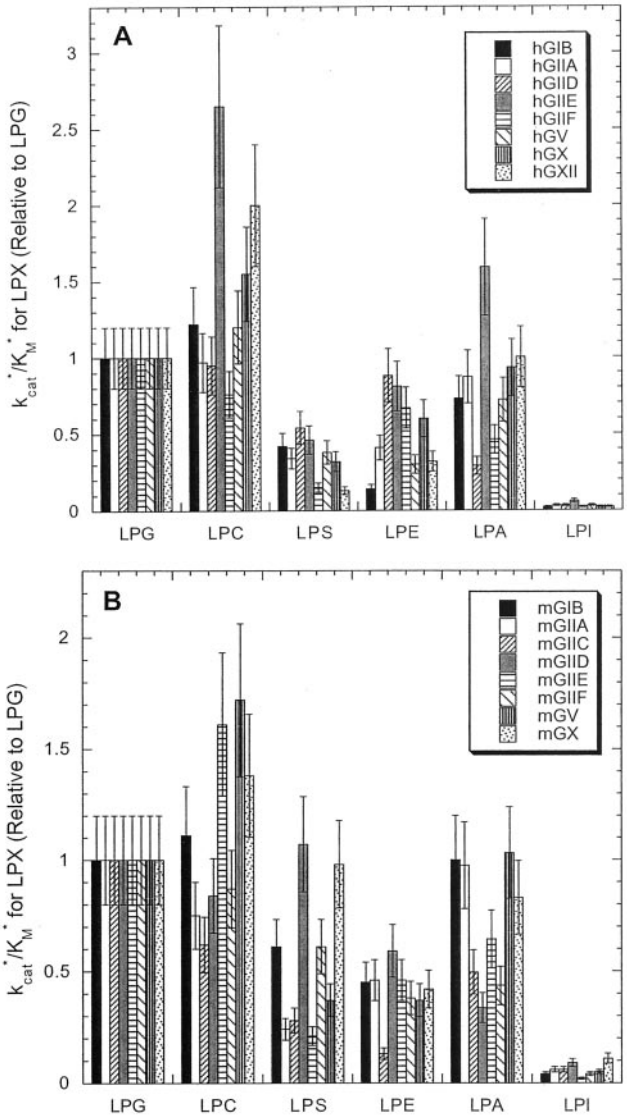

FIG. 2. **Phospholipid head group specificity of sPLA$_2$s.** *A*, human enzymes; *B*, mouse enzymes. All data are normalized to mol of LPG produced. The intensity in the minus sPLA$_2$ control was typically <3% of the signal seen in the plus sPLA$_2$ reaction for the most abundant LPX species. *Error bars* for the LPX species were in the range of 12–20% (each sPLA$_2$ was analyzed independently three or four times), and *error bars* of 20% are shown for all species.

by double channel scintillation counting with radiolabeled PA and PC (see "Experimental Procedures"). Remarkably, all human and mouse sPLA$_2$s display similar substrate preferences; modest differences can be seen in Fig. 2, *A* and *B*. POPI is generally the least preferred substrate; and for most of the enzymes, POPE, POPS, and POPA are modestly less preferred than POPG and POPC.

sn-2 Fatty Acyl Chain Preferences—We also determined the relative values of $k_{cat}*/K_m*$ for the hydrolysis of [^{14}C]SAPC *versus* [^{3}H]DPPC present as minor components in POPG vesicles using the same strategy as described above for phospholipid headgroup studies except that the ratio of arachidonate to palmitate released by sPLA$_2$ action was determined by dual channel scintillation counting. Results are listed in Table I. It is clear that none of the human and mouse sPLA$_2$s shows a significant preference for a polyunsaturated *versus* saturated *sn*-2 fatty acyl chain. hGX and mGX show the largest preference for the arachidonyl chain (2.2–2.3-fold), and mGIIA, mGIID, and hGV show the lowest preference (0.3–0.4-fold).

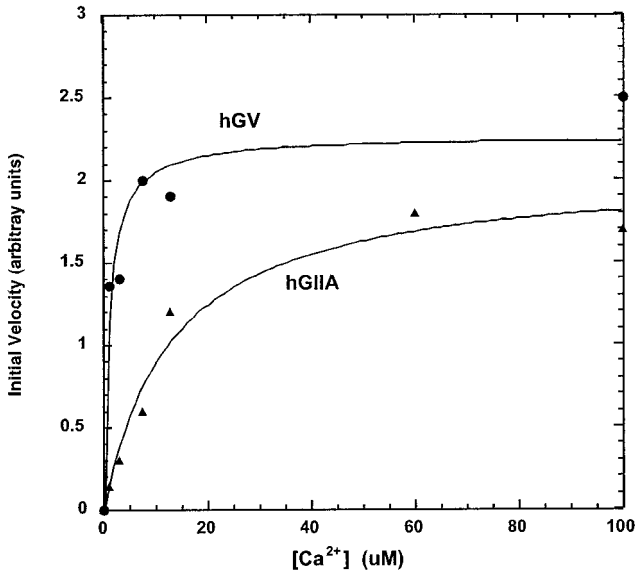

FIG. 3. **Calcium dependence of phospholipid vesicle hydrolysis.** Initial velocities (measured with the fatty acid-binding protein assay) for the hydrolysis of 30 μM POPG vesicles by 10 ng of hGIIA or 22 ng of hGV as a function of the free calcium concentration are shown. The *solid lines* are the regression fit to the standard hyperbolic binding equation. For additional information, see "Experimental Procedures." At least two independent calcium rate profiles were obtained for each sPLA$_2$.

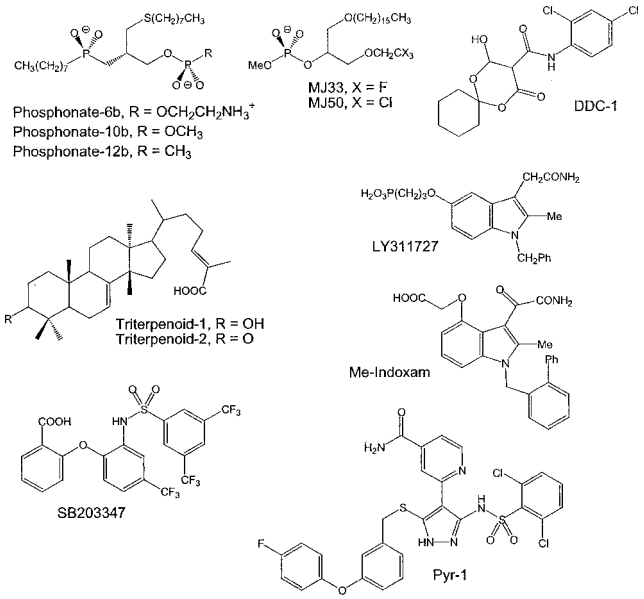

FIG. 4. **Structures of sPLA$_2$ inhibitors.**

Calcium Affinity—For all human and mouse sPLA$_2$s, the initial velocity for the hydrolysis of pure POPG vesicles was measured as a function of the free calcium concentration using the fatty acid-binding protein assay and calcium buffers appropriate for the range of needed calcium concentrations (see "Experimental Procedures"). In all cases, the initial velocity was undetectable in the absence of calcium and increased in a hyperbolic fashion with increasing calcium (examples shown in Fig. 3). The apparent values of the sPLA$_2$-Ca^{2+} equilibrium dissociation constant, $K_{Ca(app)}$, (see "Discussion") were obtained by fitting the velocity-calcium profile to the hyperbolic equation and are listed in Table I. Values of $K_{Ca(app)}$ vary significantly and are in the range of 1–225 μM. For a subset of those sPLA$_2$s that hydrolyze POPC and POPS vesicles without a lag, values of $K_{Ca(app)}$ for the hydrolysis of these vesicles are also listed and were found to be higher than the corresponding values for the hydrolysis of POPG vesicles.

Competitive Inhibitors—11 previously reported catalytic site-based sPLA$_2$ inhibitors (structures shown in Fig. 4) were tested on the human and mouse sPLA$_2$s. Estimated IC_{50} values, listed in Table IV, were obtained with at least four inhibitor concentrations that span 10–90% inhibition using a radiometric assay consisting of [^{3}H]DPPC present as a minor compound in POPG vesicles (see "Experimental Procedures"). Me-Indoxam was found to be the most generally potent inhibitor among the mouse and human sPLA$_2$s and was analyzed most extensively. Me-Indoxam inhibition assays were carried out with two assays, a fluorometric assay with 1-palmitoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphoglyerol and a radiometric assay using radiolabeled *E. coli* membranes (see "Experimental Procedures"). Anionic vesicles were used because it was anticipated that they support high affinity interfacial binding of most of the sPLA$_2$s. For each human and mouse sPLA$_2$, the most potent inhibitor of the 12 tested is listed in Table I. Dramatic differences are seen in the IC_{50} values for the different inhibitors tested against the human and mouse sPLA$_2$s. Among human sPLA$_2$s, phosphonate-10b is a selective inhibitor (>10-fold) of hGIID, and Pyr-1 is selective for hGIIA and hGIID (Table IV). Among mouse sPLA$_2$s, phosphonate-6b and -12b are selective for mGIB, and phosphonate-10b, MJ33, and MJ50 are selective for mGIIC (Table IV). LY311727 shows good selectivity for hGIIA, mGIIA, hGIIE, and mGIIE as does phosphonate-6b toward hGIB and mGIB. Me-Indoxam is related in structure to LY311727 (Fig. 4). Because Me-Indoxam is the most generally potent sPLA$_2$ inhibitor, we determined the IC_{50} values using two different assays (Table IV). Me-Indoxam is a potent inhibitor of hGIIA, mGIIA, mGIIC, hGIIE, mGIIE, hGV, and mGV, with IC_{50} values in the low nanomolar range. It displays intermediate potency against hGIB, mGIB, and mGX and is less potent against hGIID, mGIID, hGIIF, mGIIF, hGX, and hGXII. The different sPLA$_2$ assays used to analyze Me-Indoxam inhibition give somewhat different IC_{50} values, but the trends are similar (Table IV). This may be because of the possibility that the phospholipid substrates in the *E. coli* membranes and the 1-palmitoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol used for the fluorometric assay may have different interfacial K_m values.

Interfacial Binding of sPLA$_2$ to Phospholipid Vesicles—Binding of sPLA$_2$s to vesicles is often carried out by monitoring fluorescence energy transfer from one or more tryptophan residues on or near the putative membrane binding surface of the protein and a fluorescence acceptor such as *N*-dansylphosphatidylethanol present in the interface (61). However, not all of the human and mouse sPLA$_2$s have tryptophans, and we have found that the energy transfer intensity varies dramatically with the phospholipid composition of the interface.[2] Thus, we decided to adopt the procedure of Buser *et al.* (62) in which vesicles are loaded with sucrose so that they pellet in an ultracentrifuge. The amount of sPLA$_2$ enzymatic activity remaining in the supernatant (measured with a sensitive fluorometric assay) was measured as a function of the concentration of vesicles. This method is suitable for measuring relatively weak interfacial binding of sPLA$_2$s (for a complete discussion, see Ref. 23). Because we anticipated that most, if not all, of the human and mouse sPLA$_2$s would bind weakly to PC-rich vesicles and more tightly to PS vesicles (63), our studies were carried out with DO$_{et}$PC. Various amounts of DO$_{et}$PS were

[2] A. G. Singer, F. Ghomashchi, C. Le Calvez, J. Bollinger, S. Bezzine, M. Rouault, M. Sadilek, M. Lazdunski, G. Lambeau, and M. H. Gelb, unpublished observations.

TABLE IV
IC_{50} values (μM) for the inhibition of human and mouse sPLA$_2$

Estimated errors are ±50% for all IC_{50} values, based on duplicate or triplicate analysis of each sPLA$_2$/inhibitor pair. For each sPLA$_2$, the IC_{50} for the most potent inhibitor(s) is in boldface. The maximum inhibitor concentration tested is 20 μM.

	Phosphonate-6b	Phosphonate-10b	Phosphonate-12b	MJ33	MJ50	Triterpenoid-1	Triterpenoid-2	SB203347	LY311727	Pyr-1	DDC-1	Me-Indoxam[a]
hGIB	**0.06**	0.3	5	>20	>20	>20	>20	>20	>20	>20	>20	0.2/6
hGIIA	>20	0.9	0.4	>20	10	>20	>20	0.8	0.2	0.08	5	**0.03/0.006**
hGIID	0.1	**0.02**	0.6	10	5	No data	No data	0.6	10	0.04	5	7/20
hGIIE	20	>20	20	>20	>20	>20	>20	>20	0.1	1	>20	**0.008/0.01**
hGIIF	6	4	20	2	2	>20	>20	>20	>20	>20	>20	5/2
hGV	>20	6	>20	10	5	>20	>20	>20	>20	>20	>20	**0.025/0.006**
hGX	3.5	>20	10	15	10	>20	>20	2	10	20	>20	2/1
hGXII	20	20	20	20	>20	>20	>20	20	>20	>20	>20	**6/~30**
mGIB	0.20	10	**0.08**	10	5	20	5	>20	>20	>20	20	0.15/0.3
mGIIA	4.5	5	1.5	10	5	>20	≫20	2	0.08	>20	5	**0.015/0.0015**
mGIIC	2	0.6	0.6	0.5	0.5	No data	No data	10	>20	>20	5	**0.06/0.12**
mGIID	7	15	11	20	10	>20	>20	>20	>20	>20	5	**7/10**
mGIIE	>20	>20	20	>20	≫20	>20	>20	>20	0.04	9	>20	**0.02/0.015**
mGIIF	>20	20	>20	20	>20	>20	>20	20	>20	>20	>20	**7/10**
mGV	>20	>20	4	5	5	>20	>20	20	20	>20	>20	**0.025/0.005**
mGX	>20	10	10	20	>20	>20	>20	20	15	>20	>20	**0.5/0.3**

[a] The first number given is the IC_{50} measured with the 1-palmitoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol assay, and the second is for the *E. coli* membrane assay.
[b] No data means that a reliable K_d value could not be obtained because of loss of enzyme to the wall of the microfuge tube.

added to $DO_{et}PC$ vesicles to determine the sPLA$_2$ binding requirement on anionic phospholipid. We found that sucrose-loaded $DO_{et}PC$ vesicles containing <10 mol% $DO_{et}PS$ do not pellet, and thus all vesicles contained at least 10 mol % $DO_{et}PS$.

Fig. 5 shows typical interfacial binding curves (mGIIC, mGIID, and hGXII binding to 10% $DO_{et}PS/DO_{et}PC$ vesicles), and dissociation constants for all mouse and human sPLA$_2$s interacting with 10% $DO_{et}PS/DO_{et}PC$ and 30% $DO_{et}PS/DO_{et}PC$ vesicle are given in Table I. Some of the enzymes (hGIB, mGIB, hGIIA, mGIIA, and mGIID) bind weakly or not all to PC-rich vesicles (up to 2 mM phospholipid), and binding is enhanced dramatically when the amount of anionic PS is increased to 30 mol%. Other enzymes (mGIIC, hGIID, hGIIF, hGIB, mGIIF, hGV, mGV, hGX, and mGX) are able to bind to PC-rich vesicles (K_d <0.4 mM). For these latter sPLA$_2$s, binding is increased when the amount of PS is increased from 10 to 30 mol%, but the dependence on anionic $DO_{et}PS$ is less dramatic for hGIIF and mGIIF than for those sPLA$_2$s that bind weaker to PC-rich vesicles. hGXII shows an affinity for PC-rich vesicles which is intermediate between the groups of enzymes described above, and binding is increased by the addition of more $DO_{et}PS$. Interestingly, hGIIE and mGIIE bind weakly (K_d >2 mM) to vesicles regardless of the mol fraction of anionic phospholipid.

Release of Arachidonate from Mammalian Cells by Exogenous sPLA$_2$s—HEK293 and RBL-2H3 cells were labeled with [^{3}H]arachidonate, and unincorporated fatty acid was removed by washing with buffer containing bovine serum albumin. For arachidonate release studies, cells were left attached to the culture dish because we have shown that enzymatic and mechanical dislodgment of adherent cells from the growth surface renders them much more susceptible to hydrolysis by exogenously added sPLA$_2$s (37). Probably the sPLA$_2$s act more efficiently on damaged cell membranes that result from dislodgment.

Results for the action of human sPLA$_2$s on adherent HEK293 cells are shown in Fig. 6, *A* and *B*, and are summarized in Table I. The following sPLA$_2$s produce little or no arachidonic acid when added up to 1 μg/ml to HEK293 cells (hGIB, mGIB, hGIIA, mGIIA, hGIID, mGIID, hGIIE, mGIIE, hGIIF, hGIIFΔC, mGIIF, and hGXII). hGV and mGV show intermediate potency in this arachidonic acid release assay, and hGX and mGX are the most potent, comparable with fatty acid release by cobra venom sPLA$_2$. With RBL-2H3.1 cells only hGX, mGX, and cobra sPLA$_2$ gave statistically significant arachidonic acid release (Fig. 6*C*).

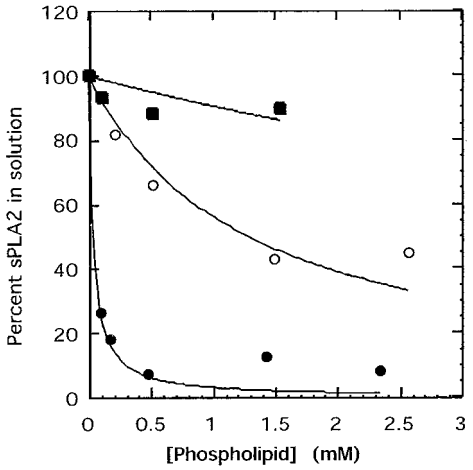

FIG. 5. **Interfacial binding of sPLA$_2$s to phospholipid vesicles.** Sucrose-loaded vesicles of 10% $DO_{et}PS$ in $DO_{et}PC$ were pelleted by ultracentrifugation, and the percent of sPLA$_2$ remaining in the supernatant was plotted as a function of the concentration of total phospholipid in the binding mixture for mGIIC (●), hGXII (○), and mGIID (■). Independent binding studies were carried out at least three times for each sPLA$_2$.

DISCUSSION

It is clear from the results in Table III that the specific activities of the set of groups I, II, V, X, and XII mouse and human sPLA$_2$s acting on pure vesicles of POPG, POPC, and POPS varies dramatically, up to 4 orders of magnitude. It is not apparent from the amino acid sequences of hGIID, mGIID, hGIIE, mGIIE, and hGXII why the turnover numbers for these enzymes are so low compared with typical values for sPLA$_2$s. These sPLA$_2$s have the Asp-His catalytic diad and a calcium binding loop. The latter is functional based on values of $K_{Ca(app)}$ in the range 11–112 μM for these five sPLA$_2$s. Overlaying their sequences onto the three-dimensional x-ray structures of other sPLA$_2$s does not reveal unusual features of the active site cavity which would be predicted to render the group IID and IIE enzymes catalytically inferior (no structural information is available for the structurally distinct hGXII). We also found for

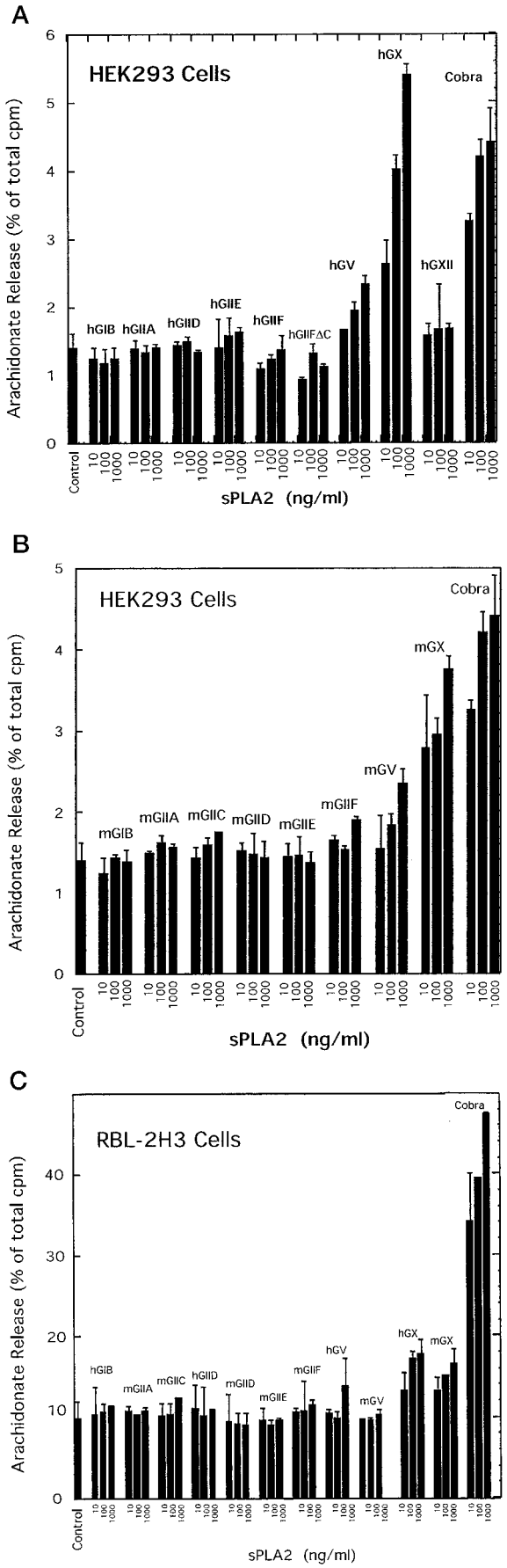

FIG. 6. **Arachidonate release from mammalian cells treated with exogenous sPLA$_2$s.** *A*, adherent HEK293 cells were treated with the indicated amounts of human sPLA$_2$ at 37 °C for 6 h. The data are expressed as the percent of total cpm (culture medium + cell-associated) released into the culture medium. *B*, same as for *A* but using mouse sPLA$_2$s. *C*, same as for *A* but using adherent RBL-2H3.1. *Error bars* show the S.D. values obtained from two or three independent experiments.

all of the low specific activity sPLA$_2$s that activity remains low on POPE and 50/50 POPE/POPC vesicles (not shown); thus these enzymes are not activated by binding to phosphatidylethanolamine-rich vesicles. One reason for the poor activity of mGIIE, hGIIE, and hGXII is their poor ability to bind to PC-rich vesicles and to those that contain 30 mol% PS. However, this cannot be the reason for the poor enzymatic activity of hGIID and mGIID, which bind well to PC/PS vesicles. Although we did not study mouse group XII, this sPLA$_2$ is also reported to have low phospholipase activity after refolding of inclusion body protein expressed in *E. coli* (64). Also, the enzymatic activity of group IID, IIE, and XII sPLA$_2$s in the culture media of mammalian cells transfected with the genes coding for these enzymes is barely detectable (38, 41, 43, 49, 64, 65). It may be noted that porcine and bovine pancreatic group IB sPLA$_2$s display true interfacial allosteric activation (k_{cat}* and K_m* type) upon binding to the vesicle interface (3), although this activation is not yet fully understood at the molecular level. It is possible that groups IID, IIE, and XII sPLA$_2$s do not show this type of interfacial activation, but this was not investigated. It is also possible that the true, physiological substrate for group IID, IIE, and XII enzymes is an atypical phospholipid that remains to be discovered or that these proteins require an auxiliary factor to activate them on membranes. These possibilities should be considered in light of the observations that forcible overexpression of mGIID and hGIIE in HEK293 cells leads to arachidonic acid release and prostaglandin production (17, 18) (however, see below). Finally, because of their low lipolytic activity, the possibility that these enzymes have physiological functions unrelated to their enzymatic activity should not be ruled out (20). Indeed, several snake venom sPLA$_2$s with little or no lipolytic activity have been described (66, 67). Interestingly, these enzymes are still myotoxic or cytotoxic or able to inhibit human immunodeficiency virus type 1 (68). Besides venoms, other catalytically inactive sPLA$_2$s such as otoconin-22 (69), otoconin-95 (7, 8), and a putative zebrafish group XII-like inactive sPLA$_2$ (41) have been found, but the functions of these are not yet identified.

It is generally stated that sPLA$_2$s require submillimolar to millimolar concentrations of calcium as a catalytic cofactor. However, the results of this study show that many of the mammalian sPLA$_2$s are fully activated even by low micromolar calcium concentrations. It is expected that the concentration of calcium required for maximal sPLA$_2$ activity will depend on the fraction of enzyme bound to the membrane and the affinity of the enzyme active site for the phospholipid substrate. This is because binding of a single phospholipid molecule to the active site requires not only that the enzyme be bound to the interface (enzyme in the aqueous phase cannot extract phospholipid from the membrane) but also that the enzyme contain calcium, as shown by direct binding studies (70) and inferred from x-ray structures of sPLA$_2$-phospholipid analog complexes (71, 72). In the present study, we have measured the dependence of the initial velocity for phospholipid vesicle hydrolysis on the concentration of free calcium, which necessarily gives the apparent constant $K_{Ca(app)}$. The synergism between phospholipid and calcium binding presumably explains why the value of $K_{Ca(app)}$ decreases for hGV and hGX as the substrate is changed from POPC, to POPS, to POPG because these sPLA$_2$s bind with increasing affinity to anionic *versus* zwitterionic phospholipid vesicles. Also, the fact that $K_{Ca(app)}$ for hGIIE, mGIIE, and

hGXII is relatively high for the action on POPG vesicles is probably related to the observation that these sPLA$_2$s bind weakly to vesicles even if they contain a high fraction of anionic phospholipid. The physiological significance of the full activation of sPLA$_2$s by micromolar calcium remains to be established, but one should not assume *a priori* that an sPLA$_2$ will be active only if the calcium concentration approaches the millimolar range.

It has been pointed out that the intrinsic specificity of the catalytic site of an interfacial enzyme is best measured in a competition experiment in which the enzyme is bound to a mixed phospholipid vesicle and chooses among the competing substrates according to their relative concentration and their relative specificity constants, $k_{cat}*/K_m*$, for the competing substrate (22). The data in Fig. 2*A* are consistent with earlier data on hGIIA obtained using pairs of differentially radiolabeled substrates (73), including the fact that hGIIA hydrolyzes PI poorly relative to PC (also seen for all of the human and mouse sPLA$_2$s). The fact that all sPLA$_2$s do not discriminate against POPA leaves open the possibility that one or more of these enzymes may be involved in generation of LPA, a mitogenic lipid mediator produced by platelets and probably other cells (74). The $k_{cat}*/K_m$ values reported previously for the action of hGIIA and hGV on PC, PE, PG, and PS vesicles (75) are for the hydrolysis of vesicles composed of a minor amount of pyrene-containing, nonpolymerized phospholipid in a polymerized phospholipid matrix (for example pyrene-PC in polymerized PC). These values are a function of the intrinsic specificity of the catalytic site of the vesicle-bound sPLA$_2$ for the various phospholipids and of the amount of enzyme bound to the vesicle, and thus they cannot be compared with the $k_{cat}*/K_m*$ shown in Fig. 2, *A* and *B*. Based on the present results it appears that the large apparent preference of hGV *versus* hGIIA for PC reported previously (75) is mostly the result of differential interfacial binding of these sPLA$_2$s to vesicles.

None of the mammalian sPLA$_2$s displays dramatic discrimination between *sn*-2 arachidonyl *versus sn*-2 palmitoyl chains. At the extreme, compared with hGV and mGV, hGX and mGX prefer the polyunsaturated fatty acyl chain over the saturated one by 6-fold because of a combination of the ~2-fold preference of the group X sPLA$_2$s for the arachidonyl chain and the ~3-fold preference of the group V sPLA$_2$s for the palmitoyl chain (Table I). The slight preference of the group X sPLA$_2$s for the arachidonyl chain is consistent with earlier studies (76, 77). We initially considered that preference for the arachidonyl chain by hGX *versus* hGV may be the reason that group X enzymes are superior to group V enzymes in their ability to release arachidonic acid when added exogenously to mammalian cells (Fig. 6, *A–C*) even though group V and X sPLA$_2$s both bind well to PC-rich membranes (see below). However, when HEK293 cells were radiolabeled with oleic acid, exogenously added hGX (10–1,000 ng/ml) gave detectable release (1–3% of total cellular oleate radiolabel), whereas 10–1,000 ng/ml hGV failed to release radiolabeled oleate into the medium after 3–6 h (not shown). In a previous study it was found that hGX is superior to rat group V sPLA$_2$ in its ability to release arachidonic acid from HEK293 and Swiss 3T3 cells (37). Cho and co-workers (78) reported that 1.5 μg/ml hGV releases 2% of the arachidonic acid radiolabel in 1 h when added exogenously to HEK293 cells; this result is consistent with the data in Fig. 6*A* showing 1% release from HEK293 cells after a 6-h treatment with 1 μg/ml hGV.

Upon examination of the data for all of the mouse and human sPLA$_2$s it is apparent that those enzymes that bind well to PC-rich vesicles (10% DO$_{et}$PS/DO$_{et}$PC) hydrolyze POPC vesicles immediately upon addition of enzyme to the assay (no lag). Those enzymes that hydrolyze POPC vesicles only after a lag phase bind relatively weakly to PC-rich vesicles (compare data in Tables I and III). This is consistent with detailed studies of porcine and bovine pancreatic sPLA$_2$s showing that the accumulation of reaction products in PC vesicles promotes interfacial binding and catalytic activity (3). We find that hGIIA does not bind to PC-rich vesicles (10% DO$_{et}$PS/DO$_{et}$PC) even with 2 mM phospholipid present, and we cannot confirm the previous result of K_d ~0.1 μM for hGIIA dissociating from dihexadecyl PC vesicles in the presence and absence of calcium (measured by surface plasmon resonance) (79).

Tryptophan on the membrane binding surface of hGV is important for the high catalytic activity of this sPLA$_2$ on PC-rich membranes (81), and addition of a tryptophan to the membrane binding surface of hGIIA renders this enzyme about 2 orders of magnitude more active on PC-rich membranes (80). We have shown recently that the presence of a tryptophan on the membrane binding surface of hGX and its absence on that of hGIIA is more important than the presence or absence of cationic residues (lysine and arginine) on the membrane binding surface of these sPLA$_2$s for allowing high affinity binding to PC-rich membranes (23). Furthermore, addition of a tryptophan to hGIIA reduces the lag phase observed during the hydrolysis of PC vesicles and allows this enzyme to liberate arachidonic acid better when added to mammalian cells (23). Based on overlaying the sequences of the mouse and human sPLA$_2$s onto the known three-dimensional x-ray structure of hGIIA, the following enzymes are predicted to contain one or more tryptophans on their membrane binding surface: hGIB, hGIID, hGV, mGIB, mGIIC, mGIID, mGV, mGX (hGXII has a distinct primary structure making it difficult to locate the putative membrane binding surface). The recently determined structure of hGX shows a single tryptophan on the putative membrane binding surface (40). With the exception of mGIB, all of these sPLA$_2$s hydrolyze PC vesicles without a discernible lag. All of the sPLA$_2$s that do not contain a membrane binding surface tryptophan, hGIIA, hGIIE, hGIIF, mGIIA, mGIIE, and mGIIF, hydrolyze PC vesicles only after a lag phase. There is no correlation between the predicted number of lysine and arginine residues on the membrane binding surface of the mouse and human sPLA$_2$s and the occurrence of the lag.

There is a dramatic correlation between the ability of the sPLA$_2$s to hydrolyze PC-rich vesicles and the ability of these enzymes to liberate arachidonic acid when added exogenously to mammalian cells. hGIB, mGIB, hGIIA, mGIIA, mGIIC, hGIID, mGIID, hGIIE, mGIIE, hGIIF, mGIIF, and hGXII all fail to liberate arachidonic acid from cells when added up to 1 μg/ml, and all display specific activities on POPC vesicles less than 0.7 μmol/(min $\times$ mg). hGV, mGV, hGX, and mGX are by far the most potent among the human and mouse sPLA$_2$s to liberate arachidonic acid from cells, and these enzymes display the highest specific activity on POPC. Furthermore, hGV, mGV, hGX, and mGX bind well to PC-rich membranes (Table I), a prelude for high catalytic turnover on these vesicles. The more efficient release of arachidonic acid by hGX *versus* hGV from HEK293 and RBL-2H3 cells (Fig. 6, *A* and *C*) is consistent with the ~6-fold higher specific activity of the former enzyme *versus* the latter on POPC vesicles (Table III). On the other hand, mGIIC, hGIID, hGIIF, and mGIIF also bind relatively well to PC-rich vesicles, but the specific activity of these enzymes for the hydrolysis of POPC vesicles is at least 10-fold less than that for hGV (at least 60-fold less than that for hGX). As noted above, mutagenesis studies have shown that tryptophan on the membrane binding surface of hGV and hGX is a key residue for supporting high affinity binding to PC-rich membranes (23, 81). The activity of exogenous hGX on mammalian

cells approaches that of cobra venom sPLA$_2$, an enzyme known for many years to display high activity on PC-rich vesicles and on mammalian cells. Altogether, the results combined with our recent studies of wild type and mutant hGIIA and hGX (23) form a strong argument that the ability to bind to and hydrolyze PC-rich vesicles is required for efficient action of exogenously added sPLA$_2$ on mammalian cells. This seems reasonable given that the extracellular face of the mammalian cell plasma membrane is highly rich in zwitterionic phospholipids (PC and sphingomyelin), whereas most of the PS and other anionic phospholipids are on the inner leaflet of the membrane.

With the exception of hGX and mGX, there is a poor correlation between the ability of the various sPLA$_2$s to liberate arachidonate when added exogenously to mammalian cells (Fig. 6) and the ability of these enzymes to liberate this polyunsaturated fatty acid when forcibly overexpressed in mammalian cells. For example, HEK293 cells transfected with hGIIA liberate arachidonate (82) even though these cells are completely resistant to even high concentrations of this sPLA$_2$ (Fig. 6). Exogenous hGV is inferior to hGX in liberating arachidonate when added exogenously to RBL-2H3 cells (Fig. 6), yet efficient arachidonate liberation is seen when these cells are transfected with hGV and hGX cDNA (83). The amount of arachidonate produced by HEK293 cells forcibly overexpressing hGIIF (19) is more than would be expect from the data with exogenously added enzyme (Fig. 6). These results suggest that those sPLA$_2$s that liberate arachidonic acid when overexpressed in mammalian cells but fail to do so when added exogenously to these cells are probably acting on a cellular membrane other than the extracellular face of the plasma membrane. Further work is needed to understand the site of action of sPLA$_2$s expressed in mammalian cells.

Although the catalytic sites of sPLA$_2$s are structurally related, underscored by the data in Fig. 2, *A* and *B*, and share a number of common amino acid residues (54), the data in Table IV shows that it is possible to find compounds that selectively inhibit a subset of the mammalian sPLA$_2$s. It should be noted that inhibition values in Table IV are apparent values in that the observed percent inhibition is a function of not only the affinity of the inhibitor for the catalytic site of the sPLA$_2$ but also of the mol fraction of inhibitor in the substrate vesicle. The latter depends on the fraction of inhibitor that partitions into the substrate vesicles, and this fraction is expected to be different for each inhibitor. However, for any single inhibitor, the relative inhibitor potency toward the set of sPLA$_2$s reflects the relative affinity of this inhibitor *versus* competing substrate for the active site of the vesicle-bound sPLA$_2$. Among the compounds tested, Pyr-1 is highly selective for hGIIA and hGIIE. On the other hand, it will require significant lead compound discovery and optimization to find highly potent and selective inhibitors for each member of the mammalian sPLA$_2$ family. LY311727, SB203347, MJ33, and Me-Indoxam-related compounds have been used in several studies, often at high concentration (>10 μM). The data in Table IV showing that these compounds cross-react with more than one member of the human or mouse sPLA$_2$ family raise concern about previous studies in which a physiological or biochemical process was attributed to a single sPLA$_2$ molecular species. LY311727 fails to inhibit hGV at concentrations up to 20 μM (Table IV), which is consistent with earlier studies using different sPLA$_2$ enzymatic assays (31, 82). We have no explanation for the discrepancy of these values with the IC$_{50}$ of 36 nM reported for inhibition of hGV by LY311727 (60) (such a large variation probably cannot be explained by the different assays used).

In summary, the interfacial kinetic and binding data for the full set of human and mouse groups I, II, V, X, and XII sPLA$_2$s show that these enzymes have dramatically different abilities to bind to phospholipid vesicles and to hydrolyze phospholipids in these vesicles. Other dramatic variations were observed for calcium activation and inhibitor potency. These variations and the fact that the tissue distribution of the mammalian sPLA$_2$s are distinct argue strongly that these enzymes are not isoforms and that they are expected to have functions other than the release of lipid mediators such as arachidonic acid for the biosynthesis of the eicosanoids (20).

Acknowledgments—We thank Pierre Escoubas and Sabine Scarzello for MALDI-TOF analysis of several sPLA$_2$s and Eric Nguyen for analysis of sPLA$_2$ inhibitors.

REFERENCES

1. Verheij, H. M., Slotboom, A. J., and De Haas, G. H. (1981) *Rev. Physiol. Biochem. Pharmacol.* **91,** 91–203
2. Waite, M. (1987) *The Phospholipases*, Plenum Publishing Corp., New York
3. Berg, O. G., Gelb, M. H., Tsai, M.-D., and Jain, M. K. (2001) *Chem. Rev.* **101,** 2613–2654
4. Berg, O. G., and Jain, M. K. (2002) *Interfacial Enzyme Kinetics*, John Wiley and Sons, West Sussex, UK
5. Valentin, E., and Lambeau, G. (2000) *Biochim. Biophys. Acta* **1488,** 59–70
6. Six, D. A., and Dennis, E. A. (2000) *Biochim. Biophys. Acta* **1488,** 1–19
7. Verpy, E., Leibovici, M., and Petit, C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 529–534
8. Wang, Y., Kowalski, P. E., Thalmann, I., Ornitz, D. M., Mager, D. L., and Thalmann, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15345–15350
9. Richmond, B. L., Boileau, A. C., Zheng, S., Huggins, K. W., Granholm, N. A., Tso, P., and Hui, D. Y. (2001) *Gastroenterology* **120,** 1193–1202
10. Vadas, P., Browning, J., Edelson, J., and Pruzanski, W. (1993) *J. Lipid Mediat.* **8,** 1–30
11. Qu, X. D., and Lehrer, R. I. (1998) *Infect. Immun.* **66,** 2791–2797
12. Madsen, L. M., Inada, M., and Weiss, J. (1996) *Infect. Immun.* **64,** 2425–2430
13. Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K., and Kudo, I. (1997) *Crit. Rev. Immunol.* **17,** 225–283
14. Balsinde, J., and Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 6758–6765
15. Mao-Qiang, M., Jain, M., Feingold, K. R., and Elias, P. M. (1996) *J. Invest. Dermatol.* **106,** 57–63
16. Schadow, A., Scholz-Pedretti, K., Lambeau, G., Gelb, M. H., Furstenberger, G., Pfeilschifter, J., and Kaszkin, M. (2001) *J. Invest. Dermatol.* **116,** 31–39
17. 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
18. Murakami, M., Yoshihara, K., Shimbara, S., Lambeau, G., Singer, A., Gelb, M. H., Sawada, M., Inagaki, N., Nagai, H., and Kudo, I. (2002) *Biochem. Biophys. Res. Commun.* **292,** 689–696
19. Murakami, M., Yoshihara, K., Shimbara, S., Lambeau, G., Gelb, M. H., Singer, A. G., Sawada, M., Inagaki, N., Nagai, H., Ishihara, M., Ishikawa, Y., Ishii, T., and Kudo, I. (2002) *J. Biol. Chem.* **277,** 19145–19155
20. Lambeau, G., and Lazdunski, M. (1999) *Trends Pharmacol. Sci.* **20,** 162–170
21. Hanasaki, K., and Arita, H. (1999) *Arch. Biochem. Biophys.* **372,** 215–223
22. Ghomashchi, F., Yu, B.-Z., Berg, O., Jain, M. K., and Gelb, M. H. (1991) *Biochemistry* **30,** 7318–7329
23. Bezzine, S., Bollinger, J. G., Singer, A. G., Veatch, S. L., Keller, S. L., and Gelb, M. H. (September 19, 2002) *J. Biol. Chem.* 10.1074/jbc.M2203137200
24. Elsbach, P., Weiss, J., Wright, G., Forst, S., van den Bergh, C. J., and Verheij, H. M. (1990) *Prog. Clin. Biol. Res.* **349,** 1–9
25. Koduri, R. S., Grönroos, J. O., Laine, V. J., Le Calvez, C., Lambeau, G., Nevalainen, T. L., and Gelb, M. H. (2002) *J. Biol. Chem.* **277,** 5849–5857
26. Beers, S. A., Buckland, A. G., Koduri, R. S., Cho, W., Gelb, M. H., and Wilton, D. C. (2002) *J. Biol. Chem.* **277,** 1788–1793
27. Gelb, M. H., and Kudo, I. (2000) *Tanpakushitsu Kakusan Koso* **45,** 1065–1071
28. Mihelich, E. D., and Schevitz, R. W. (1999) *Biochim. Biophys. Acta* **1441,** 223–228
29. Balsinde, J., Balboa, M. A., Insel, P. A., and Dennis, E. A. (1999) *Annu. Rev. Pharmacol. Toxicol.* **39,** 175–189
30. Bryant, M. D., Flick, K. E., Koduri, R. S., Wilton, D. C., Stoddard, B. L., and Gelb, M. H. (1999) *Bioorg. Med. Chem. Lett.* **9,** 1097–1102
31. Degousee, N., Ghomashchi, F., Stefanski, E., Singer, A. G., Smart, B. P., Borregaard, N., Reithmeier, R., Lindsay, T. F., Lichtenberger, C., Reinisch, W., Lambeau, G., Arm, J., Tischfield, J. T., Gelb, M. H., and Rubin, B. B. (2002) *J. Biol. Chem.* **277,** 5061–5073
32. 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., Mihelich, E. D., Olkowski, J. L., Snyder, D. W., and Sommers, J.-P. (1995) *Nat. Struct. Biol.* **2,** 458–465
33. Jain, M. K., Tao, W., Rogers, J., Arenson, C., Eibl, H., and Yu, B.-Z. (1991) *Biochemistry* **30,** 10256–10268
34. Jain, M. K., Yu, B. Z., Rogers, J. M., Smith, A. E., Boger, E. T., Ostrander, R. L., and Rheingold, A. L. (1995) *Phytochemistry* **39,** 537–547
35. Marshall, L. A., Hall, R. H., Winkler, J. D., Badger, A., Bolognese, B., Roshak, A., Flamberg, P. L., Sung, C. M., Chabot-Fletcher, M., Adams, J. L., and Mayer, R. J. (1995) *J. Pharmacol. Exp. Ther.* **274,** 1254–1262
36. Lin, H.-K., and Gelb, M. H. (1993) *J. Am. Chem. Soc.* **115,** 3932–3942
37. 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
38. Valentin, E., Koduri, R. S., Scimeca, J.-C., Carle, G., Gelb, M. H., Lazdunski,

M., and Lambeau, G. (1999) *J. Biol. Chem.* **274,** 19152–19160
39. Snitko, Y., Han, S. K., Lee, B. I., and Cho, W. (1999) *Biochemistry* **38,** 7803–7810
40. Pan, Y. H., Yu, B.-Z., Singer, A. G., Ghomashchi, F., Lambeau, G., Gelb, M. H., Jain, M. K., and Bahnson, B. (2002) *J. Biol. Chem.* **277,** 29086–29093
41. Gelb, M. H., Valentin, E., Ghomashchi, F., Lazdunski, M., and Lambeau, G. (2000) *J. Biol. Chem.* **275,** 39823–39826
42. Valentin, E., Singer, A. G., Ghomashchi, F., Lazdunski, M., Gelb, M. H., and Lambeau, G. (2000) *Biochem. Biophys. Res. Commun.* **279,** 223–228
43. Valentin, E., Ghomashchi, F., Gelb, M. H., Lazdunski, M., and Lambeau, G. (1999) *J. Biol. Chem.* **274,** 31195–31202
44. Dudler, T., Chen, W.-Q., Wang, S., Schneider, T., Annand, R. R., Dempcy, R. O., Crameri, R., Gmachl, M., Suter, M., and Gelb, M. H. (1992) *Biochim. Biophys. Acta* **1165,** 201–210
45. Cupillard, L., Mulherkar, R., Gomez, N., Kadam, S., Valentin, E., Lazdunski, M., and Lambeau, G. (1999) *J. Biol. Chem.* **274,** 7043–7051
46. Hollander, T., Aeed, P. A., and Elhammer, A. P. (1993) *Carbohydr. Res.* **247,** 291–297
47. Hassani, O., Loew, D., Van Dorsselaer, A., Papandreou, M. J., Sorokine, O., Rochat, H., Sampieri, F., and Mansuelle, P. (1999) *FEBS Lett.* **443,** 175–180
48. Kubelka, V., Altmann, F., Staudacher, E., Tretter, V., Marz, L., Hard, K., Kamerling, J. P., and Vliegenthart, J. F. (1993) *Eur. J. Biochem.* **213,** 1193–1204
49. Ishizaki, J., Suzuki, N., Higashino, K.-C., Yokota, Y., Ono, T., Kawamoto, K., Fujii, N., Arita, H., and Hanasaki, K. (1999) *J. Biol. Chem.* **274,** 24973–24979
50. Jain, M. K., and Gelb, M. H. (1991) *Methods Enzymol.* **197,** 112–125
51. Ghomashchi, F., Schuttel, S., Jain, M. K., and Gelb, M. H. (1992) *Biochemistry* **31,** 3814–3824
52. Hixon, M. S., Ball, A., and Gelb, M. H. (1998) *Biochemistry* **37,** 8516–8526
53. Ancian, P., Lambeau, G., and Lazdunski, M. (1995) *Biochemistry* **34,** 13146–13151
54. Scott, D. L., and Sigler, P. B. (1994) *Adv. Protein Chem.* **45,** 53–88
55. de Geus, P., van den Bergh, C. J., Kuipers, O., Verheij, H. M., Hoekstra, W. P. M., and De Haas, G. H. (1987) *Nucleic Acids Res.* **15,** 3743–3759
56. Noel, J. P., and Tsai, M.-D. (1989) *J. Cell. Biochem.* **40,** 309–320
57. Kelley, M. J., Crowl, R. M., and Dennis, E. A. (1992) *Biochim. Biophys. Acta* **1118,** 107–115
58. Han, S. K., Yoon, E. T., and Cho, W. (1998) *Biochem. J.* **331,** 353–357
59. Vuillard, L., Marret, N., and Rabilloud, T. (1995) *Electrophoresis* **16,** 295–297
60. Chen, Y., and Dennis, E. A. (1998) *Biochim. Biophys. Acta* **1394,** 57–64
61. Jain, M. K., and Vaz, W. L. C. (1987) *Biochim. Biophys. Acta* **905,** 1–8
62. Buser, C. A., Sigal, C. T., Resh, M. D., and McLaughlin, S. (1994) *Biochemistry* **33,** 13093–13101
63. Jain, M. K., Ranadive, G., Yu, B.-Z., and Verheij, H. M. (1991) *Biochemistry* **30,** 7330–7340
64. Ho, I.-C., Arm, J. P., Bingham, C. O., Choi, A., Austen, K. F., and Glimcher, L. H. (2001) *J. Biol. Chem.* **276,** 18321–18326
65. 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
66. Gutierrez, J. M., and Lomonte, B. (1995) *Toxicon* **33,** 1405–1424
67. Ownby, C. L., Selistre de Araujo, H. S., White, S. P., and Fletcher, J. E. (1999) *Toxicon* **37,** 411–445
68. Fenard, D., Lambeau, G., Valentin, E., Lefebvre, J., Lazdunski, M., and Doglio, A. (1999) *J. Clin. Invest.* **104,** 611–618
69. Pote, K. G., Hauer, C. R. I., Michel, H., Shabanowitz, J., Hunt, D. F., and Kretsinger, R. H. (1993) *Biochemistry* **32,** 5017–5024
70. Yu, B.-Z., Berg, O. G., and Jain, M. K. (1993) *Biochemistry* **32,** 6485–6492
71. Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., and Sigler, P. B. (1990) *Science* **250,** 1541–1546
72. Thunnissen, M. M. G. M., Ab, E., Kalk, K. H., Drenth, J., Dijkstra, B. W., Kuipers, O. P., Dijkman, R., De Haas, G. H., and Verheij, H. M. (1990) *Nature* **347,** 689–691
73. Bayburt, T., Yu, B.-Z., Lin, H.-K., Browning, J., Jain, M. K., and Gelb, M. H. (1993) *Biochemistry* **32,** 573–582
74. Sano, T., Baker, D., Virag, T., Wada, T., Yatomi, Y., Kobayashi, T., Igarashi, Y., and Tigyi, G. (2002) *J. Biol. Chem.* **277,** 21197–21206
75. Kim, K. P., Han, S. K., Hong, M., and Cho, W. (2000) *Biochem. J.* **348,** 643–647
76. Cupillard, L., Koumanov, K., Matt'ei, M. G., Lazdunski, M., and Lambeau, G. (1997) *J. Biol. Chem.* **272,** 15745–15752
77. 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
78. Kim, Y. J., Kim, K. P., Rhee, H. J., Das, S., Rafter, J. D., Oh, Y. S., and Cho, W. (2002) *J. Biol. Chem.* **277,** 9358–9365
79. Stahelin, R. V., and Cho, W. (2001) *Biochemistry* **40,** 4672–4678
80. Baker, S. F., Othman, R., and Wilton, D. C. (1998) *Biochemistry* **37,** 13203–13211
81. Han, S. K., Kim, K. P., Koduri, R., Bittova, L., Munoz, N. M., Leff, A. R., Wilton, D. C., Gelb, M. H., and Cho, W. (1999) *J. Biol. Chem.* **274,** 11881–11888
82. Murakami, M., Shimbara, S., Kambe, T., Kuwata, H., Winstead, M. V., Tischfield, J. A., and Kudo, I. (1998) *J. Biol. Chem.* **273,** 14411–14423
83. Enomoto, A., Murakami, M., Valentin, E., Lambeau, G., Gelb, M. H., and Kudo, I. (2000) *J. Immunol.* **165,** 4007–4014