Interfacial Kinetic and Binding Properties of the Complete Set of Human and Mouse Groups I, II, V, X, and XII Secreted Phospholipases A2*

Expression of the full set of human and mouse groups I, II, V, X, and XII secreted phospholipases A2 (sPLA2s) in Escherichia coli and insect cells has provided pure recombinant enzymes for detailed comparative interfacial kinetic and binding studies. The set of mammalian sPLA2s display dramatically different sensitivity to di-comitent enzymes for detailed comparative interfacial interfacial interfacial 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 sPLA2s, with Me-Indoxam being the most generally potent sPLA2 inhibitor. A dramatic correlation exists between the ability of the sPLA2s to hydrolyze phosphatidycholine-rich vesicles efficiently in vitro and the ability to release arachidonic acid when added exogenously to mammalian cells; the group V and X sPLA2s are uniquely efficient in this regard.

The mammalian family of phospholipases A2 includes the secreted forms (sPLA2s), which are 14–19-kDa (with some exceptions), Ca2+-dependent, disulfide-rich enzymes that catalyze the hydrolysis of phospholipids at the sn-2 position to release fatty acids and lysophospholipids (1–4). 10 sPLA2s 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 sPLA2s are probably also involved in phospholipid degradation in the gastrointestinal tract (9). The first nonpancreatic mammalian sPLA2 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 sPLA2s 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
keratinocyte sPLA₂ 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 sPLA₂s 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 can require a variety of proteins that bind sPLA₂s tightly. Two types of sPLA₂ receptor (M- and N-type), the cell surface proteoglycan glypican, and soluble sPLA₂-binding proteins have been identified and are likely to play a role in the physiological functions of mammalian sPLA₂s and in the toxicity of a wide variety of myotoxic and neurotoxic sPLA₂s found in reptile and invertebrate venoms (17, 20, 21).

sPLA₂s 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 sPLA₂s 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 sPLA₂s 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 sPLA₂s has important physiological consequences. For example, human group IIA sPLA₂ 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 sPLA₂, 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 S PLA₂ 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 sPLA₂ 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 sPLA₂s (mGIB, hGIB, mGIIA, hGIIB, mGIIC, hGIIC, mGIID, hGIID, mGIIE, hGIIE, mGIIF, hGIIF, mGV, hGV, mGX, hGX, and hGXII). The group III sPLA₂ gene predicts a protein consisting of a central sPLA₂ domain flanked by large N- and C-terminal extensions. This sPLA₂ was not included in the present study 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 sPLA₂s measured with a common set of conditions. The interfacial binding affinities of the mammalian sPLA₂s 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 sPLA₂ 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 sPLA₂s, 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 sPLA₂s 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-linoleoylphosphatidyl ethanol. sPLA₂ 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 Beecham Pharmaceuticals) (35); phosphonate-6b, -10b, and -12b (36); Ppy-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, mGIIB, mGIIE, hGIIF, and mGX by expression in *Drosophila* S2 cells will be reported elsewhere. Cobra venom sPLA₂ was purified from *Naja naja venom* (37).

**Preparation of Recombinant Human sPLA₂s**—Except as noted below, sPLA₂s were produced by *in vitro* refolding of inclusion body protein produced by expression in *E. coli*. All sPLA₂s were analyzed by mass spectrometry using electrospray ionization on a Bruker Autoflex–Flexi Mass–TOF–MS (Fig. S1), LC Ion Trap machine. For some sPLA₂s, 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, sPLA₂s were purified on a C18 ZipTip (Millipore) to remove traces of salts prior to electrospray mass spectrometry. The tip was rinsed twice with 50% CH₃CN and 0.1% trifluoroacetic acid, then three times with water, 0.1% trifluoroacetic acid. sPLA₂ solution (10 μl) was loaded onto the tip, which was washed twice with water and 0.1% trifluoroacetic acid and then with 80% CH₃CN to elute the protein.

hGIIE 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, plasmid encoding the first 8.4 kDa of glutathione S-transferase followed by a factor Xa protease recognition site fused to the N terminal of mature hGIB and hGIIF (i.e., without signal peptides or preproteins) (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 hGXG (37) and used to construct a pAB, plasmid as for hGIB (sequence available from the authors upon request). For hGIIFC, a PCR fragment coding for a factor Xa cleavage site (Ile-Glu-Gly-Arg) followed by the hGIIFC mature protein ending with the peptide sequence PTPNC (42) was amplified with *Pco* DNA polymerase and subcloned in-frame in pAB (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 Ndel site present in the pET21a expression plasmid (Novagen Inc.).

hGIB, hGIE, hGIIF, hGIIFC, 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 sPLA₂ are described below. Enzymatic activity was followed using a fluorometric assay with 1-palmitoyl-2-pyrene-decanoyl-sn-glycero-3-phosphethanolamin (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 then passed through 6.5 liters of prechilled refolding buffer (0.9 mM 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 buffer (400 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM CaCl₂). The buffer was changed twice for a total of 20 liters. The contents of the dialysis bag were centrifuged at 8,000 × 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/μl of hGIIF 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 mmln, 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. hGIIF 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 hGIIF is ~1.7 mg/lt 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 sPLA2 activity was detected during 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 (0.025 μg of fusion protein) to the supernatant. The 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 hGIE 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 16,583.4, and the calculated weight is 16,583.7.

hGIIFAC was expressed in E. coli as follows. A PCR fragment coding for a factor Xa cleavage site (Ile-Glu-Gly-Arg) followed by the hGIIFAC mature protein ending with the peptide sequence PPFPN (43) was amplified with Pwo DNA polymerase and subcloned in-frame in the pBAD3 vector. Bacterial induction, preparation of inclusion bodies, sulfonation of the fusion protein, and dialysis against 1% acetic acid were performed as described (38). Sulfonated hGIIFAC 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 20% acetonitrile, 0.1% trifluoroacetic acid, and then loaded on a Waters Symmetry Shield C8 column (4.6 × 100 mm, C18 reverse phase) that had been equilibrated previously with 10% solvent B in solvent A (same solvents as for hGIIF). The sample was centrifuged at 4 mg/liter of bacterial culture. The fusion protein was purified by the increase in sPLA2 activity using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (20–100%). Fractions containing hGIIFAC were pooled, dialyzed, and loaded on a Waters Symmetry Shield C8 column (4.6 × 250, 4.6 ml, 100 A, 5 μm) equilibrated with the same buffer. hGIIFAC protein was eluted with a shallow gradient of acetonitrile in 0.1% trifluoroacetic acid (25–35% over 120 min). The hGIIFAC 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 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 mM guanidine-HCl, 10 mM CaCl2, 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 sample solution was then kept at room temperature or overnight at 4 °C. The solution 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 mM guanidine-HCl, 10 mM CaCl2, 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. 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, 10 mM sodium chloride, 50 mM l-cysteine for 48 h at 4 °C). After ~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 A, 5 μm). Elution was performed at 4 ml/min using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (20–100%). Fractions containing hGV were pooled, 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 A, 5 μm) equilibrated with the same buffer. hGIIF protein was eluted with a shallow gradient of acetonitrile in 0.1% trifluoroacetic acid (25–35% over 120 min). The hGIIF 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 mM guanidine-HCl, 10 mM CaCl2, 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 ultrafiltration cell (Amicon, YM-10 membrane) with stirring under 30–40 psi of N2 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 0.2 μm HPLC solvent filter (Amicon) and then 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. hGV elutes at 36 min. The protein solution was next equilibrated with 6 M guanidine-HCl, and lauryl sulfobetaine was added to a concentration of 5 mM. The sample was concentrated in a Centricon-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 until the time of yield of pure, refolded hGV fusion protein. The observed molecular weight (MALDI-TOF mass spectrometry) is 13,577.70, and the calculated weight is 13,578.60.
The purification of hGIID was routinely performed from batches of 2 liters of induced cells. To prevent extensive oxidation of hGIID, 1 mM t-methionine was added to all of the purification buffers. The cell medium and rCI extract from 2 liters of induced cells were combined, diluted 4-fold 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 mM NaCl and 1 liter of the same buffer containing 0.4 mM NaCl. sPLA2 activity was eluted with 2 liters of 20 mM Tris, pH 7.4, containing 1 mM NaCl, 8 mM urea, and 0.1 mM HCl. Fractions containing sPLA2 activity were diluted with a C18 reverse phase HPLC column (Nomura Chemicals, 8 μm, 55 ml), equilibrated with the same buffer. The column was eluted at 8 ml/min using a linear gradient of ammonium acetate (0–2 mM, pH 6.8 to 100 mM) in 10% acetonitrile. The main fractions containing sPLA2 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 the C18 column (Nomura Chemicals, 8 μm, 55 ml, 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% trifluoroactic 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. The observed molecular weight (electrospray) is 18,701.4, and the calculated weight is 18,701.5.

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). The amplified cDNA was then subcloned into a pGEM-T easy (Promega) vector, followed by mass spectrometry analysis confirmed that hGIID produced 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 with internal calibration. The observed molecular mass determined for nonoxidized nonglycosylated hGIID (14,522.9) is consistent with the calculated mass of the hGIID polypeptide with a serine at position 60 (14,522.6). The oxidized 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 sPLA2 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 weight 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 Da.

For these assays, cells were plated at 3×10^6 cells/ml in 24-well plates and induced the day after with 500 μM CuSO4 for 3–7 days. For large scale production of hGIID, stably transfected cells were seeded in 1-liter Spinners of phosphate-buffered saline containing 1 mM KCl. The cells were again pelleted, and the rCI extract was combined with the cell-free medium.

The Interfacial Kinetics and Binding of Mammalian sPLA2s

**Interfacial Kinetics with Phospholipid Vesicles**—The initial rate of hydrolysis of phospholipid vesicles by sPLA2s 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 were performed in 250 μl of reaction mixture containing 9.7 μg of fatty acid-binding protein, 2 μl of lipids, 1 mM CaCl2, and 250 μl of 100 mM dansyldecanoic 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...
with a stream of $N_2$ at room temperature, and remaining water was contained with $3$ ml of $1$ M NaCl, $0.6$ M CaCl$_2$, and a total phospholipid minus enzyme control were subtracted from the corresponding LPX ion was added to the quenched reaction.

Enzyme Catalytic Site Phospholipid Headgroup Substrate Specificity—The concentrations of phospholipids in CHCl$_3$/CH$_2$OH solutions were determined by inorganic phosphate assay. An equimolar mixture of POPA, POPC, POPS, POPG, and POF, and PI in CHCl$_3$/CH$_2$OH 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 $1$ M NaCl, $0.6$ M CaCl$_2$, and a total phospholipid concentration of $0.25$ M at $21$ °C. A sufficient amount 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 $2$ M. The internal standard d$_5$-LPG (37), $5.5$ nmol, was added to each reaction.

To a disposable column containing $1$ ml of C18 reverse phase silica packing (Bakerbond spe$^\text{®}$, 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 wash water, the quenched pH-stat reaction was loaded on to 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 activity was detected by reacting the residue.

The quantification of LPA was also checked by an independent method as LPA eluted early from the HPLC column for electrospray ionization mass spectrometry with mass spectrometry detection. Also, the variation in the amount of d$_5$-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, 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/mmol, American Radiochemicals Inc.) in $50$ ml Tris-HCl, pH $8.0$, $0.6$ mm CaCl$_2$ at room temperature. The [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 at each time point to determine the ratio of $^{3}H$ to $^{14}C$ radioactivity 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.

**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% CO₂ 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 [3H]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 sPLA₂. 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 CHCl₃/CH₃OH (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.

Results

Production of Recombinant Mouse and Human sPLA₂s—Expressing large amounts of functional sPLA₂s 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 sPLA₂s is part of a catalytically important hydrophobic domain that is exposed when overexpressed in native form. Furthermore, the 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 sPLA₂s, and it is this approach that we have taken to express most of the mammalian sPLA₂s. In this case, the insoluble inclusion body fraction of E. coli was isolated, protein was solubilized in denaturant, and 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 sPLA₂s (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 sPLA₂s. For those sPLA₂s that did not express well when they lack an N-terminal fusion peptide, the pAB₃ expression plasmid (38) was used to generate the sPLA₂ 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 sPLA₂, 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 sPLA₂ 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 hGIIId fusion protein in high yield from pAB₃ 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 sPLA₂s (except mGIC, which was produced in S2 cells) could be obtained by E. coli expression and in vitro refolding (the methods will be published elsewhere).

hGIF and mGIF are unique among mammalian sPLA₂s in that they have a 23-amino acid C-terminal extension containing a cysteine residue. We found that solutions of mGIF and hGIF 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 mGIF and hGIF monomer (established by mass spectrometry). All subsequent studies of mGIF and hGIF were carried out with the monomeric proteins. It remains to be established in vitro whether these monomers are linked together by a disulfide bridge or whether they are disulfide-linked to a different cellular protein. We also prepared hGIFαC (hGIF lacking the C-terminal 23-amino acid extension) by bacterial expression.

All human and mouse recombinant sPLA₂s were shown to be >98% pure by SDS-PAGE (not shown). Analysis of the sPLA₂ 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. All subsequent studies establish that all disulfide bonds are formed in the refolded sPLA₂s and that trypsin did not cleave any of the proteins internally.

Sensitivity of Mouse and Human sPLA₂s to DTT—There are numerous reports in the literature in which loss of PLA₂ activity upon treatment of the cellular extract with DTT is taken as firm evidence that the activity is caused by an sPLA₂. The sensitivity of the full set of mouse and human groups I, II, V, X, and XII sPLA₂s to DTT is summarized in Table I (numerical values in Table II). The most DTT-sensitive sPLA₂s are hGIIIA and mGIIIA, mGIIIC, 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, hGIF and mGIF, 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 sPLA₂ 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 PLA₂ enzymatic activity in a cell or tissue extract is caused by an sPLA₂. In addition, DTT sensitivity data provided in Table I may be useful to provide information on the type of sPLA₂ being detected in a cell lysate.

Activity of Mouse and Human sPLA₂s on Phospholipid Vesicles—We determined the specific activities for each sPLA₂ 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 sPLA₂s 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 sPLA₂s (Table III). The initial velocities were close...
| sPLA₂ | DTT sensitivity | Catalytic efficiency | Vesicle preference (single phospholipid vesicles) | Catalytic site phospholipid preference | sn-2 Fatty acyl chain specificity | K<sub>Ca,app</sub> | Most potent inhibitor<sup>a</sup> | Interfacial binding K<sub>i</sub> | Exogenous action on mammalian cells<sup>b</sup> |
|-------|----------------|---------------------|-----------------------------------------------|-----------------------------------|----------------------------------|---------------|---------------------------|-----------------|-----------------------------|
| hGIB  | ++             | +++                 | POPG ≫ POPS ≫ POPC ≫ PC ≫ PE ≫ PS | PG~PC~PE~PA~PS | 1.8 | 8 ± 3, POPG | Phosphonate-6b | ~3 (30% PS/PC) | – |
| hGIIA | +++            | +++                 | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 0.8 | 13 ± 3, POPG | Me-Indoxam | No binding at 2 mM (10% PS/PC) | – |
| hGIID | +++            | +                   | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 2.1 | 11 ± 3, POPG | Phosphonate-10b | 0.12 (10% PS/PC) | – |
| hGHE  | +              | +                   | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 0.7 | 112 ± 39, POPG | Me-Indoxam | No binding at 2 mM (10% PS/PC) | – |
| hGIF  | ++             | ++                  | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 2.0 | 27 ± 14, POPG | MJ33 and MJ50 | 0.35 (10% PS/PC) | – |
| hGV   | +++            | +                   | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 0.3 | 1 ± 0.5, POPG | Me-Indoxam | 0.05 (10% PS/PC) | ++ |
| hGX   | +              | +                   | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 2.3 | 1.5 ± 0.4, POPG | Me-Indoxam | 1.1 (10% PS/PC) | – |
| hGII  | ++             | +                   | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 1.9 | 45 ± 13, POPG | Me-Indoxam | 0.20 (30% PS/PC) | – |
| mGIB  | +              | +++                 | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 1.3 | 8 ± 3, POPG | Phosphonate-12b | 2.0 (10% PS/PC) | – |
| mGIIA | +++            | +                   | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 0.4 | 4 ± 1, POPG | Me-Indoxam | No data (10% PS/PC) | – |
| mGIIIC | +++         | +                   | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 1.9 | 14 ± 4, POPG | Me-Indoxam | 0.03 (10% PS/PC) | – |
| mGIID | +++            | +                   | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 0.4 | 46 ± 15, POPG | DDC-1 and Me-Indoxam | <10% bound at 2 mM (10% PS/PC) | – |
| mGIE  | +              | ++                  | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 1.0 | 100 ± 40, POPG | LY311727 and Me-Indoxam | No binding at 2 mM (10% PS/PC) | – |
| mGIF  | ++             | ++                  | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 1.7 | 35 ± 10, POPG | Me-Indoxam | 0.04 (10% PS/PC) | – |
| mGV   | +++            | +                   | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 0.3 | 1 ± 0.5, POPG | Me-Indoxam | ~0.02 (30% PS/PC) | ++ |
| mGX   | ++             | ++                  | POPG > POPS > POPC > PC > PE > PS | PG~PC~PE~PA~PS | 2.2 | 48 ± 15, POPG | Me-Indoxam | 0.07 (10% PS/PC) | – |

<sup>a</sup> Actual values are in Table II.
<sup>b</sup> 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.
<sup>c</sup> Ranked according to relative k<sub>cat</sub>/K<sub>cat</sub> values for the hydrolysis of mixed phospholipid vesicles. Actual values are given in Fig. 2, A and B.
<sup>d</sup> k<sub>cat</sub>/K<sub>m</sub> values for POPG and POPC vesicles are shown in Table IV.
<sup>e</sup> For detailed values, see Table V.

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**Table I**

Interfacial kinetic and binding properties of human and mouse sPLA₂s

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**Interfacial Kinetics and Binding of Mammalian sPLA₂s**

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### Interfacial Kinetics and Binding of Mammalian sPLA₂s

#### TABLE II

Remaining enzymatic activity of human and mouse sPLA₂s after treatment with 10 mM DTT

| sPLA₂  | Room temperature | | | 50 °C | | | |
|--------|------------------|---|---|---|---|---|---|
|        | 5 min            | 30 min | 60 min | 5 min | 30 min | 60 min |
| hGIB   | 100 *           | 100 | 100 | 73 | 0 | ND b |
| hGIA   | 36              | 0 | ND | ND | ND | ND |
| hGID   | 3               | 0 | ND | ND | ND | ND |
| hGIE   | 100             | 100 | 100 | 100 | 55 | 9 |
| hGIIF  | 100             | 98 | 85 | 40 | 0 | ND |
| hGV    | 31              | 0 | ND | 0 | ND | ND |
| hGIX   | 100             | 100 | 100 | 100 | 56 | 16 |
| hGIIX  | 84              | 63 | 46 | 23 | 0 | ND |
| mGIB   | 100             | 100 | 100 | 61 | 0 | ND |
| mGHA   | 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 |

* Percent remaining enzymatic activity after exposure to DTT under the indicated conditions.

**Not determined.

#### TABLE III

Action of human and mouse sPLA₂ 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 Hank’s balanced salt solution with 1.27 mM Ca²⁺ and 0.90 mM Mg²⁺ at 37 °C.

- **sPLA₂**
- **On POPG**
- **On POPS**
- **On POPC**

| sPLA₂  | On POPG | On POPS | On POPC |
|--------|---------|---------|---------|
| hGIB   | 1,030 ± 490, NL a | 55 ± 30, NL | 0.2 ± 0.07, NL |
| hGIA   | 220 ± 90, NL | 40 ± 18, NL | 0.7 ± 0.2, L |
| hGID   | 0.9 ± 0.2, NL | 0.1 ± 0.03, NL | 0.05 ± 0.02, NL |
| hGIID b | 0.7 ± 0.2, NL | No data | No data |
| hGIE   | 0.05 ± 0.1, NL | 0.12 ± 0.2, NL | 0.12 ± 0.01, NL |
| hGIIF  | 18 ± 1, NL | 0.04 ± 0.01, NL | 0.03 ± 0.01, NL |
| hGIIFc  | 15 ± 1, NL | 0.05 ± 0.01, NL | 0.09 ± 0.02, NL |
| hGV    | 24 ± 5, NL | 10 ± 1, NL | 0 ± 1, NL |
| hGIX   | 14 ± 0.8, NL | 4 ± 2, NL | 30 ± 2, NL |
| hGIIX  | 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 |
| mGIA   | 140 ± 60, NL | 24 ± 7, NL | 0.12 ± 0.03, L |
| mGIIC  | 12 ± 5, NL | 2.5 ± 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  | 0.2 ± 0.04, NL | 0.12 ± 0.05, L |
| mGIIF  | 4 ± 1.6, NL | 0.035 ± 0.15, NL | 0.4 ± 0.2, SL |
| mGV    | 40 ± 5, NL | 3 ± 0.3, 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 b is the nonglycosylated fraction of hGIID produced in Drosophila S2 cells.

c No data means that a reliable Kₐ value could not be obtained because of loss of enzyme to the wall of the microfuge tube.

d hGIIDₐ is the catalytic site specificity (22).

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...
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 sPLA2s 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 hGIFPAC 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 sPLA2 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 sPLA2 to the vesicle interface. Catalytic site specificity of the human and mouse sPLA2s toward phospholipids with different polar headgroups was obtained by measuring the ratio of lysophospholipid 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 sPLA2 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. A qualitative summary is provided in Table I. Mass spectrometry was used to quantify all LPX species except LPA; the latter was quantified by double channel scintillation counting with radiolabeled PA and PC (see “Experimental Procedures”). Remarkably, all human and mouse sPLA2s 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 sPLA2 action was determined by dual channel scintillation counting. Results are listed in Table I. It is clear that none of the human and mouse sPLA2s shows a significant preference for a polysaturated 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 hGIV show the lowest preference (0.3–0.4-fold).
and are in the range of 1 nM for all tested on the human and mouse sPLA2s. Estimated IC50 values are the dissociation constant, Kd, and are listed in Table I. Values of Kd vary significantly and are in the range of 1–225 nM. For a subset of those sPLA2s that hydrolyze POPC and POPS vesicles without a lag, values of Kd were found to be higher than the corresponding values for the hydrolysis of POPG vesicles.

**Calcium Affinity**—For all human and mouse sPLA2s, 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 sPLA2-Ca2+ dissociation constant, K_dissociation constant, (see “Discussion”) were obtained by fitting the velocity-calcium profile to the hyperbolic equation and are listed in Table I. Values of K_dissociation constant vary significantly and are in the range of 1–225 μM. For a subset of those sPLA2s that hydrolyze POPC and POPS vesicles without a lag, values of K_dissociation constant 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 sPLA2 inhibitors (structures shown in Fig. 4) were tested on the human and mouse sPLA2s. Estimated IC50 values, listed in Table IV, were obtained with at least four inhibitor concentrations that span 10–90% inhibition using a radiometric assay consisting of [3H]DPPC present as a minor compound in POPG vesicles (see “Experimental Procedures”). Me-Indoxam was found to be the most generally potent sPLA2 inhibitor, we determined the IC50 values using two different assays (Table IV). Me-Indoxam is a potent inhibitor of 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 sPLA2 inhibitor, we determined the IC50 values using two different assays (Table IV). Me-Indoxam is a potent inhibitor of hGIIA, mGIIA, hGIIE, and mGIIE, and GIV, with IC50 values in the low nanomolar range. It displays intermediate potency against hGIB, mGIB, and mGIX and is less potent against hGIID, mGIID, hGIIF, mGIIF, hGX, and hGXXII. The different sPLA2 assays used to analyze Me-Indoxam inhibition give somewhat different IC50 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_dissociation constant values.

**Interfacial Binding of sPLA2s to Phospholipid Vesicles**—Binding of sPLA2s 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 sPLA2s have tryptophans, and we have found that the energy transfer intensity varies dramatically with the phospholipid composition of the interface. 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 sPLA2 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 sPLA2s (for a complete discussion, see Ref. 23). Because we anticipated that most, if not all, of the human and mouse sPLA2s would bind weakly to PC-rich vesicles and more tightly to PS vesicles (63), our studies were carried out with DOPEPC. Various amounts of DOPEPC were

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**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 hGIIA 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 sPLA2.

**Fig. 4.** Structures of sPLA2 inhibitors.
Interfacial Kinetics and Binding of Mammalian sPLA₂s

Table IV

IC₅₀ values (μM) for the inhibition of human and mouse sPLA₂

| Phosphonate-6b | Phosphonate-10b | Phosphonate-12b | MJ33 | MJ50 | Triterenoid-1 | Triterenoid-2 | SB203347 | LY311727 | Pyr-1 | DDC-1 | Me-Indoxam |
|----------------|----------------|----------------|------|------|---------------|---------------|----------|----------|-------|-------|-----------|
| hGIB 0.06      | 0.3            | 5              | >20  | >20  | >20           | >20           | >20      | >20      | >20   | >20   | 0.03/0.006 |
| hGIHA >20      | 0.9            | 0.4            | >20  | 10   | >20           | >20           | >20      | >20      | >20   | >20   | 0.008/0.01 |
| 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      | >20      | >20   | >20   | 0.025/0.006 |
| hGIIF 6        | 4              | 20             | >20  | 2    | >20           | >20           | >20      | >20      | >20   | >20   | 0.025/0.005 |
| hGV >20        | 6              | >20            | 10   | 5    | >20           | >20           | >20      | >20      | >20   | >20   | 0.015/0.0015 |
| hGX 3.5        | >20            | 10             | 15   | 10   | >20           | >20           | >20      | >20      | >20   | >20   | 0.06/0.12  |
| hGXII 20       | 20             | 20             | 20   | >20  | >20           | >20           | >20      | >20      | >20   | >20   | 7/10      |
| mGIIC 0.20     | 10             | 0.08           | 10   | 5    | >20           | >20           | >20      | >20      | >20   | >20   | 0.15/0.3  |
| mGIID 4.5      | 5              | 1.5            | 10   | 5    | >20           | >20           | >20      | >20      | >20   | >20   | 0.008/0.01 |
| mGIIC 2        | 0.6            | 0.6            | 0.5  | 0.5  | No data       | No data       | 0.5      | 10       | 20    | >20   | 0.015/0.0015 |
| mGIID 7        | 15             | 11             | 20   | 10   | >20           | >20           | >20      | >20      | >20   | >20   | 0.02/0.015 |
| mGIIE >20      | 20             | 20             | >20  | >20  | >20           | >20           | >20      | >20      | >20   | >20   | 0.025/0.005 |
| mGIIF >20      | 20             | 20             | >20  | >20  | >20           | >20           | >20      | >20      | >20   | >20   | 0.025/0.005 |
| mGV >20        | 20             | 20             | >20  | >20  | >20           | >20           | >20      | >20      | >20   | >20   | 0.025/0.005 |
| mGX >20        | 20             | 10             | 20   | >20  | >20           | >20           | >20      | >20      | >20   | >20   | 0.025/0.005 |

* The first number given is the IC₅₀ measured with the 1-palmitoyl-2-(10-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol assay, and the second is for the E. coli membrane assay.

No data means that a reliable Kᵣ value could not be obtained because of loss of enzyme to the wall of the microfuge tube.

![Fig. 5. Interfacial binding of sPLA₂s to phospholipid vesicles.](image)

Cobra venom sPLA₂. With RBL-2H3.1 cells only hGX, mGX, and cobra sPLA₂ gave statistically significant arachidonic acid release (Fig. 6C).

**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 sPLA₂ acting on pure vesicles of POPG, POPC, and POPV 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 sPLA₂. These sPLA₂ have the Asp-His catalytic diad and a calcium binding loop. The latter is functional based on values of Kᵣ (app) in the range 11–112 μM for these five sPLA₂. Overlaid their sequences onto the three-dimensional x-ray structures of other sPLA₂ 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...
all of the low specific activity sPLA₂s 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 sPLA₂ 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 sPLA₂s 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 sPLA₂s display true interfacial allosteric activation (kcat* and Km* 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 sPLA₂ 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 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 sPLA₂s 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 venom sPLA₂s, other catalytically inactive sPLA₂s such as otoconin-22 (69), otoconin-95 (7, 8), and a putative zebrafish group XII-like inactive sPLA₂ (41) have been found, but the functions of these are not yet identified.

It is generally stated that sPLA₂s require submillimolar to millimolar concentrations of calcium as a catalytic cofactor. However, the results of this study show that many of the mammalian sPLA₂s are fully activated even by low micromolar calcium concentrations. It is expected that the concentration of calcium required for maximal sPLA₂ 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 sPLA₂-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 KCa(app). The synergism between phospholipid and calcium binding presumably explains why the value of KCa(app) decreases for hGV and hGX as the substrate is changed from POPC, to POPS, to POPG because these sPLA₂s bind with increasing affinity to anionic versus zwitterionic phospholipid vesicles. Also, the fact that KCa(app) for hGIIE, mGIIE, and

expressed as the percent of total cpm (culture medium + cell-associated) released into the culture medium. B, same as for A but using mouse sPLA₂. 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.
hGII is relatively high for the action on POPG vesicles is probably related to the observation that these sPLA₂s bind weakly to vesicles even if they contain a high fraction of anionic phospholipids. The physiological significance of the full activation of sPLA₂ by micromolar calcium remains to be established, but one should not assume a priori that an sPLA₂ 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. 2A 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 sPLA₂s). The fact that all sPLA₂s 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 sPLA₂ 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 binding is mostly the result of differential interfacial binding of these sPLA₂s to vesicles.

None of the mammalian sPLA₂s 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 polysaturated fatty acyl chain over the saturated one by 6-fold because of a combination of the −2−fold preference of the group X sPLA₂ for the arachidonyl chain and the −3−fold preference of the group V sPLA₂ for the palmitoyl chain (Table I). The slight preference of the group X sPLA₂ 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 sPLA₂s 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 sPLA₂ 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. 6a 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 sPLA₂s it is apparent that those enzymes that bind well to PC-rich vesicles (10% DO₆ₐ/PS/DO₆ₐ/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 sPLA₂s 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% DO₆ₐ/PS/DO₆ₐ/PC) even with 2 mM phospholipid present, and we cannot confirm the previous result of $K_d$ ∼ 0.1 µM for hGIID dissociating from dihexadecyl PC vesicles in the presence and absence of calcium (measured by x-ray emission from surface plasma resonance) (79).

Tryptophan on the membrane binding surface of hGV is important for the high catalytic activity of this sPLA₂ 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 hGIIA 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 sPLA₂s 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 sPLA₂s onto the known three-dimensional x-ray structure of hGIID, the following enzymes are predicted to contain one or more tryptophans on their membrane binding surface: hGII, hGIID, hGV, mGII, mGIIC, mGIIID, 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 mGV, all of these sPLA₂s hydrolyze PC vesicles without a discernible lag. All of the sPLA₂s that do not contain a membrane binding surface tryptophan, hGIID, hGII, 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 sPLA₂s and the occurrence of the lag.

There is a dramatic correlation between the ability of the sPLA₂s to hydrolyze PC-rich vesicles and the ability of these enzymes to liberate arachidonic acid when added exogenously to mammalian cells. hGII, hGIID, hGIID, mGII, mGIIC, mGIIID, 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 mGV, all of these sPLA₂s hydrolyze PC vesicles without a discernible lag. All of the sPLA₂s that do not contain a membrane binding surface tryptophan, hGIID, hGII, 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 sPLA₂s and the occurrence of the lag.

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cells approaches that of cobra venom sPLA2, 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 sPLAs 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 hMGX, there is a poor correlation between the ability of the various sPLA2s 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 sPLA2 (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 expected from the data with exogenously added enzyme (Fig. 6). These results suggest that those sPLA2s 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 sPLA2s expressed in mammalian cells.

Although the catalytic sites of sPLA2s 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 sPLA2 family. 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 sPLA2 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 sPLA2s reflects the relative affinity of this inhibitor versus competing substrate for the active site of the vesicle-bound sPLA2. 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 sPLA2 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 sPLA2 family raise concern about previous studies in which a physiological or biochemical process was attributed to a single sPLA2 molecular species. LY311727 fails to inhibit hGX at concentrations up to 20 μM (Table IV), which is consistent with earlier studies using different sPLA2 enzyme assays (31, 82). We have no explanation for the discrepancy of these values with the IC50 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 sPLA2s show that these enzymes have dramatically different abilities to bind to phospholipid vesicles and to hydrolyze phospholipids in these vesicles. Other dramatic observations were varied for calcium activation and inhibitor potency. These variations and the fact that the tissue distribution of the mammalian sPLA2s are distinct argue strongly that these enzymes are not isozymes 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).

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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., Tzai, 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. Valentine, 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. Vyas, E., Liebermann, M., and Pestet, C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 529–534
8. Wang, Y., Kowalski, P. F., Thalhammer, I., Ortmiz, D. M., Mager, D. L., and Thalhammer, R. (1998) FEBS Lett. 435, 135–139
9. Murakami, M., Koduri, R. S., Enomoto, A., Shimbara, S., Seki, M., Yoshii, K., Singer, A., Valentin, E., Ghashami, F., Lambeau, G., Gelb, M. H., and Kudo, I. (2001) J. Biol. Chem. 276, 39981–39996
10. Vyas, E., Liebermann, M., Enomoto, A., Yoshii, K., Ishihara, M., Ishikawa, Y., Ishii, T., and Kudo, I. (2002) J. Biol. Chem. 277, 19145–19155
11. Lambeau, G., and Lazdunski, M. (1999) Trends Pharmacol. Sci. 20, 162–170
12. Hansakasi, K., and Arita, H. (1999) Arch. Biochem. Biophys. 372, 215–223
13. Ghashami, F., Yu, B.-Z., Berg, O., Jain, M. K., and Gelb, M. H. (1999) Trends Biochem. Sci. 30, 731–738
14. Bezine, S., Bollerig, 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
15. Richach, P., Weiss, J., Wright, C. S., van den Bergh, C. J., and Verheij, H. M. (1999) Prog. Clin. Biol. Res. 349, 1–9
16. Koduri, R. S., Grönroos, J. O., Laine, Y. J., Le Calvez, C., Lambeau, G., Nevalainen, T. L., and Gelb, M. H. (2002) J. Biol. Chem. 277, 5848–5857
17. 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
18. Gelb, M. H., and Kudo, I. (2000) Tanpakushitsu Kakusan Koso 45, 1085–1071
19. Mihelich, E. D., and Schevitiz, R. W. (1999) Biochim. Biophys. Acta 1441, 223–228
20. Balsinde, J., Balboa, M. A., Insel, P. A., and Dennis, E. A. (1999) Annu. Rev. Pharmacol. Toxicol. 39, 175–189
21. 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
22. Degweker, N., Ghashami, F., Singh, R., Smart, B. P., Borregaard, N., Reithmeier, R., Lindsay, T. F., Lichtenberger, C., Reinsich, W., Lambeau, G., Arm, J., Tischfeld, J. T., Gelb, M. H., and Rubin, B. B. (2002) J. Biol. Chem. 277, 5061–5073
23. Schevitiz, R. W., Bach, N. J., Carlan, D. G., Gharigue, N. Y., Claussen, D. W., Dillard, R. D., Draheim, S. E., Hartley, L. W., Jones, N. D., Mihelich, E. D., Oklowski, J. L., Snyder, D. W., and Sommers, J. P. (1995) Nat. Struct. Biol. 2, 458–465
24. Jain, M. K., Tao, W., Rogers, J., Areson, C., Eibl, H., and Yu, B.-Z. (1991) Biochemistry 30, 10256–10268
25. Jain, M. K., Yu, B.-Z., Rogers, J., Smith, A. E., Boger, E. T., Ostrander, R. L., and Rheingold, A. L. (1995) Ptochozyme 39, 537–547
26. Marshall, L. A., Hall, R. H., Winkler, J. D., Badger, A., Bolognese, B., Roshak, A., Flamberg, P. L., Sund, C. M., Chabot-Fletcher, M., Adams, J. L., and Mayer, R. J. (1995) J. Pharmacol. Exp. Ther. 274, 1254–1262
27. Lin, H.-K., and Gelb, M. H. (1993) J. Am. Chem. Soc. 115, 3832–3842
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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., Valentín, E., Ghomashchi, F., Lazdunski, M., and Lambeau, G. (2000) J. Biol. Chem. 275, 39823–39826
42. Valentín, E., Ghomashchi, F., Lazdunski, M., Gelb, M. H., and Lambeau, G. (2000) Biochem. Biophys. Res. Commun. 279, 223–228
43. Valentín, 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., Gaichl, M., Suter, M., and Gelb, M. H. (1999) Biochim. Biophys. Acta 1165, 201–210
45. Cupillard, L., Mulherkar, R., Gomez, N., Kadam, S., Valentín, 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 Dorsseleer, A., Papandreou, M. J., Sornkine, O., Rochat, H., Sampieri, F., and Marsouelle, P. (1999) FEBS Lett. 445, 175–180
48. Kubelka, V., Altman, P., Staedacher, E., Tretter, V., Marz, L., Hard, K., Kamerling, J. P., and Vrieze, 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., Schutte, 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. Anzian, 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. (1996) Biochim. J. 311, 353–357
59. Vailllard, L., Marret, N., and Rambaud, C. (1995) Electrophoresis 16, 295–297
60. Chen, Y., and Dennis, E. A. (1998) Biochim. Biophys. Acta 1394, 57–64
61. Jain, M. K., and Var, W. L. C. (1987) Biochim. Biophys. Acta 905, 1–8
62. Buser, C. A., Sigal, C. T., Resh, M. D., and McLaughlin, S. (1984) Biochemistry 23, 13090–13101
63. Jain, M. K., Ranadive, G., Yu, B.-Z., and Verheij, H. M. (1991) Biochemistry 30, 7330–7340
64. Ho, L. C., Arm, J. P., Bingham, C. O., Choi, A., Austen, K. F., and Glommer, L. H. (2001) J. Biol. Chem. 276, 18321–18326
65. Suzuki, I., 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 Lamotte, B. (1995) Toxicon 33, 1405–1424
67. Ownby, C. L., Selistre de Araujo, H. S., White, S. P., and Fletcher, J. E. (1999) Biochemistry 38, 7803–7810
68. Fanerd, D., Lambeau, G., Valentín, E., Lefebvre, J., Lazdunski, M., and Duglo, A. (1999) J. Clin. Invest. 104, 611–618
69. Pote, K. G., Hauer, C. R. I., Michel, H., Shabanowitz, J., Hunt, D. F., and Kretzinger, 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. Thummi, M. M. G., Ab, E., Kulk, 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, 575–582
74. Sano, T., Baker, D., Virag, T., Wada, T., Yatomi, Y., Kobayashi, T., Igarashi, Y., and Tigg, G. (2002) J. Biol. Chem. 277, 21197–21206
75. Kim, K. P., Han, S. K., Hong, M., and Cho, W. (2000) Biochim. J. 348, 643–647
76. Cupillard, L., Koumanov, K., Mattüz, 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, N., Nakao, K., Yamada, K., Ishizaki, J., and Arita, H. (1999) J. Biol. Chem. 274, 34203–34211
78. Kim, Y. J., Kim, K. P., Rhe, J. H., Das, S., Rafter, J. D., Oh, Y. S., and Cho, W. (2002) J. Biol. Chem. 277, 9358–9365
79. Shahabin, 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., Kodori, 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., Kuroha, H., Winstead, M. V., Tischfield, J. A., and Kudo, I. (1998) J. Biol. Chem. 273, 14411–14423
83. Enomoto, A., Murakami, M., Valentín, E., Lambeau, G., Gelb, M. H., and Kudo, I. (2000) J. Immunol. 165, 4007–4014