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Exogenously Added Human Group X Secreted Phospholipase A_2 but Not the Group IB, IIA, and V Enzymes Efficiently Release Arachidonic Acid from Adherent Mammalian Cells*

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Mammalian secreted phospholipases A_2 (sPLA2s) comprise a group of at least eight enzymes, including the recently identified group X sPLA2. A bacterial expression system was developed to produce human group X sPLA2 (hGX). Inhibition studies show that the sPLA2 inhibitor LY311727 binds modestly more tightly to human group IIA sPLA2 than to hGX and that a pyrazole-based inhibitor of group IIA sPLA2 is much less active against hGX. The phospholipid head group preference of vesicle-bound hGX was determined. hGX binds tightly to phosphatidylcholine vesicles, which is thought to be required to act efficiently on cells. Tryptophan 67 hGX makes a significant contribution to interfacial binding to zwitterionic vesicles. As little as 10 ng/ml hGX releases arachidonic acid for cyclooxygenase-2-dependent prostaglandin E_2 generation when added exogenously to adherent mammalian cells. In contrast, human group IIA, rat group V, and mouse group IB sPLA2s are virtually inactive at releasing arachidonate when added exogenously to adherent cells. Dislodging cells from the growth surface enhances the ability of all the sPLA2s to release fatty acids. Studies with CHO-K1 cell mutants show that binding of sPLA2s to glycosaminoglycans is not the basis for poor plasma membrane hydrolysis by group IB, IIA, and V sPLA2s.

The secreted phospholipase A_2 (sPLA2)[1] family comprises 14–16 kDa, calcium-dependent (K_{Ca} ~ millimolar) interfacial enzymes that liberate *sn*-2 fatty acids including arachidonic acid for the biosynthesis of eicosanoids (1–4). To date, eight mammalian sPLA2s have been identified (groups IB, IIA, IIC, IID, IIE, IIF, V, and X) that display distinct yet partial overlapping tissue distributions (5–9). Our continued awareness of the diversity of mammalian sPLA2s has cast doubt on earlier studies reporting the molecular identity of sPLA2 species expressed in cultured cells. Furthermore, understanding the physiological functions of the various mammalian sPLA2s is now a complex and challenging area of research in the eicosanoid field.

sPLA2s must bind to the lipid-water interface to gain access to phospholipid substrates (10). It is becoming apparent that the different mammalian sPLA2s display dramatically different interfacial binding properties (11–14). For example, human group IIA sPLA2 binds weakly or not at all to vesicles of the zwitterionic phospholipid phosphatidylcholine even in the presence of millimolar phospholipid, and interfacial binding is greatly enhanced (>10^6-fold) when anionic phospholipids such as phosphatidylserine or phosphatidic acid are present (15). Like group IIA sPLA2, human and porcine group IB sPLA2s also bind much tighter to anionic vesicles, but binding to millimolar concentrations of phosphatidylcholine vesicles can be detected (14, 16). Human group V sPLA2 binds at least 100-fold tighter to anionic phosphatidylglycerol vesicles than to phosphatidylcholine vesicles, but binding to the latter is of much higher affinity (interfacial dissociation constant ~ low micromolar) than for group IB and IIA sPLA2s (17). Of all the sPLA2s analyzed, cobra venom sPLA2 displays the highest affinity for zwitterionic vesicles (dissociation constant ~10^{-7} M) (16, 18). Although the interfacial binding surfaces of sPLA2s are comprised of a large external surface of the enzyme (19), contributions to binding from single amino acid residues can be

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[1] The abbreviations used are: sPLA2, secreted 14–16-kDa phospholipase A_2; cobra-GIA, sPLA2 from *N. naja naja* venom; COX-1,2, cyclooxygenase-1 and -2; dithio-DMPM, 1,2-bismyristoylthio-1,2-dideoxy-*sn*-3-glycero-3-phosphomethanol; FABP, rat liver fatty acid binding protein; HEK293, human embryonic kidney 293 cells; hGIIA-N1A, N1A mutant of human group IIA sPLA2; hGX, human group X sPLA2; LPA, LPC, LPE, LPG, LPM, and LPS, 1-palmitoyl-*sn*-glycero-3-phosphate, -phosphocholine, -phosphoethanolamine, -phosphoglycerol, -phosphomethanol, and -phosphoserine (d_{31} designates a perdeuterated palmitoyl chain); LUV, large unilamellar vesicle; mGIB, mouse group IB sPLA2; PGE_2, prostaglandin E_2; Pyr-1, 3-(2, 6-dichlorophenylsulfonylamino)-4-(2-(4-acetamido)pyridyl)-5-(3-(4-fluorophenoxy)benzyl(thio)-(1*H*)-pyrazole; POPA, C, E, G, M, and S, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate, -phosphocholine, -phosphoethanolamine, -phosphoglycerol, -phosphomethanol, and -phosphoserine; CHO, Chinese hamster ovary; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; rGV, rat group V sPLA2.

dissected. Tryptophan appears to be one of the most important residues for promoting tight binding to phosphatidylcholine vesicles (12, 17), and lysine and arginine residues are important for electrostatic binding to anionic vesicles (11, 13, 20) and for destabilizing binding to phosphatidylcholine vesicles.

Although our understanding of how sPLA2s bind to vesicles at the single amino acid level is increasing, our knowledge of how these enzymes interact with mammalian cell membranes is very limited. Understanding functional sPLA2-cell interaction is a challenging undertaking because of the complexity of cell membranes and the existence of multiple membranes in cells. To study the action of sPLA2s on mammalian cells, two approaches have been taken. Kudo and co-workers (4, 21) have overexpressed sPLA2s in human embryonic kidney cells (HEK293), which normally have little or no endogenous sPLA2s. Such studies have shown that groups IIA and V sPLA2s are able to liberate arachidonic acid that functionally couples to cyclooxygenase for production of prostaglandin E_2 (4, 21). The second approach is to add sPLA2s exogenously to cells (22–24). This approach has generally revealed that mammalian cells are highly resistant to the action of exogenously added group IIA sPLA2, with relatively high concentrations (1–10 μg/ml) being required to detect arachidonate release. This may be due in part to the predicted low affinity of group IIA sPLA2 for the phosphatidylcholine-rich extracellular face of the plasma membrane. Interestingly, very recent studies have shown that group IIA sPLA2 expressed in HEK293 cells becomes associated with the heparan sulfate-containing proteoglycan glypican and is then internalized into what appear to be caveolae (25). Such a process may allow group IIA sPLA2 to come in contact with an interface to which it can bind to release arachidonate for coupling to oxygenating enzymes for eicosanoid production. On the other hand, exogenously added group V sPLA2 is 10–30-fold more active than group IIA sPLA2 in liberating fatty acids from mammalian cells (RAW264.7 cells, CHO-K1 cells, and neutrophils) (however, see below), which may be due in part to the ability of the former enzyme to bind phosphatidylcholine vesicles (17).

In the present study, we have developed a bacterial expression system for the production of milligram amounts of properly folded human group X sPLA2 (hGX). This has enabled us to study the interfacial binding and kinetic properties of this recently discovered sPLA2. In addition, the ability of hGX to liberate arachidonate when added exogenously to cells was evaluated in comparison with recombinant human group IIA (hGIIA), rat group V (rGV), mouse group IB (mGIB), and cobra venom group IA (cobra-GIA) sPLA2s. The studies reveal striking features of the exogenous action of hGX on mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Recombinant rGV was obtained as a gift from Marcel Janssen (26). The preparation of recombinant human group V sPLA2 will be reported elsewhere. The primary structures of all sPLA2s were confirmed by electrospray ionization mass spectrometry using a Bruker/Hewlett-Packard Esquire LC ion-trap HPLC/mass spectrometry. The resolution ($\pm$1 atomic mass units at 14 kDa) is sufficient to ensure that all sPLA2 disulfides are intact. mGIB, hGIIA-N1A, and cobra-GIA (from *Naja naja naja* venom, Miami Serpentarium) were prepared as described (27–29). The N1A mutant of human group IIA sPLA2 (hGIIA-N1A) is used to facilitate removal of the initiator methionine, and its specific activity on vesicles is identical to that of the wild-type enzyme (28). Recombinant FABP (30) was purified from an *Escherichia coli* overexpressor using the procedure provided by D. C. Wilton (University of Southampton). Phospholipids are from Avanti, and 11-dansyl-undecanoic acid is from Molecular Probes. Human interleukin-1β is from R & D Systems. LY311727 was obtained as a gift from E. Mihelich (Lilly), and Pyr-1 was synthesized as described (31).

d_{31}-LPA was made and purified (32) by hydrolyzing d_{31}-LPC (Avanti) with cabbage phospholipase D (33). d_{31}-LPG and d_{31}-LPE were prepared by hydrolysis of the corresponding *sn*-1 chain perdeuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol and -ethanolamine (Avanti), respectively, with phospholipase A_2 (crude *Crotalus adamenteus* venom, Miami Serpentarium) at 37 °C in sodium borate, pH 7.0, with $CaCl_2$ (34). After work up (34), compounds were purified by flask chromatography on silica using $CHCl_3$ with increasing CH_3OH. A $CHCl_3/CH_3OH$ solution of lysophospholipid was washed with water to remove residual silica, and organic solvent was removed *in vacuo*. For d_{31}-LPS, *sn*-1 chain perdeuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (Avanti) was hydrolyzed poorly by *C. adamenteus* venom, so the phospholipid was dispersed at 10 mol % in 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol vesicles (16), and the deuterated phospholipid was hydrolyzed to completion by following the reaction in a pH-stat (35). The reaction mixture was concentrated to dryness in a Speed-Vac (Savant), and the residue was taken up in $CHCl_3/CH_3OH$, and the product was purified by preparative thin layer chromatography on a silica plate using $CHCl_3/CH_3OH/H_2O$ (65/25/4). d_{31}-LPS was eluted from the scraped silica with $CHCl_3/CH_3OH$, and the organic layer was washed with water and filtered through a syringe filter (Gelman Acrodisc, PTFE, 0.45 μm). Solvent was removed *in vacuo*. The concentrations of lysophospholipids in stock solutions were determined by standard phosphate analysis with ammonium molybdate, and the structures of all lysophospholipids were confirmed by electrospray ionization mass spectrometry.

The colorimetric sPLA2 substrate dithio-DMPM was prepared as follows. 1,2-Bismyristoylthio-1,2-dideoxy-*sn*-3-glycero-3-phosphocholine (36) (62 mg) was dissolved in a mixture of 1.47 ml of methanol, 2.42 ml of ether, and 4.96 ml of buffer (100 mM sodium acetate, 100 mM $CaCl_2$, pH 5.6) in a Teflon septum, screw cap glass vial with a stir bar. Cabbage acetone powder (20 mg) (33) was added, and the solution was vigorously stirred for 24 h at room temperature. Thin layer chromatography on a silica plate with $CHCl_3/CH_3OH/CH_3CO_2H$ (65/35/8) showed ~80% product (R_f 0.95) and ~20% starting material (R_f 0.6). $CHCl_3$, CH_3OH, and water were added to give a two-phase system, and ~100 mg of EGTA was added. After vortex mixing and separation of layers by centrifugation, the lower organic layer was concentrated to dryness *in vacuo*. The product was purified by flash chromatography on silica gel using $CHCl_3/CH_3OH$ (85/15). The product fractions were pooled, concentrated to dryness *in vacuo,* and lyophilized from benzene to give a white powder (25 mg). The product structure was confirmed by ^1H NMR.

Preparation of Recombinant hGX—A synthetic gene encoding the hGX mature protein was designed based on the protein sequence of cloned hGX sPLA2 (5) using *E. coli* preferred codons for all amino acids. The gene was prepared in a two-step PCR process using high fidelity *Pwo* polymerase (Roche Molecular Biochemicals) and three sets of two long, partially overlapping single-stranded oligonucleotides (70–102 bases long). In the first round of PCR (final volume 50 μl), each set of oligonucleotides (2 μg of each primer) with a 20-base complementary sequence was annealed (50 °C/30 s), elongated (72 °C/30 s), and denaturated (95 °C/30 s) for three cycles, and the sizes of the expected DNA fragments were analyzed on 2.5% agarose gel. In the second round of PCR (final volume 50 μl), 0.5 μl of each first PCR reaction were mixed, annealed, and elongated as above for 5 cycles. The two oligonucleotides (0.5 μg each) flanking the hGX synthetic gene were then added, and 15 additional cycles of amplification (94°/1 min, 50 °C/1 min, 72 °C/1 min) were performed. *Taq* polymerase (2.5 units, Life Technologies, Inc.) and fresh dNTP (0.2 mM) were then added to the reaction tube, and incubation was continued for 5 min at 72 °C. The final PCR product was purified with the wizard PCR prep kit (Promega), ligated into the pGEM-T easy vector (Promega), and entirely sequenced to verify the construct. The hGX synthetic gene was then used as template in a novel PCR reaction to prepare a DNA fragment coding for a factor Xa cleavage site Ile-Glu-Gly-Arg followed by the mature hGX protein, and the fragment was subcloned in frame with the truncated glutathione *S*-transferase (~8 kDa) encoded by the modified pGEX-2T vector (pAB3) as described previously for mouse group IID sPLA2 (7). The sequence of the synthetic gene is available from the authors upon request.

Expression of the hGX fusion protein was carried in *E. coli* BL21 host cells. Bacteria was grown in 2 liters of LB broth with 50 μg/ml ampicillin at 37 °C until the absorbance at 600 nm reached 0.5–0.6. Isopropylthiogalactoside was added to 1 mM, and the culture was continued for 3–3.5 h. Cells were harvested by centrifugation at 4 °C, and the cell pellet was stored at −80 °C until processed.

The cell pellet was suspended in 100 ml of cold buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.5 mM freshly added phenylmethylsulfonyl fluoride), and the suspension was sonicated on ice (15 cycles of 15 s on and 15 s off). After centrifugation at 10,000 rpm (SS-34 rotor) for 10

min at 4 °C, the pellet was resuspended in 100 ml of cold buffer containing 0.8% (v/v) Triton X-100 and 0.8% sodium deoxycholate with stirring for 20 min. Sonication followed by centrifugation was repeated as above. The pellet was stirred with 100 ml of buffer containing 1% Triton X-100 for 20 min at room temperature and centrifuged. This process was repeated with buffer without detergent. The inclusion body pellet can be stored at −80 °C or further processed.

The protein pellet was dissolved in 6 M guanidinium hydrochloride (Amresco, Ultra Pure Grade), 0.3 M Na_2SO_3, 50 mM Tris, pH 8.0 (10 mg of moist protein per ml of buffer), with stirring at room temperature, and 0.05 volume of Tannhauser reagent (37) was added. After stirring for 1 h at room temperature, the solution was dialyzed against 1% acetic acid at 4 °C (4 changes, 50 volumes each). The precipitated sulfonated fusion protein was collected by centrifugation at 10,000 rpm (GSA rotor) at 4 °C for 20 min. The pellet (~240 mg) was dried in a vacuum dessicator overnight and stored at −80 °C.

Protein (70 mg) was dissolved in 350 ml of 6 M guanidinium chloride, 50 mM Tris, pH 8, at room temperature with stirring, and the solution was dialyzed against 11 liters of pre-chilled 0.9 M guanidinium chloride, 50 mM Tris, pH 8.0, 0.5 mM freshly added cysteine, 5 mM EDTA at 4 °C, and the buffer was replaced after 6 h. The enzymatic activity was checked every 12 h using a spectrofluorimetric assay (37) until it reached a maximum (typically 1–2 days). The dialysis bag was transferred to 11 liters of pre-chilled factor Xa buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$). After several hours, the buffer was replaced once. Protein precipitate was removed by centrifugation at 15,000 × *g* for 20 min at 4 °C. Factor Xa (336 units per 70 mg of processed sulfonated fusion protein, Amersham Pharmacia Biotech) was added, and digestion was allowed to proceed for 12–15 h at room temperature. Protein can be stored at −20 °C or processed further.

Protein solution was filtered (Uniflo-25, 0.2 μm, low protein binding, Schleicher & Schuell). The entire solution was pumped at 3 ml/min directly onto a C18 reverse phase HPLC column (Vydac 218 TP1010) that was previously equilibrated with water with 0.06% trifluoroacetic acid (solvent A). After loading, the solvent program was started as follows: 10% solvent B (acetonitrile with 0.06% trifluoroacetic acid) to 30% B over 20 min, then to 60% B over 110 min. Column eluant was monitored by UV detection at 280 nm. Factor Xa-cleaved hGX elutes at ~40 min (column fractions checked by enzyme assay and SDS-polyacrylamide gel electrophoresis). The purified protein was mixed with 3 volumes of water and lyophilized. The protein was dissolved in 1 ml of water and re-purified by HPLC using a solvent program of 10% B to 30% B over 20 min then to 45% B over 110 min. Pure hGX eluted at ~45 min. The hGX fraction was diluted with 3 volumes of water and lyophilized. The approximate yield per liter of bacterial culture is 1 mg. The W67A hGX mutant was prepared as for wild-type enzyme. Mutagenesis was carried out with the QuickChange kit (Stratagene), and the full-length coding sequence was verified by DNA sequencing.

Recombinant hGX was found to have the correct N-terminal sequence and was verified to be native (all disulfides formed) by electrospray ionization mass spectrometry (calculated 13, 615.5, observed 13, 614.7, $(M + H)^+$). The molar extinction at 280 nm ($\epsilon_{1\%}$) was calculated to be 15.9 (38). The corresponding values for the other sPLA2s were calculated similarly. The specific activity of the purified protein is 8.6 μmol/min/mg using the spectrofluorimetric assay (37).

Interfacial Kinetic Studies with Phospholipid Vesicles—LUVs of POPC, POPS, and POPG were prepared by extrusion in water as described (39) and stored for up to 2 days at 4 °C (or frozen and re-extruded). Phospholipid concentrations in stock solutions were determined by phosphate analysis (ammonium molybdate method). Vesicle hydrolysis studies were carried out with the indicated concentrations of LUVs (Table I) in 1.3 ml of Hanks' balanced salt solution with Mg^{2+} and Ca^{2+} (1 mM each) containing 13 μg of FABP and 1 μM 11-dansylundecanoic acid at 37 °C with magnetic stirring. After recording the base line for ~5 min (<2 pmol/min) (excitation at 350 nm and emission at 500 nm, slits 10 nm), reactions were started by the addition of sPLA2. Assays were calibrated to give moles of oleic acid formed by addition of standard oleic acid to the reaction mixture containing all components except sPLA2.

Phospholipid Head Group Specificity Studies—These were carried out with a mixed phospholipid vesicle composed of equimole amounts of POPA, POPC, POPE, POPG, POPM, and POPS. Phospholipids in $CHCl_3$ were mixed; solvent was removed *in vacuo*, and small unilamellar vesicles were prepared by sonicating a suspension of 6 mM total phospholipid in water as described (35). The pH-stat reaction mixture consisted of 8 ml of 1 mM NaCl, 1 mM $CaCl_2$, pH 8.0, 23 °C containing 250 μM total phospholipid. A 2-ml sample was removed as the minus enzyme control and transferred to a glass tube containing a mixture of deuterated lysophospholipid internal standards (d_{31}-LPA, -C, -E, -G, and -S, 610 pmol each). Then, 1.4 μg of hGX was added to initiate the reaction. After partial reaction, 3 min, a 2-ml aliquot was transferred to a tube with internal standards, and the sample was immediately quenched by addition of 16 μl of 6 M HCl followed by 3 ml of $CHCl_3$/MeOH. The sample was vortexed and kept on ice. The control aliquot was processed similarly. Within 20 min of quenching, the samples were centrifuged to separate the layers fully, and the lower layers were transferred to new tubes. Extraction was repeated with a second portion of organic solvent, and the organic layers were combined and concentrated to dryness in a Speed-Vac (Savant). Residues were stored at −20 °C and analyzed the next day by dissolving them in 0.1 ml of methanol and injecting 2 μl onto the HPLC.

Samples were analyzed by combined HPLC-electrospray ionization mass spectrometry using the HPLC method described previously (40) with minor modifications. A Vydac TP218MS52 C18 reverse phase column was run at 0.5 ml/min with solvent A (12% water, 88% methanol, with 0.5% concentrated NH_4OH) and solvent B (12% hexanes, 88% methanol, with 0.5% concentrated NH_4OH) as follows: 0–3 min/0% B, 3–20 min/0–100% B, and 20–22 min/100% B. The HPLC/mass spectrometer was configured in splitless mode, with nebulizer nitrogen at 20 pounds/square inch, drying nitrogen at 10 liters/min and 300 °C, capillary and end plate voltages at −4,000 and −3,500, respectively (positive mode) and +3,500 and +3,000, respectively (negative mode), mass scan range at 80–1,000 atomic mass units, and the ion charge control (automatic gain control) activated with the target set at 15,000. The instrument was switched from negative to positive mode at ~6 min to enable more sensitive detection of LPC at ~9 min. Ion traces for the ions of all non-deuterated and deuterated lysophospholipids were integrated to obtain the moles of each enzymatically generated non-deuterated lysophospholipid. d_0-LPG and d_{31}-LPE are isobaric and unfortunately co-elute from the HPLC column. To resolve this problem, a equimole mixture of d_{31}-LPE and d_{31}-LPA was infused into the mass spectrometry in HPLC solvent A to obtain the ratio of ion peak integrals d_{31}-LPE/d_{31}-LPA (this ratio varied by <10% in multiple runs). The, the integral of the ion peak for d_{31}-LPA measured in the sample runs was multiplied by this ratio to estimate the ion peak integral for d_{31}-LPE. Since the d_0-LPG/d_{31}-LPE ion ratio is large in these samples, the integral of the d_0-LPG + d_{31}-LPE peak was taken as the amount of d_0-LPG ions. To maximize signal-to-noise, the ion peak integrals were derived from the sum of ions whose masses are as follows: LPA (409–410); d_{31}-LPA (439–442); LPC (496–497); d_{31}-LPC (524–528); LPE (452–453); LPG (483–484); d_{31}-LPG (513–516); LPM (423–424); LPS (496–497); d_{31}-LPS (524–528).

Microtiter Plate Assay of sPLA2s for Inhibition Analysis—To each well of a 96-well microtiter plate was added the desired concentration of sPLA2 inhibitor (4 μl in Me_2SO from serial diluted stock solutions) or 4 μl of Me_2SO only for control reactions. To each well was added 150 μl of assay buffer (50 mM Tris-HCl, pH 8.0, 0.2 mM EGTA, 0.8 mM Ellman's reagent) followed by 10 μl of vesicle substrate stock solution. The latter was prepared by mixing $CHCl_3$/CH_3OH stock solutions of dithio-DMPM and DMPM to give 10 mol % dithio-DMPM in DMPM. Solvent was removed *in vacuo* for 1 h; water was added to give 2.9 mM DMPM, and small unilamellar vesicles were made by sonication in a high power bath sonicator (35). Vesicle stock solution was stored at −20 °C and sonicated after each thaw. To each well was added 1 μl of sPLA2 stock solution (typically 200–300 ng, Hamilton syringe). The reactions were started by addition of 10.5 μl of 20 mM $CaCl_2$ using a multichannel pipettor (to give 1 mM $CaCl_2$ in excess of EGTA), and the absorbance at 412 nm was read with a microtiter plate spectrophotometer (30 readings per well, 5- intervals). Control reactions without enzyme or inhibitor were routinely run. The XI_{50} values were obtained from the analysis based on at least 6 inhibitor concentrations.

Cell Culture Studies—HEK293 cells (from ATCC) and Swiss-3T3 cells (from H. Herchman, UCLA (41)) were cultured in RPMI 1640 and DMEM (low glucose) medium, respectively, with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37 °C in a humidified atmosphere of 5% CO_2. CHO-K1, pgsA-745, and pgsD-677 cells were grown as described (23). For arachidonic acid release studies, cells (2×10^5 for Swiss-3T3, 5×10^5 for HEK293, and 5×10^4 for CHO-K1, pgsA-745, and pgsD-677 cells) were seeded in 1 ml of medium in each well of a 24-well plate and grown overnight (or 2 days for CHO-K1, pgsA-745, and pgsD-677 cells) to near confluence. [^{3}H]Arachidonic acid (0.1 μCi, 200 Ci/mmol, American Radiolabeled Chemicals) was added to each well, and the cells were incubated for 24 h. Cells were washed three times with complete medium, and 1 ml of complete medium was added to the washed cells followed by various amounts of sPLA2s. Cells were incubated at 37 °C for the

desired times; supernatants were removed and briefly microcentrifuged to remove any detached cells, and 0.5 ml of supernatants were submitted to scintillation counting. To the remaining cell layer was added 1 ml of $CHCl_3/CH_3OH$ (2/1), and the sample was submitted to scintillation counting after removal of the organic solvent *in vacuo*. [^{3}H]Arachidonic acid release is expressed as a percentage of the cell medium counts/min to the total counts/min (medium + organic extract). In some studies with HEK293 cells, interleukin-1β (1 ng/ml) was added to the culture medium 10 min before the additions of sPLA2. In some experiments, radioactive material was extracted into Dole organic solvent, and the extract submitted to silica chromatography to separate radiolabeled phospholipid from free arachidonic acid (42).

For PGE_2 production studies, parental HEK293 cells and cells expressing COX-1 or COX-2 isoforms (21) were seeded in 48-well plates (5×10^4 cells/ml in 500 μl of culture medium) and cultured for 4 days. Cells were incubated with sPLA2s (conditions in figure legends) in 100 μl of medium, and supernatants were taken for PGE2 enzyme immunoassay (Cayman Chemicals).

The FABP-based assay of mammalian cell hydrolysis was carried out at 37 °C using the same components as for the LUV studies except that vesicles were replaced by live cells. To study adherent cells, HEK293 cells were grown on glass coverslips (1 × 2 cm). One coverslip was positioned in the 1 × 1-cm fluorescence cuvette against the wall opposite the wall closest to the excitation light source, and a second coverslip was mounted against the wall opposite the wall facing the detector. Calibration with standard oleic acid revealed no change in fluorescence sensitivity due to the presence of cell-coated coverslips. After recording the base line for a few minutes, sPLA2 was added, and the signal was recorded for a few minutes (Fig. 7). Cells were dislodged from the coverslips by removing 1 ml of buffer with a Pipettor and squirting the buffer over the coverslip (repeated several times), and the fluorescence was monitored for an additional few minutes.

Studies to determine the binding of sPLA2s to HEK293 cells were carried out by adding sPLA2 (100 ng) to wells containing confluent cells in 1 ml of complete medium at 37 °C. At various times (see "Results"), aliquots (10 μl for hGIIA-N1A, 15 μl for hGX, 5 μl for mGIB, 100 μl for rGV, and 5 μl for cobra-GIA) were added to an sPLA2 fluorimetric assay mixture containing 1-palmitoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphomethanol to determine the amount of sPLA2 as described (37).

The glycosaminoglycan contents of CHO-K1, pgsA-745, and pgsD-677 cells were determined by radiolabeling cells with [^{35}S]sulfate followed by HPLC analysis (43). Cells (2×10^5) were seeded in a 10-cm dish and grown for 4 days in the presence of 100 μCi of sodium [^{35}S]sulfate (0.4 mCi/mg, Amersham Pharmacia Biotech) in sulfate-depleted Ham's F12 medium (Specialty Media Inc.), and half of the processed material was analyzed by HPLC using a TSK-DEAE-3SW column (0.75 × 15 cm, Toso Haas) that was equilibrated with buffer A (0.2 M NaCl in 10 mM potassium phosphate, pH 6.0). After washing the column for 5 min, a gradient was started (0–100% buffer B (1 M NaCl in 10 mM potassium phosphate, pH 6.0, over 100 min) at a flow rate of 1 ml/min. One-ml fractions were collected and submitted to scintillation counting. Chondroitin sulfate A and heparan sulfate standards were obtained from Sigma.

RESULTS

Preparation of Recombinant hGX—We developed a system for preparation of properly folded hGX from an *E. coli* expression system using a synthetic gene that is similar to our procedure for mouse group IID sPLA2 (7). Inclusion body protein was solubilized in denaturant, and cysteine residues were sulfonated to disrupt inter- and intramolecular disulfides (37, 44). Denaturant concentration was lowered by dialysis to initiate refolding in the presence of cysteine to remove sulfonates and allow disulfide formation. During refolding, substantial amounts of protein precipitated, which is typical with this procedure (37, 44). Attempts to improve the yield of refolded protein by rapid dilution in buffer containing non-detergent sulfobetaine (45) were unsuccessful. Refolded and soluble fusion protein was treated with recombinant factor Xa to liberate the native N terminus of hGX. This step proceeded in 20–30% yield, and intact fusion protein remained if the amount of factor Xa was further increased. This suggests that remaining uncleaved fusion protein may not be present as solitary monomers in aqueous solution. Folded hGX eluted from a reverse phase HPLC column as a sharp peak and was judged to be pure by SDS-polyacrylamide gel electrophoresis. The molecular weight of this material agrees with the calculated value to within 0.8 atomic mass units indicating that recombinant hGX has no free SH groups as expected for a properly folded protein with eight disulfides. Likewise, the mass of other sPLA2s used in this study were all shown to be <1 atomic mass unit away from the calculated values. It is important to note that all sPLA2s used in this study do not contain peptide extensions on their N and C termini. The x-ray structures of several sPLA2s show that the N terminus is part of a hydrogen-bonding network involving catalytic residues (46), and N-terminal extended sPLA2s generally have lower lipolysis specific activities.

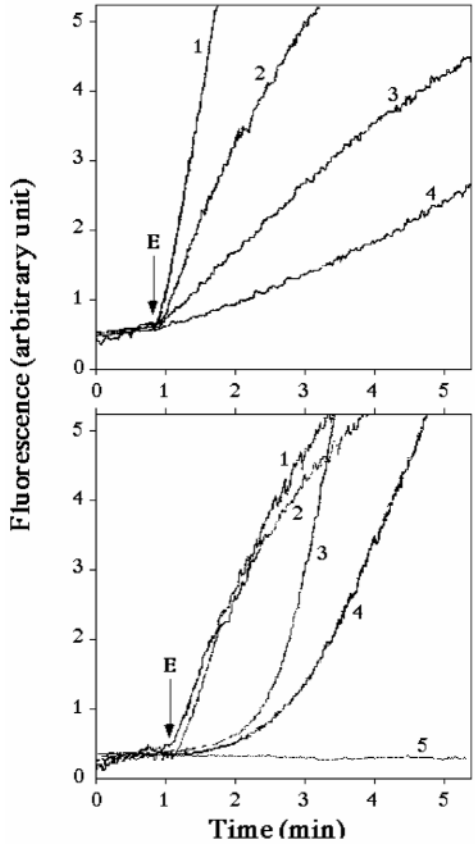

FIG. 1. **Reaction progress curves for the hydrolysis of phospholipid LUVs by sPLA2 obtained with the FABP assay.** Enzyme was added at the *arrow*. One fluorescence unit corresponds to 74 pmol of oleic acid. *Top panel*, *curve 1*, 10 μM POPG + 84 ng hGX-W67A; *curve 2*, 10 μM POPC + 140 ng hGX; *curve 3*, 30 μM POPC + 504 ng hGX-W67A; *curve 4*, 10 μM POPC + 504 ng hGX-W67A. *Lower panel, curve 1*, 30 μM POPG + 0.85 ng hGIIA-N1A; *curve 2*, 10 μM POPG + 7 ng mGIB; *curve 3*, 30 μM POPC +700 ng mGIB; *curve 4*, 10 μM POPC + 700 ng mGIB; *curve 5*, 30 μM POPC + 3,400 ng hGIIA-N1A.

Hydrolysis of Phospholipid Vesicles by sPLA2s—As a first step to characterize the interfacial properties of hGX in comparison to other sPLA2s, we determined the specific initial reaction velocities of this enzyme acting on vesicles of single phospholipid species of the type POP*X*, where *X* designates choline, glycerol, and L-serine (Table I). The use of the real time fluorescence assay with FABP and 11-dansylundecanoic acid allows accurate initial velocities to be obtained (47). This is especially important in the case of interfacial catalysis since the reaction progress curve may contain a lag phase ((48) and see below), and thus fixed time point assays may greatly overestimate the initial velocity. Representative reaction progress curves are shown in Fig. 1. The specific activities of sPLA2s acting on vesicles are a reflection of not only the affinity of the

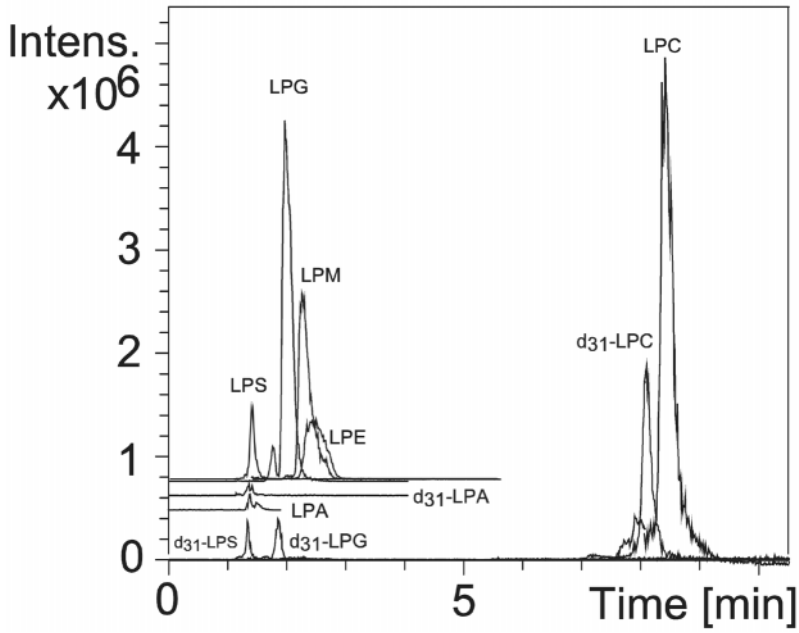

FIG. 2. **HPLC/mass spectrometry analysis of lysophospholipids produced from the action of hGX on mixed phospholipid vesicles.** For each lysophospholipid species, ions whose masses are in the specified range (see "Experimental Procedures") were detected, and these ion intensities (*Intens.*) were added to give the plotted ion intensity as a function of HPLC elution time.

TABLE I

Specific activities for the sPLA2-catalyzed hydrolysis of phospholipid LUVs

All specific activities are micromoles of fatty acid/min/mg sPLA2. Standard errors in all numbers were determined to be <20% from duplicate or triplicate analyses.

sPLA2	10 μM POPG	30 μM POPG	10 μM POPS	30 μM POPS	10 μM POPC	30 μM POPC	Dislodged HEK293 cells[a]
mGIB	20	14	1.9	2.1	Lag, 0.18[a]	Lag, 0.27	77×10^{-3}
hGIIA-N1A	144	144	28	37	$<5 \times 10^{-4}$	$<5 \times 10^{-4}$	5.5×10^{-3}
rGV	5.1	6.4	0.4	0.4	2.2	1.7	183×10^{-3}
hGX	9.1	8.5	1.2	1.5	0.9	0.9	50×10^{-3}
hGX-W67A	4.7	4.8	1.2	1.4	Lag, 0.04	0.12	ND[c]
Cobra-GIA	10	12	6.7	6.2	17	30	664×10^{-3}

[a] The FABP assay detects oleate, linoelate, palmitate, and stearate with similar reponses, and response to arachidonate is ~3-fold lower per mol.
[b] In cases where a lag was seen, the specific activity after the lag phase is given.
[c] ND, not.

sPLA2 for the vesicle (interfacial binding) but also of the catalytic efficiency for the hydrolysis of the POP*X* species by the sPLA2 bound at the lipid-water interface (active site specificity) (2).

Information about active site specificities were obtained by determining the amount of lysophospholipid species formed by the action of sPLA2 tightly bound to the interface of a mixed POP*X* vesicle (49). Such specificity is a function of the kinetic parameters for the action of the enzyme at the interface on substrate at the interface (k_{cat}* and K_m*) (2).

In the present study, the preferences of the active site of hGX bound to vesicles for phospholipids with different polar head groups was determined by allowing the enzyme to partially hydrolyze a vesicle composed of equimole amounts of POPA, POPC, POPE, POPG, POPM, and POPS. The amount of each lysophospholipid species formed was quantified in a single HPLC/mass spectrometry analysis (Fig. 2). This method is superior to the previously reported radiometric approach (49) since the latter method can only be carried out with a pair of phospholipids (one tritiated and the other labeled with carbon 14), and the former method is carried out with readily available nonradiolabeled phospholipid. Under low conversion conditions, *i.e.* when each of the phospholipid species has been hydrolyzed by approximately <10%, the ratio of lysophospholipids formed approximates the ratio of initial velocities for the hydrolysis of competing pairs of phospholipids in the mixed vesicle. These ratios are equal to the ratio of interfacial k_{cat}*/K_m* values for each pair of substrates since the ratio X_1/X_2 for each pair is unity in the equimole phospholipid mixed vesicle. The use of an equimole mixture of deuterated lysophospholipid internal standards eliminates problems of quantification of enzyme-generated non-deuterated lysophospholipids due to differential losses of material during sample preparation and due to differential ionization efficiencies in the mass spectrometer. The latter are significant; LPC ionizes much more efficiently than does LPA for example (Fig. 2). Table II gives the ratio of values of interfacial k_{cat}*/K_m for every pair of phospholipids in the mixed vesicle.

The results in Tables I and II are summarized as follows. All of the mammalian sPLA2s display highest activity on anionic POPG vesicles. The fact that the specific activity for each enzyme is invariant to a change in the POPG concentration from 10 to 30 μM (Table I) implies that the enzymes are fully bound to vesicles at these lipid concentrations. It is becoming apparent that most, if not all, sPLA2s bind tightly to anionic vesicles including phosphatidylmethanol vesicles (50). The active site of hGX displays the rank order preference POPE > POPG > POPC > POPS > POPA (Table II) which is in marked contrast to that seen with hGIIA (PG ≈ PA ≫ PS ≈ PE > PC) (51) and with hGV (PC ≈ PG ≈ PA > PE > PS) (52). Thus, the high activity of hGX on POPG vesicles is the result of a combination of its high affinity for these vesicles and its ability to well accommodate POPG in its active site. The same can be said for mGIB, rGV, hGIIA-N1A, and cobra-GIA also binds tightly to POPG vesicles. All sPLA2s studied bind tightly to anionic POPS vesicles (same specific activity with 10 and 30 μM lipid, Table I). The lower specific activities of the mammalian sPLA2s acting on POPS vesicles compared with those for

TABLE II
Relative interfacial substrate specificity constants (k_{cat}/K_m*) for the hydrolysis of phospholipids with different polar head groups by hGX*

	POPE	POPG	POPC	POPS	POPA
POPE	1.0[a]				
POPG	2.78	1.0			
POPC	11.0	4.0	1.0		
POPS	16.3	5.9	1.5	1.0	
POPA	30.4	10.9	2.8	1.9	1.0

[a] Each entry is the k_{cat}*/K_M* for the phospholipid in the column divided by the k_{cat}*/K_M* of the phospholipid in the row. Errors in all numbers are <15% based on duplicate analyses.

POPG vesicle hydrolysis is the result of active site specificity for POPG *versus* POPS (Table II (51, 52)). In light of the possible role of sPLA2s in producing the second messenger lysophosphatidic acid from PA (53), it is interesting to note that hGX displays a dramatic discrimination against PA compared with hGIIA and hGV.

Dramatic differences are seen among the sPLA2s for the hydrolysis of POPC vesicles. hGIIA-N1A is $>3 \times 10^6$-fold more active on POPG vesicles than on POPC vesicles, which is consistent with earlier studies (12). This is due to the combination of poor interfacial binding of hGIIA-N1A to zwitterionic vesicles and to poor active site accommodation of POPC. A hallmark of cobra-GIA is its ability to bind tightly to phosphatidylcholine vesicles (16), and the data in Table I reflect this fact. As with hGV (17, 52), rGV is fully bound to 10 and 30 μM POPC LUVs and displays high activity on these vesicles (POPC is well accommodated by its active site (52)). The interfacial kinetics of mGIB acting on POPC are complex. The initial velocity is close to zero, and the velocity accelerates over several minutes (Fig. 1). Such a lag has been reported for the action of porcine pancreatic sPLA2 on phosphatidylcholine vesicles and is due in part to the fact that reaction products (fatty acid and lysophospholipid) promote the binding of enzyme to the interface (54). Thus the real time sPLA2 assay reveals that mGIB binds weakly to POPC vesicles that lack reaction products. The data in Table I establish that hGX, like rGV and cobra-GIA, binds tightly to POPC vesicles. The lower specific activity for the hydrolysis of POPC *versus* POPG LUVs by hGX is the result of active site discrimination (Table II).

It has been recently shown that tryptophan on the interfacial binding surfaces of hGV and of the V3W mutant of hGIIA plays an important role in supporting interfacial binding to phosphatidylcholine (12, 17). Trp-67 on the putative interfacial binding surface of hGX plays an important role in supporting the high affinity of this enzyme for POPC vesicles. hGX-W67A, unlike wild-type enzyme, displays a lag phase when acting on 10 μM POPC vesicles (Fig. 1). The fact that the lag is not seen with 30 μM LUVs (Fig. 1) strongly suggests that the lag is due to product-induced binding of enzyme to POPC vesicles, which in turn suggests that hGX-W67A is not fully bound to 10 μM POPC LUVs. The data in Table I and the absence of a lag also show that Trp-67 is not very important for the binding of hGX to anionic POPG and POPS vesicles.

Inhibition Studies with LY311727 and Pyr-1—LY311727 (55) and Pyr-1 (31) were tested as inhibitors of hGX and hGIIA-N1A using a colorimetric, scooting mode assay (56) of sPLA2 comprised of 10 mol % dithio-DMPM in DMPM vesicles, which was carried out with a microtiter plate reader. The advantages of using a scooting-mode vesicle assay in which enzyme is tightly bound to the vesicle to analyze inhibitors of sPLA2 have been extensively documented (57–59). The concentrations of LY311727 required to produce a 2-fold reduction in the initial velocity of hydrolysis of dithio-DMPM (IC_{50} values) are 0.6 ± 0.2 μM for hGIIA-N1A and 1.5 ± 0.2 μM for hGX (data not shown). These concentrations correspond to mole fractions of 0.0034 and 0.0086 (XI_{50} values) of inhibitor in the presence of 175 μM phospholipid in the assay. This modest 2.5-fold selectivity of LY311727 in favor of hGIIA-N1A *versus* hGX is a lower limit since hGIIA-N1A displays an active site preference for substrates with anionic head groups such as phosphomethanol, whereas hGX prefers phosphoethanolamine (see above). This factor will raise the XI_{50} for LY311727 acting on hGIIA-N1A *versus* hGX as the inhibitor is competing with the tighter binding dithio-DMPM substrate in the case of hGIIA-N1A. These factors complicate the use of LY311727 in cell studies to establish the involvement of a particular sPLA2 because the inhibitor is competing with a complex mixture of cellular phospholipids that display differential affinity for the active sites of sPLA2s. The only solution to this problem is the availability of sPLA2 inhibitors that are more selective than LY311727.

The IC_{50} values for the inhibition of hGIIA-N1A and hGX by Pyr-1 are 0.09 ± 0.03 and 11 ± 3 μM, respectively (XI_{50} values of 5.1×10^{-4} and 0.062) (data not shown). The 120-fold selectivity of Pyr-1 for hGIIA-N1A *versus* hGX is sufficient to justify the use of this inhibitor for distinguishing between these two enzymes in cell studies.

Action of Exogenously Added sPLA2s on HEK293 and Swiss-3T3 Cells—The ability of exogenously added sPLA2s to liberate arachidonic acid from membrane phospholipids of HEK293 and Swiss-3T3 cells was studied by addition of various amounts of enzymes to the complete culture medium and measuring the amount of tritium released into the medium after 6 h from [^{3}H]arachidonic acid-labeled cells. In these studies, cells were left attached to the bottom of the plastic tissue culture dishes. Results are summarized in Fig. 3. Under these conditions, no detectable release of arachidonate from HEK293 and Swiss-3T3 cells was seen 6 h after the addition of up to 1,000 ng/ml amounts of hGIIA-N1A. Cells were also resistant to the action of rGV; only a trace of arachidonate release was detected with 1,000 ng/ml enzyme. In addition, no significant arachidonate release was detected when HEK293 cells in Hanks' balanced salt solution with 1 mM $CaCl_2$ and $MgCl_2$ rather than complete medium were treated with 100 ng/ml rGV for 4–6 h (not shown). mGIB at 10 and 100 ng/ml produced no detectable arachidonate release, but significant release occurred with 1,000 ng/ml enzyme. Interestingly, hGX, like cobra-GIA, efficiently released arachidonate from both mammalian cells (confirmed to be free [^{3}H]arachidonate by chromatography on silica, see "Experimental Procedures"). Even 10 ng/ml hGX was sufficient to give significant release, and release was comparable to that released by cobra-GIA over the whole concentration range. As shown in Fig. 3, addition of 1 ng/ml interleukin-1β to HEK293 cells causes a slight increase in [^{3}H]arachidonate release in all cases including in the absence of added sPLA2.

Time course studies for the release of arachidonate from HEK293 and Swiss-3T3 cells are shown in Fig. 4. Arachidonate release by exogenous hGX and cobra-GIA when added to cells at 100 ng/ml have virtually identical progress curves. hGX-W67A behaves similarly to wild-type enzyme on Swiss-3T3 cells but shows a statistically significant delay in arachidonate release from HEK293 cells (Fig. 4).

In order to examine whether arachidonate release by exogenous hGX is converted to PGE_2 by the downstream cyclooxygenases, we took advantage of HEK293 cells stably transfected with human COX-1 and COX-2 (21). As shown in Fig. 5*A*, as little as 10 ng/ml exogenous hGX elicited PGE_2 generation via COX-2 in marked preference to COX-1. With 1 μg/ml hGX, the level of PGE_2 reached 1.7 ng/10^6 cells after 4 h from COX-2-expressing cells, whereas only 0.06 ng/10^6 of PGE_2 was produced from COX-1-expressing cells (Fig. 5*B*). In contrast, 10 μM

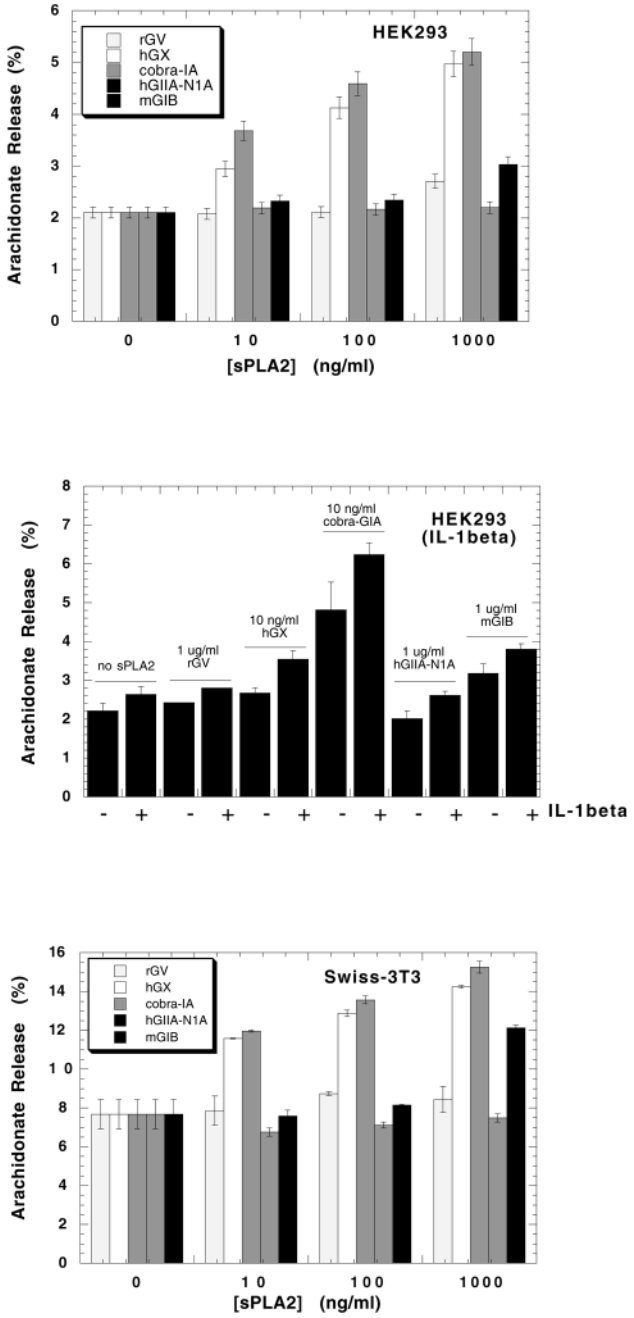

FIG. 3. **Release of [^{3}H]arachidonate into the medium from HEK293, in the presence and absence of interleukin-1β, and from Swiss-3T3.** Release after 6 h of treatment with the indicated concentrations of sPLA2s is expressed as a percentage of the cell medium counts/min to the cell-associated counts/min (organic extract). *Error bars* show the S.D. from duplicate or triplicate runs. For the *middle plot*, different amounts of sPLA2 were used in the presence (+) and absence (−) of 1 ng/ml human interleukin-1β.

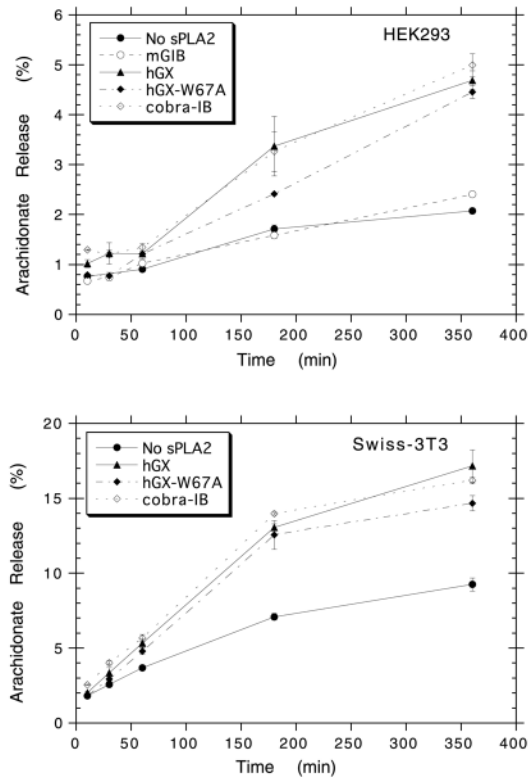

FIG. 4. **Time course of arachidonate release from HEK293 (*top panel*) and Swiss-3T3 (*lower panel*) cells by 100 ng/ml exogenous sPLA2s.** *Error bars* show the S.D. from duplicate runs (invisible error bars are hidden by *symbols*).

exogenously added arachidonate was converted to 3.7 and 4.1 ng of PGE_2 per 10^6 COX-1- and COX-2-expressing cells, respectively. Cells that had not been transfected with COX-1 and COX-2 produced virtually no detectable PGE_2 when treated with 1 μg/ml hGX. As shown in Fig. 5*C*, 1 μg/ml hGIIA-N1A and rGV produced minimal amounts of PGE_2 when added exogenously to COX-2 expressing HEK293 cells.

Studies with Normal and Glycosaminoglycan-deficient CHO-K1 Cells—It has already been mentioned that endogenously expressed hGIIA and probably hGV become attached to heparan sulfate-containing glypican on the surface of HEK293 cells (25). Both of these sPLA2s are highly basic proteins (calculated pI of 9.4 and 8.7, respectively) and bind to heparan sulfate and heparin *in vitro* (23, 60). On the other hand, hGX is acidic (calculated pI of 5.1) and fails to bind to heparin even at low ionic strength (not shown). To test the hypothesis that binding of hGIIA-N1A and rGV to cell surface heparan sulfate protects the plasma membrane from lipolysis by exogenously added sPLA2s, we studied arachidonate release from CHO-K1 cells and CHO-K1 lines that have mutations in glycosaminoglycan biosynthetic enzymes and are deficient in heparan sulfate (pgsD-677) and in total glycosaminoglycan (pgsA-745) (61). To confirm the phenotype of these cells, sulfated glycosaminoglycans were radiolabeled by culturing cells in sulfate-depleted medium containing radiolabeled sulfate. Total cellular glycosaminoglycan was isolated using standard methods and analyzed by ion exchange HPLC (see under "Experimental Procedures"). Results were virtually identical to those reported previously (43) and show that whereas chondroitin sulfate and heparan sulfate are readily detected in samples prepared from wild-type CHO-K1 cells, no detectable heparan sulfate is present in pgsD-677 cells, and these mutant cells contain 2–3-fold higher levels of chondroitin sulfate compared with wild-type cells. pgsA-745 cells contain no detectable heparan sulfate and may contain 1–2% of the wild-type level of chondroitin sulfate.

Up to 1,000 ng/ml exogenously added hGIIA-N1A failed to produce detectable free arachidonate over a 6-h period when added to CHO-K1, pgsD-677, and pgsA-745 cells (Fig. 6). Exogenous rGV and mGIB were also poorly active on all three cell lines when tested up to 1,000 ng/ml for 6 h. Like with HEK293 and Swiss-3T3 cells, hGX and cobra-GIA effectively released arachidonate from wild-type and mutant CHO-K1 cells. These results establish that binding of the basic sPLA2s hGIIA-N1A and rGV to heparan sulfate is not the basis for protection of the

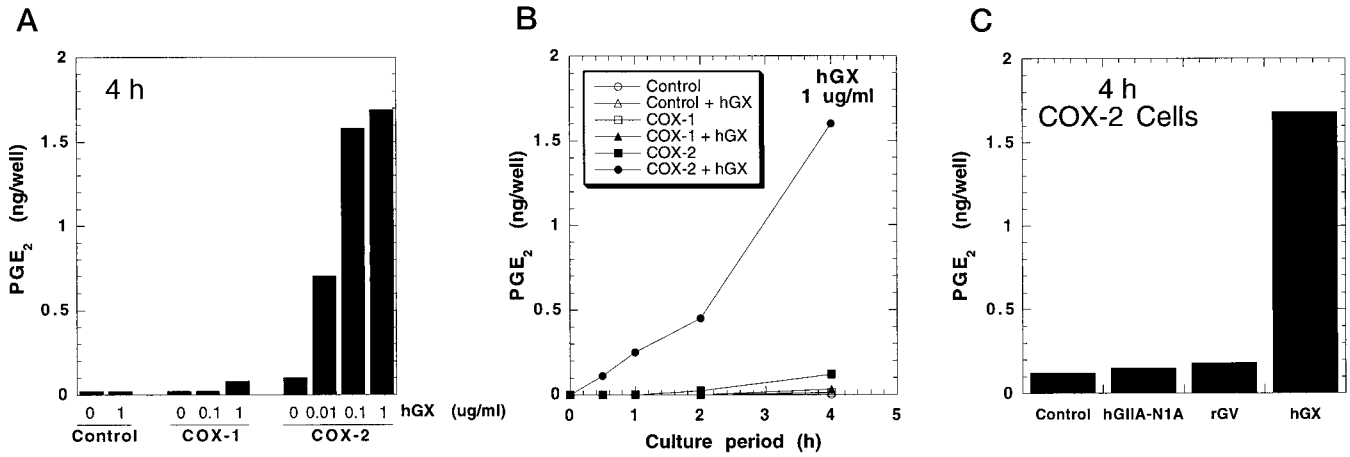

FIG. 5. **PGE_2 generation by exogenous hGX via COX-2.** *A,* HEK293 cells expressing either human COX-1 or COX-2 and control cells were incubated for 4 h with various concentrations of hGX in culture medium containing 10% serum. *B,* cells were cultured for the indicated periods with or without 1 μg/ml hGX. *C,* COX-2-expressing HEK293 cells were cultured for 4 h with 1 μg/ml of the indicated sPLA2 (no sPLA2 for control). Representative results of three independent experiments are shown.

extracellular face of the plasma membrane from lipolysis by these sPLA2s.

Distribution of sPLA2s between the Medium and the Surface of HEK293 Cells—The various sPLA2s were added at 100 ng/ml to wells containing a layer of surface-attached HEK293 cells in complete medium. After various periods of incubation at 37 °C, an aliquot of the medium was withdrawn and assayed for sPLA2 activity using a fluorimetric assay (see under "Experimental Procedures"). With mGIB, rGV, hGX, and cobra-GIA, the activity did not detectably decrease over 4 h when compared with an aliquot taken from a glass tube in which 100 ng/ml sPLA2 was added to complete medium in the absence of cells (data not shown). The sPLA2 activity decreased by less than 10% over 4 h when enzyme was added to complete medium in a well in the absence of cells. These results show that these sPLA2s do not significantly bind to HEK293 cells or to the plastic well surface. With hGIIA-N1A, activity decreased in a time-dependent manner in wells with or without confluent HEK293 cells, but the decrease was reproducibly slower in the presence of cells (data not shown). After 10 min, the activity in the supernatant decreased to 38% in wells with cells and to 15% in wells without cells, and activity continued to fall to near 0% in wells with and without cells. These results show that hGIIA-N1A binds to the plastic well surface and that the presence of a layer of HEK293 cells slows this nonspecific binding. The only conclusion that can be drawn from these studies is that most of the hGIIA-N1A does not bind rapidly to HEK293 cells (*i.e.* in <10 min).

Arachidonate Release from Dislodged HEK293—In previous studies with hGIIA-N1A and human group V sPLA2, it was found that 1 μg/ml of the former and 50 ng/ml of the latter were able to release fatty acids when added exogenously to RAW264.7 macrophage-like cells, NIH-3T3 cells, and CHO-K1 cells (FABP assay) (17, 23). The marked discrepancy between these results and those in Figs. 3, 4, and 6 showing no arachidonate release by hGIIA-N1A and rGV may be due to the fact that in the previous study, cells were detached from the culture dish with trypsin or with an EDTA-based cell dissociation solution, whereas in the present study, cells were left attached to the dish surface. To investigate this further, we extended the use of the FABP real time fatty acid release assay for the study of adherent cells by mounting coverslips containing attached HEK293 cells in the fluorescence cuvette (see under "Experimental Procedures"). As shown in Fig. 7, after addition of exogenous rGV (1 μg/ml) to attached cells, no fatty acid release was detected. This assay can readily detect 10 pmol of oleic acid. When the cells were dislodged from the coverslips by pipetting the liquid up and down a few times, fatty acid release was immediately detected without addition of more enzyme (Fig. 7). Similar results were obtained with hGIIA-N1A except that larger amounts of sPLA2 (3 μg/ml) were needed to see slow fatty acid release after dislodging the cells (Fig. 7). These results confirm our previous studies that human group V sPLA2 and hGIIA-N1A release fatty acids from dislodged mammalian cells. No fatty acid release was detected when HEK293 cells bound to coverslips were treated with 1–3 μg/ml mGIB, cobra-GIA, and hGX, but fatty acid release was readily detected after cells were dislodged (not shown). The specific activities for the hydrolysis of dislodged HEK293 cells by all of the sPLA2s are listed in Table I. hGIIA-N1A stands out as having the lowest activity on dislodged cells, which is presumably due to its very low affinity for phosphatidylcholine-rich interfaces and its low active site preference for this zwitterionic class of phospholipids.

We also found that CHO-K1 cells become more sensitive to exogenous sPLA2s when they are dislodged from coverslips (not shown). When the dislodged CHO-K1 cells in the reaction mixture with rGV were pelleted by low speed centrifugation and the supernatant transferred back to the fluorescence cuvette, no fatty acid release was detected. This shows that sPLA2-catalyzed fatty acid release is due to the action of the sPLA2 on the cell membrane rather than on microvesicles that may be shed from cells during the dislodgement process.

We also studied dislodged HEK293 cells that were labeled with [^{3}H]arachidonate since the radiometric method is more sensitive than the FABP assay for free fatty acids. When adherent radiolabeled HEK293 cells were incubated in complete medium for 2 h at 37 °C, 0.4 ± 0.1% of cellular counts/min was released into the medium. A dramatic increase in counts/min released into the medium (11 ± 4%) occurred when cells were dislodged as above and allowed to incubate for 2 h at 37 °C. Most of this released counts/min is intact phospholipid, probably microvesicles shed from the cells following dislodgement, since 93% of the counts/min was retained on silica gel (see under "Experimental Procedures"). When cells were dislodged in the presence of 100 ng/ml hGX or cobra-GIA, released counts/min after 2 h was 11 ± 0.5 and 18 ± 1%, respectively. When this released counts/min was extracted and submitted to silica chromatography, it was found that 44 and 49% of it was [^{3}H]arachidonate in the presence of hGX and cobra-GIA, re-

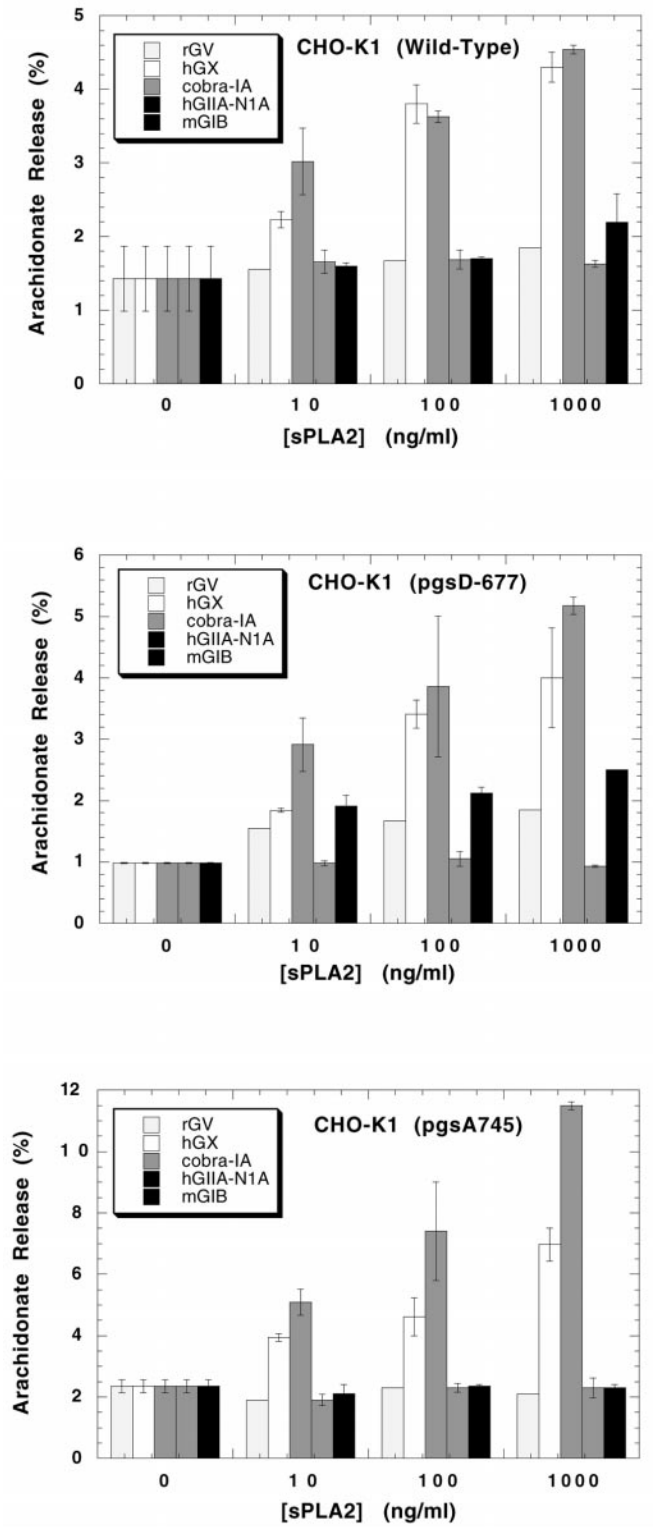

FIG. 6. **Release of [^{3}H]arachidonate into the medium from CHO-K1, pgsD-677, and pgsD-745 cells.** Release after 6 h of treatment with the indicated concentrations of sPLA2s is expressed as percentage of the cell medium counts/min to the cell-associated counts/min (organic extract). *Error bars* show the S.D. from duplicate or triplicate runs.

spectively. In contrast, 90% of the counts/min released from dislodged cells in the presence of 1 μg/ml hGIIA-N1A is intact phospholipids.

To assess whether [^{3}H]arachidonate release from dislodged HEK293 cells in the presence of exogenous hGX and cobra-GIA is coming from dislodged cells or from microvesicles, radiolabeled cells were dislodged and submitted to centrifugation to pellet the cells leaving the microvesicles in the supernatant (62). The supernatant (1 ml) as well as the cell pellet after resuspending the cells in 1 ml of complete medium were treated with 100 ng/ml hGX, cobra-GIA, or human group V sPLA2 for 4 h at 37 °C. Reaction mixtures were extracted and submitted to silica chromatography to obtain the counts/min of free [^{3}H]arachidonate. With hGX, [^{3}H]arachidonate from the microvesicle fraction amounted to 2.3 ± 0.1%, based on total sample counts/min (compared with 0.8 ± 0.05% in the absence of sPLA2), and [^{3}H]arachidonate from the dislodged cells amounted to 9.5 ± 0.6% (compared with 2 ± 0.3% from the control). The corresponding numbers with cobra-GIA are 3.2 ± 0.2 and 16.8 ± 1.5% from microvesicles and cells, respectively. With human group V sPLA2, the numbers are 0.9 ± 0.07 and 5.6 ± 1% from microvesicles and cells, respectively. Thus the majority of the free [^{3}H]arachidonate comes from the dislodged cells. This result is consistent with the FABP-based assay and CHO-K1 cells described above.

DISCUSSION

Interfacial Kinetic Analysis of hGX in Comparison to Other sPLA2s—hGX and rGV can fully bind to micromolar concentrations of phosphatidylcholine vesicles, whereas mGIB binds weakly, and hGIIA-N1A does not bind to zwitterionic vesicles. Examination of the amino acids present on the interfacial binding surfaces of these enzymes provides a starting point to explain differences in their interfacial binding behavior. Since the tertiary structures of sPLA2s are well conserved (46), a model of rGV and hGX sPLA2s can be constructed by overlaying their amino acid sequences onto the structure of hGIIA (46). Spectroscopic studies have shown that the interfacial binding surfaces of sPLA2s comprise the surface of the protein that surrounds the opening to the active site slot (10, 19, 63). Like human, mouse, and rat group V sPLA2s (W31A), human, mouse, and rat group X sPLA2s contain a tryptophan on their putative interfacial binding surfaces (9). As for the tryptophan to alanine mutant of human group V sPLA2 (17), hGX-W67A displays significantly lower affinity for zwitterionic POPC vesicles (Fig. 1), although the effect of mutation seems to be smaller for hGX than for human group V sPLA2. The W67A mutation does not seem to cause a structural change that significantly reduces the catalytic power of the active site since hGX-W67A is only 1.8-fold less active than hGX on anionic POPG and POPS vesicles (Table I). With anionic vesicles, electrostatic interfacial binding is possible, which may alleviate the need for tryptophan.

Our working hypothesis is that tryptophan inserts into the region of the bilayer at the boundary between the phospholipid hydrocarbon and the polar head group regions. Such a proposal is based on the observations that indole analogs reside in membranes in the region close to the glycerol backbone (64) and that tryptophan in transmembrane protein helices is not present in the center of the bilayer but tends to appear near the lipid-water interface (65). Because tryptophan has an indole NH group and π electrons, it is more polar than the other hydrophobic amino acids and thus would prefer the region of the bilayer with intermediate polarity. The smaller effect of the W67A mutation on the ability of hGX to hydrolyze mammalian cell membranes (Fig. 4) *versus* POPC vesicles (Table I) is presumably a consequence of other phospholipids such as small amounts of anionic phospholipids that may be present in the outer leaflet of the plasma membrane of mammalian cells.

The dramatically higher affinity of hGX and rGV *versus* hGIIA-N1A for POPC vesicles is due to other factors in addition to the presence of tryptophan on the former but not on the

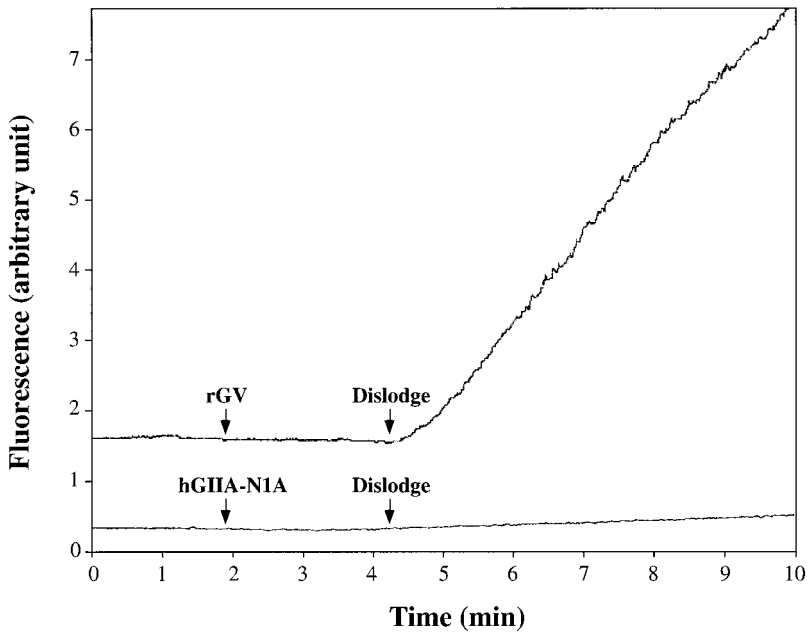

FIG. 7. **Reaction progress curves for the hydrolysis of HEK293 cells by sPLA2 obtained with the FABP assay.** Coverslips containing adherent cells were placed in the cuvette, and enzyme was added at the *1st arrow* (1 μg of rGV, 3 μg of hGIIA-N1A). At the *2nd arrow*, the cells were dislodged by pipetting the buffer up and down a few times (see "Experimental Procedures"). One fluorescence unit corresponds to 166 pmol of oleic acid.

latter. Previous mutagenesis studies identified Arg-7, Lys-10, and Lys-15 (Arg-7, Lys-10, and Lys-16 in the common numbering system (66)) of hGIIA as being the most important basic residues for promoting high affinity binding to anionic vesicles (11), and presumably these residues disfavor binding to zwitterionic phosphatidylcholine vesicles. These residues lie on the same face of the N-terminal α-helix that packs against the phospholipid bilayer (19, 63, 67). hGX contains glycine residues replacing Arg-7 and Lys-10 and a proline residue in place of Lys-15, and these changes are expected to contribute to the enhanced binding of hGX to POPC vesicles. rGV contains Lys-15 but has a serine replacing Arg-7 and a glutamate replacing Lys10, changes that are expected to compress the difference in binding affinity for anionic *versus* zwitterionic vesicles.

The amino acids surrounding the ethanolamine polar head group of a phosphatidylethanolamine analog bound to hGIIA as seen in the x-ray structure (66) are well conserved in human group V and hGX sPLA2s except at position 52 of hGIIA. Lys-52 of hGIIA may contribute to the preference of this enzyme for anionic head groups of POPG and POPA (51). This residue is replaced with glycine in rGV and with threonine in hGX, which may explain why these enzymes tolerate head groups that lack negative charge.

Action of Exogenous sPLA2s on Mammalian Cells—Among the mammalian sPLA2s studied, hGX, like cobra-GIA, is uniquely able to release efficiently arachidonic acid when added exogenously to adherent HEK293, Swiss-3T3, and CHO-K1 cells. Despite the fact that hGX, rGV, and cobra-GIA bind tightly to POPC vesicles and presumably to the phosphatidylcholine-rich extracellular face of the plasma membrane, rGV does not affect arachidonate release from the adherent cells HEK293, Swiss-3T3, and CHO-K1. rGV is a highly basic protein (pI 8.7), whereas hGX and cobra-GIA are acidic proteins (pI 5.1 and 4.93, respectively). One possibility is that rGV cannot access the plasma membrane because it is trapped on some non-membrane, anionic extracellular component (however, see below). This component cannot be sulfated glycosaminoglycan because rGV also failed to release arachidonate from CHO-K1 cells (Fig. 6) which lack this polysaccharide.

The possibility that rGV is bound to the plasma membrane but does not encounter phospholipids that are accommodated in its active site can be ruled out by the fact that this enzyme prefers phosphatidylcholine, the most abundant phospholipid in the outer leaflet of the plasma membrane. rGV failed to release arachidonate from HEK293 cells in balanced salt solution suggesting that a component of the complete culture medium is not preventing the enzyme from hydrolyzing cellular phospholipids. hGIIA-N1A is a highly cationic protein (pI 9.4) that cannot bind to phosphatidylcholine vesicles and cannot accommodate well the phosphocholine head group in its active site; all three factors probably contribute to the inability of this enzyme to release arachidonate from adherent cells. Interestingly, 10–100 ng/ml amounts of human group V sPLA2 released fatty acids and caused leukotriene B_4 production when added exogenously to human peripheral blood neutrophils, whereas exogenous hGIIA-N1A was ineffective (17). This is the expected result based on the high affinity of group V sPLA2 but not hGIIA-N1A for phosphatidylcholine-rich vesicles. Fatty acid release from neutrophils was monitored with the same FABP assay that was used to show that rGV is unable to release fatty acids from the adherent HEK293 cells (Fig. 7), thus ruling out any differences due to assay sensitivity. Although this suggests that group V sPLA2 is readily able to access the plasma membrane of neutrophils, it is possible that the process of neutrophil purification leads to some damaged cells or cell fragments that serve as preferential substrate for group V sPLA2 (see below). Further studies are needed to understand why exogenous group V sPLA2 releases fatty acids from neutrophils but not the adherent cells reported herein.

Arachidonate released by as little as 10 ng/ml hGX is available for PGE_2 production by COX-2 but not by COX-1 (Fig. 5). PGE_2 production required overexpression of COX-2 in HEK293 cells, consistent with the previous report that expression of hGX in these cells fails to induce expression of endogenous COX-2 (whereas expression of hGIIA and hGV does cause COX-2 induction) (21). Exogenous rGV and hGIIA-N1A fail to elicit PGE_2 production in cells expressing COX-1 or COX-2 (Fig. 5), consistent with the inability of these enzymes to liberate arachidonate when added exogenously. The results in Fig. 5 are consistent with our recent study (68) showing that expression of hGX in HEK293 cells leads to PGE_2 production when COX-2, but not COX-1, is co-expressed in these cells. Since arachidonate released by exogenous or expressed hGX presumably occurs from the plasma membrane, some of the free fatty acid may leave the cells by binding to albumin pres-

ent in the culture medium. If only a portion of the liberated arachidonate enters the cell, this would explain the observed lack of hGX/COX-1 coupling because we previously showed (21) that low levels of exogenously added arachidonate are used by COX-2 in preference to COX-1.

An important finding of the present study is that dislodging HEK293 cells from the surface where they were attached renders them sensitive to the action of exogenous sPLA2s (Fig. 7). The inability to detect cobra-GIA- and hGX-catalyzed fatty acid release from adherent HEK293 cells using the FABP protein assay despite the fact that these enzymes released [^{3}H]arachidonate from radiolabeled adherent cells (Figs. 3, 4, and 6) is due to the lower sensitivity of the FABP assay. With the radiometric assay, arachidonate release is monitored over hours, whereas fatty acid release over minutes must occur to be detected with the FABP assay (the fluorimetric signal is not stable enough to detect gradual fatty acid release over hours). In any case, it is clear that cell dislodgement greatly increases their susceptibility to a variety of sPLA2s. Given that a high fraction of the sPLA2s, including hGX, are present in the extracellular fluid above adherent HEK293 cells (see "Results"), any changes in the surface of cells that occur during dislodgement that leads to an increased fraction of membrane-bound sPLA2 will enhance fatty acid release. This presumably explains why even hGX is more active on dislodged cells. In our previous study (23) we found that hGIIA-N1A-catalyzed fatty acid release from dislodged wild-type and pgsD-677 and pgsD-745 mutant CHO-K1 cells occurs at the same rate (measured with the FABP assay), and thus cell surface glycosaminoglycan does not modulate the activity of this sPLA2 on dislodged cells. hGIIA-catalyzed release of fatty acids from microvesicles shed from activated platelets has been proposed as a mechanism for arachidonic acid liberation for eicosanoid biosynthesis (53). In the present study we found that fatty acid release after cell dislodgement occurs from cells or cell fragments that are pelleted by low speed centrifugation and not from microvesicles that remain in the supernatant. By using HEK293 cells labeled with [^{3}H]arachidonate, we detected significant amounts of intact phospholipid released into the culture medium after cell dislodgement (presumably as microvesicles).

The factors that contribute to enhanced sensitivity of dislodged cells to sPLA2s are unknown. Dislodgement-dependent sPLA2 activation could be due to changes in cell shape that renders the membrane more susceptible to attack by sPLA2s. Such changes could involve a change in phospholipid asymmetry across the plasma membrane. For example an increase in exposure of phosphatidylserine or other anionic lipids to the extracellular face of the plasma membrane is expected to enhance the binding of virtually all sPLA2s to the interface, especially for hGIIA-N1A. Changes in cell shape will affect membrane curvature, which may modulate the penetration of sPLA2s into the interface. Cell dislodgement may also lead to cell damage, leading to membrane fragments that are preferred substrates for sPLA2s. The possibility that these putative fragments are microvesicles has been ruled out (see "Results") at least for the studies reported herein. The important conclusions of the present study are that the numerous previous studies reporting the activity of sPLA2s on cells that have been dislodged from the surface of the growth vessel should now be interpreted with caution, in light of the results shown in Fig. 7. Furthermore, hGX is the only mammalian sPLA2 found so far that can liberate significant amounts of arachidonate when added exogenously to adherent cells (HEK293, Swiss-3T3, and CHO-K1).

The possibility that the extracellular matrix acts as a boundary to protect adherent cells from the action of basic but not acidic sPLA2s may be considered. Dislodging the cells may partially strip away matrix components and expose them to sPLA2s. Perhaps basic but not acidic sPLA2s bind to anionic components other than sulfated glycosaminoglycans (based on the findings in Fig. 6) which prevents these enzymes from reaching the cell membrane. In this context, it may be noted that hGIIA binds to the extracellular matrix proteins decorin, biglycan, and versican (69, 70). At least in the case of decorin, hGIIA seems to bind by electrostatic interaction (disrupted by high salt) to the protein core rather than the glycosaminoglycan chains.[2] The problem with the idea that the extracellular matrix protects adherent cells from the action of exogenous sPLA2s is the observation that a high fraction of sPLA2 remains in the extracellular fluid above a layer of adherent cells (see "Results"). This might be due to saturation of non-membrane sPLA2-docking sites, but then it is difficult to explain why excess unbound sPLA2 does not find its way to the plasma membrane. Overall, the simplest explanation seems to be that the unique ability of hGX and cobra-GIA to release arachidonate efficiently from adherent mammalian cells may be due to a higher fraction of these enzymes bound to the plasma membrane, relative to the other mammalian sPLA2s studied. This in turn suggests that hGX and cobra-GIA bind tighter to the plasma membrane than do the other sPLA2s. Although our studies (Fig. 1 and Table I) show that both hGX and rGV are fully bound to 10 μM POPC vesicles (thus the dissociation equilibrium constants, K_d, for the enzyme-POPC vesicle complex are <10 μM), they do not rule out the possibility that the absolute value of K_d for hGX is less than that for rGV. Methods for determining K_d values for protein-vesicle complexes of much less than micromolar are lacking (10), and efforts are underway to develop surface plasmon resonance techniques for this purpose (18). The possibility of an unknown component(s) in the plasma membrane of mammalian cells that enhances the interfacial binding of hGX and cobra-GIA also needs to be considered. The fact that hGIIA-N1A releases fatty acids from neutrophils only 10-fold slower than does human group V sPLA2 (17) despite the fact that the former enzyme binds more than 10^4-fold weaker than the latter to phosphatidylcholine vesicles argues that the neutrophil plasma membrane phospholipid composition or some element in the membrane promotes interfacial binding of both sPLA2s, which promotes interfacial catalysis on these non-adherent cells.

Despite the fact that exogenously added groups IIA and V sPLA2s are ineffective at releasing fatty acids from adherent cells, expression of these enzymes in HEK293 cells by transfection leads to serum- and interleukin 1β-dependent arachidonate release in preference to oleate release (4). These contrasting results seem paradoxical because groups IIA and V sPLA2s are expected to be secreted by the classical secretory pathway when expressed by transfection in cells and should therefore encounter the same barrier that exogenously added sPLA2 experience in reaching the plasma membrane. Very recent studies by Kudo and co-workers (25) show that hGIIA expressed by transfection appears on the proteoglycan glypican present on the surface of HEK293 cells, with only a minor amount of hGIIA ending up in the culture medium. Glypican is a glycosylphosphatidylinositol-linked protein that may be taken up by cells via entry into caveolae, and this may explain why hGIIA is seen not only on the cell surface but also as punctate domains inside the cell (25). Perhaps glypican-mediated transport of group IIA sPLA2 into cells brings this enzyme in contact with an arachidonylphospholipid-rich membrane

[2] P. Sartipy, B. Johansen, K. Gåsvik, and E. Hurt-Camejo, submitted for publication.

containing a relatively large fraction of acidic phospholipids where it can readily bind so that efficient arachidonate release occurs. However, it is difficult to understand why exogenously added hGIIA-N1A does not bind to glypican and affect arachidonate release in HEK293 cells, even in the presence of serum and interleukin-1β (see "Results"). Perhaps the binding of this sPLA2 to the surface of growth vessels prevents it from reaching glypican. Thus, it appears that hGIIA processed in HEK293 cells by the secretory pathway is somehow channeled to glypican without passing through the extracellular fluid; perhaps the hGIIA-glypican complex forms in secretory vesicles or that hGIIA is secreted from cells bound to another protein that facilitates its delivery to glypican. In contrast, expression of hGX by transfection in HEK293 cells leads to significant amounts of arachidonate and oleate release, and enzyme ends up in the extracellular culture medium (68). The amount of arachidonate release, 2–3% of the total cpm over 4 h, is similar to the release we observed with exogenous hGX (Figs. 3, 4, and 6). These results are consistent with the present study showing that exogenous hGX efficiently releases fatty acids from the plasma membrane of adherent HEK293 cells. The fact that hGX produced by transfection or added exogenously and efficiently releases arachidonate from HEK293 cells in the absence of interleukin-1β (Ref. 68 and the present study) is consistent with different mechanisms of action of hGX *versus* hGIIA and group V sPLA2s on mammalian cells. Thus a picture is emerging whereby the different mammalian sPLA2s with different interfacial binding properties and degrees of surface cationic charge operate by distinct mechanisms in mammalian cells for fatty acid release.

The inability of exogenously added hGIIA-N1A and rGV to release fatty acids from adherent cells may have important biological implications. For example, it has recently been shown that human tears contain large amounts (>30 μg/ml) of hGIIA where it is thought to function as an anti-Gram-positive bactericidal agent (71). The inability of this enzyme to act on adherent cells would protect the corneal epithelial cells from membrane degradation by such high concentrations of hGIIA. Another role of hGIIA could be to act on injured or apoptotic cells that presumably become non-adherent as suggested previously (72). Kudo and co-workers (73) have shown that low microgram per ml concentrations of hGIIA can liberate arachidonate from rat pheochromocytoma PC12 cells, mouse mast cells, and human monocytic U937 cells after they become apoptotic by stimulation with Fas antigen. Furthermore, this activity of hGIIA may be glypican-independent as mutation of basic residues of hGIIA that reduce its affinity for sulfated glycosaminoglycans has little effect on fatty acid release from apoptotic cells by exogenous enzyme. Recent studies by Bell and co-workers (62) have shown that hGIIA acts poorly on mouse S49 lymphoma cells (grown in suspension), but sPLA2-catalyzed membrane hydrolysis increases after treating the cells with lysophosphatidylcholine or by causing a rise in intracellular Ca^{2+} by addition of ionophore. Clearly there is much to be learned about how mammalian cell membranes become modified to allow interfacial catalysis by group IIA and V sPLA2s. The important finding of the present study is that hGX can efficiently act on non-apoptotic and non-damaged adherent cells. Thus it seems clear that the secretion of this enzyme from cells must be tightly regulated.

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