

Arachidonic Acid Release from Mammalian Cells Transfected with Human Groups IIA and X Secreted Phospholipase A₂ Occurs Predominantly during the Secretory Process and with the Involvement of Cytosolic Phospholipase A₂-α*[§]

Received for publication, December 1, 2003, and in revised form, March 5, 2004
Published, JBC Papers in Press, March 8, 2004, DOI 10.1074/jbc.M313019200

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Stable expression of human groups IIA and X secreted phospholipases A₂ (hGIIA and hGX) in CHO-K1 and HEK293 cells leads to serum- and interleukin-1β-promoted arachidonate release. Using mutant CHO-K1 cell lines, it is shown that this arachidonate release does not require heparan sulfate proteoglycan- or glycosylphosphatidylinositol-anchored proteins. It is shown that the potent secreted phospholipase A₂ inhibitor Me-Indoxam is cell-impermeable. By use of Me-Indoxam and the cell-impermeable, secreted phospholipase A₂ trapping agent heparin, it is shown that hGIIA liberates free arachidonate prior to secretion from the cell. With hGX-transfected CHO-K1 cells, arachidonate release occurs before and after enzyme secretion, whereas all of the arachidonate release from HEK293 cells occurs prior to enzyme secretion. Immunocytochemical studies by confocal laser and electron microscopies show localization of hGIIA to the cell surface and Golgi compartment. Additional results show that the interleukin-1β-dependent release of arachidonate is promoted by secreted phospholipase A₂ expression and is completely dependent on cytosolic (group IVA) phospholipase A₂. These results along with additional data resolve the paradox that efficient arachidonic acid release occurs with hGIIA-transfected cells, and yet exogenously added hGIIA is poorly able to liberate arachidonic acid from mammalian cells.

Phospholipases A₂ (PLA₂s)¹ are a class of enzymes that release fatty acids from the *sn*-2 position of glycerophospholip-

ids. Biomedical interest in these enzymes stems from the finding that the liberation of arachidonic acid for the biosynthesis of the eicosanoids (prostaglandins, leukotrienes, and others) is mediated in mammalian cells by one or more PLA₂s. Current evidence favors a role for cytosolic phospholipase A₂-α (cPLA₂-α, also known as group IVA PLA₂) as a major component of the arachidonate releasing signal transduction pathway (1–3). Mammals also contain a large number of secreted phospholipases A₂ (sPLA₂s) (4, 5), and the possible participation of these enzymes in arachidonate release is under active investigation. For example, group V sPLA₂ is present in the macrophage-like cell line p388D1 and contributes a portion of the arachidonate released in response to lipopolysaccharide (6). Exogenous addition of groups V and X sPLA₂s to a variety of mammalian cells leads to arachidonate release (7–10). There is some evidence to suggest that the action of cPLA₂-α is a prerequisite for sPLA₂ function in cells (11, 12) and even for the vice versa scenario (13–15), but such cross-talk between PLA₂s remains poorly understood.

To assess the arachidonate releasing capacity of PLA₂s in mammalian cells, CHO and HEK293 cell lines that stably express the various enzymes have been established (16–20). The behavior of human group IIA (hGIIA) and human group X (hGX) sPLA₂s when transfected in HEK293 and CHO cells has been extensively studied. hGIIA is secreted from HEK293 cells, and most of the extracellular enzyme is associated with the cell surface. This sPLA₂ is highly basic and is thought to bind to the anionic heparan sulfate chains of cell surface glypican (21), a GPI-linked proteoglycan. hGX is not a highly basic protein and accumulates in the extracellular medium when transfected in HEK293 cells (18). Whereas serum stimulates arachidonate release from both hGIIA- and hGX-transfected HEK293 cells, only the former cells show an additional increase in fatty acid release if the pro-inflammatory cytokine interleukin-1β (IL-1β) is added along with serum (18). The biochemical basis for this cytokine effect is not known. In this cell model, it has been suggested that glypican-bound hGIIA is internalized into intracellular punctate domains near the cell nucleus. That this is due to import from the cell surface into caveolae and caveolae-derived vesicles is proposed based on confocal microscopy studies showing punctate co-localization of hGIIA and caveolin-2 throughout the cytosol including the perinuclear region (21). These observations raise the possibility that the site of fatty acid release by hGIIA is within intracellular vesicles. In contrast, the non-heparan sulfate-binding hGX is thought to act on the extracellular face of the plasma membrane of HEK293 cells and does not accumulate in intracellular domains (18). It is not

* This work was supported by National Institutes of Health Grants HL36835 and HL50040 (to M. H. G.) and a program grant from the National Health and Medical Research Council of Australia (to R. G. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental figures and text.

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¹ The abbreviations used are: PLA₂, phospholipase A₂; BSA, bovine serum albumin; cPLA₂-α, human cytosolic PLA₂ α-isoform (group IVA); hGIIA, human group IIA secreted phospholipase A₂; GPI, glycosyl phosphatidylinositol; hGX, human group X secreted phospholipase A₂; IL, interleukin; sPLA₂, secreted PLA₂; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; FBS, fetal bovine serum; AB, apical-to-basolateral; BA, basolateral-to-apical.

yet known whether there is a biochemical connection between IL-1 β -induced arachidonate release and intracellular action of hGIIA.

The possibility that hGIIA acts on a cellular membrane other than the extracellular face of the plasma membrane is appealing. This is because the extracellular face of the mammalian cell plasma membrane is rich in the zwitterionic phospholipids phosphatidylcholine and sphingomyelin, and it is well established that hGIIA displays extremely low enzymatic activity toward phosphatidylcholine-rich vesicles *in vitro* because this enzyme cannot bind to the zwitterionic interface (22, 23). Indeed, relatively large amounts (typically >10 μ g/ml) of hGIIA need to be added exogenously to mammalian cells, including HEK293 cells, to cause detectable arachidonate release (23). Thus, it was surprising to find that HEK293 cells stably transfected with hGIIA, which produce ng/ml amounts of enzyme, are able to liberate arachidonate. This suggests that hGIIA releases arachidonic acid within a membrane compartment that is accessible to enzyme produced by transfection but not by enzyme added exogenously to cells. The very poor ability of exogenously added hGIIA to liberate arachidonic acid from HEK293 cells is seemingly at odds with the glypican-shuttling model described above. One would anticipate that exogenously added hGIIA would bind to the heparan sulfate chains of cell surface glypican and be imported into caveolae and caveolae-derived vesicles. For example cholera toxin readily labels caveolae when added exogenously to mammalian cells (24). Perhaps hGIIA must be preloaded onto glypican during the secretory process, and exogenously added hGIIA cannot access the heparan sulfate chains of cell surface glypican. But it is difficult to conceive that amounts of exogenously added hGIIA 3 orders of magnitude higher than those produced by transfected HEK293 cells would not lead to loading of cell surface heparan sulfate chains. It is this paradox that prompted us to further investigate the mode of action of hGIIA in transfected CHO and HEK293 cells.

In marked contrast to hGIIA, hGX binds with high affinity to phosphatidylcholine-rich membranes *in vitro* (23), and ng/ml amounts of exogenously added hGX are sufficient to liberate arachidonic acid from HEK293 cells and other mammalian cells (8, 9, 25). Given these observations and the fact that hGX does not bind to heparan sulfate, it has been suggested that hGX acts on the extracellular face of the plasma membrane once secreted from hGX-transfected HEK293 cells (18). Thus, we also included hGX-transfected CHO and HEK293 cells in side-by-side comparative studies with hGIIA-transfected cells. Our results show that arachidonate release by hGIIA in transfected CHO and HEK293 cells occurs prior to secretion to the extracellular fluid. Arachidonate release by hGX occurs before and after secretion from CHO cells and prior to secretion from HEK293 cells. sPLA₂ action prior to secretion resolves the paradox of why hGIIA is able to release arachidonic acid in transfected cells but not when added exogenously to cells.

EXPERIMENTAL PROCEDURES

Materials—The cPLA₂- α inhibitors pyrrophenone (26), pyrrolidine-1 (27), and AZ-1 (compound 22 of (28)) and the sPLA₂ inhibitor Me-Indoxam (29) were prepared as described. Porcine intestinal mucosa low molecular mass heparin (~3,000 Da) and heparin are from Sigma (H3400 and H3149, respectively). The Golgi-specific probe NBD-C6-ceramide is from Molecular Probes (Eugene, OR). pgsA-745 and GPI-LA1 CHO cells were obtained as gifts from J. Esko (University of California) and F. G. van der Goot (University of Geneva), respectively. The synthesis of Me-Indoxam radiolabeled in the benzylic position, [³H]Me-Indoxam, is given in the Supplemental Material. [³H]Arachidonate (200 Ci/mmol) is from American Radiochemicals Inc. Human recombinant IL-1 β is from R & D Systems and was used from small frozen aliquots, thus avoiding freeze-thaw cycles. Recombinant human cPLA₂- α was prepared as described (30), and anti-cPLA₂- α antisera

were obtained from C. C. Leslie (National Jewish Medical Research Center) and M. Wakelam (University of Birmingham).

Cell Culture and Preparation of Stable Transfectants—CHO-K1 and HEK293 cells were obtained from the American Type Culture Collection and cultured at 37 °C in a humidified atmosphere of 5% CO₂ in Ham's F-12 medium with 2 mM glutamine (Sigma N6658) containing 10% FBS for CHO-K1 cells and in RPMI 1640 medium with 2 mM glutamine (Invitrogen catalog number 11875-093) containing 10% FBS for HEK293 cells. For both media, 2 mM glutamine was added to working solution every 2 weeks. To prevent, cross-clone contamination, pipette tips with cotton plugs were used when handling multiple cell clones. The cells were split using trypsin/EDTA (trypsin was removed from the cell monolayer prior to adding fresh medium to minimize the degradation of cell surface proteins).

The plasmid for hGIIA expression, pRc-CMV/neo-hGIIA, contains the sequence GCGGCCGCC-ATG-hGIIA-TGA-TAAAGATCT (the NotI and XbaI sites are underscored, the full-length hGIIA cDNA from start to stop codon is italicized) ligated into the NotI and XbaI sites of the pRc-CMV/neo vector (Invitrogen). The plasmid for hGX expression, pRc-CMV/neo-hGX, contains the sequence GCGGCCGCC-ATG-hGX-TGA-TCTAGA. Note that the hGX cDNA contains two potential start sites, and pRc-CMV/neo-hGX uses the downstream start site, which corresponds to the sole start site of the mouse group X sPLA₂ cDNA. Thus, the N-terminal sequence of the hGX produced is MLLL. Plasmids for transfection were prepared in endotoxin-free form from bacterial cultures using the SNAP MidiPrep Kit (Invitrogen K1910-01), and DNA was quantified by optical density at 260 nm. CHO-K1 cells were transfected using OPTI-MEM® I Reduced Serum Medium and LipofectAMINE (both from Invitrogen) using the optimized procedure for these cells provided by Invitrogen. Stable transfectants were selected by limiting dilution in 96-well plates using medium containing 600 μ g/ml Geneticin (Invitrogen) (an antibiotic kill curve was carried out to establish that this concentration was effective for selection). HEK293 cells were transfected as above except the optimized procedure for these cells was used (provided by Invitrogen), and the Geneticin concentration was 500 μ g/ml. Maintenance cultures of stably transfected CHO-K1 and HEK293 cells were routinely grown in medium containing Geneticin, but antibiotic was omitted when cells were plated for studies or during the initial plating of frozen stocks.

Site-specific mutagenesis was prepared using the QuikChange kit (Stratagene), and full-length coding sequences were submitted to DNA sequencing to verify that no additional mutations were introduced.

Arachidonic Acid Release Studies—CHO-K1 cells were plated at 5×10^4 cells/ml in 24-well plates (Nunc catalog number 143982) (1 ml medium/well). When cells reached ~80% confluence, 0.1 μ Ci of [³H]arachidonate was added per well, and the cells were placed in the incubator for 20–24 h. The cells were washed three times with complete medium (medium carefully removed with a pipette rather than by aspiration) and then covered with 1 ml of medium containing the desired additives (described in the figure legends). When IL-1 β was used, it was added from a freshly prepared stock of 0.1 ng/ μ l in PBS containing 0.2% bovine serum albumin. The cells were placed in the incubator for the desired time. After the desired time, the medium was removed and centrifuged for 7 min at 3,000 rpm to pellet any dislodged cells. A 0.5-ml aliquot of the supernatant was submitted to scintillation counting. To the cell pellet in the well was added 0.5 ml of trypsin/EDTA, and the plate was placed in the incubator for 30 min. The cells were resuspended by pumping the solution up and down several times with a pipettor, and all of the liquid was submitted to scintillation counting to give the cell-associated cpm. The percentage of [³H]arachidonate release to the medium was calculated as $100 \times (\text{dpm in medium})/(\text{dpm in medium} + \text{cell associated dpm})$.

[³H]Arachidonate release from HEK293 cells was carried out by plating cells in 24-well plates. When cells reached ~80% confluency, 0.1 μ Ci of [³H]arachidonate was added to 1 ml of complete medium in each well. The cells were placed in the incubator for 20–24 h. The medium was removed with a pipette, and the cells were covered with 1 ml of complete medium. After 5–10 min the medium was removed, and the cells were covered with 1 ml of medium containing the desired additives (described in the figure legends). The cells were placed in the incubator for the desired time period, and the percentage of total [³H]arachidonate released to the medium was determined as for CHO-K1 cells.

In some studies, the cells were extracted with organic solvent to determine the amount of tritium in the neutral lipid fraction. The cells were labeled with [³H]arachidonate and stimulated as described above. After removal of the medium, the cell pellets were treated with trypsin/EDTA as above, and the cell suspension was transferred to an Eppendorf tube. An aliquot of the cell suspension (125 μ l) was transferred to

a new Eppendorf tube, and 370 μ l of CHCl₃, MeOH, 10 N HCl (200/200/1 by volume) was added followed by 460 μ l of CHCl₃, MeOH, H₂O (1/2/0.8) and then 240 μ l of CHCl₃, and finally 240 μ l of H₂O. After mixing with a vortex mixer for \sim 1 min, the tubes were centrifuged for 2 min at \sim 10,000 $\times g$ to separate the solvent layers. Most of the lower layer was transferred to a glass tube, and solvent was removed with a stream of N₂. Oleic acid (10 pmol) was added followed by 1 ml of low boiling petroleum ether/ethyl ether/glacial acetic acid (70/30/1, by volume). The sample was mixed on a vortex mixer for \sim 30 s and loaded onto a small column of silica gel in a glass wool-plugged Pasteur pipette. The eluant was collected into a scintillation vial along with the eluant after washing the column with an additional 4 ml of the same solvent. The solvent was removed with a stream of air, and the residue was submitted to scintillation counting.

In some studies, the cells were extracted with organic solvent, and the extract was analyzed for [³H]arachidonic acid after TLC on a silica plate. The cells were labeled with [³H]arachidonate and stimulated as described above. After removal of the medium, the cell pellets were treated with trypsin/EDTA as above, and the cell suspension was transferred to an Eppendorf tube. A portion of the cell suspension was extracted with organic solvent and prepared for silica gel chromatography as described above. To the extract was added \sim 50 μ g of nonlabeled arachidonic acid, and the solution was spotted on one lane of a 20 \times 20-cm silica TLC plate. The plate was developed in a tank with *n*-hexane/diethyl ether/glacial acetic acid (70:30:1 by volume). The arachidonic acid spot was located by placing the plate in a tank of iodine vapor. The spot was scrapped from the plate, the silica was transferred to a vial containing scintillation fluid and submitted to scintillation counting.

sPLA₂ Enzymatic Assay—sPLA₂ enzymatic activity of cell culture medium was measured using a variation of the fluorimetric assay (31). The substrate, 1-palmitoyl-2-(10-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (Molecular Probes) was dissolved at 1 mg/ml in toluene/isopropanol (1/1), and the concentration was determined from the absorbance at 342 nm (ϵ 40,000 M⁻¹ cm⁻¹). A 0.2-ml aliquot of substrate stock solution was concentrated to dryness with a stream of N₂ followed by the addition of 1 ml of absolute ethanol (any undissolved material was removed by centrifugation at \sim 14,000 $\times g$ for 2 min). After re-determination of the substrate concentration, a working solution of substrate mixture was prepared by diluting the ethanol stock of substrate into 30 ml of buffer (50 mM Tris, 100 mM NaCl, 1 mM EGTA, pH 7.4) while mixing on a vortex mixer to give a final substrate concentration of 1 μ M. The 1-ml assay mixture is prepared by mixing 980 μ l of substrate mixture with 10 μ l of fatty acid free BSA (10 mg/ml, w/v in water; Sigma) and 10 μ l of CaCl₂ (1 M in water) in a fluorescence cuvette equipped with a magnetic stir bar. After the addition of tissue culture medium (typically 20–40 μ l for hGIIA and 100–200 μ l for hGX-transfected cells) or recombinant sPLA₂, the fluorescence was monitored with excitation at 342 nm and emission at 395 nm at room temperature with stirring. The assay is calibrated to give moles of product by the addition of a standard solution of 10-pyrenedecanoic acid in ethanol to the complete assay mixture lacking sPLA₂.

Extraction of Cell Surface hGIIA with Salt—The cells were covered with complete medium supplemented with various amounts of NaCl (see figure legends). The cells were placed in the incubator for 15 min, and then the medium was transferred to an Eppendorf tube and centrifuged for 7 min and 3,000 rpm to pellet any dislodged cells. The supernatant was submitted to the fluorimetric sPLA₂ assay (see above) or stored at -20 °C prior to assay.

Confocal Laser Fluorescence Microscopy—Cells for microscopy were grown on nitric acid-washed coverslips (12-mm diameter, #1.5; Electron Microscopy Sciences catalog number 72230-01, autoclaved before use) to 50–60% confluence, stimulated if desired, and fixed as follows. The medium was removed by aspiration, and the cells were washed once with PBS at room temperature. The cells were covered with ice-cold formaldehyde (3% w/v, prepared by dilution of an ampule of 16% formaldehyde; Electron Microscopy Sciences catalog number 15710, with PBS). After 20 min at room temperature, the cells were washed three times with PBS at room temperature. For permeabilization, Triton X-100 (0.2% v/v in PBS) was added, and the cells were incubated at room temperature for 10 min. The cells were washed twice with PBS and then incubated for 10 min at room temperature with 1% BSA in PBS. After removing the blocking agent, the cells were incubated for 2 h at room temperature with 1% BSA containing anti-hGIIA antiserum (1/500 dilution, antiserum prepared as described (2)). The cells were washed three times with PBS and then incubated with 1% BSA in PBS for 10 min at room temperature. After removing the blocking agent, secondary antibody (1/200 dilution of affinity purified, fluorescein iso-

thiocyanate-conjugated, anti-rabbit IgG F(ab')₂, Organon Teknika Corp., Durham, NC) was added in 1% BSA in PBS. After 1 h at room temperature, the cells were washed four times with PBS. Coverslips were mounted on glass slides with mounting solution (0.1 M *n*-propyl gallate in 9:1 glycerol:PBS) and sealed to the slide with nail polish. The slides were viewed within 15 h with a Bio-Rad MRC-600 confocal microscope equipped with a 60 \times oil immersion objective. NBD-C6 ceramide was used as a Golgi marker. CHO-K1 cells were fixed as described above. After washing the cells, the coverslips were placed at 4 °C, and 1 ml of freshly prepared NBD-C6 ceramide (5 μ M in 1% BSA in PBS) was added per well. After 30 min at 4 °C, the cells were washed several times with 3% BSA in PBS to remove excess reagent and viewed by confocal microscopy.

In some experiments the cells were permeabilized with saponin. After the third PBS wash (see above), the cells were permeabilized with 1% saponin (w/v) (Sigma catalog number S7900), 1% (w/v) BSA in PBS for 30 min at room temperature. The cells were washed once with 1% BSA in PBS and then covered with 1% BSA in PBS containing the primary antiserum. After a 1-h incubation at room temperature, the cells were washed three times with 1% BSA in PBS at room temperature. The cells were covered with 1% BSA in PBS with the secondary antibody and incubated at room temperature for 1 h. The cells were washed four times with PBS at room temperature and prepared for confocal microscopy as described above.

Electron Microscopy—hGIIA-transfected HEK293 cells (clone 1) were grown in 10-cm dishes to \sim 70% confluence. The medium was removed and replaced with fresh complete medium with or without 4 ng/ml IL-1 β . After 4 h at 37 °C, the medium was removed, and the cell layer was washed twice with PBS. In some experiments, the cells were incubated with complete medium containing 0.5 mg/ml heparin for 20 h at 37 °C in the presence or absence of IL-1 β . The cells were covered either with PBS containing 8% paraformaldehyde (paraformaldehyde; Electron Microscopy Sciences catalog number 15710). Fixation was allowed to proceed for 2 h at room temperature. The fixative was removed, and the cells were washed twice with PBS. The plates were filled with PBS, covered, sealed with Parafilm, and stored at 4 °C until processed further for electron microscopy (within 7 days). Fixed cells were processed for frozen sectioning as described (32). The sections were labeled with polyclonal antibodies to hGIIA (Cayman) followed by 10-nm protein A-gold (University of Utrecht, Utrecht, The Netherlands). For double labeling, sections were labeled with antibodies to hGIIA PLA2 followed by 5-nm protein A-gold, fixed, and then labeled with antibodies to caveolin 1 (Transduction Laboratories) followed by 10-nm protein A-gold.

Cell Permeability of Me-Indoxam—Uptake of Me-Indoxam into HEK293 cells was measured by incubating cells with [³H]Me-Indoxam (680 Ci/mol, prepared as described in the Supplemental Material) followed by centrifugation of the cells through a layer of serum to rapidly separate them from the extracellular medium (33). Nontransfected HEK293 cells were dislodged from the plate with trypsin/EDTA and washed two times with PBS. Trypsinized cells were washed three times with 4–5 ml of PBS, and the cell number was determined with a hemocytometer. The desired number of cells in 100 μ l of medium without serum containing the indicated amount of radiolabeled compound (see legend to Fig. 9S) were placed in the CO₂ incubator at 37 °C for 20 min. The solution was carefully layered onto 400 μ l of 100% FBS in the bottom of a 0.5-ml Eppendorf tube, and the samples were centrifuged at 10,000 $\times g$ for 30 s in a swinging bucket rotor at room temperature. The tubes were immediately placed in a dry ice/ethanol bath, and the frozen tubes were cut with a razor blade (as a cutting guide, the tube was placed in a 1.5-ml Eppendorf tube whose bottom was cut so that the smaller tube protruded 2 mm from the bottom edge of the larger tube). The tube bottom was placed in scintillation fluid to determine the amount of radioactivity. Additional radioactive compounds used were 1-[³H]-D-mannitol (20 Ci/mmol; American Radiochemicals Inc.) and 4-[³H]-L-propranolol (24.4 Ci/mmol; PerkinElmer Life Sciences). Cell permeability of Me-Indoxam was also measured using the Caco-2 (TC7 clone) cell monolayer assay using standard procedures (details given as in the Supplemental Material).

Immunoblot Analysis of cPLA₂- α —This is described in the Supplemental Material.

RESULTS

Arachidonate Release in CHO-K1 Cells Transfected with hGIIA and hGX—CHO-K1 cells were transfected with plasmids driving constitutive expression of hGIIA and hGX, and 12 stable transfectants were established. The culture medium

from each clone was assayed for sPLA₂ enzymatic activity using a sensitive, real time fluorimetric assay. Five to six hGIIA clones and hGX clones produced detectable sPLA₂ enzymatic activity, and these clones were used for subsequent experiments. The amount of [³H]arachidonate released into the culture medium was determined after cells were cultured under various conditions. The release of [³H]arachidonate is expressed as percentage of the total dpm (medium and cell associated) that appears in the medium. In this way, fatty acid release is normalized to remove differences because of variation in the amount of radiolabeled arachidonate incorporated into cellular phospholipids and to variation in cell number/culture well.

As shown in Fig. 1A, both nontransfected and hGIIA-transfected CHO-K1 cells (clones 4 and 5 shown) liberate arachidonate into the medium in a time-dependent manner. FBS alone and IL-1 β alone increase arachidonate release slightly (\sim 1.3- and \sim 2-fold, respectively), and both together lead to a larger release of arachidonate than that produced during stimulation with either agent alone. Stable expression of hGIIA in CHO-K1 cells leads to higher arachidonate release than that produced in nontransfected cells (Fig. 1A). The differential between transfected and nontransfected cells is less than 2-fold but statistically significant; the data in Fig. 1A show the standard deviation of triplicate experiments for each clone. Clone 4 liberates more arachidonate than does clone 5, and this correlates with the observation that clone 4 releases more hGIIA enzymatic activity than clone 5 into the culture medium (*inset* in Fig. 1A). These same trends in arachidonate release and sPLA₂ enzymatic activity were observed in six independent experiments, each done in triplicate. hGIIA-dependent increase in arachidonate release correlated to the amount of sPLA₂ enzymatic activity was also seen in the three other CHO-K1 clones examined (not shown).

We measured [³H]arachidonate released into the culture medium by CHO-K1 cells. Albumin present in the culture medium presumably serves as a sink by binding fatty acid; this was further investigated with HEK293 cells (see below). We also examined the cells for the presence of radiolabel in the neutral lipid fraction. This was accomplished by extracting the cell pellet with chloroform:methanol and passing the extract through a small column of silica gel. For CHO-K1 cells in the absence of FBS/IL-1 β stimulation, $1.2 \pm 0.2\%$ of the total radioactivity is in the neutral lipid fraction, and this number increases to $2.0 \pm 0.2\%$ when cells are stimulated with FBS/IL-1 β . The corresponding numbers for hGIIA-transfected CHO-K1 cells (clone 1) are 1.2 ± 0.2 and $1.8 \pm 0.2\%$ in the absence and presence of FBS/IL-1 β , respectively (1.0 ± 0.1 and $1.8 \pm 0.2\%$ for clone 4). Given these small values compared with the amount of radioactivity released into the culture medium and the fact that the numbers are independent of whether the cells are transfected with hGIIA, we did not examine whether this cell-associated neutral lipid radioactivity is due to free arachidonic acid, arachidonyl-triglyceride, or some other neutral arachidonyl species.

CHO-K1 cells were also transfected with the hGIIA mutant in which the catalytic site histidine has been changed to glutamine (hGIIA-H47Q). Recombinant hGIIA-H47Q displays 2–4% of wild type enzymatic activity (34). Nine clones of hGIIA-H47Q transfected CHO-K1 cells were stimulated with FBS and IL-1 β using the standard protocol, and the average percentage of [³H]arachidonate release was $4.5 \pm 0.2\%$ after 8 h. In a side-by-side analysis, nontransfected CHO-K1 cells released $4.6 \pm 0.2\%$ [³H]arachidonate (five independent measurements) after 8 h, and hGIIA-transfected CHO-K1 cells (clone 4) released $6 \pm 0.1\%$ [³H]arachidonic acid (four inde-

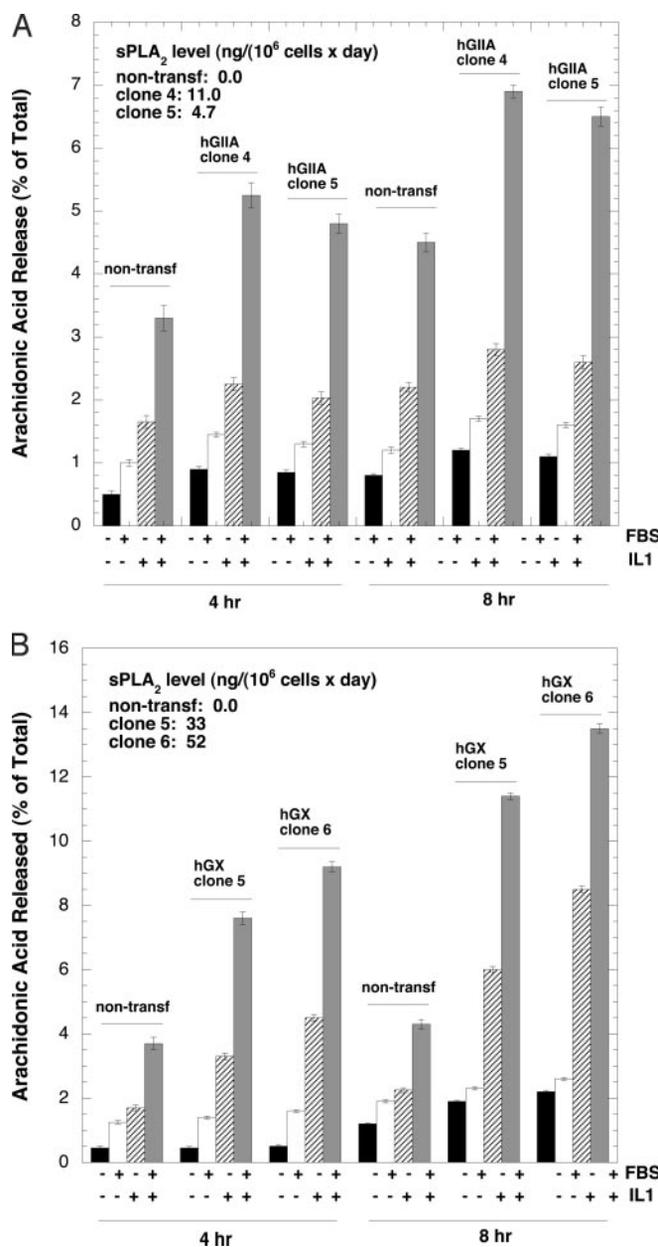


FIG. 1. Arachidonate release by CHO-K1 cells transfected with hGIIA (A) or hGX (B). Arachidonate release is expressed as a percentage of total [³H]arachidonate (medium + cell associated) released into the medium after 4 or 8 h of culture in the presence and absence of FBS and IL-1 β (1 ng/ml) as indicated. Nontransfected CHO-K1 cells, clones 4 and 5 of hGIIA-transfected cells, and clones 5 and 6 of hGX-transfected cells are shown. The amount of sPLA₂ secreted into the culture medium after 3 days of culture was quantified (see *insets*) by the fluorimetric enzyme assay (after washing the cells with 1 M NaCl in the case of hGIIA transfectants). The data shown are representative of six independent experiments, each done in triplicate; the error bars show the standard deviation from one of the triplicate analyses.

pendent measurements) after 8 h. Thus, it is clear that enzymatically compromised hGIIA is not able to augment arachidonic acid release in these cells.

Although we could not assess the expression level of hGIIA-H47Q by enzymatic assay, immunocytochemical analysis of two clones studied by confocal immunofluorescence microscopy of the clones showed qualitatively similar levels of hGIIA expression as clones expressing wild type hGIIA (see below). Thus, the increase in arachidonate liberation shown in Fig. 1A requires the expression of enzymatically active hGIIA.

We also established several clones of CHO-K1 stably trans-

fecting with hGX, and arachidonate release data is shown for clones 5 and 6 in Fig. 1B. Time-dependent arachidonate release is seen for all clones, and as for hGIIA-transfectants, stimulation of the cells with FBS and IL-1 β leads to more arachidonate release than that measured when either agent is added alone (Fig. 1B). FBS/IL-1 β -stimulated arachidonate release was more pronounced in hGX transfectants compared with hGIIA transfectants (Fig. 1, compare A and B). hGX clone 6 produces more arachidonate than hGX clone 5, which correlates with the higher enzymatic activity measured in the culture medium of clone 6 versus clone 5 cells (*inset* to Fig. 1B). The results in Fig. 1B were reproducible after six independent trials (each done in triplicate). A similar correlation between enhanced [³H]arachidonate release and hGX expression level was seen in the three additional hGX clones examined (not shown).

A Portion of Expressed hGIIA but Not hGX Is Cell Surface-bound—CHO-K1 cells expressing hGIIA (clones 1, 4, and 5) were cultured until confluent in medium containing FBS, and the amount of hGIIA enzymatic activity in the culture medium was quantified using the fluorimetric assay. After removal of the medium, the cell layer was covered with medium containing 1 M NaCl for 15 min at 37 °C, and additional hGIIA was recovered in the salt wash. It was found that $47 \pm 10\%$ of the total recovered enzyme was liberated into the culture medium in the absence of salt. These results suggest that approximately half of the secreted hGIIA is electrostatically bound to one or more anionic components on the cell surface. This is presumably due to the fact that hGIIA is a highly cationic protein, with 23 surface lysine and arginine residues (calculated pI of 9.4). Consistent with this electrostatic attachment, much more, $94 \pm 5\%$ (average of three experiments), of the mutant hGIIA in which three basic residues have been mutated to anionic residues, hGIIA-R7E/K10E/K16E, was secreted into the culture medium in the absence of high salt. When CHO-K1 cell clones expressing hGX were treated as above, almost all of the hGX, $97 \pm 5\%$ (average of three experiments), was found in the culture medium in the absence of high salt, as expected given that this sPLA₂ is a slightly acidic protein (calculated pI of 5.1).

hGIIA is known to bind tightly to heparin (35), a highly anionic polymer. We measured the fraction of extracellular hGIIA that could be captured in the culture medium in the presence of heparin. hGIIA-transfected HEK293 cells (clone 1) were first washed with culture medium without high salt to remove hGIIA in the medium and then were cultured for 24 h in the presence of various concentration of heparin added to the culture medium. The amount of hGIIA enzymatic activity released into the medium was quantified by fluorimetric assay, and the amount of remaining cell-associated hGIIA was quantified in the same way after cells were further washed with medium containing 1 M NaCl. The results in Fig. 1S (Supplemental Material) show that increasing heparin leads to a progressive increase in the amount of hGIIA in the culture medium. A dose of 0.25–0.50 mg/ml heparin is sufficient to remove the maximal amount of hGIIA from the cell surface (~85%, recall that ~50% of the secreted enzyme is in the medium, in the absence of heparin or high salt). Low molecular mass heparin (~3,000 Da) was less efficient at removing cell surface hGIIA; 3–4 mg/ml was required to remove a maximal amount of 85% of the hGIIA (not shown).

Glycosaminoglycan and GPI-linked Proteins Are Not Required for hGIIA- and hGX-dependent Arachidonate Release—We also expressed hGIIA and hGX in two CHO-K1 mutant cell lines. pgsA-745 cells have a defect in the glycosaminoglycan biosynthetic pathway and contain no detectable (<5%) total glycosaminoglycan (as assessed by high pressure liquid chromatography analysis of [³⁵S]sulfate-labeled cells,

(36, 37)). GPI-LA1 cells are defective in the GPI biosynthetic enzyme that *N*-deacetylates *N*-acetylglucosamine phosphatidylinositol (38). These cells lack GPI-linked proteins (based on the absence of cell surface alkaline phosphatase and the major GPI-linked protein GPI-130 (38)).

After screening for hGIIA expression (by enzymatic activity measurement) in pgsA-745 and GPI-LA1 cells, three clones from each group were submitted to [³H]arachidonate release analysis in the presence and absence of FBS and IL-1 β . We found no statistically significant difference in [³H]arachidonate release from these mutant cells compared with hGIIA-transfected wild type CHO-K1 clones (normalized for different amounts of sPLA₂ enzymatic activity measured in the high salt wash of cells cultured for the same time period) (not shown). There was also no statistically significant difference in the amount of arachidonate liberated from nontransfected wild type, pgsA-745, and GPI-LA1 CHO cells in response to FBS and IL-1 β (not shown). Finally, there was no statistically significant difference in arachidonate release from hGX-transfected wild type, pgsA-745, and GPI-LA1 clones (again after normalization for sPLA₂ enzymatic activity) (not shown). These results show that the amount of arachidonate liberated by hGIIA and hGX in CHO-K1 cells is not altered by the absence of glycosaminoglycan and GPI-link proteins.

We also examined the distribution of secreted hGIIA between the medium and the cell surface of pgsA-745 and GPI-LA1 cells when washed cells were treated with medium containing various concentrations of added NaCl (0.12–1 M). As shown in Fig. 2S (Supplemental Material), the salt dependence of the release of hGIIA from the cell surface was similar for wild type and pgsA-745 cells, and release of hGIIA from GPI-LA1 cells required a slightly lower salt concentration. Approximately 0.2–0.25 M NaCl is required to release half as much enzyme as that released with 1 M salt, and inclusion of concentrations of NaCl higher than 1 M would not be expected to liberate more hGIIA. Although this data does not rule out the binding of hGIIA to proteoglycan or a GPI-linked protein on the surface of wild type CHO-K1 cells, it is clear that such components are not obligatory for the association of secreted hGIIA with the extracellular cell surface. The fact that heparin can dissociate hGIIA from the cell surface does not establish that this enzyme is bound to a glycosaminoglycan polymer on the cell surface. Because hGIIA binds tightly to heparin, the presence of this anionic polymer in the culture medium can remove cell surface hGIIA by mass action, regardless of the cell component(s) to which the enzyme is bound, as long such cell surface binding is not irreversible.

Arachidonate Release from CHO-K1 Cells Is Not Due to Extracellular hGIIA—To investigate whether hGIIA bound to the extracellular face of the plasma membrane of CHO-K1 cells is involved in arachidonate release, we measured the release of [³H]arachidonate into the culture medium by hGIIA-transfected CHO-K1 cells (clones 1 and 4) in the presence of sufficient heparin to cause maximum release of hGIIA to the culture medium. As shown in Fig. 2, when hGIIA-transfected cells were stimulated with FBS and IL-1 β , [³H]arachidonate released into the culture medium was enhanced compared with nontransfected cells. Remarkably, when these experiments were repeated in the presence of heparin (present during the 24-h period in which cells were labeled with [³H]arachidonate and during the stimulation period), no inhibition of [³H]arachidonate release was observed for both nontransfected and hGIIA-transfected cells (Fig. 2). The concentration of heparin used, 1 mg/ml, is more than sufficient to cause maximal release of hGIIA into the culture medium (Fig. 1S). Use of 4 mg/ml heparin resulted in a slight increase in released [³H]arachi-

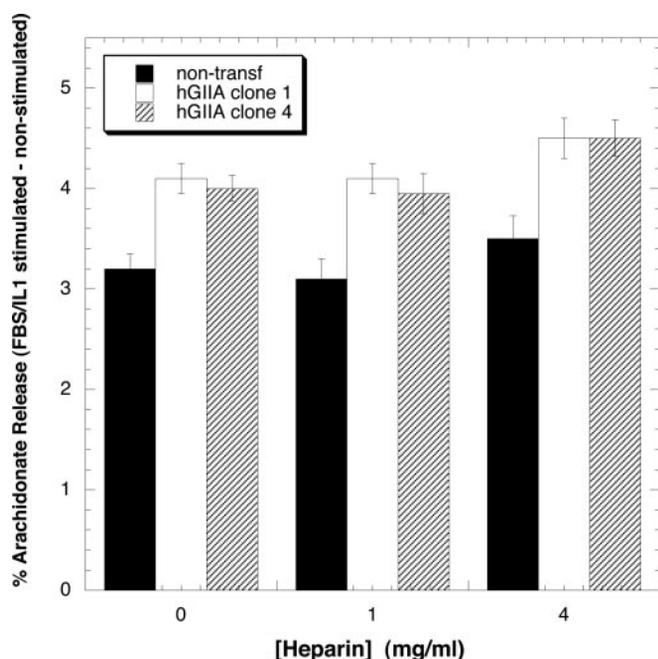


FIG. 2. Effect of extracellular heparin on arachidonate release by CHO-K1 cells transfected with hGIIA. hGIIA-transfected CHO-K1 cells (clones 1 and 4) were cultured for 24 h in the presence of [³H]arachidonate and in the presence of the indicated amount of heparin. The cells were washed to remove unincorporated [³H]arachidonate and then stimulated with FBS + IL-1 β for 6 h in the presence of the indicated amount of heparin in the medium. Release of [³H]arachidonate into the culture medium was measured as described under "Experimental Procedures." Plotted is the difference in [³H]arachidonate release (% of total) between stimulated (+ FBS, + IL-1 β) and nonstimulated (- FBS, - IL-1 β) cells. The data shown are representative of two independent experiments, each done in triplicate; the error bars show the standard deviation from one of the triplicate analyses.

donate for both transfected and nontransfected cells (Fig. 2). This lack of inhibition of [³H]arachidonate release by extracellular heparin was observed in six independent experiments, each done in triplicate, and maximal release of hGIIA enzymatic activity into the culture medium by heparin was confirmed by submitting the same culture medium used for scintillation counting to the fluorimetric sPLA₂ enzymatic assay. These results, together with the fact that [³H]arachidonate release in these cells is proportional to the amount of expressed hGIIA (Fig. 1A), argue strongly that externalized hGIIA is not responsible for [³H]arachidonate release. Strictly speaking, we cannot rule out the possibility that the small amount of externalized hGIIA that may remain cell surface-bound in the presence of heparin is responsible for [³H]arachidonate release. However, the studies described below with Me-Indoxam strongly argue against this possibility.

The amount of [³H]arachidonate released from FBS/IL-1 β -stimulated CHO-K1 cells expressing hGIIA-R7E/K10E/K16E was not statistically different from that from cells expressing similar levels of wild type hGIIA (not shown). This result, together with the observation that essentially all of the mutant enzyme accumulates in the culture medium, argues that cell surface hGIIA is not responsible for the arachidonate release.

As shown in Fig. 3A, [³H]arachidonate release by hGIIA in transfected CHO-K1 cells was completely insensitive to concentrations of Me-Indoxam that are more than sufficient to fully block the enzymatic activity of hGIIA (~1,000-fold the IC₅₀ measured for inhibition of hGIIA by Me-Indoxam in an *in vitro* assay (29)). We ruled out the trivial explanation that Me-Indoxam was degraded in the cell culture well. Thus, Me-Indoxam was added to complete culture medium above

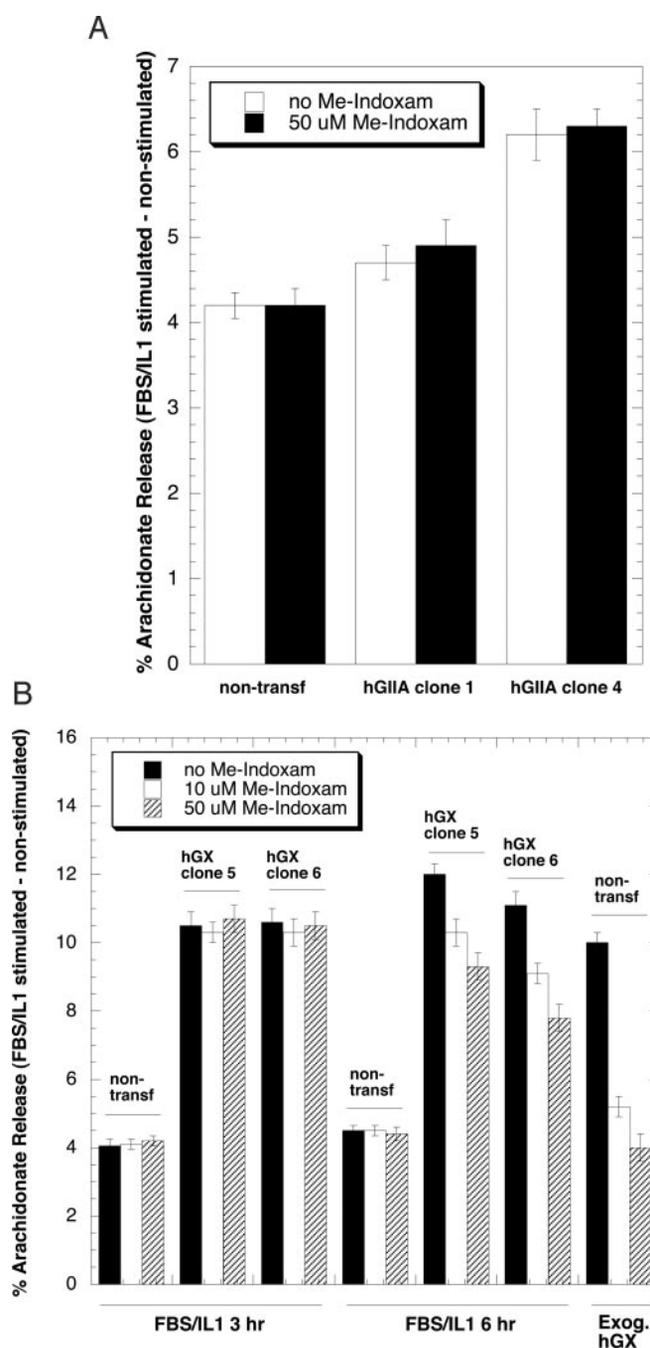


FIG. 3. Effect of Me-Indoxam on arachidonate release by CHO-K1 cells transfected with hGIIA or hGX. A, hGIIA-transfected CHO-K1 cells (clones 1 and 4) were stimulated for 6 h in the presence of FBS and IL-1 β and the indicated concentration of Me-Indoxam (which was also present during the 24-h [³H]arachidonate labeling period), and release of [³H]arachidonate into the culture medium was measured as described under "Experimental Procedures." B, as for A but with hGX-transfected CHO-K1 cells (clones 5 and 6), and stimulation with FBS and IL-1 β was for 3 or 6 h as indicated. The data on the right side of the figure is for nontransfected CHO-K1 cells treated for 3 h with 100 ng/ml exogenously added recombinant hGX in the presence of the indicated concentrations of Me-Indoxam. Plotted is the difference in [³H]arachidonate release (% of total) between stimulated (+ FBS, + IL-1 β) and nonstimulated (- FBS, - IL-1 β) cells. The data shown are representative of two independent experiments, each done in triplicate; the error bars show the standard deviation from one of the triplicate analyses.

CHO-K1 cells for 6 h. Dilution of an aliquot of this medium into the fluorimetric hGIIA assay solution to give the IC₅₀ concentration of Me-Indoxam (previously determined for the Me₂SO stock solution of Me-Indoxam) produced 48% inhibition (no

inhibition was observed when medium lacking added Me-Indoxam was substituted); thus, Me-Indoxam is completely stable under the conditions of Fig. 3A. A second hGIIA inhibitor LY311727 (39), which is structurally related to Me-Indoxam, also failed to inhibit arachidonate release in hGIIA-transfected CHO-K1 cells when tested up to 50 μ M (not shown).

The studies described below conclusively show that Me-Indoxam does not pass through the plasma membrane of mammalian cells. Thus, the lack of inhibition of hGIIA-dependent arachidonate release in CHO-K1 cells by the extracellular, cell-impermeable agents Me-Indoxam and heparin strongly argues that the free fatty acid is produced by enzyme before it is secreted to the outside of the cell. It would be expected that Me-Indoxam would inhibit arachidonate release by hGIIA added exogenously to CHO-K1 cells. It was found that large amounts of exogenously added hGIIA are needed to get detectable [³H]arachidonate release from CHO-K1 cells (see the Introduction). The addition of 15 μ g/ml hGIIA to CHO-K1 cells in the presence of serum led to $4.8 \pm 0.3\%$ [³H]arachidonate in 12 h, whereas the release was $3.8 \pm 0.3\%$ in the absence of added hGIIA. Co-addition of 10 μ M Me-Indoxam along with hGIIA led to complete inhibition ($3.6 \pm 0.2\%$ measured [³H]arachidonate release). This shows that exogenously added hGIIA is accessible to Me-Indoxam and that the small amount of [³H]arachidonate released over a prolonged period is due to the action of hGIIA. It may also be noted that the addition of recombinant hGIIA in the low μ g/ml range to some mammalian cells and tissues such as guinea pig bronchoalveolar lavage cells and lung pleural strips led to fatty acid release that was completely blocked by submicromolar to low micromolar concentration of LY315920 (40), a compound very similar in structure to Me-Indoxam. We have shown previously that the addition of a tryptophan to the interfacial binding surface of hGIIA dramatically promotes the binding of this enzyme to phosphatidylcholine rich vesicles *in vitro* and also allows the enzyme to more efficiently act on the plasma membrane of mammalian cells when added exogenously (23). As shown in Fig. 3S (Supplemental Material), exogenous addition of the hGIIA-V3W mutant (10 μ g/ml) leads to statistically significant release of [³H]arachidonate into the culture medium (in just 4 h), and this release is fully blocked by 1 and 10 μ M Me-Indoxam.

Arachidonate Release by hGX from CHO-K1 Cells Is Partially Due to Extracellular Enzyme—We tested the ability of the cell-impermeable inhibitor Me-Indoxam to block [³H]arachidonate release from CHO-K1 cells transfected with hGX (heparin was not studied because hGX does not bind to heparin; however, see below). The results summarized in Fig. 3B show that 10 or 50 μ M Me-Indoxam has no effect on the amount of [³H]arachidonate released into the culture medium when hGX-transfected CHO-K1 cells (clones 5 and 6) were stimulated with FBS and IL-1 β for 3 h, but partial and dose-dependent inhibition is seen when released [³H]arachidonate is quantified after 6 h of stimulation. Me-Indoxam at 10 μ M blocks most of the [³H]arachidonate released by exogenously added hGX, and the inhibition is complete with 50 μ M compound (Fig. 3B). It is remarkable that Me-Indoxam inhibits [³H]arachidonate by hGX and not by hGIIA (see above) because this compound inhibits hGIIA with an *in vitro* IC₅₀ of 6–30 nM compared with a value of 1–2 μ M for hGX (29).

Localization of hGIIA in CHO-K1 Cells by Immunofluorescence Confocal Microscopy—hGIIA-transfected CHO-K1 cells were formaldehyde-fixed and permeabilized with Triton X-100, and hGIIA was visualized by laser scanning confocal microscopy using a highly specific anti-hGIIA antiserum (2). The results shown in Fig. 4 (A and C) show intense fluorescence from regions that appear to be the Golgi based on positioning

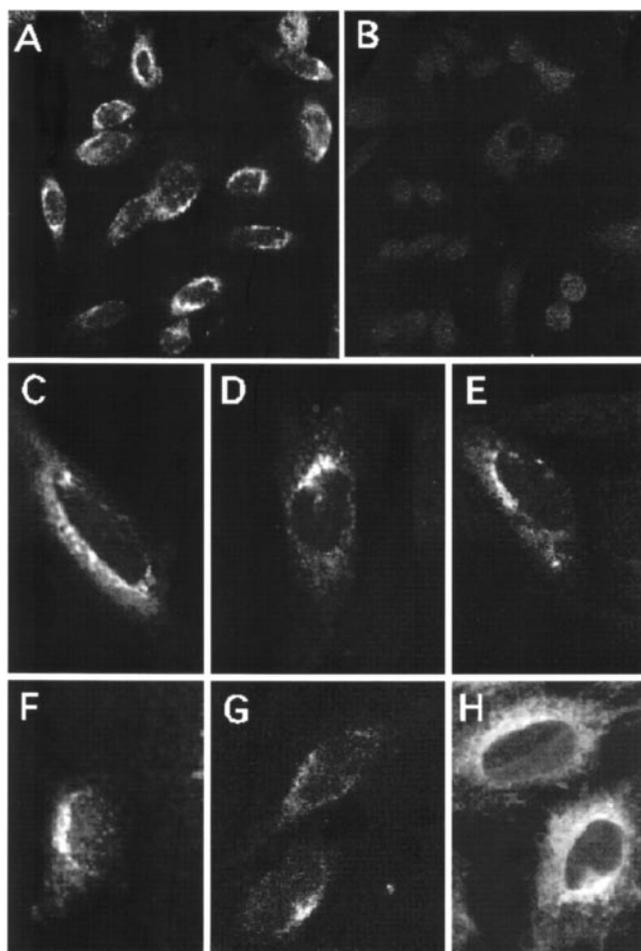


FIG. 4. Confocal immunofluorescence microscopy of hGIIA-transfected CHO-K1 cells. A, hGIIA-transfected wild type cells; B, nontransfected wild type cells; C, hGIIA-transfected wild type cells; D, hGIIA-transfected wild type cells stimulated for 5 h with FBS and IL1 β ; E, hGIIA-transfected wild type cells treated for 12 h with 4 mg/ml low molecular mass heparin; F, hGIIA-transfected pgsA-745 cells; G, hGIIA-H47Q-transfected wild type cells; H, wild type cells stained with NBD-C6-ceramide. A and B are with a 20 \times objective, and C–H are with a 60 \times objective.

near the nuclear envelope but not fully surrounding the nuclear envelope. This staining is similar to that seen for the Golgi-specific marker NBD-C6-ceramide (Fig. 4H). Faint, perhaps punctate staining is also seen in other regions of the cell, but this was indistinguishable from the faint staining seen with nontransfected cells (Fig. 4B). Stimulation of hGIIA-transfected CHO-K1 cells with FBS and IL-1 β for 5 h (Fig. 4D) or treatment of the cells with heparin (Fig. 4E) did not change the staining pattern. Also, hGIIA-transfected pgsA-745 CHO-K1 cells (Fig. 4F) and wild type CHO-K1 cells transfected with the enzymatically deficient mutant hGIIA-H47Q (Fig. 4G) showed similar staining as for hGIIA-transfected wild type cells. Thus, the localization of hGIIA is tentatively assigned to Golgi (confirmed by electron microscopy studies given below) presumably as part of the classical secretory process.

Arachidonate Release in HEK293 Cells Transfected with hGIIA and hGX—HEK293 clones stably transfected with hGIIA and hGX were prepared and submitted to the same arachidonate release assay as for the CHO-K1 cells. Fig. 5A shows the amount of [³H]arachidonate released into the medium (again expressed as a percentage of total [³H]arachidonate) from three different hGIIA-HEK293 clones in the presence and absence of FBS and with increasing amounts of IL-1 β . As with CHO-K1 cell transfectants, hGIIA-transfected

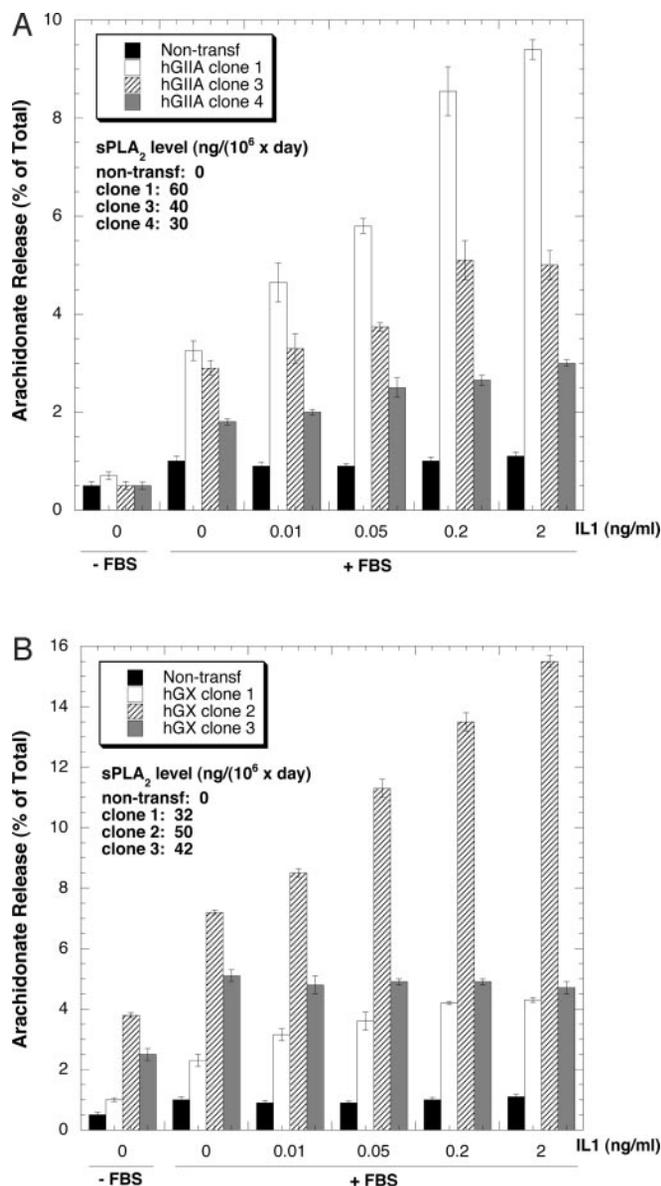


FIG. 5. Arachidonate release by HEK293 cells transfected with hGIIA (A) or hGX (B). Arachidonate release is expressed as a percentage of total [³H]arachidonate (medium + cell associated) released into the medium after 4 h of culture in the presence and absence of FBS and IL-1 β (0–2 ng/ml) as indicated. The data for nontransfected and three different transfected clones are shown. The inset shows the amount of sPLA₂ secreted from the transfected cells (quantified by the fluorimetric enzymatic assay). The data shown are representative of five independent experiments, each done in triplicate; the error bars show the standard deviation from one of the triplicate analyses.

HEK293 cells show an increase in [³H]arachidonate in the presence of FBS and a further increase when IL-1 β is added. Note that [³H]arachidonate release from nontransfected HEK293 cells is enhanced ~2-fold by FBS, no further increase in fatty acid release occurs with IL-1 β , and release of [³H]arachidonate in the presence of FBS is ~3-fold less than from nontransfected CHO-K1 cells in the presence of FBS and IL-1 β (Fig. 1A). In hGIIA-transfected HEK293 cells, [³H]arachidonate release depends on the dose of IL-1 β , with half-maximal fatty acid release occurring with ~0.05 ng/ml for all three transfected clones. Despite this common dose response, the different clones show different maximal responsiveness to IL-1 β . Clones 1 and 3 both show an ~6-fold increase in [³H]arachidonate release in response to FBS alone, but clone 1 shows a ~3-fold maximal increase in [³H]arachidonate in response to

IL-1 β , whereas only a ~1.5-fold increase is seen for clone 3. Clone 4 shows a smaller response to FBS, ~3-fold increase in [³H]arachidonate, and a further increase in fatty acid release caused by IL-1 β reaches ~1.5-fold. These results in Fig. 5A were reproducible after five independent experiments, each done in triplicate. The amount of FBS-induced [³H]arachidonate release from the three hGIIA-transfected HEK293 cell clones correlates with the amount of hGIIA protein being expressed (as measured by enzymatic activity assay; inset of Fig. 5A). Finally, [³H]arachidonate release from these clones was time-dependent over 0–6 h (not shown).

It is possible that albumin present in FBS is responsible for the increase in [³H]arachidonate release when cells are stimulated with medium containing 10% FBS compared with serum-free medium (also for the IL-1 β -dependent increase because the commercial preparation of this cytokine contains small amounts of albumin) because albumin serves as an extracellular sink for free fatty acids. However, this is not the case because the addition of 0.5% BSA to serum-free medium in the presence or absence of IL-1 β does not alter the amount of [³H]arachidonate release from hGIIA- and hGX-transfected HEK293 (not shown). Thus, it appears that IL-1 β alone and factor(s) in FBS other than albumin are able to stimulate fatty acid release from these cells.

As for CHO-K1 cells, we quantified the amount of tritium in the neutral lipid fraction obtained by organic solvent extraction of the HEK293 cell pellets. Nontransfected HEK293 cells were found to contain $0.8 \pm 0.1\%$ of the total radiolabel in the neutral lipid fraction, and this number did not change, $0.7 \pm 0.1\%$, when cells were stimulated with FBS/IL-1 β . For hGIIA-transfected HEK293 cells clones 1, 3, and 4, 1.0 ± 0.1 , 0.9 ± 0.1 , and $1.0 \pm 0.2\%$, respectively, of the total tritium was found in the neutral lipid fraction derived from the cell pellets, and the corresponding numbers after FBS/IL-1 β stimulation were 1.0 ± 0.1 , 1.1 ± 0.2 , and $0.9 \pm 0.1\%$, respectively, for the three different clones. Thus, as for CHO-K1 cells, essentially all of the increase in [³H]arachidonate resulting from transfecting cells with hGIIA is released to the culture medium.

Fig. 5B shows similar experiments carried out with hGX-transfected HEK293 cells. As for hGIIA-transfected cells, FBS causes an increase in [³H]arachidonate release in hGX-transfected cells. Interestingly, hGX-HEK293 clone 3 shows no additional release in [³H]arachidonate in the presence of IL-1 β . In contrast, IL-1 β , in a dose-dependent fashion, enhances [³H]arachidonate from hGX-HEK293 clones 1 and 2, with a maximal increase of ~2-fold in both cases. [³H]Arachidonate release was time-dependent over 0–6 h (not shown). The amount of [³H]arachidonate released from the three hGX-transfected HEK293 cell clones is correlated with the amount of hGX that they expressed as determined by enzymatic activity assay (inset of Fig. 5B).

Secreted hGIIA but Not hGX Is Bound to the Cell Surface of HEK293 Cells—hGIIA-transfected HEK293 cells (clone 1) were washed with complete medium and then covered with complete medium containing 4 ng/ml IL-1 β and various concentrations of heparin. After 6 h at 37 °C, the medium was removed, and the cells were treated with complete medium containing 1 M NaCl for 15 min at 37 °C. The results are shown in Fig. 4S (Supplemental Material). In the absence of heparin, it was found that ~3-fold more enzyme activity was found in the high salt wash compared with medium in which NaCl was not added, indicating that approximately 75% of the secreted hGIIA is cell surface-bound (assuming that the salt removes all of the bound enzyme). Extracellular heparin allows transfer of hGIIA to the medium, with less than ~0.25 mg/ml heparin being required to cause 50% of the maximal effect (Fig. 4S); 1

mg/ml heparin removes >90% of the secreted hGIIA from the cell surface. Essentially identical results were obtained with a different hGIIA-HEK293 clone (clone 3; not shown). The amount of hGIIA enzymatic activity secreted into the culture medium in the presence or absence of heparin did not depend on the presence of IL-1 β (not shown), suggesting that this cytokine does not modulate the amount of enzyme secreted from cells.

In the case of hGX-transfected HEK293 cells, enzymatic activity was detected in medium without added salt but not in the high salt wash (not shown). Furthermore, the amount of hGX activity in the medium was not influenced by the presence of heparin. Thus, as in the case of CHO-K1 cells, essentially all of the hGX is released into the culture medium. As for hGIIA-transfected cells, IL-1 β did not alter the amount of hGX enzymatic activity secreted from cells (not shown).

Arachidonate Release from HEK293 Cells Is Not Due to Extracellular hGIIA and hGX—To investigate whether [³H]arachidonate release from HEK293 cells transfected with hGIIA or hGX is due to extracellular enzyme, we carried out a series of experiments analogous to those performed with transfected CHO-K1 cells. We first tested whether exogenously added hGIIA and hGX could release [³H]arachidonate from nontransfected HEK293 cells. We have reported previously that release of [³H]arachidonate into the medium is not detected when HEK293 cells are treated with exogenous 1 μ g/ml hGIIA, whereas even 10 ng/ml amounts of hGX gives detectable release (29). This was investigated in more detail in the present study. As shown in Fig. 5S (Supplemental Material), no additional release of [³H]arachidonate into the medium above that seen in the absence of added hGIIA was detected when nontransfected HEK293 cells were pretreated with 10 μ g/ml hGIIA for 6 h in the absence of IL-1 β or when cells were treated with IL-1 β for 15 h followed by incubation with IL-1 β and 10 μ g/ml hGIIA for an additional 6 h. In addition, when the cell pellets were extracted with chloroform/methanol and [³H]arachidonate was quantified after purification on a silica gel plate, only a small amount of radiolabeled fatty acid was detected compared with that released into the culture medium regardless of whether cells were treated with hGIIA in the presence or absence of IL-1 β (Fig. 5S). When HEK293 cells were incubated with hGIIA in complete medium for 6 h at 37 °C, 25–30% of the added enzyme could be recovered based on enzymatic assay of a small aliquot of culture medium using the fluorimetric assay. Although some enzyme is lost, presumably because of binding to the cell surface and/or the walls of the culture well, this cannot be the reason that exogenously added hGIIA fails to release [³H]arachidonate from HEK293 cells. On the other hand, as shown in Fig. 5S, significant release of [³H]arachidonate into the medium was detected when HEK293 cells were treated for 6 h with small amounts of recombinant hGX (100 ng/ml). Fatty release was perhaps slightly enhanced when IL-1 β was also present during the period of treatment with hGX.

As shown in Fig. 3S (right side), detectable [³H]arachidonate release was observed when 10 μ g/ml hGIIA-V3W was added to nontransfected HEK293 cells (as for CHO-K1 cells). The addition of 1 and 10 μ M Me-Indoxam to the culture medium leads to complete inhibition of [³H]arachidonate release from nontransfected HEK293 cells treated with exogenous hGIIA-V3W (as for CHO-K1 cells). These results with exogenous hGIIA-V3W and hGX (see above) argue that the period of labeling of cells with [³H]arachidonate (20–24 h) is sufficient to allow those phospholipids that are exposed to exogenous sPLA₂s to become radiolabeled. Furthermore, if nontransfected HEK293 cells were labeled with [³H]arachidonate for an even longer period

(36 h), no measurable [³H]arachidonate release into the medium was observed when cells were treated with 10 μ g/ml wild type hGIIA for 6 h, regardless of whether cells were pretreated with 2 ng/ml IL-1 β for 16 h prior to the addition of the sPLA₂. Fatty acid release was readily detected when cells were treated for 6 h with 100 ng/ml hGX (not shown).

As shown in Fig. 6S (Supplemental Material), as expected, the amount of hGIIA in the culture medium depends on the length of time that hGIIA-transfected HEK293 cells were cultured. When HEK293 cells are cultured in complete medium for 15 h, the amount of enzyme in the culture medium is ~4-fold higher than the amount secreted after the cells are washed to remove accumulated extracellular hGIIA and then cultured for an additional 6 h. The same is true when heparin is present in the culture medium either during the first 15-h period or the second 6-h period except that the amount of released hGIIA is much larger than when heparin is not present as expected (Fig. 6S). Thus, these cells continuously produce extracellular hGIIA over the combined 21-h period, and this hGIIA is continuously trapped in the culture medium by the presence of heparin.

Next we studied the effect of heparin on [³H]arachidonate release from HEK293 cells transfected with hGIIA. The cells were labeled with [³H]arachidonic acid for 15 h in the presence of FBS, washed, and then stimulated for 6 h in the presence of FBS and in the presence or absence of IL-1 β . Heparin was present only during the 15-h period, only during the 6-h period, during both periods, or not present at all. Only the [³H]arachidonate released during the 6-h period was measured. The results are shown in Fig. 7SA for clone 1 and in Fig. 7SB for clone 3 (Supplemental Material). Heparin shows a complex effect. When it is present only during the 15-h labeling period, it causes a slight increase in [³H]arachidonate release compared with the no heparin control. On the other hand, when heparin is present only during the 6-h stimulation period, it causes partial inhibition of [³H]arachidonate release. When heparin is present all the time (15 + 6 h), inhibition of [³H]arachidonate release is observed and to a similar degree as the inhibition seen when heparin is present only during the 6-h stimulation period. Inhibition of [³H]arachidonate release by heparin is seen both in the presence and absence of IL-1 β . Finally, the degree of inhibition of [³H]arachidonate release by heparin depends on the transfected HEK293 clone. For clone 1, heparin inhibition is approximately 60% (Fig. 7SA), whereas for clone 3 it is only approximately 20% (Fig. 7SB).

We also studied the effect of heparin on [³H]arachidonate release from HEK293 cells transfected with hGX, and the results are summarized in Fig. 8SA for clone 2 (IL-1 β -sensitive) and in Fig. 8SB for clone 3 (IL-1 β -insensitive) (Supplemental Material). The results are similar to those obtained with hGIIA-transfected HEK293 cells (Figs. 7SA and 7SB). Heparin causes a slight activation in [³H]arachidonate released when it is present during the 15-h labeling period and causes partial inhibition when present during the 6-h stimulation period or when present all the time (21 h). These results with heparin for hGIIA and hGX-transfected HEK293 cells (Figs. 7S and 8S) were reproducible after three independent experiments, each done in triplicate.

We tested Me-Indoxam for its ability to inhibit [³H]arachidonate release from hGIIA-transfected HEK293 cells. It is clear from the results in Fig. 6A that 0.5–10 μ M Me-Indoxam has no effect on the amount of [³H]arachidonate released into the medium from cells stimulated with FBS/IL-1 β . This is true for clone 1 (Fig. 6A) and clone 3 (not shown), which are responsive to IL-1 β , and with clone 4, which is less responsive to cytokine (not shown). In addition, 50 μ M LY311727 did not reduce

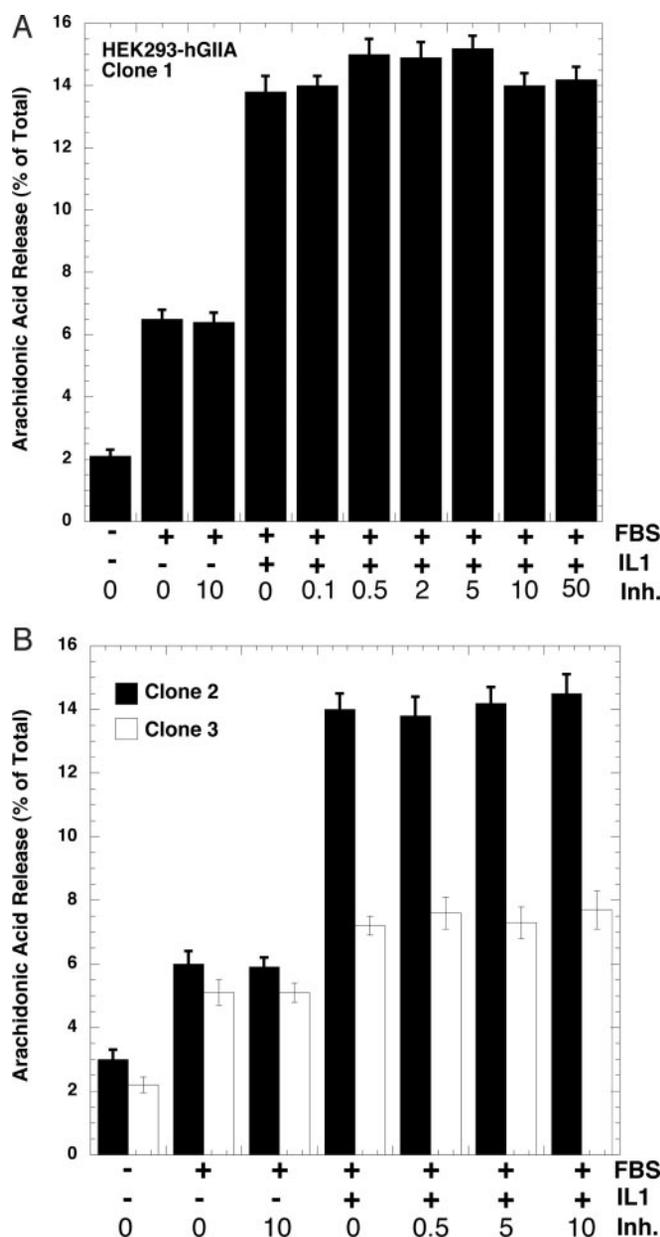


FIG. 6. [³H]Arachidonic acid release from HEK293 cells in the presence of sPLA₂ inhibitors. *A*, HEK293-hGIIA cells (clone 1) in the presence and absence of FBS, IL-1 β , and inhibitor (*Inh.*) as noted. Me-Indoxam was used at 0–10 μ M, as noted; the last bar is with 50 μ M LY311727. *B*, same as for *A* except with HEK293-hGX cells (clones 2 and 3). The data shown are representative of five independent experiments, each done in triplicate; the error bars show the standard deviation from one of the triplicate analyses. See text for additional information.

[³H]arachidonate release (not shown). For all three HEK293-hGIIA clones, there was also no effect of 0.1–10 μ M Me-Indoxam on [³H]arachidonate release induced by serum in the absence of IL-1 β (not shown). Similarly, as shown in Fig. 6B, 0.5–10 μ M Me-Indoxam has no effect on the amount of [³H]arachidonate released into the medium from hGX-transfected HEK cells stimulated with serum and IL-1 β (clones 2 and 3) or serum alone (not shown). Also, no inhibition of [³H]arachidonate from hGIIA- and hGX-transfected cells was seen when 10 μ M Me-Indoxam was included throughout the period in which cells were labeled with [³H]arachidonate and during the stimulation period (not shown).

We used two different methods to gauge the ability of Me-Indoxam to enter the cytosol of mammalian cells. In one assay,

nontransfected HEK293 cells were incubated with [³H]Me-Indoxam for 20 min at 37 $^{\circ}$ C, and the cells were rapidly separated from the culture medium by centrifugation through a layer of 100% FBS (33). The well established cell-impermeable compound 1-[³H]-D-mannitol was used as a negative control, and the well established cell permeable compound 4-[³H]-L-propranolol was used as a positive control. The results in Fig. 9S (Supplemental Material) show that the amounts of 1-[³H]-D-mannitol and [³H]Me-Indoxam that reach the bottom of the centrifuge tube is independent of the number of HEK293 cells in the incubation. Fig. 9S also shows the expected behavior if the intracellular concentration of [³H]Me-Indoxam reaches the extracellular concentration of 5 μ M. This curve was calculated assuming a cell radius of 10 microns. In contrast, the amount of 4-[³H]-L-propranolol to reach the tube bottom is proportional to the cell number, as expected for this readily cell-permeable compound. The data argue that the radius of these HEK293 cells is larger than 10 microns. Thus, it is clear that Me-Indoxam, like mannitol, fails to enter the cytosol of HEK293 cells. [³H]Me-Indoxam almost certainly partitions into the extracellular leaflet of the plasma membrane, and thus some compound might have been expected to pellet with the cells at the bottom of the centrifuge tube. However, the derivation given as Supplemental Material shows that membrane-bound [³H]Me-Indoxam is expected to dissociate into the extracellular medium with a half-life on the order of 1 μ s. Thus, all of the outer membrane leaflet compound is expected to desorb from the cell membranes during the time that it takes for the cells to pass through the serum layer, where the concentration of free [³H]Me-Indoxam is close to 0.

We also examined the cell permeability of Me-Indoxam using the well established Caco-2 cell monolayer assay. These cells are derived from the human gut epithelium and from tight intercellular junctions that are essentially impermeable to water-soluble low and high molecular mass compounds. Measurements were made in both the apical-to-basolateral (AB) direction and in the reverse direction (BA) because it is well established that transporters and pumps can modulate the passage of compounds across the Caco-2 cell monolayer. In this assay Me-Indoxam had very low apparent permeability values (P_{app}) of 0.08 and 0.1×10^{-6} cm/s (duplicate runs in the AB direction) and 0.20 and 0.17×10^{-6} cm/s in the BA direction. These values are comparable with those measured for mannitol, 0.1 – 0.2×10^{-6} cm/s, which cannot cross the cell membrane either passively or actively and is thought to slowly leak through the tight junctions between adjacent cells. In contrast, propranolol is thought to rapidly cross the cell membrane by passive diffusion and gave P_{app} values of 53.6 and 52.5×10^{-6} cm/s (AB) and 29.8×10^{-6} cm/s (BA). Vinblastine is a well established substrate for P-glycoprotein that actively transports compounds in the BA direction. This compound gave values of P_{app} of 1.87 and 2.2×10^{-6} cm/s (AB) and 47.6 and 39.5×10^{-6} cm/s (BA). Ranitidine is considered a poorly permeable standard and gave P_{app} values of 0.84 and 0.90×10^{-6} cm/s (AB) and 3.5 and 3.1×10^{-6} cm/s (BA). The recovery of all compounds was >79% (obtained from the concentrations of test compound measured on both the donor and acceptor sides of the cell monolayer). Additional information about the Caco-2 assay is given as Supplemental Material. These data showing essentially no passage of Me-Indoxam across the caco-2 cell monolayer other than transfer between cells are consistent with the HEK293 cell uptake studies measured by the centrifugation method.

Localization of hGIIA in HEK293 Cells by Immunofluorescence-Confocal and Immunogold Electron Microscopies—We

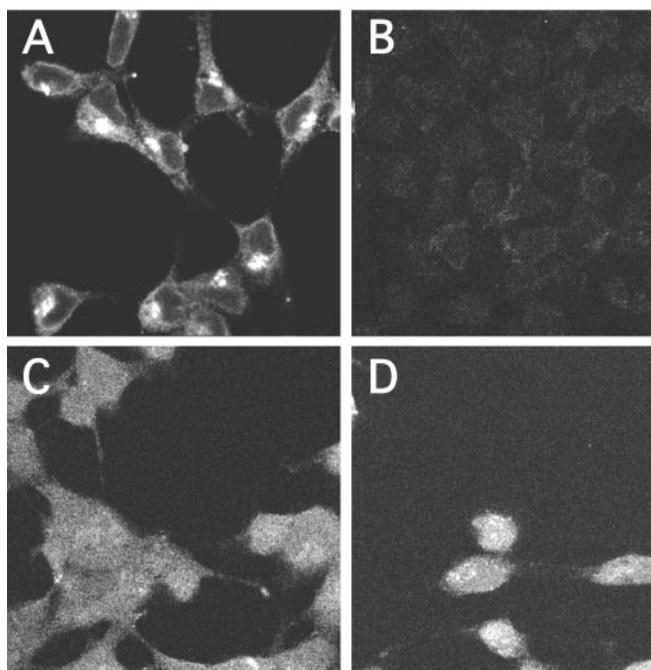


FIG. 7. Confocal immunofluorescence microscopy of hGIIA-transfected HEK293 cells. *A*, hGIIA-transfected HEK293 cells fixed at room temperature and permeabilized with Triton X-100 at room temperature; *B*, nontransfected HEK293 cells prepared as in *A*; *C*, hGIIA-transfected HEK293 cells fixed at 4 °C and permeabilized with 1% saponin at room temperature; *D*, same as *C* but fixed at room temperature.

studied the localization of hGIIA in transfected HEK293 cells by immunofluorescence confocal microscopy, and the results are shown in Fig. 7. When the cells were fixed with formaldehyde (at room temperature) and permeabilized with Triton X-100 (same conditions as used for CHO-K1 cells; Fig. 4), hGIIA localized to a region tentatively assigned to the Golgi compartment. When the cells were fixed with formaldehyde (at room temperature or 4 °C) and permeabilized with 1% saponin (conditions reported previously (21)), hGIIA was detected throughout the cytosol (Fig. 7, *C* and *D*) with some punctate staining appearing in Fig. 7*D*. Similar diffuse-type cytosolic staining was seen with hGIIA-transfected CHO-K1 cells permeabilized with 1% saponin (not shown). This diffuse staining is probably the result of the relatively high amount of saponin used; most studies use 0.1% saponin.

We also investigated the intracellular distribution of hGIIA at the electron microscopic level by preparing ultra thin sections of hGIIA-transfected HEK293 cells (clone 1) and labeling with a hGIIA-specific antibody. The intracellular distribution of hGIIA in HEK293 treated with serum or with serum and IL-1 β and in the absence and presence of extracellular heparin was similar (Fig. 8). Intracellular labeling was confined to the Golgi complex (Fig. 8, *A* and *B*). hGIIA labeling was also seen on the cell surface (Fig. 8*C*). Although the labeling density was low, detailed examination of the labeled sections revealed only sparse background labeling elsewhere, and this labeling was indistinguishable from the labeling of the nontransfected cells. Caveolae, identified by labeling for caveolin-1, were rare in HEK293 cells but generally showed negligible labeling for hGIIA in double labeled sections (Fig. 8*D*).

Involvement of cPLA₂- α in arachidonate release in hGIIA and hGX-transfected HEK293 and CHO-K1 cells—We tested two structurally distinct, potent, and selective inhibitors of cPLA₂- α , pyrrophenone (27, 41) and AZ-1 (compound 22 in Ref. 28), for their possible effect on [³H]arachidonate release from

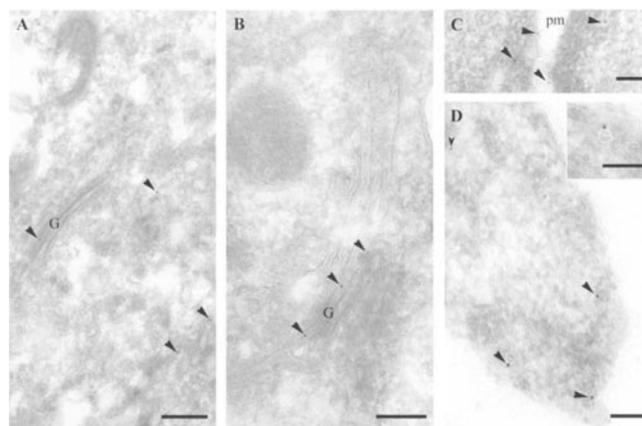


FIG. 8. Immunoelectron microscopic location of hGIIA in HEK293 cells. HEK293 cell lines expressing hGIIA PLA₂ were either untreated (*A* and *D*) or treated with IL-1 β alone (*B*) or IL-1 β and heparin (*C*). They were then fixed in paraformaldehyde and processed for frozen sectioning. Ultra thin sections were immunolabeled with antibodies to hGIIA followed by 10-nm protein A gold. Specific labeling (*arrowheads*) in all treatments was associated with the Golgi stack (*G*) and associated membranes (*A* and *B*). Specific but variable labeling was observed close to the plasma membrane (*pm*) (*C*). In addition, ultra thin sections were immunolabeled with antibodies to hGIIA followed by 5-nm protein A gold, fixed, and then immunolabeled with antibodies to caveolin 1 followed by 10-nm protein A gold (*D*). Caveolin-1 antibodies labeled surface caveolae (*arrowheads*; *inset* shows higher magnification view of one caveola), but negligible labeling for hGIIA (*small arrowhead*) was observed in these structures. *Bars*, 200 nm.

hGIIA- and hGX-transfected HEK293 cells. As shown in Fig. 9*A*, [³H]arachidonate release from nontransfected cells is insensitive to IL-1 β (consistent with the results in Fig. 5*A*) or to the addition of pyrrophenone or AZ-1. In hGIIA-transfected HEK293 cells clone-1 (which is highly sensitive to IL-1 β ; Fig. 5*A*), in the absence of IL-1 β , [³H]arachidonate release is ~2-fold higher than in nontransfected cells, and pyrrophenone blocks approximately 50% of this increase. The degree of inhibition by pyrrophenone is the same at all three doses (0.2, 2, and 10 μ M), suggesting that pyrrophenone is inhibiting all of the cPLA₂- α . The addition of IL-1 β to clone 1 approximately doubles the amount of [³H]arachidonate release, and all three doses of pyrrophenone dramatically reduce [³H]arachidonate release, bringing it close to the level seen in the presence of pyrrophenone and in the absence of IL-1 β (Fig. 9). The second cPLA₂- α inhibitor AZ-1 also causes substantial inhibition of [³H]arachidonate release but is less potent than pyrrophenone (Fig. 9*A*). With hGIIA-transfected HEK293 cells (clone 4), which show a smaller response than clone 1 to IL-1 β (Fig. 8*A*), the two cPLA₂- α inhibitors cause a similar response as seen with clone 1 (Fig. 9*A*).

As shown in Fig. 9*B*, addition of the cPLA₂- α inhibitors to the IL-1 β -responsive hGX-transfected HEK293 cell clone 2 blocked all of the additional [³H]arachidonate release induced by IL-1 β . As noted earlier, [³H]arachidonate release in the absence of IL-1 β was larger than in hGIIA-transfected HEK293 cells, and this was completely insensitive to the cPLA₂- α inhibitors. [³H]Arachidonate release from hGX-transfected HEK293 cells clone 1, which is much less sensitive to IL-1 β (Fig. 5*B*), is virtually insensitive to the cPLA₂- α inhibitors (Fig. 9*B*).

As shown in Fig. 10*S*, HEK293 cells express cPLA₂- α as detected by immunoblot analysis. The level of cPLA₂- α does not seem to vary between nontransfected cells and those transfected with hGIIA (clones 1 and 4) or hGX (clones 2 and 3).

We also studied the effect of pyrrolidine-1, an analog of pyrrophenone that is also a potent inhibitor of cPLA₂- α (27, 42), on [³H]arachidonate release from hGIIA-transfected CHO-K1 cells. Nontransfected cells released $3.8 \pm 0.2\%$ more [³H]ara-

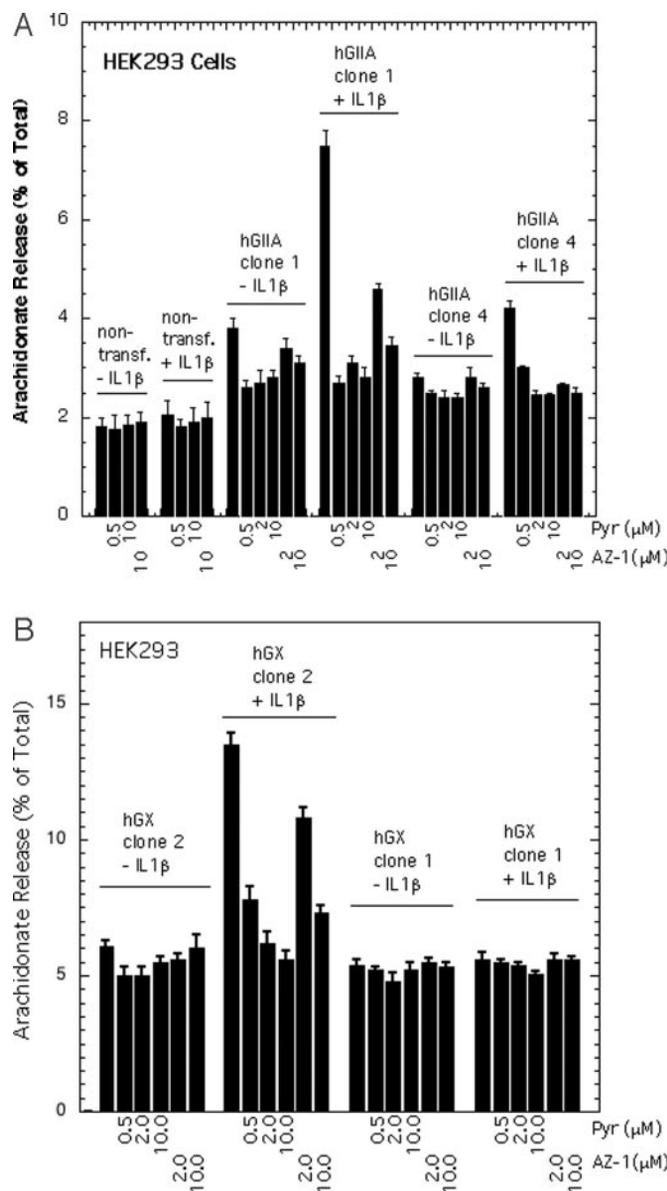


FIG. 9. Effect of cPLA₂-α inhibitors on arachidonate release by HEK293 cells transfected with hGIIA (A) or hGX (B). Arachidonate release after 4 h of culture in the presence and absence of IL-1β (2 ng/ml) and in the presence or absence of the cPLA₂-α inhibitors pyrrophenone (Pyr) or AZ-1 as indicated (concentrations given along the x axis). A shows data for nontransfected cells and for HEK293 cells transfected with hGIIA (clones 1 and 4). B shows data for nontransfected cells and for HEK293 cells transfected with hGX (clones 1 and 2). Serum was present in all experiments. The data shown are representative of three independent experiments, each done in triplicate; the error bars show the standard deviation from one of the triplicate analyses.

chidonate in the presence of serum and IL-1β compared with nonstimulated cells, and this difference was reduced to $2.2 \pm 0.1\%$ in the presence of $5 \mu\text{M}$ pyrrolidine-1. hGIIA-transfected CHO-K1 cells (clone 4) showed a $4.8 \pm 0.2\%$ difference in [³H]arachidonate release between stimulated *versus* nonstimulated cells and this difference was reduced to $2.4 \pm 0.2\%$ in the presence of $5 \mu\text{M}$ pyrrolidine-1. Thus, approximately 50% of the [³H]arachidonate from nontransfected cells appears to be due to cPLA₂-α, which is consistent with the fact that these cells were shown by immunoblot analysis to contain cPLA₂-α (43). Inhibition of cPLA₂-α reduces most of the increase in [³H]arachidonate release induced by transfection of cells with hGIIA (as for HEK293 cells).

DISCUSSION

[³H]Arachidonate Release by sPLA₂s Prior to Enzyme Secretion—It has already been noted that exogenously added hGIIA is several orders of magnitude less efficient at liberating arachidonic acid from HEK293 cells compared with cells constitutively expressing this enzyme by stable transfection. The simplest explanation for this result is that arachidonate release occurs by hGIIA prior to secretion to the extracellular medium. It has been recently reported that exogenously added hGIIA is internalized into HEK293 cells only when IL-1β is added as shown by immunoblot analysis of cell pellet associated hGIIA only in the presence of cytokine (7). We did not directly study the reinternalization of hGIIA into HEK293 cells, but it is clear from the results in Fig. 5S that the addition of relatively large amounts of exogenous hGIIA to HEK293 cells in the presence or absence of pretreatment with IL-1β failed to produce detectable [³H]arachidonate release either to the medium or as cell-associated free fatty acid. Perhaps some [³H]arachidonate is released but is not detected because of rapid reincorporation into phospholipid. However, the release of [³H]arachidonate to the culture medium is readily detected in hGIIA-transfected cells (Fig. 5A). Thus, it is suggested that the [³H]arachidonate released from transfected cells is not due to reinternalization of secreted hGIIA.

Additional strong evidence for this latter statement comes from studies with Me-Indoxam. It is clear that the addition of this sPLA₂ inhibitor to hGIIA-transfected HEK293 and CHO-K1 cells has no effect on [³H]arachidonate release. Control studies clearly show that Me-Indoxam is not destroyed by cell-conditioned medium, and this compound blocks all of the [³H]arachidonate release induced by exogenously added sPLA₂s (small amounts of hGX, enormous amounts of exogenously added hGIIA, and lower amounts of the hGIIA-V3W mutant). By two independent assays, it is shown that Me-Indoxam does not cross the plasma membrane of cells. The glypican-shuttling mechanism or any other secretion followed by re-entry mechanism for hGIIA action requires that the sPLA₂ comes in contact with the extracellular fluid. This would allow hGIIA to bind Me-Indoxam even though this compound cannot cross the plasma membrane. Thus, the complete lack of inhibition by Me-Indoxam rules out mechanisms in which [³H]arachidonate release is by hGIIA acting on the extracellular face of the plasma membrane or within an intracellular vesicle compartment whose aqueous lumen is derived from the extracellular medium. Me-Indoxam did not reduce [³H]arachidonate release even when it was included in the culture during the several hour fatty acid labeling period. If secreted enzyme produced during the labeling period is reinternalized and contributes to [³H]arachidonate release during the stimulation period, it should have been blocked by Me-Indoxam. In a previous study, it was reported that $10 \mu\text{M}$ LY311727 (an sPLA₂ inhibitor structurally related to Me-Indoxam but less potent on hGIIA) partially inhibits [³H]arachidonate release from serum/IL-1β-stimulated, hGIIA-transfected HEK293 cells (19). In our hands, even $50 \mu\text{M}$ LY311727 fails to produce detectable inhibition of hGIIA-transfected CHO-K1 and HEK293 cells when examined in more than 10 experiments by two laboratory workers.

We have confirmed the previous findings (19) that extracellular heparin traps secreted hGIIA in the culture medium by preventing its association with the surface of CHO-K1 cells (Fig. 1S) and of HEK293 cells (Figs. 4S and 6S). Inhibition of [³H]arachidonate release by extracellular heparin would argue in favor of the re-entry model for action of this sPLA₂ in transfected cells. Extracellular heparin has no effect on [³H]arachidonate release by hGIIA-transfected CHO-K1 cells

(Fig. 2), consistent with fatty acid release occurring prior to exposure of hGIIA to the extracellular fluid. The effect of heparin with HEK293 cells is complex. Although inhibition of [³H]arachidonate release by extracellular heparin was observed with hGIIA-transfected HEK293 cells (Fig. 7S, A and B) several features strongly argue that this is not the result of trapping of hGIIA. Firstly, removal of the majority of the secreted hGIIA by pretreatment of cells with heparin for 15 h has no effect on the level of [³H]arachidonate released during the subsequent 6-h period in which cells were stimulated with serum and IL-1 β . If [³H]arachidonate release occurred by hGIIA shuttled intracellularly from the cell surface, the amount of fatty acid release during the 6-h stimulation period should be less if there is less sPLA₂ on the cell surface. Secondly, inhibition by extracellular heparin was partial (only ~20% for hGIIA-HEK293) despite the fact that sufficient heparin was added to fully remove hGIIA from the cell surface. Thirdly, and perhaps most significantly, is the observation that heparin also partially inhibited [³H]arachidonate release by hGX-transfected HEK293 cells (Fig. 8S, A and B) despite the fact that this sPLA₂ has no detectable affinity for heparin (8). Thus, the presence of extracellular heparin somehow reduces [³H]arachidonate release following stimulation with serum and IL-1 β by a nonspecific mechanism, the details of which are not understood. Thus, caution is advised in the use of extracellular heparin, a highly anionic and polymeric substance, to explore the mode of action of sPLA₂s.

hGIIA has been localized to punctate domains throughout the cytosol of transfected HEK293 cells, when studied by immunofluorescence-confocal microscopy using 1% saponin to permeabilize the cells (21). It was suggested that these punctate domains are caveolae or caveolae-derived vesicle compartments based on co-location of hGIIA with caveolin-2. Although we could see staining of hGIIA throughout the cytosol with some punctate appearance in transfected HEK293 cells permeabilized with 1% saponin (Fig. 7), hGIIA appears to reside in the Golgi compartment when cells were permeabilized with 0.2% Triton X-100 in both HEK293 (Fig. 7) and CHO-K1 cells (Fig. 4). Furthermore, there was no effect of extracellular heparin on the microscopic images. It should be pointed out that caveolin-1 is considered to be a reliable marker of caveolae, but caveolin-2 is more of a Golgi marker than a marker of caveolae (see for example Refs. 44–48). Some caveolin-2 is found in caveolae, in association with caveolin-1, but even in these cases, most of the caveolin-2 detected by immunofluorescence resides in the Golgi complex (45–47). A recent study showed co-localization of human group V sPLA₂ with caveolin-2 in punctate domains in the macrophage-like cell line p388D1 (49). Given the above concerns, the localization of human group V sPLA₂ in caveolae is called into question. Treatment of cells with extracellular heparin reduces the amount of immunofluorescence staining of hGIIA and group V sPLA₂ in punctate domains (21, 49), and this is cited as evidence that the punctate localization of sPLA₂ derives from re-entry of extracellular enzyme. It may be noted that heparin treatment did not eliminate punctate staining (49). Furthermore, because heparin forms high molecular mass, multi-protein/polymer aggregates with basic sPLA₂s (50), it is possible that the effect of heparin is to prevent the transfer of extracellular sPLA₂ into detergent-permeabilized cells, given that formaldehyde fixation is typically not a complete process. In this context, we have found that intracellular hGIIA staining is seen when CHO-K1 and HEK293 cells are treated first with exogenous hGIIA followed by formaldehyde fixation and detergent permeabilization and that such intracellular staining is prevented by treating the cells with extracellular heparin (not shown).

To further explore the localization of hGIIA in transfected HEK293 cells, we turned to immunogold electron microscopy using ultra thin sections. The results in Fig. 8 show that these cells contain caveolae; however, we see localization of hGIIA only at the cell surface and Golgi stacks. Although this is a negative result for localization of hGIIA in caveolae, the electron microscopic images fit well with the overwhelming amount of additional data in this study, ruling out the re-entry model for hGIIA action in HEK293 and CHO-K1 cells.

Although we did not study the role of glypican, a GPI-anchored, heparan sulfate-containing proteoglycan, in promoting [³H]arachidonate release in hGIIA-transfected HEK293 cells, the observation that fatty acid release is fully intact in mutant CHO-K1 cells that lack all glycosaminoglycans and GPI anchors establishes that glypican shuttling is not essential for function of hGIIA in CHO-K1 cells.

The addition of Me-Indoxam has no effect on [³H]arachidonate released early from hGX-transfected CHO-K1 cells, but this sPLA₂ inhibitor partially blocks fatty acid released later in time (Fig. 3B). The simplest explanation for this unusual behavior is that hGX-catalyzed [³H]arachidonate release occurs in part by enzyme in the secretory compartment prior to externalization of enzyme, and a portion occurs by extracellular enzyme acting on the extracellular face of the plasma membrane. As time goes on, the extracellular concentration of hGX rises (the amount of secreted hGX, detected with the fluorimetric assay, increases linearly over the 0–24-h time period) until it is high enough to lead to fatty acid release from the plasma membrane. The latter process depends on the extracellular concentration of enzyme; mass action dictates that dilution of hGX by extracellular medium reduces the fraction of enzyme bound to the plasma membrane. In contrast, the concentration of membrane “seen” by hGX residing inside secretory compartments is independent of the volume of culture medium, and thus fatty acid release by hGX prior to secretion occurs at all time points. With hGX-transfected HEK293 cells, Me-Indoxam has no effect on [³H]arachidonate release, arguing that the extracellular concentration of this sPLA₂ never builds to a high enough level to allow plasma membrane phospholipid hydrolysis. In the case of hGIIA, which binds orders of magnitude more weakly than hGX to the phosphatidylcholine-rich outer plasma membrane (8, 23), [³H]arachidonate release occurs in CHO-K1 and HEK293 cells only prior to enzyme secretion. This model nicely explains why exogenously added hGIIA is orders of magnitude less efficient at phospholipid hydrolysis than hGIIA produced by transcription/translation within the cell; the concentration of membrane that hGIIA “sees” is orders of magnitude higher when enzyme is in the secretory compartment compared with the extracellular medium. *In vitro* kinetic studies show that hGX is orders of magnitude more active than hGIIA on phosphatidylcholine-rich because only the former enzyme can binding tightly to such vesicles (8, 23, 29). Thus, one might anticipate that the intracellular membrane in which the sPLA₂ acts is rich in phosphatidylcholine based on the observation that hGX is more efficient than hGIIA at liberating [³H]arachidonate from transfected CHO-K1 and HEK293 cells despite the fact that the specific activity of hGX is approximately 10-fold lower than that of hGIIA when both are tested on their most preferred substrate (29).

Role of cPLA₂- α in IL-1 β -dependent [³H]Arachidonate Release and Cross-talk between cPLA₂- α and sPLA₂—We have confirmed the previous observations that serum and IL-1 β enhance [³H]arachidonate release in hGIIA-transfected CHO-K1 and HEK293 cells (19). Early studies showed that serum but not IL-1 β enhances [³H]arachidonate release in hGX-transfected HEK293 cells (18). This leads to the hypoth-

esis that the IL-1 β enhancement of fatty acid release and glypican shuttling are somehow linked. However, in the present study, we have found that there is a large clonal variation in the IL-1 β augmentation of fatty acid release. Some hGIIA-transfected HEK293 cells are more responsive to IL-1 β than others (Fig. 5A), and some hGX-transfected HEK293 clones show clear and reproducible IL-1 β augmentation of [³H]arachidonate release (Fig. 5B). In addition, we verified that hGX-transfected cells secrete hGX and not hGIIA based on the observation that the extracellular sPLA₂ activity is blocked by Me-Indoxam with an IC₅₀ in the low micromolar range, consistent with the published IC₅₀ of 1–2 μ M for Me-Indoxam acting on hGX, whereas the IC₅₀ for Me-Indoxam acting on hGIIA is much lower, 0.01–0.03 μ M (29). All together, the newer results would seem to rule out a IL-1 β -glypican shuttling connection. The reason for this clonal variation is not known, nor is the reason for the IL-1 β effect. However, the present results with two structurally distinct and potent cPLA₂- α inhibitors show that the IL-1 β -dependent but not the IL-1 β -independent component of [³H]arachidonate release involves cPLA₂- α (Fig. 9). Furthermore, neither serum nor IL-1 β leads to an increase in [³H]arachidonate release in cells not transfected with hGIIA or hGX (Fig. 5), thus one concludes that the IL-1 β -dependent fatty acid release involves the action of both cPLA₂- α and sPLA₂, whereas the IL-1 β -independent components involves the action of sPLA₂ alone. Based on immunoblot analysis, it has been suggested that HEK293 cells lack cPLA₂- α (51), but other immunoblot results and the effect of cPLA₂- α inhibitors (Fig. 10S) (52) clearly show the presence of cPLA₂- α in these cells.

In the macrophage-like cell line p388D1, it has been suggested that cPLA₂- α action leads to an intracellular pool of free [³H]arachidonate accumulation, whereas the group V sPLA₂ is mainly responsible for accumulation of [³H]arachidonate in the extracellular medium (53). In our studies with CHO-K1 and HEK293 cells, it is clear that essentially all of the released [³H]arachidonate is accounted for by that which accumulates in the culture medium. Also the fact that the cPLA₂- α inhibitors reduce accumulation of [³H]arachidonate in the medium in the present as well as in previous studies (2, 26, 28, 42) argues that free [³H]arachidonate liberated by cPLA₂- α transfers from the cells to the culture medium. Also, lack of transfer of liberated [³H]arachidonate from the inside of the cell to the culture medium is clearly not the reason why HEK293 cells transfected with hGIIA release [³H]arachidonate much more efficiently than cells treated with exogenous addition of hGIIA.

The data presented in this paper provide a particularly clear example of cross-talk between hGIIA or hGX and cPLA₂- α . A second clear example of this cross-talk comes from the recent study of Han *et al.* (15). They showed that expression of either hGIIA or group V sPLA₂ in cPLA₂- α -deficient murine mesangial cells does not lead to H₂O₂-stimulated [³H]arachidonate release, but expression of these sPLA₂s augments [³H]arachidonate release in mesangial cells that express cPLA₂- α above the fatty acid release seen in cells containing cPLA₂- α but not the sPLA₂s. Both mitogen-activated protein kinase and protein kinase C are involved in this sPLA₂-dependent cPLA₂- α activation (15). Other studies have shown that exogenous addition of sPLA₂ to mammalian cells leads to activation of mitogen-activated protein kinases along with activation of cPLA₂- α (for example see Refs. 13, 14, and 54). In other cells, it has been proposed that the lipolytic action of cPLA₂- α is required for the lipolytic action of sPLA₂s (for example see Refs. 55 and 56).

It must be stressed that none of these studies showing sPLA₂-cPLA₂- α cross-talk reveals the directionality of this cross-talk, *i.e.* does cPLA₂- α allow sPLA₂ to release fatty acids

or vice versa? However, it may be noted that Han *et al.* (15) and Murakami *et al.* (18) showed that in H₂O₂-activated mesangial cells and in serum/IL-1 β -stimulated, hGIIA-transfected HEK293 cells, respectively, arachidonate is released in preference to oleate. Because cPLA₂- α displays approximately 10-fold selectivity for phospholipids with an *sn*-2 arachidonyl chain *versus* and *sn*-2 oleoyl chain (1), whereas hGIIA displays essentially no selectivity (29, 57), it would appear that in mesangial and HEK293 cells, cPLA₂- α is responsible for most of the arachidonate release in the presence of H₂O₂ and serum/IL-1 β , respectively. The mechanism by which the action of sPLA₂ activates cPLA₂- α is under active investigation.

Acknowledgments—We are grateful to Gerard Lambeau and Mahendra Jain for helpful discussions during this study.

Note Added in Proof—Recently, Arm and co-workers have shown that disruption of the mouse group V sPLA₂ gene leads to an ~50% reduction in the amount of arachidonic acid liberated from zymosan-stimulated peritoneal macrophages (58), and yet earlier studies showed that the disruption of the cPLA₂- α gene leads to complete loss of arachidonic acid from these cells (59). Thus, it appears that group V sPLA₂ action somehow augments the function of cPLA₂- α in peritoneal macrophages. We have found that pyrrophenone blocks all of the arachidonate release from zymosan-stimulated mouse peritoneal macrophages, but 1 and 10 μ M Me-Indoxam is without effect (data not shown). These results are consistent with the gene disruption studies (58, 59) and further suggest that mouse group V is able to augment the action of cPLA₂- α and that the sPLA₂ acts prior to release from the cells. Thus our conclusions in the present study derived from transfected cells appear to be relevant to at least some non-transfected cells/agonist systems.

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