Title

Antisense inhibition of group II phospholipase A2 expression blocks the production of prostaglandin E2 by P388D1 cells.

Journal

Journal of Biological Chemistry, 268(29)

ISSN

0021-9258

Authors

Barbour, SE
Dennis, EA

Publication Date

1993-10-01

DOI

10.1016/s0021-9258(20)80622-x

Peer reviewed

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 268, No. 29, Issue of October 15, pp. 21875-21882, 1993
Printed in U.S.A.

Antisense Inhibition of Group II Phospholipase A_2 Expression Blocks the Production of Prostaglandin E_2 by P388D_1 Cells*

(Received for publication, January 27, 1993, and in revised form, May 17, 1993)

Suzanne E. Barbour‡ and Edward A. Dennis§

From the Department of Chemistry, 0601, University of California at San Diego, La Jolla, California 92093-0601

Macrophage-like P388D_1 cells release [^{3}H]arachidonic acid and produce prostaglandin E_2 (PGE_2) upon stimulation with bacterial lipopolysaccharide (LPS) and platelet-activating factor (PAF). To determine whether group II phospholipase A_2 (PLA_2) is involved in this release, we treated P388D_1 cells with antisense inhibitors specific for group II PLA_2 RNA. Treatment with oligonucleotide ASGII decreased PLA_2 activity in P388D_1 cell homogenates by ~60% and reduced the release of [^{3}H]arachidonic acid and PGE_2 from activated cells to nearly resting cell levels. The inhibition by antisense oligonucleotide ASGII was blocked when its sense complement, SGII, was included in the incubation mixture. Stably transfected P388D_1 cells expressing an antisense construct for group II PLA_2 also produced reduced quantities of PGE_2 in response to LPS and PAF. These data suggest that prostaglandin production by activated P388D_1 cells involves phospholipid hydrolysis by group II PLA_2. Oligonucleotide ASGII also blocked the appearance of a heparin-releasable group II PLA_2 in the culture supernatants of P388D_1 cells. The disappearance of this protein correlated with reduced PGE_2 production by activated cells, indicating that an extracellular heparin-associated pool of group II PLA_2 is involved in prosta-glandin production by P388D_1 cells.

Phospholipase A_2 (PLA_2)[1] comprises a family of lipolytic enzymes which attacks the *sn*-2 carbonyl of phospholipids to produce fatty acids and lysophospholipids and is implicated in the release of arachidonic acid for prostaglandin biosynthesis in P388D_1 cells (1). The best studied PLA_2s, the secretory PLA_2s ($sPLA_2$), are 14-kDa calcium-dependent enzymes (2). Based on conserved disulfide bonding patterns, these proteins have been divided into groups I, II, and III (3). As the "secretory" name implies, these proteins are secreted into bee and reptile venoms, into mammalian pancreatic exudates, and by a variety of mammalian cells (2, 4–8). Recently, an 85-kDa arachidonate-specific PLA_2 has been purified and cloned from the cytosol of mammalian cells (9–14). This cytosolic PLA_2 ($cPLA_2$) is translocated to membranes (where its substrate is localized) in response to physiological (submicromolar) levels of calcium and is activated by phosphorylation (12–15). These observations have led to the assumption that $cPLA_2$ rather than $sPLA_2$ is involved in receptor-mediated activation of mammalian cells.

Phospholipase A_2 activities have been implicated in many cell activation systems; however, there has been no direct demonstration of a requirement for PLA_2 catalysis in these systems. In some studies, PLA_2 activity is inferred from the release of [^{3}H]arachidonic acid from the phospholipids of prelabeled cells (see Refs. 16 and 17 for example). This approach does not distinguish between $sPLA_2$ and $cPLA_2$ activities and ignores other possible mechanisms for the release of fatty acid from intact phospholipids. Other investigators have relied on a variety of nonspecific inhibitors (*p*-bromophenacyl bromide, glucocorticoids, and mepacrine, for example) to demonstrate $sPLA_2$ activity (see Refs. 18 and 19 for example). More specific, mechanism-based inhibitors have been described for $sPLA_2$, but many of these require complicated syntheses, are available in extremely small quantities, and are either not taken up by cells or are cytotoxic (20, 21). In other studies, increases in PLA_2 mRNA and protein levels have been used as indirect indications of $sPLA_2$ activation (5, 6, 8). It is possible that the induction of $sPLA_2$ protein is nonspecific and PLA_2 activity may not be involved in these cell activation systems (7).

Previous work from our laboratory has demonstrated that P388D_1 cells stimulated with bacterial lipopolysaccharide (LPS) and platelet-activating factor (PAF) produce prostaglandin E_2 (PGE_2) (1). The release of PGE_2 is inhibited by manoalogue, indicating the possible involvement of a group II PLA_2 in the response. To test this hypothesis, we have designed specific antisense RNA inhibitors of group II PLA_2. Antisense RNA technology offers the potential of designing potent PLA_2 inhibitors with absolute specificity (22). The inhibition is based on the binding of complementary nucleotides to group II PLA_2 mRNA. Using such inhibitors, we demonstrate that group II PLA_2 activity is involved in the release of PGE_2 from activated P388D_1 cells. In addition, evidence is provided to suggest that this release is mediated by an extracellular pool of group II PLA_2 that is localized to cell surface proteoglycans.

EXPERIMENTAL PROCEDURES

Materials—P388D_1 cells were obtained from ATCC (Rockville, MD). LPS Re 595 was the kind gift of R. Ulevitch (Research Institute of Scripps Clinic, La Jolla, CA). R. Kramer (Eli Lilly Co.) kindly provided *Escherichia coli* strain SN17 which was used to prepare the substrate for the PLA_2 assay. A polyclonal antibody against human synovial fluid group II PLA_2 (W98–7UJ-39A) was the generous gift of C. Teater and J.L. Bobbitt (Eli Lilly Co.). Antibodies R385, R377, and MB 5.2 (developed against rat group II PLA_2) were the kind gifts of K. Inoue and I. Kudo (University of Tokyo). A cocktail of these anti-group II PLA_2 antibodies was prepared for immunoblotting. C. Leslie (National Jewish Center for Immunology and Respiratory Medicine) kindly provided the antiserum against the murine $cPLA_2$.

Cell Culture—P388D_1 cells were maintained at 37 °C, 10% CO_2, 95% humidity (cell culture conditions) in DME-10, which consisted of low

* This work was supported by National Institutes of Health Grant HD 26171. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of University of California President's Postdoctoral Fellowship and National Institutes of Health Individual National Research Service Award GM 15279.

§ To whom correspondence should be addressed.

[1] The abbreviations used are: PLA_2, phospholipase A_2; $sPLA_2$, secretory PLA_2; $cPLA_2$, 85-kDa cytosolic PLA_2; LPS, lipopolysaccharide; PAF, 1-*O*-alkyl-2-acetyl-*sn*-glyceroyl-3-phosphorylcholine (platelet-activating factor); PGE_2, prostaglandin E_2; PCR, polymerase chain reaction; HUVEC, human umbilical vein endothelial cell; COX, cyclooxygenase; SFM, serum-free medium; RIA, radioimmunoassay; PAGE, polyacrylamide gel electrophoresis; DME, Dulbecco's modified Eagle's medium.

endotoxin Iscove's modified Dulbecco's medium (DME, Whittaker Bioproducts, Walkersville, Md) supplemented with 10% low endotoxin fetal calf serum (HyClone, Logan, UT), with 50 μg/ml gentamycin sulfate (Sigma), 2.5 μg/ml amphotericin, nonessential amino acids (Irvine Scientific Co., Santa Ana, CA) and 2 mM glutamine. The cells used in these experiments were between passages 15 and 30. Cells were plated at 10^6/well in six-well plates, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free medium (SFM), composed of DME, 1 × nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Preparation of Oligonucleotides—An antisense oligonucleotide to group II PLA_2 (referred to as ASGII, sequence 5′ GAT CCT CTG CCA CCC ACA CC 3′) with phosphorothioate linkages, was utilized in these experiments. The original sample of this oligonucleotide was kindly provided by C. Frank Bennett (ISIS Pharmaceuticals, Carlsbad, CA).

Subsequent samples of ASGII, its sense complement (oligonucleotide SGII), and PCR primers were synthesized on a Milligen Cyclone DNA synthesizer using phosphoramidite chemistry. Phosphorothioate analogs were prepared by substituting a sulfurizing reagent (Glen Research, Sterling, VA) for iodine in the oxidation reaction. This reagent was originally described by Beaucage (23). Phosphorothioate oligonucleotides were synthesized according to a protocol supplied by Milligen/Biosearch (Waltham, MA). All oligonucleotides were purified by ethanol precipitation (two times, using 2.5 M NaCl) and showed single sharp bands upon denaturing acrylamide gel electrophoresis.

Oligonucleotide Treatment—$P388D_1$ cells were transfected with oligonucleotide in the presence of 5 μg/ml Lipofectin (Life Technologies, Inc.) as reported by Chiang *et al.* (24). Lipofectin was mixed with oligonucleotide or water, and complexes were allowed to form at room temperature for 10–15 min. During this incubation, the $P388D_1$ cell monolayers were washed two times with SFM. Each well received 1 ml of SFM and then 100 μl of Lipofectin-oligonucleotide complexes were added with gentle agitation. Control cells received no treatment or Lipofectin alone. Typically, the final concentration of oligonucleotide in the incubation mixture was 250 nM. The transfection was allowed to proceed for 6 h under cell culture conditions. Cell viability was assessed by measuring the release of lactate dehydrogenase into the cell supernatants using a kit from Sigma and was greater than 95% during the course of these experiments.

Cell Activation and Measurement of Released PGE_2—Our standard regimen for activating $P388D_1$ cells with LPS and PAF (1) was used with the following modifications. After 6-h incubation in the presence of oligonucleotide and Lipofectin, 1 ml of LPS Re 595 (400 ng/ml in SFM), or SFM alone was added to each well. This addition decreased the concentration of oligonucleotide in the incubation to 125 nM. The cells were incubated with LPS for 60 min under cell culture conditions, washed two times with SFM, and then incubated in 1 ml of SFM in the presence of 250 nM oligonucleotide (no Lipofectin) for 30 min (wash step). After the wash step, the supernatants were removed and replaced with 1 ml of SFM or 20 nM PAF (Sigma) in SFM. PAF stimulation was continued for 10–30 min under cell culture conditions.

Cell culture supernatants were harvested and centrifuged at 1000 × *g* to remove nonadherent cells. A radioimmunoassay (RIA) was used to detect the PGE_2 released into the culture supernatants (Advanced Magnetics, Cambridge, MA). RIA data were analyzed by nonlinear regression using custom software. All data points were taken in triplicate and are reported with the standard deviation of the mean.

PLA_2 Assays of Cell Homogenates—After activation or oligonucleotide treatment, the $P388D_1$ cell monolayers were scraped into 0.64 ml of phosphate-buffered saline, and cell homogenates were prepared by sonicating the cell suspensions on ice. The protein concentrations of the unfractionated cell homogenates were measured using the Bio-Rad Protein Assay (Bio-Rad).

Calcium-dependent PLA_2 activity was measured using radiolabeled *E. coli* membranes. [^{3}H]Oleic acid-labeled *E. coli* membranes were prepared by standard procedures (25). This assay is commonly used to measure the activities of group II PLA_2s (25, 26). Equivalent amounts of cell homogenate protein were incubated with ~50,000 cpm of *E. coli* membranes in PLA_2 assay buffer (25 mM Tris-HCl, pH 9.0, 10 mM calcium chloride, 5 mg/ml bovine serum albumin) in a final volume of 200 μl for 80 min at 37 °C. The reaction was stopped, and liberated fatty acids were extracted by the additions of 100 μl each of 2 N HCl and 20 mg/ml bovine serum albumin, respectively. After 20 min on ice, the reaction mixtures were centrifuged at 10,000 × *g*. The percentage hydrolysis was determined by the percentage of input counts released into the supernatants.

Calcium-dependent PLA_2 activity was also measured using a mixed micellar substrate consisting of 100 μM dipalmitoylphosphatidylcholine and 200 μM Triton X-100 in 80 mM glycine, pH 9.0, 5 mM $CaCl_2$, 70% glycerol. These assay conditions have been optimized to measure the membrane-associated, calcium-dependent PLA_2 activity that we have purified previously from $P388D_1$ cells (27).

$P388D_1$ cells also express a cytosolic calcium-independent PLA_2 which we have purified and characterized (28).[2] This enzyme may be distinct from the calcium-independent plasmenyl phospholipid-specific PLA_2s described by Gross and co-workers (29, 30). Calcium-independent PLA_2 activity in $P388D_1$ cell homogenates was measured using a mixed micellar substrate consisting of 100 μM dipalmitoylphosphatidylcholine and 400 μM Triton X-100 in 25 mM Tris-HCl, 5 mM EDTA, pH 7.5. The calcium-independent PLA_2 from $P388D_1$ cells preferentially hydrolyzes this micellar substrate.

In both the calcium-dependent and calcium-independent mixed micelle PLA_2 assays, approximately 100,000 cpm of 1-palmitoyl-2-[^{14}C]palmitoyl phosphatidylcholine (Du Pont-New England Nuclear) was added to each assay as a tracer. Assay tubes were incubated for 3 h (lysates of stably transfected cells) or 1 h (lysates of oligonucleotide-treated cells) at 40 °C and processed according to the Dole assay method (27). The data are presented as the percentage of input counts/min recovered in the flow-through fraction of the silica column.

Cytosolic PLA_2 ($cPLA_2$) activity was measured using the assay described by Kramer *et al.* (9). This assay uses the 2-arachidonoyl phosphatidylcholine substrate that is preferred by $cPLA_2$. Assay tubes were incubated at 40 °C for 15 min and were processed using the Dole assay method (27). Unlabeled oleic acid was included in the Dole assay workup as a carrier.

It is difficult to develop assays with absolute specificity for a particular type of PLA_2 (31). However, "selective" methods (based on substrate preferences and calcium dependence) were used to distinguish between the PLA_2 activities in $P388D_1$ cells. As $cPLA_2$ preferentially hydrolyzes phospholipids containing *sn*-2 arachidonic acid, it is likely that this enzyme does not contribute to the hydrolysis of the *E. coli* membranes. Conversely, the group II PLA_2 from human synovial fluid is not active in the assay for $cPLA_2$ (data not shown). This is most likely due to the inclusion of 2-mercaptoethanol in the assay buffer. The assay for the calcium-independent PLA_2 is probably the most selective method that we have used, as neither group II PLA_2 nor $cPLA_2$ activity should contribute to substrate hydrolysis in the absence of calcium.

Release of [^{3}H]Arachidonic Acid—$P388D_1$ cells were labeled with [^{3}H]arachidonic acid during the 6-h transfection. Preliminary experiments indicated that the presence of Lipofectin and oligonucleotides did not alter the uptake of arachidonic acid by the cells. $P388D_1$ cell monolayers were overlayed with 1 ml of SFM containing 0.5 μCi 5,6,8,9,11,12,14,15-[^{3}H]arachidonic acid (Du Pont-New England Nuclear). Lipofectin and oligonucleotide were added directly to the supernatants, and the cells were incubated for 6 h under cell culture conditions. After 6 h of incubation, the cell monolayers were washed four times with 10% fetal calf serum in phosphate-buffered saline and then cell activation was performed as described above.

Preliminary experiments indicated a 10-min end point for the release of [^{3}H]arachidonic acid from $P388D_1$ cells stimulated with LPS and PAF. The cell culture supernatants were harvested at 10 min, centrifuged at 1000 × *g*, and [^{3}H]arachidonic acid release was quantitated by scintillation counting. The data are presented as the percentage of total counts released. Total counts were measured by detergent lysis of $P388D_1$ cell monolayers.

Heparin Treatment—After treatment with oligonucleotides or activation, the culture media was removed from the cell monolayers and replaced with SFM containing 33 μg/ml endotoxin-free heparin (Sigma). The cells were treated with heparin for 16 h under cell culture conditions. The following day, the culture supernatants were harvested and centrifuged at 1000 × *g* to remove nonadherent cells. In some experiments, an aliquot of the culture media was assayed for PLA_2 activity. The culture media was then lyophilized to dryness and resuspended in 50 μl of water.

Immunoblotting—The proteins in whole cell lysates or in the culture supernatants of heparin-treated cells were separated by SDS-PAGE (32) and then electroblotted onto Trans-Blot Transfer Membranes (Bio-Rad). The membranes were blocked with blotto (5% non-fat dry milk in Tris-buffered saline) at room temperature for at least 2 h and then incubated overnight with blotto containing the primary antibody. The blots were developed using biotinylated secondary antibodies and avidin-coupled horseradish peroxidase (Amersham Corp.), and the peroxidase signal was detected using either the DAB Peroxidase Substrate

[2] L. J. Ackermann and E. A. Dennis, manuscript in preparation.

Kit (Vector Laboratories, Burlingame, CA) or ECL Western Blotting Detection Reagents (Amersham Corp.).

DNA Cloning and Sequencing—RNA was prepared from P388D_1 cell monolayers by the method of Chomczynski and Sacchi (33). First strand cDNA synthesis was performed using the SuperScript Preamplification kit from Life Technologies, Inc., with random priming. As the cDNA sequence of the murine group II PLA_2 had not been reported, we designed primers for the PCR based on the published nucleotide sequences of the rat spleen and rat platelet group II PLA_2s (34, 35). Primer I (5′ ATG AAG GTC CT(A,G) (C,T)TG CTA GCA GT(T,G) 3′) and primer III (5′ GCC ACA TCC ACG TTT CTC CAG ACG 3′) were complementary to nucleotides 1–24 and 219–243, respectively (34, 35). PCR was performed as described (36) with the following amplification regimen: 1 min, 94 °C; 1 min 52 °C; 3 min, 72 °C (30 times) followed by 15 min at 72 °C.

Based on the rat spleen group II PLA_2 cDNA sequence, we predicted that this protocol would amplify an ~240-base pair product from P388D_1 cell RNA. The fragment was isolated by agarose gel electrophoresis and cloned into Bluescript KS– (Stratagene, La Jolla, CA). Single-stranded DNA was prepared according to standard protocols (37) and sequenced using the Sequenase kit from United States Biochemical Corp.

Preparation of Cells Stably Expressing Antisense RNA for Group II PLA_2—The 240-base pair fragment amplified from P388D_1 cell RNA was subcloned into plasmid pRc/CMV (Invitrogen Corp., San Diego, CA) in an antisense orientation to produce plasmid asgII. The orientation of the insert in asgII was confirmed by DNA sequencing.

P388D_1 cells were transfected with pRc/CMV or asgII using calcium phosphate precipitation. Transfected cells were maintained in DME-10 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml Geneticin (Life Technologies, Inc.) to select for stable expression of the vectors. The PLA_2 activities of antibiotic-resistant colonies of cells were assessed using the PLA_2 assays described above. Although we made several attempts to clone the asgII-transfected cells, the phenotype was not stable and we were unsuccessful. These problems may arise because cells lacking group II PLA_2 activity are not viable. Our experiments were performed using colonies of antibiotic-resistant cells, not clones.

RESULTS

Cloning and Sequencing of Murine Group II PLA_2—P388D_1 cells express calcium-dependent and calcium-independent PLA_2 activities (28). To determine whether a group II PLA_2 is among the calcium-dependent activities, we used the PCR to amplify its cDNA from P388D_1 cell RNA. As the nucleotide sequence of murine group II PLA_2 has not been reported, the PCR primers were designed based on the published cDNA sequences of the rat spleen and rat platelet group II PLA_2s (34, 35). As expected based on the sequence of the rat group II PLA_2, the primers amplify an ~240-base pair cDNA fragment that codes for amino acids from the initiator methionine to the active site histidine/aspartic acid pair of the group II PLA_2 from P388D_1 cells (data not shown). The nucleotide sequence of this fragment is >95% homologous to the rat group II PLA_2 sequence. These data indicate that P388D_1 cells express a group II PLA_2.

Transient Transfection of P388D_1 Cells with Oligonucleotides—The release of PGE_2 from activated P388D_1 cells is blocked by a nonspecific inhibitor of secretory PLA_2s (1). This observation raises the possibility that group II PLA_2 is involved in the generation of PGE_2. To test this hypothesis, we examined the effect of a group II PLA_2-specific antisense oligonucleotide, ASGII, on the release of PGE_2 from activated P388D_1 cells. Oligonucleotide ASGII is complementary to nucleotides 148–168 (in the calcium binding loop) of the murine group II PLA_2. Chiang *et al.* (24) have demonstrated that oligonucleotides complexed with Lipofectin are more readily taken up by cells than are free oligonucleotides. We used this strategy to perform transient transfections of P388D_1 cells.

P388D_1 cells were treated with Lipofectin plus oligonucleotides or buffer, activated with LPS and PAF, and whole cell homogenates were prepared. PLA_2 activity in the homogenates was assessed using radiolabeled *E. coli* membranes as substrates. As noted under "Experimental Procedures," others have used this assay to measure the activities of cell-associated group II PLA_2s. A representative experiment is shown in Fig. 1. Untreated cells and cells treated with Lipofectin alone hydrolyzed 2–3% of *E. coli* phospholipids during the 80-min incubation. Hydrolysis was 3-fold lower in homogenates from cells treated with the antisense oligonucleotide (ASGII). In contrast, homogenates from cells treated with the sense complement of oligonucleotide ASGII (SGII) hydrolyzed the *E. coli* phospholipids to the same extent as control cell homogenates. Co-incubation with SGII neutralized the activity of oligonucleotide ASGII and prevented the decrease in PLA_2 activity, indicating that oligonucleotide ASGII inhibits through an antisense mechanism.

P388D_1 cells express several PLA_2 activities. Hence, it was important to establish that group II PLA_2 is the major activity that is altered upon treatment with ASGII. Table I addresses the specificity of the effects of oligonucleotide ASGII on PLA_2 activities in P388D_1 cells. Although the assays used to generate the data in Table I are not absolutely specific for each PLA_2, they should allow us to distinguish between the activities based on their substrate preferences.

As indicated by the representative experiment shown in Fig. 1 and the data presented in Table I, treatment with 0.25 μM ASGII reduced the hydrolysis of *E. coli* membranes by the lysates of resting P388D_1 cells to approximately one-third of control. Lysates from cells activated with LPS and PAF showed a similar approximately two-thirds decrease in hydrolysis of the *E. coli* membrane substrate (data not shown). These data imply that ASGII blocks the synthesis of the group II PLA_2 in P388D_1 cells. Since ASGII is specific for group II PLA_2, the residual PLA_2 activity observed in the *E. coli* assay may be due to other phospholipases. Alternatively, residual hydrolysis could be due to pre-existing pools of group II PLA_2 whose synthesis was not affected by the antisense reagent.

We also observed small, but significant, reductions in substrate hydrolysis in assays optimized to examine the activities of $cPLA_2$ and a calcium-independent PLA_2. It is possible that these effects reflect group II PLA_2 activity under these assay conditions. Alternatively, the ~25% decreases in substrate hydrolysis in both the $cPLA_2$ and calcium-independent PLA_2 assays may be due to nonspecific effects of ASGII treatment. The

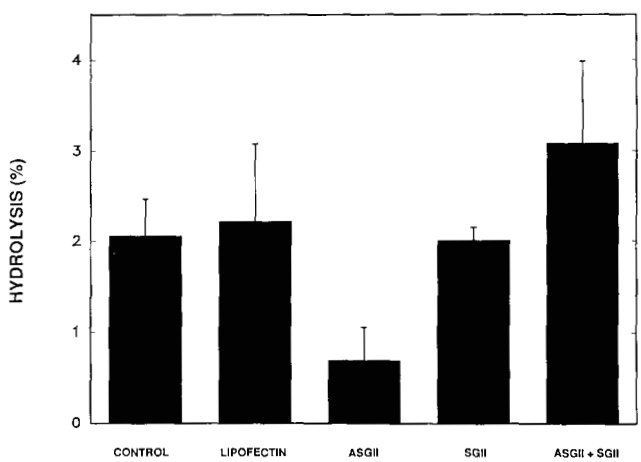

FIG. 1. **ASGII inhibition of PLA_2 activity in P388D_1 cell homogenates.** P388D_1 cells were treated with oligonucleotides plus Lipofectin, activated with LPS and PAF, cell homogenates were prepared, and PLA_2 activity was measured using the *E. coli* assay. *CONTROL*, untreated cells; *LIPOFECTIN*, Lipofectin only; *ASGII*, antisense oligonucleotide; *SGII*, sense oligonucleotide, *ASGII + SGII*, both oligonucleotides.

TABLE I
PLA_2 activities in ASGII-treated P388D_1 cells

PLA_2 assay[a]	Substrate	Ca^{2+}	Activity
			% of control[b]
Group II	*E. coli* membranes	5 mM	37 ± 9 (*n*=6)
$cPLA_2$	Arachidonoyl-PC/ dioleoyl glycerol (2/1)	1 mM	74 ± 8 (*n*=8)
Ca^{2+}-independent	Dipalmitoyl-PC/ Triton X-100 (1/4)		75 ± 1 (*n*=6)

[a] Assays were optimized to measure the activities of the PLA_2s from P388D_1 cells (as described under "Experimental Procedures").
[b] Control cells were treated with Lipofectin alone.

former conclusion is supported by the immunoblot shown in Fig. 2. Treatment of P388D_1 cells with ASGII had no effect on the expression of $cPLA_2$.

Inhibition of PGE_2 and [^{3}H]Arachidonic Acid Release by Oligonucleotide ASGII—We next examined the effect of oligonucleotide ASGII on the production of PGE_2 by activated P388D_1 cells. Cells were preincubated with oligonucleotides and then treated with LPS and PAF. The culture supernatants were collected and assayed for PGE_2 release. As in our previous experiments (1), resting P388D_1 cells released significant amounts of PGE_2 (Fig. 3*A*). Prostaglandin production was increased 7-fold when the cells were activated by LPS and PAF. Lipofectin alone had no effect on the production of PGE_2 by activated P388D_1 cells (data not shown). The release of PGE_2 from activated cells was reduced to resting cell levels by treatment with oligonucleotide ASGII. Oligonucleotide SGII had little effect on the production of PGE_2 by P388D_1 cells. However, the inhibition of PGE_2 release by ASGII was prevented when SGII was included in the incubation, again indicating that ASGII inhibits through an antisense mechanism. The dose-response curve for oligonucleotide ASGII-mediated inhibition of PGE_2 release from activated cells is shown in Fig. 3*B*. PGE_2 release was inhibited up to 3-fold by 400 nM ASGII. There was no evidence of cytolysis of P388D_1 cells treated with these doses of oligonucleotide.

Oligonucleotide ASGII also reduced the production of PGE_2 by resting P388D_1 cells. However, this inhibition was not reversed when the sense oligonucleotide was included in the transfection, indicating that ASGII inhibition of PGE_2 production in resting P388D_1 cells may not proceed in an antisense manner. ASGII treatment of P388D_1 cells did not alter the activity of prostaglandin endoperoxide synthetase, as determined by the metabolism of exogenous arachidonic acid to PGE_2 (data not shown). Hence, nonspecific inhibition of prostaglandin synthesis does not contribute to the reduction in PGE_2 release from resting P388D_1 cells. We have also observed nonspecific inhibition of [^{3}H]arachidonic acid release by SGII (see below). At present, we do not know the mechanism of this inhibition.

We next examined the effect of oligonucleotide ASGII on the release of [^{3}H]arachidonic acid from the phospholipids of prelabeled P388D_1 cells (Fig. 4). P388D_1 cells were labeled with [^{3}H]arachidonic acid during the incubation with oligonucleotide, washed, and activated with LPS and PAF. Activated

FIG. 2. **Group II PLA_2-specific antisense oligonucleotide 3358 has no effect on the expression of $cPLA_2$.** P388D_1 cells were treated with Lipofectin and oligonucleotides as described under "Experimental Procedures." After transfection, cell homogenates were prepared and proteins were separated by 12% SDS-PAGE under reducing conditions. Shown is an immunoblot for $cPLA_2$. The blot was developed using the ECL Western Blotting Detection kit from Amersham Corp. *Lane A*, untreated cells; *lane B*, Lipofectin; *lane C*, ASGII; *lane D*, SGII; *lane E*, ASGII + SGII.

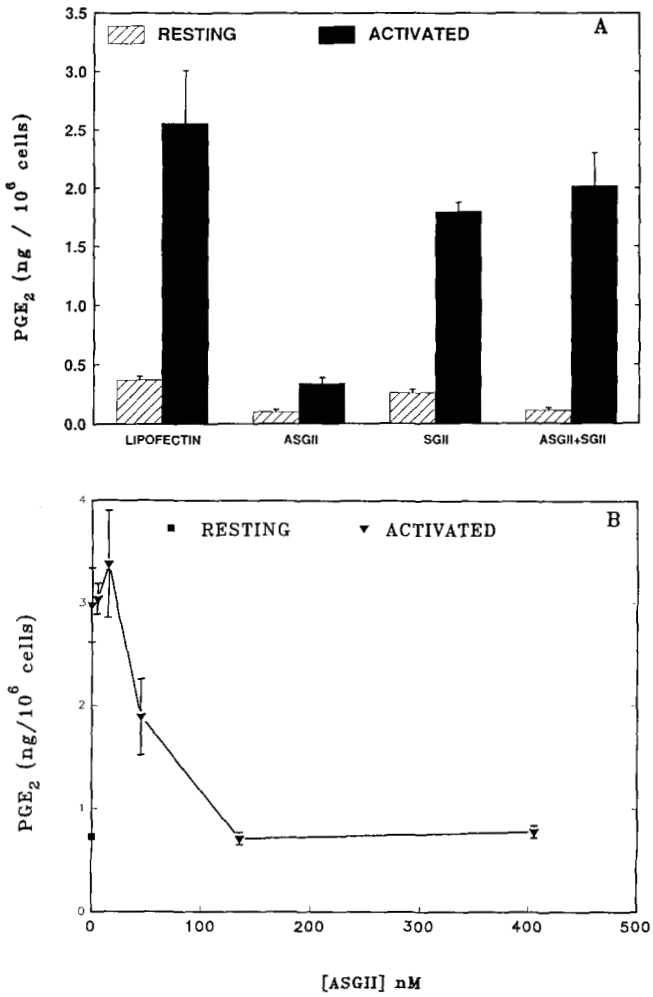

FIG. 3. **ASGII inhibition of PGE_2 release from activated P388D_1 cells.** *A*, P388D_1 cells were treated with oligonucleotides and Lipofectin as indicated and treated with SFM alone (resting cells, *hatched bars*) or treated with LPS and PAF (activated cells, *solid bars*). After 30 min of PAF treatment, the culture supernatants were recovered, and PGE_2 was measured by RIA. The data shown are the mean of three determinations. *B*, dose response of P388D_1 cells to oligonucleotide ASGII. *Open square*, resting cells; *solid triangle*, activated cells.

P388D_1 cells released 2-fold more [^{3}H]arachidonic acid from their phospholipids than did resting cells. Oligonucleotide ASGII reduced LPS/PAF-stimulated release of [^{3}H]arachidonic acid to resting cell levels. As in the PGE_2 experiments reported above, the antisense inhibitor was less effective at blocking the release of [^{3}H]arachidonate from resting cell phospholipids. Treatment with SGII alone inhibited the release of [^{3}H]arachidonic acid from activated cells to a modest extent (presumably, this is not an antisense inhibition as it was not blocked in the presence of ASGII). SGII also blocked ASGII-mediated inhibition of [^{3}H]arachidonic acid release from both resting and activated P388D_1 cells, indicating that ASGII-mediated inhibition occurs through an antisense mechanism.

Stable Expression of Antisense Group II PLA_2 RNA in P388D_1 Cells—We also developed a system for the stable expression of antisense group II PLA_2 RNA in P388D_1 cells. The 240-base pair fragment of the murine group II PLA_2 was cloned into a mammalian expression vector in antisense orientation. P388D_1 cells were transfected with vector alone (control) or the antisense vector and maintained in Geneticin to select for plasmid expression. Fifteen colonies of antibiotic-resistant cells from each transfection were screened for PLA_2 activity. The average hydrolysis of *E. coli* membranes was 2.4 and 1.8% for

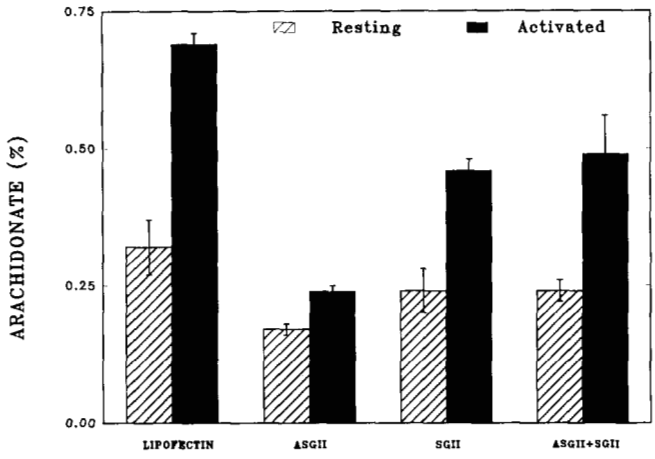

FIG. 4. **ASGII inhibition of [^{3}H]arachidonic acid release from activated P388D_1 cells.** P388D_1 cells were prelabeled with [^{3}H] arachidonic acid during the transfection (6 h). Labeled cells were washed and activated with LPS and PAF. After 10-min incubation with PAF, the culture supernatants were collected and released ^{3}H was measured. The data presented are the means of three determinations. Although the figure is labeled "arachidonate," the radioactive material contains arachidonic acid metabolites as well.

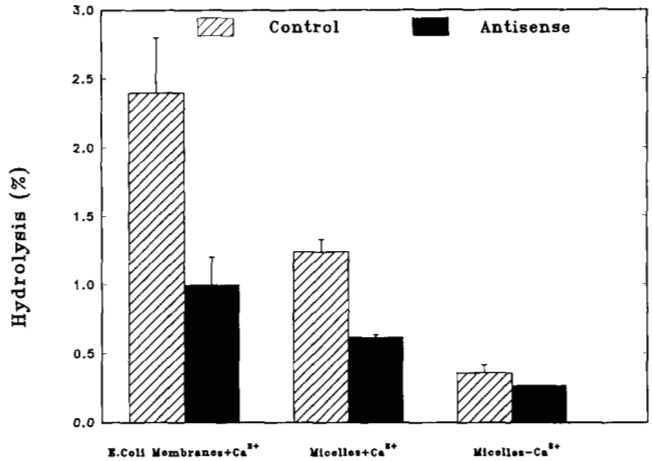

FIG. 5. **PLA_2 activity of stably transfected P388D_1 cells.** Stably transfected P388D_1 cells were prepared as described under "Experimental Procedures." Cell homogenates were prepared and PLA_2 activity was measured in three different PLA_2 assays: *E. coli*, calcium-dependent PLA_2 assay with *E. coli* membrane substrate. This assay is commonly used to measure group II PLA_2 activity; *Micelles+Ca*, calcium-dependent PLA_2 assay with mixed micelle substrate. This assay was optimized to measure a calcium-dependent membrane-associated PLA_2 activity in P388D_1 cells; *Micelles-Ca*, calcium-independent PLA_2 assay with mixed micelle substrate. This assay was optimized to the calcium-independent cytosolic PLA_2 activity in P388D_1 cells. *Hatched bars*, P388D_1 cells transfected with plasmid pRc/CMV alone; *solid bars*, P388D_1 cells transfected with antisense plasmid.

control and antisense cells, respectively. One colony of each transfection was selected for further characterization. In the case of the antisense cells, we selected the colony which showed the greatest reduction in PLA_2 activity for further study.

At the time these experiments were performed, we did not have access to anti-group II PLA_2 antibodies which cross-reacted with the murine enzyme. Hence, we characterized the stably transfected P388D_1 cells by measuring PLA_2 activity under several assay conditions. The results are shown in Fig. 5. As noted above, the *E. coli* membrane assay is commonly used to measure the activity of group II PLA_2. The antisense cell lysate showed an ~60% reduction in PLA_2 activity in this assay, indicating that the expression of group II PLA_2 is reduced in these cells. We also measured PLA_2 activity of the cell lysates using optimal assay conditions for the calcium-dependent membrane-associated PLA_2 from P388D_1 cells, (Fig. 5, *micelles+Ca^{2+}*). The molecular weight, calcium dependence, and heparin binding ability of this enzyme indicate that it is a group II PLA_2.[3] As in the *E. coli* assay, substrate hydrolysis was reduced by ~60% when antisense cell lysates were assayed using a mixed micellar substrate in the presence of calcium. Taken together, these data suggest that antisense cells express reduced levels of group II PLA_2.

In contrast, PLA_2 activity was the same in the homogenates of antisense cells and cells expressing vector alone when the mixed micelle assay was performed under conditions optimized to measure the activity of the soluble, calcium-independent PLA_2 activity in P388D_1 cells (Fig. 5, *micelles-Ca^{2+}*). As the antisense cells were selected on the basis of reduced PLA_2 activity, this is an important control. It indicates that we have chosen a colony that has reduced expression of group II PLA_2 and that other PLA_2s are not affected.

Inhibition of PGE_2 Release by Stably Expressed Antisense RNA—PGE_2 release was almost completely absent in resting antisense cells (Table II). These data imply a role for group II PLA_2 in the basal turnover of phospholipids by P388D_1 cells. We have attempted an extended (24 h) incubation with oligonucleotide ASGII in the transient transfection system to deplete group II PLA_2 from resting P388D_1 cells (data not shown). This incubation reduced PLA_2 activity in the *E. coli* assay to near control levels, but it also resulted in cytolysis. Interestingly, the cells remained viable when both ASGII and the sense oligonucleotide were included in the incubation. This observation substantiates our notion group II PLA_2 may be involved in the phospholipid metabolism of resting P388D_1 cells.

TABLE II
Stable expression of plasmid asgII inhibits the release of PGE_2 from P388D_1 cells

Cell type	PGE_2 (resting)[a]	PGE_2 (activated)[a]
	ng/mg protein	
Control	1.2 ± 0.2	8.9 ± 0.5
Antisense	0.2 ± 0.1	3.2 ± 0.4

[a] Control and antisense cells were treated with SFM (resting) or activated with LPS and PAF.

PGE_2 release from activated cells expressing the antisense construct was reduced ~3-fold compared with cells transfected with vector alone. This observation corroborates the data obtained in the transient expression system and indicates that group II PLA_2 is involved in the release of PGE_2 from activated P388D_1 cells. The inhibition of PGE_2 release from activated P388D_1 cells was less potent in the stable transfection system than with oligonucleotide ASGII. There are several possible explanations for this observation. For stable expression, the vector must integrate into the host cell DNA. Hence, the copy number of antisense RNAs in the stably transfected cells is probably lower than in the oligonucleotide-treated cells, and the stably transfected cells may not make enough antisense RNA to compete with LPS/PAF-induced synthesis of group II PLA_2 RNA. Furthermore, since these experiments were performed using isolated colonies of antibiotic-resistant cells, not clones, it is possible that some of the antisense cells express group II PLA_2 and hence are capable of responding to LPS and PAF. Finally, if group II PLA_2 is involved in the basal turnover of phospholipids in P388D_1 cells, then PLA_2-null cells may not be viable and all cells in the colonies may express low levels of the enzyme.

Identification of a Heparin-releasable Pool of Group II PLA_2

[3] E. A. Dennis and R. J. Ulevitch, unpublished observations.

—Although we made several attempts, we could not demonstrate group II PLA_2 protein secreted into the culture supernatants of resting or activated $P388D_1$ cells (data not shown). Others have suggested that group II PLA_2 may be localized to the plasma membranes of mammalian cells through associations with cell surface heparins (38–41). If this is true, then we should be able to compete the protein away from the membranes and into the culture supernatants by treating $P388D_1$ cells with exogenous heparin. Murakami *et al.* (41) have performed similar experiments in human umbilical vein endothelial cells (HUVEC). $P388D_1$ cells were treated with Lipofectin and oligonucleotides, activated with LPS and PAF and then treated with heparin as described under "Experimental Procedures." Immunoblots for group II PLA_2 protein were performed on the culture supernatants of heparin-treated cells. As shown in Fig. 6, heparin treatment of $P388D_1$ cells resulted in the appearance of group II PLA_2 protein in the culture media. This "released" PLA_2 was capable of hydrolyzing *E. coli* phospholipids, indicating that it is an active pool of enzyme (data not shown). These data suggest that group II PLA_2 is associated with the cell surface heparins of $P388D_1$ cells.

Control, Lipofectin-, and SGII-treated cells expressed a heparin-releasable group II PLA_2 (Fig. 6). Cells treated with both the antisense and sense oligonucleotides also expressed heparin-associated group II PLA_2, but this protein was absent from the culture supernatants of $P388D_1$ cells treated with ASGII alone. As these data are consistent with the inhibition of PGE_2 release by oligonucleotide ASGII, they suggest that an extracellular heparin-associated pool of group II PLA_2 may be responsible for prostaglandin production. There was also a reduction in the expression of an ~28-kDa protein. It is possible that this band represents a group II PLA_2 dimer (which we have observed on previous occasions). The heavily stained band at ~40 kDa is probably the soluble form of the low affinity receptor for IgG (42). Oligonucleotide treatment did not alter the expression of this protein. Hence, this band is a control for the nonspecific effects of ASGII on protein expression.

DISCUSSION

Role of Group II PLA_2 in PGE_2 Production in $P388D_1$ Cells—The goal of our work was to determine whether group II PLA_2 is involved in the release of PGE_2 from $P388D_1$ cells. Antisense oligonucleotide ASGII reduces PLA_2 activity in the homogenates of $P388D_1$ cells activated with LPS and PAF and inhibits the release of PGE_2 and [^{3}H]arachidonic acid from activated $P388D_1$ cells. This release is also inhibited by the constitutive expression of antisense group II PLA_2 RNA in a stable transfection system. These data indicate that the arachidonic acid that is converted to PGE_2 by activated $P388D_1$ cells is (at least in part) the product of group II PLA_2 hydrolysis. Even at a saturating dose of ASGII (250 nM, see Fig. 3*B*), we observed a 3-fold increase in PGE_2 production by activated $P388D_1$ cells (Fig. 3*A*). This residual activation suggests that in addition to stimulating the synthesis of group II PLA_2, LPS/PAF activation may modulate the activity of existing pools of the enzyme (which are not affected by the antisense reagent). Alternatively, other mechanisms for the production of PGE_2 (including the activation of $cPLA_2$, see below) could be active in our system as well.

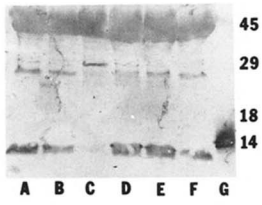

FIG. 6. **ASGII inhibition of the expression of heparin-releasable group II PLA_2.** $P388D_1$ cells were treated with Lipofectin and oligonucleotides, activated with LPS and PAF and incubated for 16 h in the presence of 33 μg/ml endotoxin-free heparin. The culture supernatants were harvested, lyophilized to dryness, resuspended in water, and proteins were separated by 18%, nonreducing SDS-PAGE. Shown is an immunoblot for group II PLA_2. The blot was developed using the DAB Peroxidase Substrate Kit from Vector Laboratories. *Lane A*, control; *lane B*, Lipofectin; *lane C*, ASGII; *lane D*, SGII; *lane E*, ASGII + SGII; *lane F*, control; *lane G*, human synovial fluid PLA_2.

LPS-mediated induction of group II PLA_2 mRNA and protein have been demonstrated in many cell systems. LPS has been shown to augment PLA_2 activity in HL-60, RAW 264.7 cells, and resident peritoneal macrophages (43–45). Although these studies did not address the issue, it is possible that the increased PLA_2 activity was a group II PLA_2. LPS treatment of rat liver or vascular smooth muscle cells leads to increased synthesis of group II PLA_2 mRNA and protein (8, 46). In addition, rats treated with LPS have elevated levels of plasma PLA_2 activity and nearly 100% of this activity is neutralized by anti-group II PLA_2 antibodies (47). The inhibition of PGE_2 release from PAF-stimulated $P388D_1$ cells is greater if ASGII is included in the 30-min wash after LPS priming than if it is included in the LPS step only (data not shown). This observation suggests that LPS induces the active turnover of group II PLA_2 in our system as well. We are currently performing experiments to directly assess the turnover of group II PLA_2 mRNA and protein in activated $P388D_1$ cells.

It was also possible that the increase in PGE_2 production by activated $P388D_1$ cells was due to the induction of cyclooxygenase (COX) enzymes. In contrast to our observations in $P388D_1$ cells, cyclooxygenase, but not PLA_2 (as assessed by the release of [^{3}H]arachidonic acid), is actively synthesized and turned over in LPS-treated human monocytes (16, 17). In addition, recent studies have demonstrated the induction of prostaglandin H synthase-2 (PGH synthase-2 or COX-2) upon LPS priming of alveolar macrophages (48–50). Neither COX-1 nor COX-2 mRNA levels are increased in $P388D_1$ cells treated with LPS and PAF.[4] In addition, exogenous arachidonic acid was metabolized to PGE_2 to the same extent by both resting and activated $P388D_1$ cells.[5] These data suggest that the induction of group II PLA_2, not cyclooxygenase enzymes, is largely responsible for increased PGE_2 release from activated $P388D_1$ cells.

Association of Group II PLA_2 with Cell Surface Heparins—As group II PLA_2 is a secreted protein, one must question how it gains access to its membrane phospholipid substrate. One possibility is that secreted group II PLA_2 re-associates with the plasma membrane. Immunofluorescence studies have demonstrated that secreted group II PLA_2 protein is localized to both the extracellular matrix and the plasma membranes of rat vascular smooth muscle cells (51). In addition, group II PLA_2 has been purified from the membranes of rat spleen cells and is associated with the particulate fraction of rat mesanglial cell homogenates (52–53). Murakami *et al.* (54) reported that activated rat mast cells release both soluble and membrane-bound ("ecto-enzyme") forms of group II PLA_2 and postulated that the extracellular group II PLA_2 might be involved in mast cell degranulation. The addition of exogenous group II PLA_2 to activated HL-60 cells or HUVEC augments the production of PGE_2, demonstrating that extracellular group II PLA_2 can attack membrane phospholipid (41, 55).

The purification protocols for group II PLA_2s sometimes include heparin affinity columns (40). We have purified a heparin

[4] H. R. Herschman, personal communication.

[5] S. E. Barbour and E. A. Dennis, unpublished observations.

binding calcium-dependent PLA_2 activity from $P388D_1$ cells.[3] In addition, exogenous heparin inhibits the uptake of circulating group II PLA_2 by rat endothelial cells, presumably by competing with cell surface proteoglycans for binding to the enzyme (39). Murakami *et al.* (41) have recently reported the association of group II PLA_2 with the cell surface heparan sulfates of HUEVC. Hence, group II PLA_2 could associate with the plasma membranes of $P388D_1$ cells in a manner analogous to lipoprotein lipase by binding to cell surface heparins (56).

We address this question by treating $P388D_1$ cells with exogenous heparin and then screening for group II PLA_2 protein in the culture supernatants. This treatment is analogous to the release of lipoprotein lipase from the surface of capillary endothelium (56). Heparin treatment of $P388D_1$ cells results in the appearance of group II PLA_2 protein in the culture supernatants, suggesting that group II PLA_2 associates with the proteoglycans of $P388D_1$ cells. This may be a nonspecific interaction between group II PLA_2 and the sulfated oligosaccharides on the surfaces of $P388D_1$ cells. Alternatively, there may be a specific proteoglycan receptor for group II PLA_2. A cell surface receptor for group I PLA_2 has been demonstrated in several cell types (57).

Oligonucleotide ASGII blocked the appearance of group II PLA_2 in the culture supernatants of heparin-treated $P388D_1$ cells. As this inhibition paralleled the ASGII-mediated decrease in PGE_2 production by activated cells, an extracellular proteoglycan-associated pool of group II PLA_2 may be responsible for PGE_2 release. Murakami *et al.* (41) have reported similar observations in HUVEC where treatment with heparin or an antibody directed against the heparin-binding domain of group II PLA_2 reduced the release of PGE_2 from tumor necrosis factor-activated cells. We have also observed a dose-dependent decrease in PGE_2 production upon heparin treatment of resting and activated $P388D_1$ cells concomittant with the release of active group II PLA_2 protein to the culture supernatants.[5] Taken together, these data suggest that proteoglycan-associated group II PLA_2 may generate the substrate for the cyclooxygenase.

The extracellular association of group II PLA_2 with proteoglycans may be of functional significance. The activity of porcine pancreatic PLA_2 is inhibited upon association with heparin (58). It is possible that the cell surface heparins of $P388D_1$ cells regulate the activity of group II PLA_2. This hypothesis is particularly intriguing, since group II PLA_2 is not subject to post-translational modifications (phosphorylation, for example), which might control its activity. We have demonstrated an early (2 min) endpoint for the release of PGE_2 by $P388D_1$ cells treated with LPS and PAF.[6] This rapid down-regulation of PGE_2 production could be the result of the inhibition of group II PLA_2 activity by cell surface heparins.

Based on our data, we predict that group II PLA_2 is synthesized and secreted by activated $P388D_1$ cells and then re-associates with proteoglycans in the plasma membrane to gain access to its substrate. This extracellular pool of group II PLA_2 then hydrolyzes membrane phospholipids to liberate arachidonic acid. Unesterified arachidonic acid is then shunted to the cyclooxygenase enzyme system for the production of PGE_2. If the active site of group II PLA_2 is oriented toward the cytoplasm, then "shunting" would involve diffusion of arachidonic acid to the cyclooxygenase enzyme system. Alternatively, phospholipid hydrolysis may occur in the extracellular space. This would require the cells to import free arachidonic acid and then shunt it to the cyclooxygenase. Preliminary results indicate that $P388D_1$ cells rapidly import exogenous arachidonic acid.[7] We are currently performing experiments to determine the validity of these hypotheses.

Role of Group II PLA_2 in $P388D_1$ Cell Activation—Our data on the involvement of group II PLA_2 in the activation of $P388D_1$ cells may be surprising in light of recent evidence linking the 85-kDa $cPLA_2$ to receptor-mediated activation of mammalian cells (15, 53, 59–61). As $cPLA_2$ preferentially hydrolyzes arachidonic acid from the *sn*-2 position of phospholipid and is activated by nanomolar concentrations of calcium (11, 13), one might expect $cPLA_2$ to be involved in the release of PGE_2 from activated $P388D_1$ cells, rather than group II PLA_2. Recent evidence supports our contention that group II PLA_2 activity is of physiological significance in the activation of mammalian cells. The overexpression of group II PLA_2 enhances the release of arachidonic acid from activated mouse fibroblasts (62). In addition, Marshall and McCarte-Roshak (63) have recently demonstrated group II PLA_2 activity at submicromolar calcium concentrations, indicating that the enzyme may be activated by physiologically significant levels of calcium. It has been suggested that both $cPLA_2$ and group II PLA_2 are involved in the release of PGE_2 from activated mesanglial cells and HUVEC (41, 53).

Although we have demonstrated the involvement of group II PLA_2 in the release of PGE_2 from activated $P388D_1$ cells, we cannot rule out the possibility that $cPLA_2$ is also involved in our system. $P388D_1$ cells express $cPLA_2$. However, neither the expression nor the activity (determined using the assay described in Ref. 9) of $cPLA_2$ is increased in $P388D_1$ cells activated with LPS and PAF.[5] These data suggest that $cPLA_2$ may not be involved in our activation system. We are currently using antisense technology to investigate the role of this enzyme in the phospholipid metabolism of resting and activated $P388D_1$ cells.

In summary, we have shown that phospholipid hydrolysis by group II PLA_2 provides the arachidonic acid substrate for the production of PGE_2 by activated $P388D_1$ cells. PGE_2 release is inhibited by both the transient expression of a phosphorothioate oligonucleotide (ASGII) and the constitutive expression of antisense RNA from plasmid asgII. Oligonucleotide ASGII also blocks the expression of a pool of group II PLA_2 which is released from $P388D_1$ cells upon heparin treatment, suggesting that an extracellular pool of the enzyme may be involved in PGE_2 production.

Acknowledgments—We thank C. Frank Bennett for the original sample of oligonucleotide ASGII. H. R. Herschman is gratefully acknowledged for sharing unpublished data. In addition, we thank C. Frank Bennett and Raymond Deems for critical reading of this manuscript.

[6] R. Asmis and E. A. Dennis, unpublished observations.

[7] J. Balsinde, I. D. Bianco, and E. A. Dennis, unpublished results.

REFERENCES

1. Glaser, K. B., Asmis, R., and Dennis, E. A. (1990) *J. Biol. Chem.* **265,** 8658–8664
2. Dennis, E. A. (1983) in *The Enzymes* (Boyer, P., ed) Vol. 16, Third Ed., pp. 307–353, Academic Press, New York
3. Davidson, F. F., and Dennis, E. A. (1990) *J. Mol. Evol.* **31,** 228–238
4. Lyons-Giordano, B., Davis, G. L., Galbraith, W., Pratta, M. A., and Arner, E. C. (1989) *Biochem. Biophys. Res. Commun.* **164,** 488–495
5. Oka, S., and Arita, H. (1991) *J. Biol. Chem.* **266,** 9956–9960
6. Schalkwijk, C., Pfeilschifter, J., Marki, F., and van den Bosch, H. (1991) *Biochem. Biophys. Res. Commun.* **174,** 268–275
7. Crowl, R. M., Stoller, T. J., Conroy, R. R., and Stoner, C. R. (1991) *J. Biol. Chem.* **266,** 2647–2651
8. Nakano, T., Ohara, O., Teraoka, H., and Arita, H. (1990) *FEBS Lett.* **261,** 171–174
9. Kramer, R. M., Roberts, E. F., Manetta, J., and Putnam, J. E. (1991) *J. Biol. Chem.* **266,** 5268–5272
10. Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P. L., Sportsman, J. R., Becker, G. W., Kang, L. H., Roberts, E. F., and Kramer, R. M. (1991) *J. Biol. Chem.* **266,** 14850–14853
11. Clark, J. D., Milona, N., and Knopf, J. L. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7708–7712
12. Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y.,

Milona, N., and Knopf, J. L. (1991) *Cell* **65,** 1043–1051
13. Wijkander, J., and Sundler, R. (1991) *Eur. J. Biochem.* **202,** 873–880
14. Channon, J. Y., and Leslie, C. C. (1990) *J. Biol. Chem.* **265,** 5409–5413
15. Lin, L.-L., Lin, A. Y., and Knopf, J. L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6147–6151
16. Fu, J.-Y., Masferrer, J. L., Siebert, K., Raz, A., and Needleman, P. (1990) *J. Biol. Chem.* **265,** 16737–16740
17. Hoffman, T., Lee, Y. L., Lizzio, E. F., Tripathi, A. K., Bonvini, E., and Puri, J. (1992) *Biochem. Pharmacol.* **44,** 955–963
18. Knauer, M. F., Longmuir, K. J., Yamamoto, R. S., Fitzgerald, T. P., and Granger, G. A. (1990) *J. Cell. Physiol.* **142,** 469–479
19. Neale, M. L., Fiera, R. A., and Matthews, N. (1988) *Immunology* **64,** 81–85
20. Washburn, W. N., and Dennis, E. A. (1991) *J. Biol. Chem.* **266,** 5042–5048
21. Yu, L., Deems, R. A., Hajdu, J., and Dennis, E. A. (1990) *J. Biol. Chem.* **265,** 2657–2664
22. Stout, J. T., and Caskey, C. T. (1987) *Methods Enzymol.* **151,** 519–530
23. Iyer, R. P., Egan, W., Regan, J. B., and Beaucage, S. L. (1990) *J. Am. Chem. Soc.* **112,** 1253–1254
24. Chiang, M.-Y., Chan, H., Zounes, M. A., Freier, S. M., Lima, W. F., and Bennett, C. F. (1991) *J. Biol. Chem.* **266,** 18162–18171
25. Kramer, R. M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E. P., Tizard, R., and Pepinsky, R. B. (1989) *J. Biol. Chem.* **264,** 5768–5775
26. Wright, G. W., Ooi, C. E., Weiss, J., and Elsbach, P. (1990) *J. Biol. Chem.* **265,** 6675–6681
27. Ulevitch, R. J., Watanabe, Y., Sano, M., Lister, M. D., Deems, R. A., and Dennis, E. A. (1988) *J. Biol. Chem.* **263,** 3079–3085
28. Ross, M. I., Deems, R. A., Jesaitis, A. J., Dennis, E. A., and Ulevitch, R. J. (1985) *Arch. Biochem. Biophys.* **238,** 247–258
29. Hazen, S. L., Stuppy, R. J., and Gross, R. W. (1990) *J. Biol. Chem.* **265,** 10622–10630
30. Gross, R. W., Ramanadham, S., Kruszka, K. K., Han, X., and Turk, J. (1993) *Biochemistry* **32,** 327–336
31. Reynolds, L. J., Washburn, W. N., Deems, R. A., and Dennis, E. A. (1991) *Methods Enzymol.* **197,** 3–23
32. Laemmli, U. K. (1970) *Nature* **227,** 680–685
33. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
34. Komada, M., Kudo, I., Mizushima, H., Kitamura, N., and Inoue, K. (1989) *J. Biochem. (Tokyo)* **106,** 545–547
35. Ishizaki, J., Ohara, O., Nakamura, E., Tamaki, M., Ono, T., Kanda. A., Yoshida, N., Teroaka, H., Tojo, H., and Okamoto, M. (1989) *Biochem. Biophys. Res. Commun.* **162,** 1030–1036
36. Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds. (1990) *PCR Protocols: A Guide to Methods and Applications*, Academic Press, San Diego, CA
37. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
38. Kudo, I., Chang, H. W., Hara, S., Murakami, M., and Inoue, K. (1989) *Dermatologica* **179,** Suppl. 1, 72–76
39. Murakami, M., Kudo, I., and Inoue, K. (1989) *Biochim. Biophys. Acta* **1005,** 270–276
40. Hara, S., Kudo, I., Chang, H. W., Matsuta, K., Miyamoto, T., and Inoue, K. (1989) *J. Biochem. (Tokyo)* **105,** 395–399
41. Murakami, M., Kudo, I., and Inoue, K. (1993) *J. Biol. Chem.* **268,** 839–844
42. Sautes, C., Mazieres, N., Galinha, A., Tartour, E., Bonnerot, C., Amigorena, S., Teillaud, C., Spagnoli, R., and Fridman, W. H. (1992) *Immunol. Res.* **11,** 181–190
43. Leslie, C. C., Voelker, D. R., Channon, J. Y., Wall, M. M., and Zelarney, P. T. (1988) *Biochim. Biophys. Acta* **963,** 476–492
44. Mohri, M., Spriggs, D. R., and Kufe, D. (1990) *J. Immunol.* **144,** 2678–2682
45. Aderem, A. A., Cohen, D. S., Wright, S. D., and Cohn, Z. A. (1986) *J. Exp. Med.* **164,** 165–179
46. Inada, M., Tojo, H., Kawata, S., Tarui, S., and Okamoto, M. (1991) *Biochem. Biophys. Res. Commun.* **174,** 1077–1083
47. Nakano, T., and Arita, H. (1990) *FEBS Lett.* **273,** 23–26
48. O'Sullivan, M. G., Huggins, E. M. Jr., Meade, E. A., De Witt, D. L., and McCall, C. E. (1992) *Biochem. Biophys. Res. Commun.* **187,** 1123–1127
49. O'Sullivan, M. G., Chilton, F. H., Huggins, E. M., and McCall, C. E. (1992) *J. Biol. Chem.* **267,** 14547–14550
50. Lee, S. H., Soyoola, E., Chanmugam, P., Hart, S., Sun, W., Zhong, H., Liou, S., Simmons, D., and Hwang, D. (1992) *J. Biol. Chem.* **267,** 25934–25938
51. Kurihara, H., Nakano, T., Takasu, N., and Arita, H. (1991) *Biochim. Biophys. Acta* **1082,** 285–292
52. Ono, T., Tojo, H., Kuramitsu, S., Kagamiyama, H., and Okamoto, M. (1988) *J. Biol. Chem.* **263,** 5732–5738
53. Schalkwijk, C. G., de Vet, E., Pfeilschifter, J., and van den Bosch, H. (1992) *Eur. J. Biochem.* **210,** 169–176
54. Murakami, M., Kudo, I., Suwa, Y., and Inoue, K. (1992) *Eur. J. Biochem.* **209,** 257–265
55. Hara, S., Kudo, I., and Inoue, K. (1991) *J. Biochem. (Tokyo)* **110,** 163–165
56. McLean, L. R., Demel, R. A., Socorro, L., Shinomiya, M., and Jackson, R. L. (1986) *Methods Enzymol.* **129,** 738–763
57. Hanasaki, K., and Arita, H. (1992) *J. Biol. Chem.* **267,** 6414–6420
58. Dicianni, M. B., Lilly-Stauderman, M., Mc Lean, L. R., Balasubramaniam, A., and Harmony, J. A. K. (1991) *Biochemistry* **30,** 9090–9097
59. Hulkower, K. I., Hope, W. C., Chen, T., Anderson, C. M., Coffey, J. W., and Morgan, D. W. (1992) *Biochem. Biophys. Res. Commun.* **184,** 712–718
60. Lin, L.-L., Lin, A. Y., and De Witt, D. L. (1992) *J. Biol. Chem.* **267,** 23451–23454
61. Spaargaren, M., Wissink, S., Defize, L. H. K., de Laat, S. W., and Boonstra, J. (1992) *Biochem. J.* **287,** 37–43
62. Pernas, P., Masliah, J., Olivier, J.-L., Salvat, C., Rybkine, T., and Bereziat, G. (1991) *Biochem. Biophys. Res. Commun.* **178,** 1298–1305
63. Marshall, L. A., and McCarte-Roshak, A. (1992) *Biochem. Pharmacol.* **44,** 1849–1858