Antisense Inhibition of Group I\(\text{II}\) Phospholipase A\(\text{2}\) Expression Blocks the Production of Prostaglandin E\(\text{2}\) by P388D\(\text{1}\) Cells*

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Macrophase-like P388D\(\text{1}\) cells release \[^{3}H\]arachidonic acid and produce prostaglandin E\(\text{2}\) (PGE\(\text{2}\)) upon stimulation with bacterial lipopolysaccharide (LPS) and platelet-activating factor (PAF). To determine whether group II phospholipase A\(\text{2}\) (PLA\(\text{2}\)) is involved in this release, we treated P388D\(\text{1}\) cells with antisense inhibitors specific for group II PLA\(\text{2}\) RNA. Treatment with oligonucleotide ASG\(\text{II}\) decreased PLA\(\text{2}\) activity in P388D\(\text{1}\) cell homogenates by \(\approx 60\%\) and reduced the release of \[^{3}H\]arachidonic acid and PGE\(\text{2}\) from activated cells to nearly resting cell levels. The inhibition by antisense oligonucleotide ASG\(\text{II}\) was blocked when its sense complement, SCG\(\text{II}\), was included in the incubation mixture. Stably transfected P388D\(\text{1}\) cells expressing an antisense construct for group II PLA\(\text{2}\) also produced reduced quantities of PGE\(\text{2}\) in response to LPS and PAF. These data suggest that prostaglandin production by activated P388D\(\text{1}\) cells involves phospholipid hydrolysis by group II PLA\(\text{2}\). Oligonucleotide ASG\(\text{II}\) also blocked the appearance of a heparin-releasable group II PLA\(\text{2}\) in the culture supernatants of P388D\(\text{1}\) cells. The disappearance of this protein correlated with reduced PGE\(\text{2}\) production by activated cells, indicating that an extracellular heparin-associated pool of group II PLA\(\text{2}\) is involved in prostaglandin production by P388D\(\text{1}\) cells.

Phospholipase A\(\text{2}\) (PLA\(\text{2}\))\(^{1}\) comprises a family of lipolytic enzymes which attacks the \(\alpha\)-2 carbonyl of phospholipids to produce fatty acids and lysophospholipids and is implicated in the release of arachidonic acid for prostaglandin biosynthesis in P388D\(\text{1}\) cells.\(^{1}\) The best studied PLA\(\text{2}\)s, the secretory PLA\(\text{2}\)s (sPLA\(\text{2}\)), 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 bile and reptile venoms, into mammalian pancreatic exudates, and by a variety of mammalian cells (2–4, 8–10). Recently, an 85-kDa arachidonate-specific PLA\(\text{2}\) has been purified and cloned from the cytosol of mammalian cells (9–14). This cytosolic PLA\(\text{2}\) (cPLA\(\text{2}\)) 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\(\text{2}\) rather than sPLA\(\text{2}\) is involved in receptor-mediated activation of mammalian cells.

Phospholipase A\(\text{2}\) activities have been implicated in many cell activation systems; however, there has been no direct demonstration of a requirement for PLA\(\text{2}\) catalysis in these systems. In some studies, PLA\(\text{2}\) 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\(\text{2}\) and cPLA\(\text{2}\) 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 PLA\(\text{2}\) activity (see Refs. 18 and 19 for example). More specific, mechanism-based inhibitors have been described for sPLA\(\text{2}\), 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\(\text{2}\) mRNA and protein levels have been used as indirect indications of sPLA\(\text{2}\) activation (5, 6, 8). It is possible that the induction of sPLA\(\text{2}\) protein is nonspecific and PLA\(\text{2}\) activity may not be involved in these cell activation systems (7).

Previous work from our laboratory has demonstrated that P388D\(\text{1}\) cells stimulated with bacterial lipopolysaccharide (LPS) and platelet-activating factor (PAF) produce prostaglandin E\(\text{2}\) (PGE\(\text{2}\)) (1). The release of PGE\(\text{2}\) is inhibited by manoamine, indicating the possible involvement of a group II PLA\(\text{2}\) in the response. To test this hypothesis, we have designed specific antisense RNA inhibitors of group II PLA\(\text{2}\). Antisense RNA technology offers the potential of designing potent PLA\(\text{2}\) inhibitors with absolute specificity (22). The inhibition is based on the binding of complementary nucleotides to group II PLA\(\text{2}\) mRNA. Using such inhibitors, we demonstrate that group II PLA\(\text{2}\) activity is involved in the release of PGE\(\text{2}\) from activated P388D\(\text{1}\) cells. In addition, evidence is provided to suggest that this release is mediated by an extracellular pool of group II PLA\(\text{2}\) that is localized to cell surface proteoglycans.

**EXPERIMENTAL PROCEDURES**

**Materials**—P388D\(\text{1}\) cells were obtained from ATCC (Rockville, MD). LPS Re 596 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\(\text{2}\) assay. A polyclonal antibody against human synovial fluid group II PLA\(\text{2}\) (W98-7UJ-39A) was the generous gift of C. Teater and J.L. Bobbitt (Eli Lilly Co.). Antibodies B385, B377, and B5.2 (developed against rat group II PLA\(\text{2}\)) were the kind gifts of K. Inoue and I. Kudo (University of Tokyo). A cocktail of these anti-group II PLA\(\text{2}\) antibodies was prepared for immunoblotting. C. Leslie (National Jewish Center for Immunology and Respiratory Medicine) kindly provided the antiseraum against the marine cPLA\(\text{2}\).

**Cell Culture**—P388D\(\text{1}\) cells were maintained at 37°C, 10% CO\(\text{2}\), 95% humidity (cell culture conditions) in DME-10, which consisted of low

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\(^{1}\) The abbreviations used are: PLA\(\text{2}\), phospholipase A\(\text{2}\); sPLA\(\text{2}\), secretory PLA\(\text{2}\); cPLA\(\text{2}\), cytosolic PLA\(\text{2}\); LPS, lipopolysaccharide; PAF, 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine (platelet-activating factor); PGE\(\text{2}\), prostaglandin E\(\text{2}\); 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 Iseove's modified Dulbecco's medium (DME, Whittaker Bio-products, Walkersville, Md) supplemented with 10% low endotoxin fetal calf serum (HyClone, Logan, UT), with 50 μg/ml gentamicin sulfate (Sigma), 2.5 μg/ml amphotericin, nonessential amino acids (Irvine Scientific Co., Santa Ana, CA) and 2 mM glucose. The cells used in these experiments were between passages 15 and 30. Cells were plated at 10^5/well in six-well plates, allowed to adhere overnight, and used for the experiments the following day. All experiments were conducted in serum-free medium (DME), composed of DME, 1 x nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glucose.

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 SGI), 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 Beauchage (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 x NaCl) and showed single sharp bands upon denaturing acrylamide gel electrophoresis.

Oligonucleotide Treatment—P388D, cells were transfected with oligonucleotide in the presence of 5 μg/ml Lipofectin (Life Technologies, Inc., Grand Island, NY). Transfection was mixed with 20 ng/ml of oligonucleotide or water, and complexes were allowed to form at room temperature for 10–15 min. During this incubation, the P388D, cell monolayers were washed two times with SFM. Each well received 1 ml of SFM and then 160 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 90% during the course of these experiments.

Cell Activation and Measurement of Released PGE_2—Our standard regimen for activating P388D, cells with LPS and PAF (1) was used with the following modifications. After a 6-h incubation in the presence of oligonucleotide and Lipofectin, 1 ml of LPS Re 585 (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 containing 200 ng/ml Lipofectin (SFM). PEG STIMULATION was continued for 10–30 min under cell culture conditions.

Cell culture supernatants were harvested and centrifuged at 1000 x 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, cell monolayers were scraped into 0.64 ml of 10% calf serum (HyClone, Logan, UT), with 50 μg/ml gentamycin sulfate, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The culture medium was then lyophilized to dryness and resuspended in 21876 2 mM glutamine. The cells used in these experiments the following day. All experiments were conducted in serum-free medium (DME), composed of DME, 1 x nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glucose.

Calcium-dependent PLA_2 activity was measured using micellar substrate consisting of 100 μM dipalmitylophosphatidylcholine and 200 μM Triton X-100 in 80 mM glycine, pH 9.0, 5.0 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, cells (27).

P388D, cells also express a cytotoxic calcium-independent PLA_2, which has not been purified (28). This enzyme may be distinct from the calcium-independent phospholipid-specific PLA_2s described by Gross and co-workers (29, 30). Calcium-independent PLA_2 activity in P388D, cell homogenates was measured using a mixed micellar substrate consisting of 100 μM dipalmitoylphosphatidylcholine and 400 μM Triton X-100 in 20 mM Tris-HCl, 5 mM EDTA, pH 7.5. The calcium-independent PLA_2 from P388D, 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-([1-3H]arachidonic acid (Du Pont-New England Nuclear) was added to each assay as a tracer. Assay tubes were incubated for 3 h (lyases 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.

Calcium-independent PLA_2 activity was measured using a protocol (27) 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 well 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, 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 used, as neither II nor I PLA, nor nonadherent cells should contribute to substrate hydrolysis in the absence of calcium.

Release of [3H]Arachidonic Acid—P388D, cells were labeled with [3H]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, cell monolayers were overlayed with 1 ml of SFM containing 0.5 μCi 5,6,8,9,11,12,14,15-[3H]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 three times with SFM containing 10% fetal calf serum (FCS) in SFM. PEG STIMULATION was continued for 10–30 min under cell culture conditions.

Cell culture supernatants were harvested and centrifuged at 1000 x 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.

Calcium-dependent PLA_2 activity was measured using radiolabeled E. coli membranes ([3H]Oic 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 50–5000 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. The reaction was started with addition of labeled calcium and incubated at 37 °C for 15 min. The reaction was terminated by addition of the 100 μl of each of 2 X Tricine and 20 mg/ml bovine serum albumin, respectively. After 20 min on ice, the reaction mixtures were centrifuged at 10,000 x 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

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DNA Cloning and Sequencing—RNA was prepared from P388D1 cell monolayers by the method of Chomczynski and Sacchi (33). First-strand cDNA synthesis was performed using the SuperScript Preapmlification kit from Life Technologies, Inc., with random priming. As the cDNA was sequenced, the primers for the PCR were estimated based on the published nucleotide sequences of the rat spleen and rat platelet group II PLAs (34, 35). Primer I (5' ATGAAG GTC CT(A,G) (C,T)TG CTAGCA GT(T,G) 3') and primer III (5' GCC ACA TCC ACG TTT CTC CAG AC 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 PLA2 cDNA sequence, we predicted that this protocol would amplify an ~240-base pair product from P388D1 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 PLA2—The 240-base pair fragment amplified from P388D1 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.

P388D1 cells were transfected with pRC/CMV or asgII using calcium phosphate precipitation. Transfected cells were maintained in DME-10 antibiotic media, supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, and 400 µg/ml G-nutcin (Life Technologies, Inc.) to select for stable expression of the vector. The PLAs activities of antibiotic-resistant colonies of cells were assessed using the PLAs 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 PLA2 activity are not viable. Our experiments were performed using colonies of antibiotic-resistant cells, not clones.

RESULTS

Cloning and Sequencing of Murine Group II PLA2—P388D1 cells express calcium-dependent and calcium-independent PLAs activities (28). To determine whether a group II PLA2 is among the calcium-dependent activities, we used the PCR to amplify its cDNA from P388D1 cell RNA. As the nucleotide sequence of murine group II PLA2 has not been reported, the PCR primers were designed based on the published cDNA sequences of the rat spleen and rat platelet group II PLAs (34, 35). As expected based on the sequence of the rat group II PLA2, 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 PLA2 from P388D1 cells (data not shown). The nucleotide sequence of this fragment is ~95% homologous to the rat group II PLA2 sequence. These data indicate that P388D1 cells express a group II PLA2.

Transient Transfection of P388D1 Cells with Oligonucleotides—The release of PGE2 from activated P388D1 cells is blocked by a nonspecific inhibitor of secretory PLAs (1). This observation raises the possibility that group II PLA2 is involved in the generation of PGE2. To test this hypothesis, we examined the effect of a group II PLA2-specific antisense oligonucleotide, ASGII, on the release of PGE2 from activated P388D1 cells. Oligonucleotide ASGII is complementary to nucleotides 148–168 (in the calcium binding loop) of the murine group II PLA2. 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 P388D1 cells.

P388D1 cells were treated with Lipofectin plus oligonucleotides or buffer, activated with LPS and PAF, and whole cell homogenates were prepared. PLA2 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 PLAs. 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-inubation with SGII neutralized the activity of oligonucleotide ASGII and prevented the decrease in PLA2 activity, indicating that oligonucleotide ASGII inhibits through an antisense mechanism.

P388D1 cells express several PLAs activities. Hence, it was important to establish that group II PLA2 is the major activity that is altered upon treatment with ASGII. Table I addresses the specificity of the effects of oligonucleotide ASGII on PLAs activities in P388D1 cells. Although the assays used to generate the data in Table I are not absolutely specific for each PLA2, 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 P388D1 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 PLA2 in P388D1 cells. Since ASGII is specific for group II PLA2, the residual PLA2 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 PLA2 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 cPLA2 and a calcium-independent PLA2. It is possible that these effects reflect group II PLA2 activity under these assay conditions. Alternatively, the ~25% decreases in substrate hydrolysis in both the cPLA2 and calcium-independent PLA2 assays may be due to nonspecific effects of ASGII treatment. The
Antisense Inhibition of Phospholipase A₂

TABLE I

<table>
<thead>
<tr>
<th>PLA₂ assay*</th>
<th>Substrate</th>
<th>Ca²⁺</th>
<th>Activity</th>
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<tr>
<td>Group II</td>
<td>E. coli membranes</td>
<td>5 mM</td>
<td>37 ± 9 (n=6)</td>
</tr>
<tr>
<td>cPLA₂</td>
<td>Arachidonoyl-PC/dioleoyl glycerol (2/1)</td>
<td>1 mM</td>
<td>74 ± 5 (n=8)</td>
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<tr>
<td>Ca²⁺-independent</td>
<td>Dipalmitoyl-PC/Triton X-100 (1/4)</td>
<td></td>
<td>75 ± 1 (n=6)</td>
</tr>
</tbody>
</table>

* Assays were optimized to measure the activities of the PLA₂s from P388D₁ cells (as described under "Experimental Procedures").

† Control cells were treated with Lipofectin alone.

Plaque inhibition of PGE₂ and [³H]Arachidonic Acid Release by Oligonucleotide ASGII—We next examined the effect of oligonucleotide ASGII on the production of PGE₂ by activated P388D₁ cells. Cells were preincubated with oligonucleotides and then treated with LPS and PAF. The culture supernatants were collected and assayed for PGE₂ release. As in our previous experiments (1), resting P388D₁ cells released significant amounts of PGE₂ (Fig. 3A). Prostaglandin production was increased 7-fold when the cells were activated by LPS and PAF. Lipofectin alone had no effect on the production of PGE₂ by activated P388D₁ cells (data not shown). The release of PGE₂ from activated cells was reduced to resting cell levels by treatment with oligonucleotide ASGII. Oligonucleotide SGII had little effect on the production of PGE₂ by P388D₁ cells. However, the inhibition of PGE₂ 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 PGE₂ release from activated cells is shown in Fig. 3B. PGE₂ release was inhibited up to 3-fold by 400 nM ASGII. There was no evidence of cytolytic activity of P388D₁ cells treated with these doses of oligonucleotide.

Oligonucleotide ASGII also reduced the production of PGE₂ by resting P388D₁ cells. However, this inhibition was not reversed when the sense oligonucleotide was included in the transfection, indicating that ASGII inhibition of PGE₂ production in resting P388D₁ cells may not proceed in an antisense manner. ASGII treatment of P388D₁ cells did not alter the activity of prostaglandin endoperoxide synthetase, as determined by the metabolism of exogenous arachidonic acid to PGE₂ (data not shown). Hence, nonspecific inhibition of prostaglandin synthesis does not contribute to the reduction in PGE₂ release from resting P388D₁ cells. We have also observed nonspecific inhibition of [³H]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 [³H]arachidonic acid from the phospholipids of prelabeled P388D₁ cells (Fig. 4). P388D₁ cells were labeled with [³H]arachidonic acid during the incubation with oligonucleotide, washed, and activated with LPS and PAF. Activated P388D₁ cells released 2-fold more [³H]arachidonic acid from their phospholipids than did resting cells. Oligonucleotide ASGII reduced LPS/PAF-stimulated release of [³H]arachidonic acid to resting cell levels. As in the PGE₂ experiments reported above, the antisense inhibitor was less effective at blocking the release of [³H]arachidonate from resting cell phospholipids. Treatment with SGII alone inhibited the release of [³H]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 [³H]arachidonic acid release from both resting and activated P388D₁ cells, indicating that ASGII-mediated inhibition occurs through an antisense mechanism.

Stable Expression of Antisense Group II PLA₂ RNA in P388D₁ Cells—We also developed a system for the stable expression of antisense group II PLA₂ RNA in P388D₁ cells. The 240-base pair fragment of the murine group II PLA₂ was cloned into a mammalian expression vector in antisense orientation. P388D₁ 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 PLA₂ activity. The average hydrolysis of E. coli membranes was 2.4 and 1.8% for...
**Antisense Inhibition of Phospholipase A₂**

**Fig. 4.** ASGII inhibition of [³H]arachidonic acid release from activated P388D₁ cells. P388D₁ cells were prelabeled with [³H]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 [³H] 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.

**Table II**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>PGE₂ (resting)ᵃ</th>
<th>PGE₂ (activated)ᵃ</th>
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<tr>
<td>Control</td>
<td>1.2 ± 0.2</td>
<td>8.9 ± 0.5</td>
</tr>
<tr>
<td>Antisense</td>
<td>0.2 ± 0.1</td>
<td>3.2 ± 0.4</td>
</tr>
</tbody>
</table>

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

Inhibition of PGE₂ Release by Stably Expressed Antisense RNA—PGE₂ release was almost completely absent in resting antisense cells (Table II). These data imply a role for group II PLA₂ in the basal turnover of phospholipids by P388D₁ cells. We have attempted an extended (24 h) incubation with oligonucleotide ASGII in the transient transfection system to deplete group II PLA₂ from resting P388D₁ cells (data not shown). This incubation reduced PLA₂ activity in the E. coli assay to near control levels, but it also resulted in cytolysis. Interest-

<sup>³</sup> E. A. Dennis and R. J. Ulevitch, unpublished observations.

**Fig. 5.** PLA₂ activity of stably transfected P388D₁ cells. Stably transfected P388D₁ cells were prepared as described under "Experimental Procedures." Cell homogenates were prepared and PLA₂ activity was measured in three different PLA₂ assays: E. coli, calcium-dependent PLA₂ assay with E. coli membrane substrate. This assay is commonly used to measure group II PLA₂ activity; Micelles-Ca, calcium-dependent PLA₂ assay with mixed micelle substrate. This assay was optimized to measure a calcium-dependent membrane-associated PLA₂ activity in P388D₁ cells; Micelles-Ca, calcium-independent PLA₂ assay with mixed micelle substrate. This assay was optimized to the calcium-independent cytosolic PLA₂ activity in P388D₁ cells. Hatchet bars, P388D₁ cells transfected with plasmid pRoCMV alone; solid bars, P388D₁ cells transfected with antisense plasmid.
Although we made several attempts, we could not demonstrate group II PLAr protein secreted into the culture supernatants of resting or activated P388D1 cells (data not shown). Others have suggested that group II PLAr may be localized to the plasma membranes of mammalian cells through association 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 P388D1 cells with exogenous heparin. Murakami et al. (41) have performed similar experiments in human umbilical vein endothelial cells (HUVEC). P388D1 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 PLAr protein were performed on the culture supernatants of heparin-treated cells. As shown in Fig. 6, heparin treatment of P388D1 cells resulted in the appearance of group II PLAr protein in the culture media. This "released" PLAr 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 PLAr is associated with the cell surface heparins of P388D1 cells. Control, Lipofectin-, and SGII-treated cells expressed a heparin-releasable group II PLAr (Fig. 6). Cells treated with both the antisense and sense oligonucleotides also expressed heparin-associated group II PLAr, but this protein was absent from the culture supernatants of P388D1 cells treated with ASGII alone. As these data are consistent with the inhibition of PGE2 release by oligonucleotide ASGII, they suggest that an extracellular heparin-associated pool of group II PLAr 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 PLAr 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 PLAr in PGE2 Production in P388D1 Cells**

The goal of our work was to determine whether group II PLAr is involved in the release of PGE2 from P388D1 cells. Antisense oligonucleotide ASGII reduces PLAr activity in the homogenates of P388D1 cells activated with LPS and PAF and inhibits the release of PGE2 and [3H]arachidonic acid from activated P388D1 cells. This release is also inhibited by the constitutive expression of antisense group II PLAr RNA in a stable transfection system. These data indicate that the

![Image](image-url)
binding calcium-dependent PLA₂ activity from P388D₁ cells.³
In addition, exogenous heparin inhibits the uptake of circulating
group II PLA₂ by rat endothelial cells, presumably by competing
with cell surface proteoglycans for binding to the enzy-
me (39). Murakami et al. (41) have recently reported the
association of group II PLA₂ with the cell surface heparan
sulfates of HUEVC. Hence, group II PLA₂ could associate with
the plasma membranes of P388D₁ cells in a manner analogous
to lipoprotein lipase by binding to cell surface heparins (56).
We address this question by treating P388D₁ cells with ex-
ogenous heparin and then screening for group II PLA₂ protein
in the culture supernatants. This treatment is analogous to the
release of lipoprotein lipase from the surface of capillary endo-
thelium (56). Heparin treatment of P388D₁ cells results in the
appearance of group II PLA₂ protein in the culture superna-
tants, suggesting that group II PLA₂ associates with the pro-
teoglycans of P388D₁ cells. This may be a nonspecific interac-
tion between group II PLA₂ and the sulfated oligosaccharides
on the surfaces of P388D₁ cells. Alternatively, there may be a
specific proteoglycan receptor for group II PLA₂. A cell surface
receptor for group II PLA₂ has been demonstrated in several cell
types (37).
Oligonucleotide ASGII blocked the appearance of group II
PLA₂ in the culture supernatants of heparin-treated P388D₁
cells. As this inhibition paralleled the ASGII-mediated decrease in
PGE₂ production by activated cells, an extracellular proteo-
glycan-associated pool of group II PLA₂ may be responsible for
PGE₂ release. Murakami et al. (41) have reported similar ob-
servations in HUEVC where treatment with heparin or an antibody
directed against the heparin-binding domain of group II
PLA₂ reduced the release of PGE₂ from tumor necrosis fac-
tor-activated cells. We have also observed a dose-dependent
decrease in PGE₂ production upon heparin treatment of resting
and activated P388D₁ cells concomitant with the release of
active group II PLA₂ protein to the culture supernatants.⁵
Taken together, these data suggest that proteoglycan-associ-
gated group II PLA₂ may generate the substrate for the cy-
clooxygenase.
The extracellular association of group II PLA₂ with proteo-
glycans may be of functional significance. The activity of por-
cine pancreatic PLA₂ is inhibited upon association with hepa-
rin (58). It is possible that the cell surface heparins of P388D₁
cells regulate the activity of group II PLA₂. This hypothesis is
particularly intriguing, since group II PLA₂ is not subject to
post-translational modifications (phosphorylation, for ex-
ample), which might control its activity. We have demonstrated
an early (2 min) endpoint for the release of PGE₂ by P388D₁
cells treated with LPS and PAF.⁶ This rapid down-regulation of
PGE₂ production could be the result of the inhibition of group
II PLA₂ activity by cell surface heparins.
Based on our data, we predict that group II PLA₂ is synthe-
sized and secreted by activated P388D₁ cells and then re-asso-
ciates with proteoglycans in the plasma membrane to gain
access to its substrate. This extracellular pool of group II
PLA₂ then hydrolyzes membrane phospholipids to liberate arachi-
donic acid. Unesterified arachidonic acid is then shunted to the
cyclooxygenase enzyme system for the production of PGE₂. If
the active site of group II PLA₂ is oriented toward the cyto-
plasm, then “shunting” would involve diffusion of arachidonic
acid to the cyclooxygenase enzyme system. Alternatively, phos-
pholipid 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
P388D₁ cells rapidly import exogenous arachidonic acid.⁷
⁶ R. Asmis and E. A. Dennis, unpublished observations.
We are currently performing experiments to determine the va-
ility of these hypotheses.
Role of Group II PLA₂ in P388D₁ Cell Activation—Our data on
the involvement of group II PLA₂ in the activation of P388D₁
cells may be surprising in light of recent evidence linking the
85-kDa cPLA₂ to receptor-mediated activation of mammalian cells (15, 53, 59–61). As cPLA₂ preferentially hydrolyzes
arachidonic acid from the sn-2 position of phospholipid and is activated by nanomolar concentrations of calcium (11, 13), one
might expect cPLA₂ to be involved in the release of PGE₂ from
activated P388D₁ cells, rather than group II PLA₂. Recent evi-
dence supports our contention that group II PLA₂ activity is of
physiological significance in the activation of mammalian cells.
The overexpression of group II PLA₂ enhances the release of
arachidonic acid from activated mouse fibroblasts (62). In ad-
dition, Marshall and McCarte-Roshak (63) have recently dem-
onstrated group II PLA₂ activity at submicromolar calcium con-
centrations, indicating that the enzyme may be activated by
physiologically significant levels of calcium. It has been sug-
gested that both cPLA₂ and group II PLA₂ may be involved in the
release of PGE₂ from activated mesangial cells and HUEVC
(41, 53).
Although we have demonstrated the involvement of group II
PLA₂ in the release of PGE₂ from activated P388D₁ cells, we
cannot rule out the possibility that cPLA₂ is also involved in
our system. P388D₁ cells express cPLA₂. However, neither the
expression nor the activity (determined using the assay
described in Ref. 9) of cPLA₂ is increased in P388D₁ cells acti-
vated with LPS and PAF.⁵ These data suggest that cPLA₂ may
not be involved in our activation system. We are currently
using antisense technology to investigate the role of this en-
zyme in the phospholipid metabolism of resting and activated
P388D₁ cells.
In summary, we have shown that phospholipid hydrolysis by
group II PLA₂ provides the arachidonic acid substrate for the
production of PGE₂ by activated P388D₁ cells. PGE₂ release is
inhibited by both the transient expression of a phosphorothio-
atide oligonucleotide (ASGII) and the constitutive expression of
antisense RNA from plasmid asgII. Oligonucleotide ASGII also
blocks the expression of a pool of group II PLA₂ which is re-
leased from P388D₁ cells upon heparin treatment, suggesting
that an extracellular pool of the enzyme may be involved in
PGE₂ production.
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Antisense Inhibition of Phospholipase A₂


