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The Rat Ovarian Phospholipase A_2 System: Gene Expression, Cellular Localization, Activity Characterization, and Interleukin-1 Dependence*

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ABSTRACT

We have previously demonstrated that interleukin-1β (IL-1β), a putative intermediary in the ovulatory process, is a potent stimulator of ovarian PG biosynthesis. In this communication, we examine the possibility that this IL-1 effect reflects in part the induction of arachidonic acid mobilization by phospholipase A_2 (PLA_2). Molecular probing of whole ovarian material revealed the immature rat ovary to be a site of modest secretory PLA_2 ($sPLA_2$) gene expression. However, no change in ovarian $sPLA_2$ gene expression was noted during the periovulatory period. Comparable probing for cytosolic PLA_2 ($cPLA_2$) failed to disclose a quantifiable signal. However, *in situ* hybridization localized both $sPLA_2$ and $cPLA_2$ ($sPLA_2 > cPLA_2$) transcripts to the granulosa cell layer of the ovarian follicle. Treatment of cultured whole ovarian dispersates with IL-1β produced significant ($P < 0.01$) increments in the steady state levels of transcripts corresponding to both $sPLA_2$ (1.7-fold increase) and $cPLA_2$ (5-fold increase), an effect reversed by an IL-1 receptor antagonist, suggesting mediation via a specific IL-1 receptor. Treatment with cycloheximide, a protein synthesis inhibitor, resulted in significant attenuation of the ability of IL-1β to up-regulate $sPLA_2$ and $cPLA_2$ gene expression as well as medium PLA_2 activity. Treatment with aminoguanidine, an inhibitor of inducible nitric oxide synthase, led to augmentation of the ability of IL-1β to up-regulate $sPLA_2$ and $cPLA_2$ gene expression as well as medium PLA_2 activity. Total cellular PLA_2 activity proved time, cell density, and calcium dependent, with an optimal pH of 8.0–9.0 and K_m values in the low micromolar range (2–5 μM). Our observations 1) establish the rat ovary as a site of $sPLA_2$ and $cPLA_2$ gene expression, 2) localize the corresponding transcripts to the granulosa cell layer, and 3) establish IL-1β as an up-regulatory agent for ovarian $sPLA_2$ and $cPLA_2$ gene expression as well as for ovarian PLA_2 activity. These findings also indicate that the IL-1 effect is 1) receptor mediated, 2) contingent in part upon *de novo* protein biosynthesis, and 3) inhibited by nitric oxide. These observations support the proposition that PLA_2 may be a key component in the IL-1-stimulated biosynthesis of ovarian PGs. (*Endocrinology* **138:** 322–331, 1997)

PHOSPHOLIPASE A_2 (PLA_2) is the enzyme that catalyses the hydrolysis of fatty acids esterified at the *sn*-2 position of phospholipids. Arachidonic acid (AA), a precursor in the eicosanoid biosynthetic cascade, is predominantly found at this position. Thus, cleavage of the AA residue will, after additional processing, give rise to potent proinflammatory mediators (*i.e.* PGs and leukotrienes). Consequently, it is the PLA_2-mediated release of arachidonic acid that constitutes the rate-limiting event in eicosanoid production (1).

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Evidence to date (reviewed in Refs. 2–4) suggests that mammalian PLA_2 is a heterogeneous family of enzymes that includes two classes of proteins: secretory, low mol wt (14 kDa; $sPLA_2$) and cytosolic, high mol wt (85–110 kDa; $cPLA_2$). The former is further subclassified into a digestive group I (synthesized and secreted mainly by the pancreas) and a nondigestive group II (synthesized and secreted by many cell types). PLA_2 isoenzymes are not only concerned with the hydrolysis of phospholipids, but may also play important roles in cellular growth and differentiation (1), acting via an ever-growing number of specific cell surface receptors (5, 6).

The relevance of PLA_2 to ovarian physiology is suggested by the importance of PG biosynthesis to the ovulatory cascade (7). Moreover, ovarian PG biosynthesis is stimulated by interleukin-1β (IL-1β) (8–10), a putative player in the ovulation process (11–15). We, therefore, set out to characterize rat ovarian PLA_2 activity, $sPLA_2$ and $cPLA_2$ gene expression, cellular localization, and IL-1β dependence.

Materials and Methods

Animals

Immature Sprague-Dawley female rats from Zivic-Miller Laboratories (Zelienople, PA) were killed by CO_2 asphyxiation on day 25 of life. The project was approved by the institutional animal care and use committee.

Reagents and hormones

Phosphatidylcholine L-α-1-stearoyl-2-arachidonyl (arachidonyl-5,6,8,9,11,12,14,15-N-^{3}H; PCSA; 88 Ci/mmol), phosphatidylcholine L-α-dipalmitoyl (2-palmitoyl-9,10-N-^{3}H; PCDP; 42 Ci/mmol], palmitic acid (9,10-N-^{3}H; PA; 39 Ci/mmol), $PGF_{2\alpha}$ (5,6,8,9,11,12,14,15-N-^{3}H; 200 Ci/mmol), PGE_2 (5,6,8,9,11,12,14,15-N-^{3}H; 154 Ci/mmol], AA (5,6,8,9,11,12,14,15-N-^{3}H; 100 Ci/mmol), and [α-^{32}P]UTP (800 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). The corresponding unlabeled materials, ribonuclease A (RNase A), PMSG, aminoguanidine hemisulfate salt (AG), cycloheximide (CHX), and other chemicals (unless specified otherwise) were purchased from Sigma Chemical Co. (St. Louis, MO). McCoy's 5a (serum-free) medium, penicillin-streptomycin solution, BSA, deoxyribonuclease, and trypan blue stain were obtained from Life Technologies (Grand Island, NY). Collagenase (*Clostridium histolyticum*; CLS type I; 144 U/mg) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Hexane, methanol, and 2-propanol were obtained from J. T. Baker, Inc. (Phillipsburg NJ). Chloroform was purchased from Fisher Scientific (Fairlawn, NJ).

Recombinant human IL-1β (2×10^7 U/mg) was generously provided by Drs. Errol B. de Souza and C. E. Newton of DuPont Merck Pharmaceutical Co. (Wilmington, DE). A recombinantly expressed preparation of the naturally occurring human IL-1 receptor antagonist (IL-1RA) was generously provided by Dr. Daniel E. Tracey, Upjohn Co. (Kalamazoo, MI). Highly purified hCG (CR-127, 14,900 IU/mg) was generously provided by Dr. R. E. Canfield through the Center for Population Research, NICHHD, NIH (Bethesda, MD). RNase T1 was obtained from Pharmacia (Piscataway, NJ). AmpliTaq polymerase was purchased from Perkin-Elmer (Norwalk, CT). Alkaline phosphatase-conjugated antidigoxigenin polyclonal antibody Fab fragments, digoxigenin-UTP, nitroblue tetrazolium, and 5-bromo-4-chloro-3-inodyl-phosphate were purchased from Boehringer Mannheim (Indianapolis, IN). Ecl136II was obtained from New England Biolabs (Beverly, MA). T7 RNA polymerase, pGEM7Zf$^+$, and other molecular grade reagents were obtained from Promega (Madison, WI).

Tissue culture procedures

Whole ovarian dispersates were prepared and maintained as previously described (16).

Cell-free PLA_2 enzyme assay

Cellular or extracellular PLA_2 activity was determined by measuring the release of ^{3}H-labeled fatty acid from the *sn*-2 position of ^{3}H-labeled PCSA or PCDP substrates (AA or PA, respectively). Whole ovarian dispersates (5×10^5 viable cells/tube) were initially cultured as previously described (16) for 48 h in the absence or presence of the specified treatments. Media were then collected, and the cells were washed once with 100 mM HEPES buffer (pH 8.0) and suspended in 1 ml of the same buffer. Cells were sonicated on ice (twice) for 5 sec (Vibra Cell, Sonics and Materials, Danbury, CT). A sonicate volume representing a specified number of cells or a medium aliquot was then incubated with radiolabeled PCSA or PCDP (5 μM except as noted, dissolved in methanol) for the duration indicated at 37 C in a total assay volume of 1 ml. Except as noted, final parameters in the assay were 100 mM HEPES (pH 8), 5% methanol, and 2 mM $CaCl_2$. The enzymatic reaction was terminated with 7% formic acid (final pH 3.5), and the resultant products were extracted with ethyl acetate and detected by HPLC as follows. Extracts were evaporated to dryness in a Speed-Vac centrifuge (Savant Instruments, Farmingdale, NY), and the residue was resuspended in 250 μl hexane-isopropanol-acetic acid (95:5:0.025). Sample constituents were fractionated using a Waters (Milford, MA) HPLC system on a normal phase diol column (10 mm; LiChrosorb Diol, EM Reagents, Gibbstown, NJ) with a concave gradient of hexane-isopropanol (95:5 to 60:40) at 2 ml/min. The column was calibrated with authentic [^{3}H]AA, [^{3}H]PGE_2, [^{3}H]$PGF_{2\alpha}$, and [^{3}H]PA. Radiolabeled metabolites of PCSA or PCDP were detected and quantified by on-line scintillation counting with a Radiomatic Flow Detector (Packard Instrument Co., Downers Grove, IL). The ratio of labeled product to labeled substrate was multiplied by the initial substrate concentration (5 μM) to calculate the rate of enzymatic conversion to product (picomoles per 10^5 cells/h). Values were also corrected for substrate availability and product recovery.

Nucleic acid probes

A plasmid containing a 750-bp *Sma*I/*Eco*RI insert of the complementary DNA (cDNA) encoding rat type II $sPLA_2$ (17) was kindly provided by Dr. J. Ishizaki from Shionogi Research Laboratories (Osaka, Japan). For the purpose of RNase protection assays, a *Bam*HI fragment was excised and subcloned into pGEM7Zf$^+$. This latter construct was then linearized with Ecl136II and transcribed with T7 RNA polymerase in the presence of [α-^{32}P]UTP to yield a 535-nucleotide antisense riboprobe that, upon hybridization, was projected to generate a 452-nucleotide protected fragment.

A full-length cDNA of the rat 85-kDa $cPLA_2$ (18) was kindly provided by Dr. Yuji Owada from Tohoku University (Sendai, Japan) in a transcribable vector (pBluescript II SK$^+$). As the transcribed region contained a polyadenylated tail and several A-rich regions, the cDNA was modified to ensure the generation of a high specific activity riboprobe. Specifically, *Bam*HI was used to remove some of the 3′-end of the cDNA. After self-ligation, the product was linearized with *Pvu*II and transcribed with T7 RNA polymerase to yield a 328-nucleotide antisense riboprobe that, upon hybridization, was projected to generate a 253-nucleotide protected fragment corresponding to the translated region of the cDNA.

The ribosomal protein large 19 (RPL19) probe was generated and employed as previously described (19).

RNA extraction

Total RNA of cultured cells and whole ovarian material was extracted with RNAzol-B (Tel Test, Friendswood, TX) according to the manufacturer's protocol.

RNase protection assay

Linearized DNA templates were transcribed with T7 RNA polymerase to specific activities of 800 Ci/mmol [α-^{32}P]UTP ($cPLA_2$ and $sPLA_2$) or 160 Ci/mmol [α-^{32}P]UTP (RPL19). The riboprobes were gel-purified as previously described (20) in an effort to eliminate transcribed products that were shorter than the full-length probes. The assay was performed as previously described (21).

The hormonally independent RPL19 messenger RNA signal was used to normalize the $sPLA_2$ and $cPLA_2$ messenger RNA data for possible variation in RNA loads. Specifically, the net (respective background subtracted) PLA_2 to net RPL19 ratio was calculated for each sample.

In situ hybridization

Sense strand for $sPLA_2$ was created by linearizing the plasmid with *Hin*dIII. Transcription with SP6 created a riboprobe of 698 bp. The probe concentration for the sense and antisense $sPLA_2$ was 4 ng/μl. The washes were performed as described previously (19). Additional washes [at room temperature for 10 min in 0.2 × SSC (standard saline citrate) and for 10 min in 0.1 × SSC at 47 C] were necessary to reduce the background. Three *in situ* hybridizations of three *in vivo* sets of ovaries were carried out.

The probe concentration for $cPLA_2$ was 8 ng/μl. The sense probe for the type I IL-1 receptor (19), in the same concentration, was used a negative control. The posthybridization washes were performed as described previously (19). Three *in situ* hybridizations of three *in vivo* sets of ovaries were carried out. In one set, the $cPLA_2$ signal was weak; in the other two sets, the signal was clear, and the signal to noise ratio was excellent.

For each *in situ* experiment, use was also made of other unrelated riboprobes as internal controls (glucose transporter 3, PG endoperoxidase synthase-2, IL-1β, and type I IL-1 receptor), which labeled different pools of follicles. The specificity of the assay was validated with respect to the antisense probes by identifying both signal-positive and signal-negative follicles within the same ovary.

Statistical analysis

Except as noted, each experiment was replicated a minimum of three times. Data points are the mean ± SE, and statistical significance (Fisher's protected least significance difference) was determined by ANOVA and Student's *t* test. Statistical values were calculated using Statview 512$^+$ for MacIntosh (Brain Power, Calabasas, CA).

Results

$sPLA_2$ and $cPLA_2$ gene expression in vivo during a simulated estrous cycle

To assess $sPLA_2$ and $cPLA_2$ gene expression during a simulated estrous cycle, immature rats were PMSG-primed/hCG-triggered. Rats were killed at the indicated time points, and ovarian transcripts for $sPLA_2$ were detected by a solution hybridization RNase protection assay. As shown (Fig. 1), faint protected fragments for $sPLA_2$ were apparent in whole ovarian material throughout the periovulatory period. However, no statistically significant changes were noted relative to time zero. Probing for $cPLA_2$ yielded signals too faint for meaningful quantification (not shown).

$cPLA_2$ and $sPLA_2$ gene expression in vitro: effect of treatment with IL-1β

Given the negligible *in vivo* expression of $cPLA_2$ as detected in whole ovarian material, we probed for $cPLA_2$ transcripts *in vitro*. Whole ovarian dispersates were cultured for 48 h in the absence or presence of IL-1β with or without IL-1RA. As shown (Fig. 2), treatment with IL-1β produced a 5-fold increase ($P < 0.01$) in the relative expression of $cPLA_2$ over that in untreated controls, an effect reversed by IL-1RA. These observations support the views that the rat ovary is a site of $cPLA_2$ expression, that ovarian $cPLA_2$ transcripts are IL-1β dependent, and that this IL-1β effect is receptor mediated. The IL-1β-mediated increase in $sPLA_2$ transcripts is also shown below (Figs. 6 and 10).

Cellular localization of cPLA2 and $sPLA_2$ transcripts: in situ hybridization studies

To establish the identity of the ovarian cell population responsible for $cPLA_2$ and $sPLA_2$ gene expression, ovaries were obtained from untreated and periovulatory 25-day-old rats and processed for *in situ* hybridization. As shown (Fig. 3), probing with an antisense $cPLA_2$ riboprobe localized the signal to the granulosa cell (*black arrow*) layer in both periovulatory (Fig. 3A) and untreated immature (Fig. 3B) ovaries. The signal appeared to intensify toward the antrum. As shown in an enlarged view (Fig. 3C), the granulosa cell signal was confined by the basement membrane (*black arrow*). Probing with an antisense $sPLA_2$ riboprobe yielded a more robust signal, which was similarly localized to the granulosa cell layer in both untreated (Fig. 4A) and periovulatory (Fig. 4B) immature ovaries. The signal appeared more intense in antral cells. Probing with the corresponding sense riboprobes

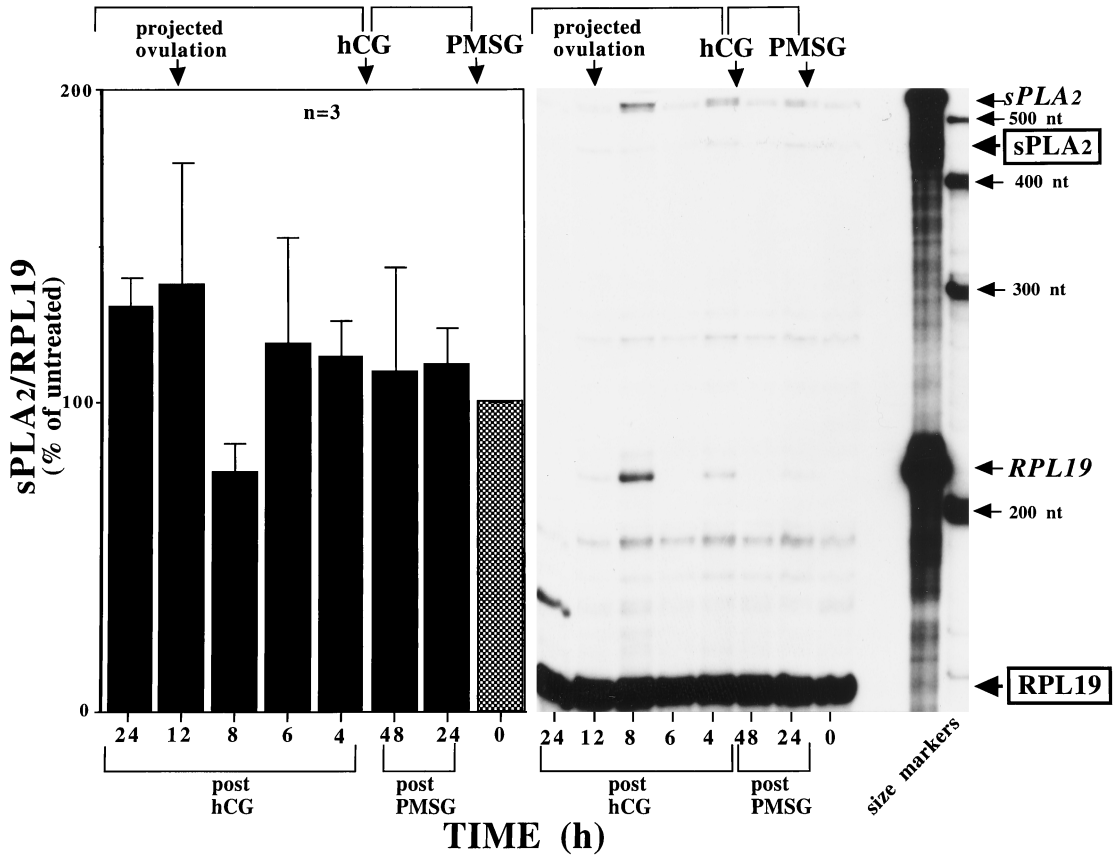

FIG. 1. $sPLA_2$ and $cPLA_2$ gene expression in PMSG/hCG-primed rats. Intact 25-day-old rats were injected (sc) with PMSG (15 IU/rat). Ovulation was triggered 48 h later with hCG (15 IU/rat). The animals were killed at the indicated time points, and total RNA was extracted and subjected to a solution hybridization/RNase protection assay with ^{32}P-labeled rat antisense riboprobes for $sPLA_2$ and RPL19. The intensity of the signals was quantified as described. The *bar graph* depicts the mean ± SE of three experiments. Data were normalized to the value obtained for the untreated rats (time zero). In the representative autoradiograph, the full-length riboprobes are marked in *italics*, and the protected fragments are indicated in *boldface*.

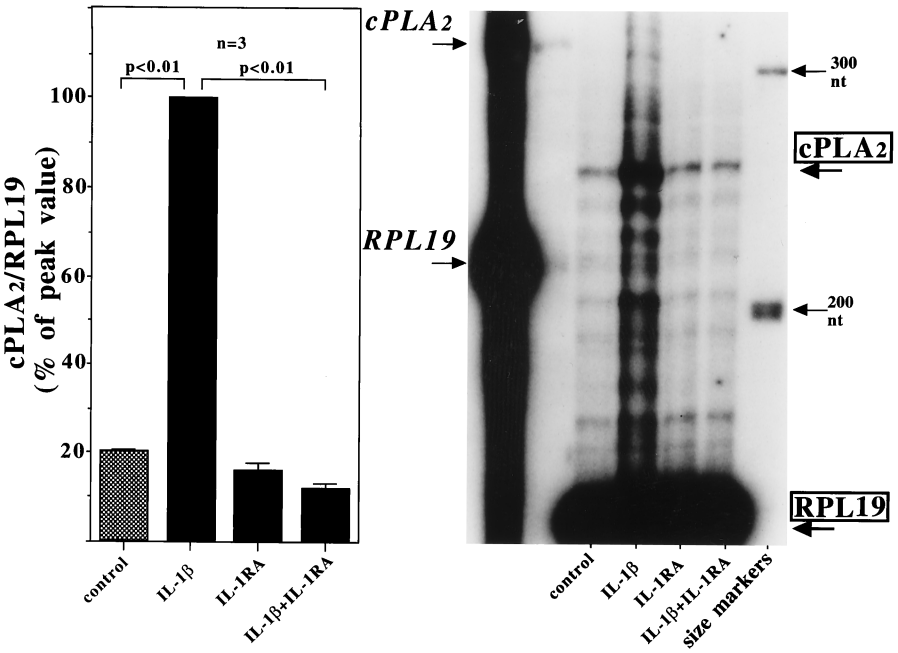

FIG. 2. $cPLA_2$ gene expression by cultured whole ovarian dispersates from immature rats: effect of treatment with IL-1β. Whole ovarian dispersates (1.5×10^6 cells/dish) were cultured for 48 h in the absence or presence of IL-1β (50 ng/ml) with or without IL-1RA (5 μg/ml). The resultant RNA samples were subjected to a RNase protection assay using ^{32}P-labeled rat antisense riboprobes for $cPLA_2$ and RPL19. The *left panel* depicts the mean ± SE of three experiments. In each individual experiment, data were normalized relative to the peak value. In the representative autoradiograph (*right panel*), the protected fragments are indicated in *boldface*.

proved negative (not shown). Thus, PLA_2 transcripts ($sPLA_2$ > $cPLA_2$) are granulosa cell exclusive in both the untreated and periovulatory state.

IL-1β-induced PLA_2 expression and activity by cultured whole ovarian dispersates: dependence on protein synthesis

We examined the role of *de novo* protein synthesis on basal and IL-1β-induced transcripts encoding $cPLA_2$ and $sPLA_2$. As shown (Fig. 5), treatment with cycloheximide (0.1 μg/ml), an established inhibitor of protein biosynthesis, produced significant ($P < 0.001$) blockade of IL-1β-induced (but not basal) $cPLA_2$ gene expression. Significant ($P < 0.05$) attenuation of both basal and IL-1β-induced $sPLA_2$ gene expression were also documented (Fig. 6). The relative specificity of the cycloheximide effect was suggested by its lack of effect on RPL19 expression (Fig. 5). These findings suggest that the induction of PLA_2 transcripts (but not basal PLA_2 expression) is contingent in part upon *de novo* protein biosynthesis.

Medium PLA_2 activity was assessed using a PA-containing substrate (PCDP) that is cleaved only by the $sPLA_2$ isoenzyme (2). A comparable $cPLA_2$-selective substrate does not exist. As shown (Fig. 7), medium PLA_2 activity was stimulated by IL-1β, similar to the IL-1β-mediated stimulation of $sPLA_2$ and $cPLA_2$ transcripts. Concurrent treatment with cycloheximide resulted in substantial inhibition of both basal and IL-1β-induced medium PLA_2 activity ($P < 0.01$ and $P < 0.05$, respectively). These observations suggest that the PLA_2 activity detected in medium conditioned by ovarian cells is dependent on the *de novo* synthesis and secretion of a protein.

IL-1β-induced PLA_2 expression and activity by cultured whole ovarian dispersates: dependence on nitric oxide

To determine whether the ability of IL-1β to induce ovarian $sPLA_2$ and $cPLA_2$ gene expression and medium PLA_2 activity is contingent upon endogenously produced nitric oxide, whole ovarian dispersates were cultured for 48 h in the absence or presence of IL-1β (50 ng/ml) with or without AG, an established inhibitor of inducible ovarian nitric oxide synthase (22). As shown (Fig. 8), treatment with AG resulted in a modest, but significant ($P < 0.01$), increase in IL-1β-stimulated medium PLA_2 activity. Moreover, treatment with AG alone had no effect, but modestly increased the IL-1β-induced expression of $cPLA_2$ ($P < 0.05$; Fig. 9) and $sPLA_2$ ($P = 0.068$; Fig. 10). These observations suggest a slight negative effect of nitric oxide on the ability of IL-1β to induce $cPLA_2$ (and possibly $sPLA_2$) gene expression.

Characterization of total cellular PLA2 activity: time, cell density, calcium, and pH dependence

To establish the optimal duration of the PLA_2 assay, cell sonicates from untreated whole ovarian dispersates were assayed for total cellular (cytosolic and secretory) PLA_2 activity. An AA-containing substrate (PCSA) was used because it is cleaved by both PLA_2 isoenzymes. As shown (Fig. 11A), the assay was linear with time for up to 90 min ($r = 0.97$). No further increments in activity could be detected thereafter. Accordingly, subsequent assays were routinely terminated after 1 h. The assay was also linear for cell density (Fig. 11A, *inset*) in the range used for subsequent assays ($50–200 \times 10^3$ cells). To determine the optimal calcium concentration for the PLA_2 assay, cell sonicates were assayed at the indicated Ca^{2+} concentration. As shown (Fig. 11B), total PLA_2 activity was Ca^{2+} dependent, increasing from 0.2–20 mM Ca^{2+}.

As $cPLA_2$ activity is calcium independent, and $sPLA_2$ activity requires mM concentrations of Ca^{2+} (3), the doubling of PLA_2 activity in the presence of 20 mM Ca^{2+} (Fig. 11B) suggests that whole ovarian dispersates contain equivalent quantities of each isoform. To determine the optimal pH for the PLA_2 assay, cell sonicates were assayed at the indicated pH (Fig. 11C). PLA_2 activity proved to be pH dependent, increasing from pH 6.5 to pH 8.0 with no further significant increments thereafter. These findings suggest an optimal pH

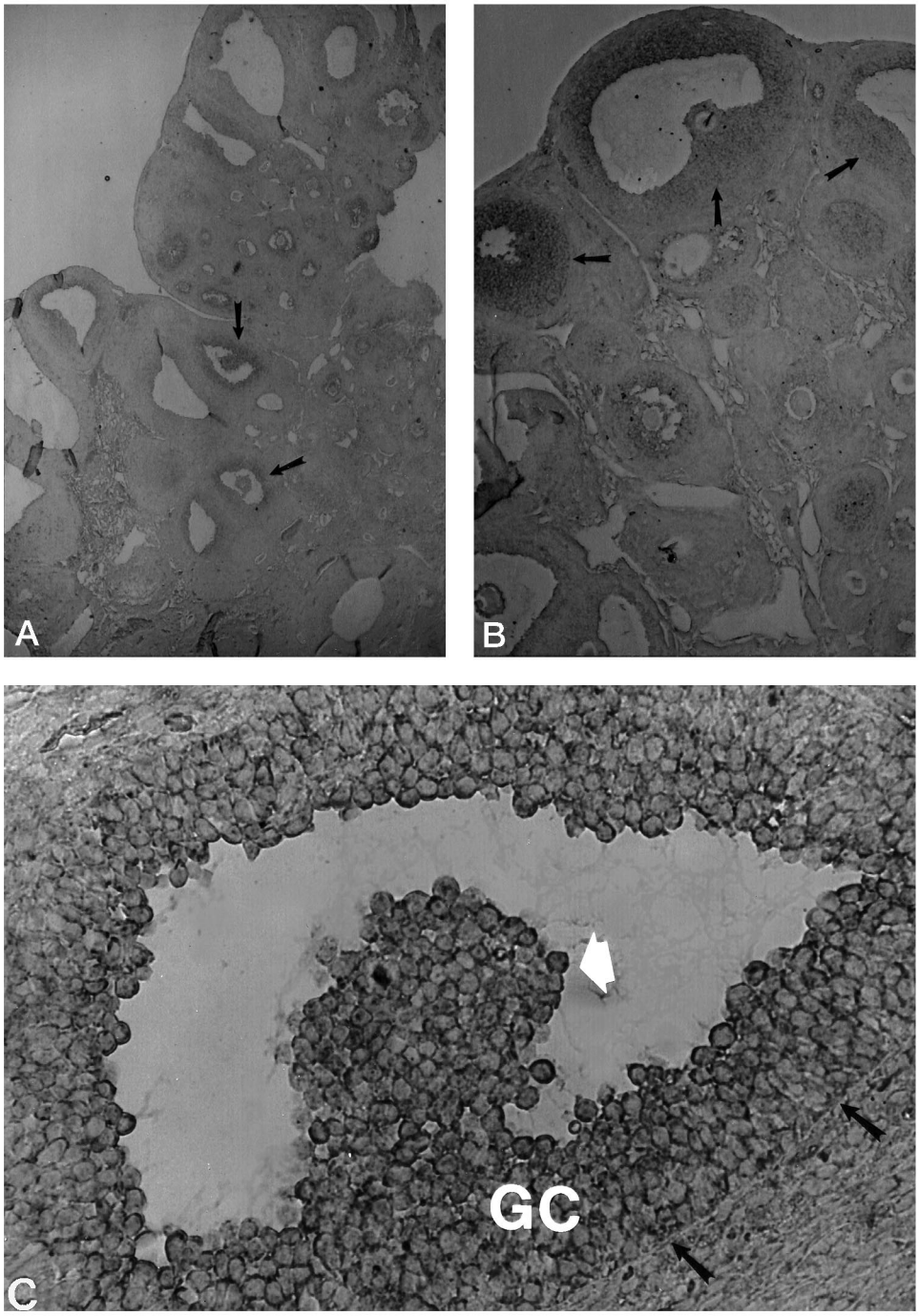

FIG. 3. Cellular localization of $cPLA_2$ transcripts. Ovaries were obtained from untreated or PMSG-primed 25-day-old rats and processed for *in situ* hybridization using a digoxigenin-labeled rat $cPLA_2$ antisense riboprobe. A, ×40 magnification of a section from a PMSG/hCG (8 h)-primed ovary. The *black arrows* denote follicles with positively stained granulosa cells. B, ×100 magnification of a section from an untreated ovary. The *black arrows* denote follicles with positively stained granulosa cells. C, ×400 magnification of a section from a PMSG/hCG (8 h)-primed ovary. GC, Granulosa cells (the *white arrow* denotes the cumulus complex; *black arrows* denote the basement membrane).

of 8.0–9.0 for total cellular (cytosolic and secretory) PLA_2 activity in the ovary, which is similar to reports for extraovarian sites (3).

Ovarian cellular PLA_2 activity: kinetic parameters

To study the kinetics of ovarian PLA_2, cell sonicates were assayed for total PLA_2 activity in the presence of increasing concentrations of PCSA substrate. As shown (Fig. 12), provision of increasing concentrations of substrate (0.2–5 μM) resulted in a progressive increase in the product generated ([^{3}H]AA). The velocity of the reaction approached saturation at 5 μM substrate. K_m values for control (two separate experiments) and IL-1β-treated (three separate experiments) cells were calculated using double reciprocal plots (a representative is shown in Fig. 12, *inset*). Values were variable, but in the same range for both groups: K_m = 2.13 and 1.7 μM and 1.06, 4.0, and 5.9 μM for control and IL-1β, respectively. These values must be viewed as crude estimates of the actual values, because the PLA_2 reaction does not necessarily comply with the constraints imposed by the Michaelis-Menten equation. In fact, a substantial body of evidence supports the view that the kinetics of the PLA_2 reaction are complex because of

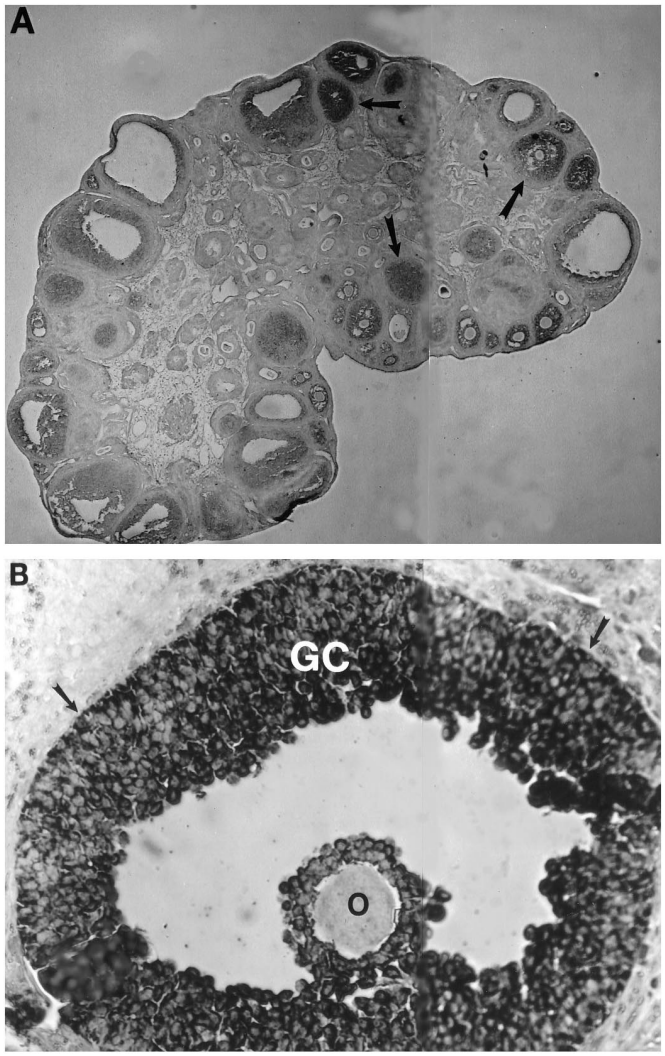

FIG. 4. Cellular localization of $sPLA_2$ transcripts. Ovaries from 25-day-old rats were processed for *in situ* hybridization using a digoxigenin-labeled rat $sPLA_2$ antisense riboprobe. A, ×4 magnification of a section from an untreated ovary. The *black arrows* denote follicles with positively stained granulosa cells. B, ×400 magnification of a section from a PMSG/hCG (24 h)-primed ovary. The *black arrows* denote the follicular basement membrane. O, Oocyte; GC, granulosa cells.

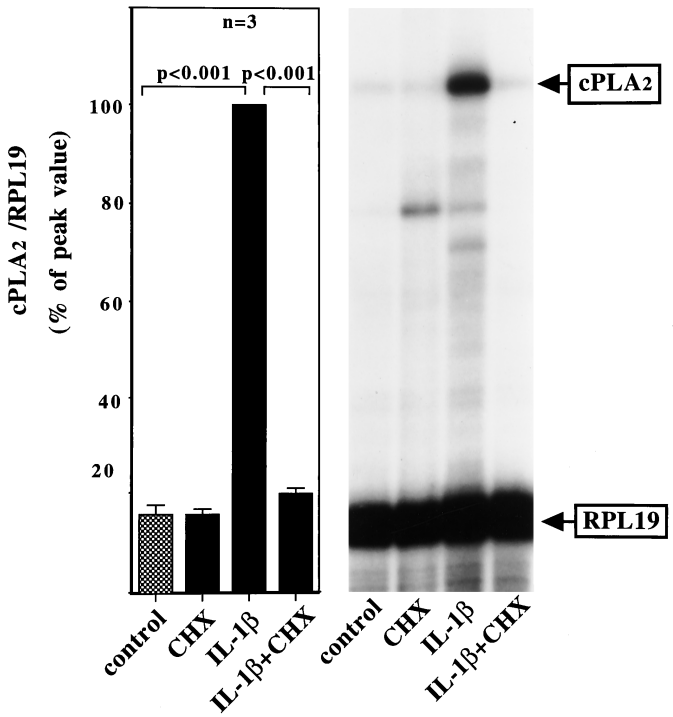

FIG. 5. IL-1β-induced $cPLA_2$ expression by cultured whole ovarian dispersates: protein synthesis dependence. Whole ovarian dispersates (1.5×10^6 cells/dish) were cultured for 48 h in the in the absence or presence of IL-1β (50 ng/ml) with or without CHX (0.1 μg/ml). The resultant RNA samples were subjected to a RNase protection assay using ^{32}P-labeled rat antisense riboprobes for $cPLA_2$ and RPL19 as described. The *left panel* depicts the mean ± SE of three experiments. In each experiment, data were normalized to the peak value. In the representative autoradiograph (*right panel*), the protected fragments are indicated in *boldface*.

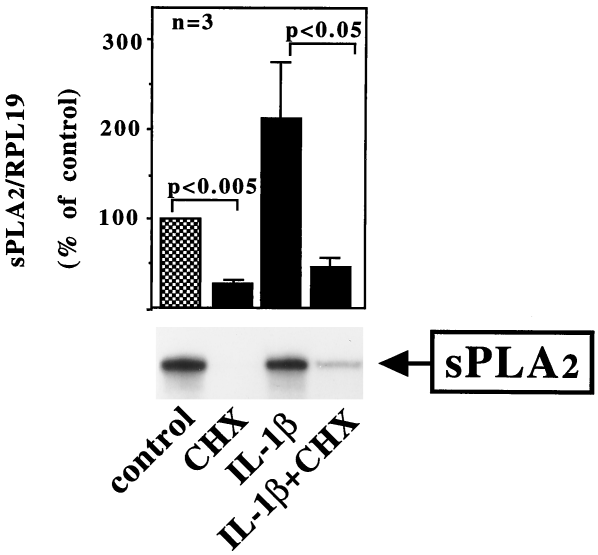

FIG. 6. IL-1β-induced $sPLA_2$ expression by cultured whole ovarian dispersates: protein synthesis dependence. Whole ovarian dispersates were cultured and analyzed as described in Fig. 5, except that rat antisense $sPLA_2$ riboprobes were used. Data were normalized relative to untreated controls (*top panel*). In the representative autoradiograph (*bottom panel*), the protected fragments for $sPLA_2$ are shown.

the need to establish optimal lipid-water interfacing (2). However, these data suggest that the kinetics of ovarian PLA_2 activity are similar (both quantitatively and qualitatively) to the kinetics of PLA_2 activity in other tissues (2).

Discussion

The role of increased PLA_2 activity as an inflammatory mediator is well established (23). Increased PLA_2 activity was noted in a host of pathological processes (*e.g.* septic shock, connective tissue inflammatory diseases, premature labor, hypertension, and pancreatitis). However, this family of enzymes also plays many physiological roles, including (but not limited to) the facilitation of fat digestion, lung surfactant metabolism, cell membrane homeostasis, insulin release, sperm maturation, and lipoprotein metabolism (2, 3). This

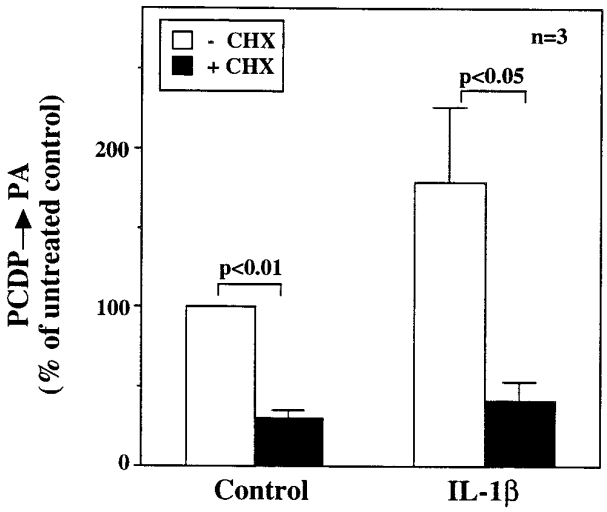

FIG. 7. IL-1β-induced medium $sPLA_2$ activity in cultured whole ovarian dispersates: protein synthesis dependence. Whole ovarian dispersates (1.5×10^6 cells/dish) were cultured for 48 h in the absence or presence of IL-1β (50 ng/ml) with or without CHX (0.1 mg/ml). At the conclusion of this treatment interval, conditioned media were assayed for PLA_2 activity by conversion of PCDP substrate to PA. The results represent the mean ± SE of three independent experiments. Data were normalized relative to untreated control values.

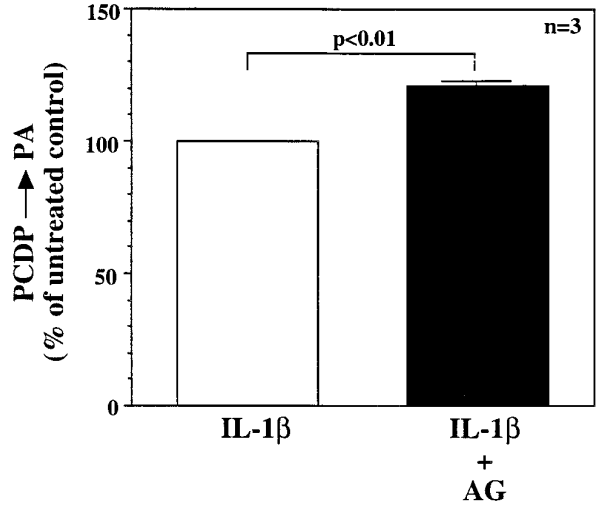

FIG. 8. IL-1β-induced medium PLA_2 activity in medium conditioned by whole ovarian dispersates: nitric oxide dependence. Whole ovarian dispersates (1.5×10^6 cells/dish) were cultured for 48 h in the presence of IL-1β (50 ng/ml) with or without AG (0.4 mM). At the conclusion of this treatment interval, conditioned media were assayed for PLA_2 activity as described in Fig. 7. The results represent the mean ± SE of three independent experiments. Data were normalized relative to IL-1β-treated cells.

communication concerns itself with the potential relevance of PLA_2 to ovarian physiology.

Our current observations document a nearly constant periovulatory level of $sPLA_2$ transcripts and a negligible $cPLA_2$ signal. It is possible that the modest fluctuation in the steady state levels of ovarian PLA_2 transcripts reflects a dilutional effect by non-PLA_2-expressing components of the ovary. Consequently, no meaningful information can be deduced as to PLA_2 economy in individual ovarian follicles. Perhaps

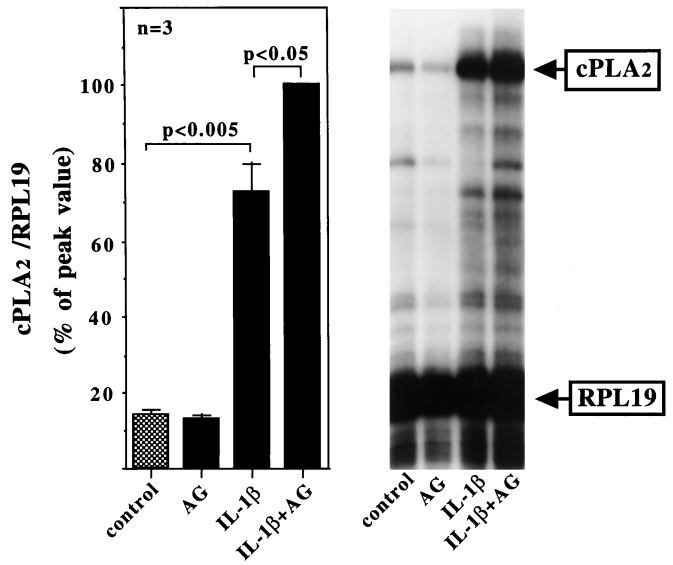

FIG. 9. IL-1β-induced $cPLA_2$ gene expression: nitric oxide dependence. Whole ovarian dispersates (1.5×10^6 cells/dish) were cultured for 48 h in the absence or presence of IL-1β (50 ng/ml) with or without AG (0.4 mM). The resultant RNA samples were subjected to a RNase protection assay using ^{32}P-labeled rat antisense riboprobes for $cPLA_2$ and RPL19 as described. The *left panel* depicts the mean ± SE of three experiments. In each experiment, data were normalized to the peak value. In the representative autoradiograph (*right panel*), the protected fragments are indicated in *boldface*.

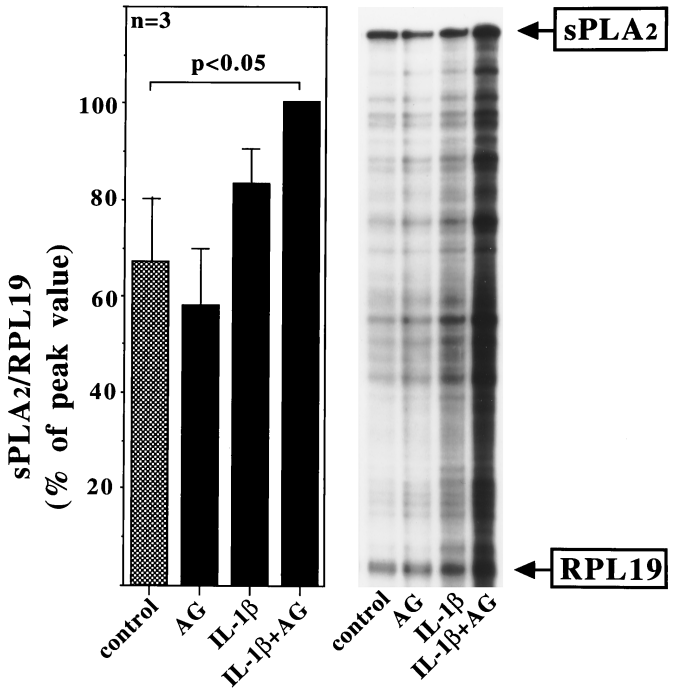

FIG. 10. IL-1β-induced $sPLA_2$ gene expression: nitric oxide dependence. Whole ovarian dispersates (1.5×10^6 cells/dish) were cultured and analyzed as described in Fig. 9, except that rat antisense $sPLA_2$ riboprobe was used.

PLA_2 is selectively expressed in rapidly growing follicles that are destined to ovulate. Such a hypothesis could be tested in an *in vitro* paradigm capable of sustaining follicular growth and maturation. Alternatively, the relevant PLA_2 transcript

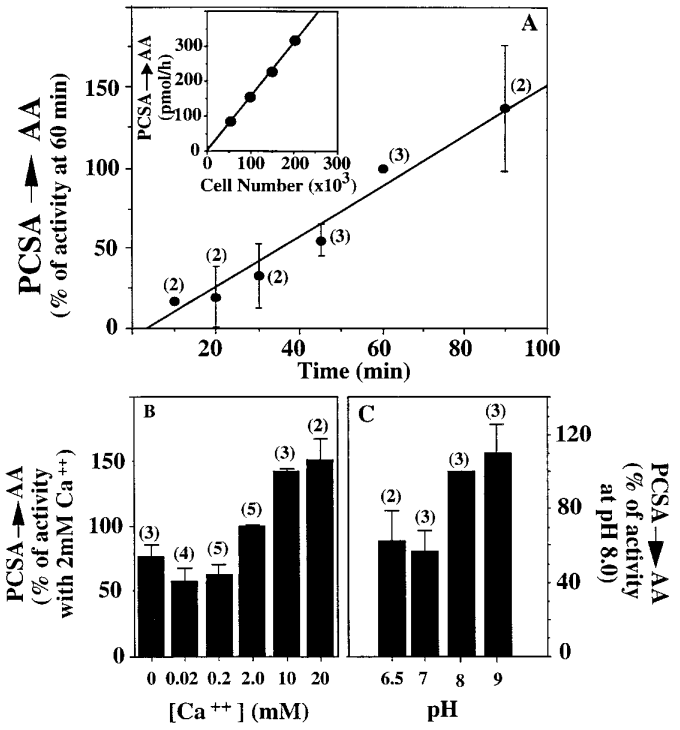

FIG. 11. Characterization of ovarian cellular PLA_2 activity. Whole ovarian dispersates (5×10^5 viable cell/tube) were cultured for 48 h in the absence of any treatment. At the conclusion of the incubation period, total cellular (cytosolic and secretory) PLA_2 activity was determined by conversion of PCSA to AA. A, Time dependence experiments. PCSA, 5 μM; $CaCl_2$, 2 mM (pH 8.0). Data reflecting the mean ± SE of the indicated number of experiments were normalized relative to the 60 min point. Similar conditions were used for a cell density dependence experiment (*inset*). B, Ca^{2+} concentration dependence experiments. PCSA, 5 μM; pH 8; duration of incubation, 1 h. Data reflecting the mean ± SE of the indicated number of experiments were normalized relative to a $CaCl_2$ concentration of 2 mM. C, pH dependence experiments. PCSA, 5 μM; $CaCl_2$, 2 mM; duration of incubation, 1 h. pH titration was accomplished using HEPES buffer over the range of 6.5–9.0. Data reflecting the mean ± SE of the indicated number of experiments (mean ± difference for n = 2) were normalized relative to pH 8.0.

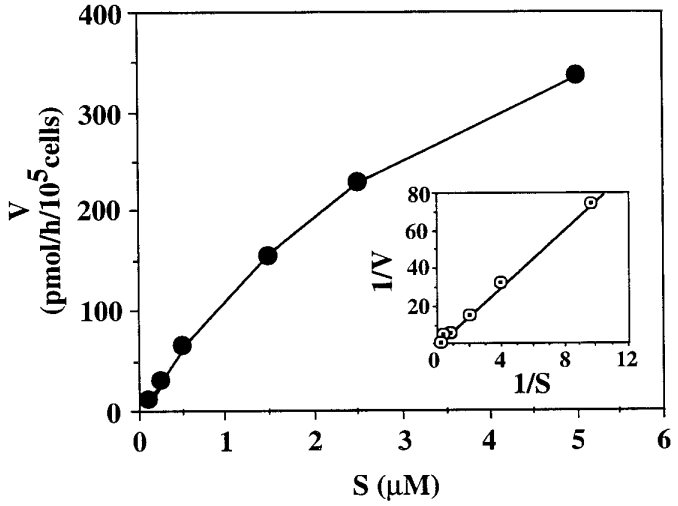

FIG. 12. Ovarian cellular PLA_2 activity: kinetic parameters. Sonicates corresponding to 1.5×10^5 cells from either untreated or IL-1β-treated whole ovarian dispersates (a representative IL-1β-treated dispersate is shown) were assayed for PLA_2 activity (1-h incubation) by conversion of PCSA to AA. Increasing concentrations (0.2–5 μM) of substrate (S) were added in duplicate. The *inset* depicts a corresponding double reciprocal (Lineweaver-Burk) plot. V, Reaction velocity.

is one other than the varieties probed for. Indeed, the growing family of PLA_2s may feature other representatives in the ovary that have yet to be evaluated. This possibility is strongly supported by the realization that ovarian PLA_2 activity is increased under *in vivo* circumstances in response to the LH surge, as reported by Bonney and Wilson (24).

Compelling evidence points to PG endoperoxide synthase (PGS-2) as the enzyme that is intimately associated with ovulation. Indeed, pharmacological (25–30) or genetic (31) ablation of PGS-2 has been shown to arrest follicular rupture. Specifically, Hedin *et al.* (32) have unequivocally shown that the hCG-induced synthesis of PGs before ovulation is associated with a transient induction of PGS-2. In contrast, $sPLA_2$ expression did not increase during the periovulatory period. Possibly, then, $sPLA_2$ (unlike PGS-2) is constantly expressed in the ovary, setting the stage for the rate-limiting PGS-2 action detected during a narrow periovulatory window (32). Alternatively, other species of PLA_2 may be at play, in that PLA_2 is clearly up-regulated at midcycle in response to the LH surge, as documented by Bonney and Wilson (24).

One of the central observations made in this communication concerns the apparent IL-1β dependence of PLA_2 expression and activity. As both PGs (7) and IL-1β (11–14) may be involved in the ovulatory process, our present observations support the proposition that PLA_2 may play a role in the context of follicular rupture. Moreover, PLA_2 may be a component in the ability of IL-1β to stimulate the biosynthesis of ovarian PGs (8). Although our findings constitute the first such report for the ovary, the ability of IL-1β to induce PLA_2 activity and to stimulate PG biosynthesis has been amply documented at multiple extraovarian sites (33–41).

IL-1β-induced gene expression for both PLA_2 subtypes requires *de novo* protein biosynthesis, as treatment with cycloheximide significantly decreased basal $sPLA_2$ expression and medium PLA_2 activity as well as the ability of IL-1β to induce $cPLA_2$ and $sPLA_2$ transcripts. We speculate that the IL-1β action requires the induction of its receptor (type I) before other end points can be affected. However, other intermediary proteins could be involved as well.

The relevance of nitric oxide to ovarian physiology was suggested by *in vivo* experiments demonstrating the ability of aminoguanidine (an inhibitor of inducible nitric oxide synthase) to suppress hCG-triggered ovulation in the rat (42). IL-1β has been shown to markedly increase ovarian nitric oxide synthase activity (22, 43). In light of the above, we examined the impact of a nitric oxide vacuum (created by aminoguanidine) on the IL-1β-induced expression of $cPLA_2$ and $sPLA_2$. Our results suggest that nitric oxide production may modestly down-regulate the PLA_2 system. As the ovarian IL-1 system is characterized by its self-amplification property (44), nitric oxide may be an intraovarian regulator that can limit IL-1β activity by decreasing PLA_2 activity. Conceivably, without such a re-

straint, IL-1β activity may lead to premature triggering of the ovulatory cascade.

Although the precise intraovarian role of PLA_2 remains an evolving subject, there is little doubt that several representatives of the PLA_2 family are expressed within the mammalian ovary and are subject to regulation. Both $cPLA_2$ and $sPLA_2$ transcripts are present in granulosa cells from untreated and periovulatory immature ovaries. It seems likely that $sPLA_2$ is constitutively expressed (given its stronger *in vitro* and *in situ* signals), whereas $cPLA_2$ may be considered inducible (given the relatively weak signal that is markedly stimulated by IL-1β). Activity for both isoforms is present in cell sonicates based on calcium dependence studies. Moreover, pancreatic PLA_2 ($sPLA_2$ group I) has recently been introduced as a new player in ovarian physiology (45). A modest increase in ovarian PLA_2 activity in response to the LH surge has also been reported (24). Although not evaluated in this report, PLA_2 activity is reportedly subject to significant fluctuations during luteal regression in pseudopregnant and pregnant rats (46). Our present observations provide additional support for the growing significance of PLA_2 in ovarian physiology.

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