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Arachidonic Acid Stimulates Phosphoinositide Hydrolysis and Human Placental Lactogen Release in an Enriched Fraction of Placental Cells

PHILIP ZEITLER AND STUART HANDWERGER

Departments of Pediatrics and Physiology, Duke University Medical Center, Durham, North Carolina 27710

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SUMMARY

Previous investigations in this laboratory have indicated that arachidonic acid stimulates a rapid, dose-dependent, and reversible increase in human placental lactogen (hPL) release which is not dependent on cyclooxygenase or lipoxygenase metabolism. To investigate further the mechanism by which arachidonic acid stimulates the release of hPL, the effects of arachidonic acid on phosphoinositide hydrolysis were examined in an enriched cell culture population of term human syncytiotrophoblast. Phosphoinositide hydrolysis was assayed by three methods: the release of ^{3}H from perfused cells prelabeled with [^{3}H]myoinositol, the measurement of inositol phosphate accumulation, and the distribution of radioactivity in phospholipids separated by two-dimensional thin layer chromatography after exposure of ^{32}P-labeled placental cells to arachidonic acid. Arachidonic acid stimulated a concentration-dependent, rapid, and reversible increase in the release of both [^{3}H]myoinositol and hPL from perfused placental cells. This effect was not inhibited by prior incubation of cells with indomethacin (20 μM). In contrast, palmitic acid and oleic acid stimulated phosphoinositide hydrolysis only at a high concentration (100 μM). Arachidonic acid also stimulated the rapid appearance of inositol monophosphate in placental cells. The effect of arachidonic acid was specific for hydrolysis of phosphoinositides and phosphatidylserine and did not involve other phospholipids. Since phosphoinositide hydrolysis is associated with hormone release in a variety of secretory systems, these results suggest that the stimulation of hPL release by arachidonic acid may be mediated, at least in part, by the activation of phospholipase C.

INTRODUCTION

hPl[1] is a gestational polypeptide secreted by the placenta which has striking chemical similarity to human growth hormone and prolactin. However, hPL secretion is not responsive to factors known to regulate these pituitary hormones (for review, see Ref. 1). In addition, the evidence suggests that the secretion of hPL may differ from that of other hormones in a number of ways. While removal of calcium from the extracellular environment results in a decrease in the rate of hormone secretion from most cell types, removal of extracellular calcium stimulates a marked increase in hPL release (2). Furthermore, unlike most polypeptide hormones, hPL is not stored in intracellular granules (3).

Recent investigations from this laboratory indicate that arachidonic acid stimulates a rapid, dose-dependent increase in the release of hPL from placental explants and dispersed placental cells (4, 5). Stimulation of hPL release occurred when explants or cells were exposed directly to arachidonic acid or to phospholipase A_2, an enzyme which cleaves endogenous arachidonic acid from the 2-acyl position of membrane phospholipids. The cyclooxygenase inhibitors indomethacin and flufenamic acid and the cyclooxygenase/lipoxygenase inhibitors eicosatetraenoic acid and BW755c did not prevent the stimulation of hPL release by either arachidonic acid or phospholipase A_2, suggesting that the action of arachidonic acid is due to the fatty acid itself or a non-cyclooxygenase, non-lipoxygenase metabolite.

Several studies indicate that arachidonic acid may affect the activity of a number of cellular enzymes, including phospholipase C, a cytoplasmic phospholipid phosphodiesterase isolated from rat brain, lymphocytes, and liver (6, 7), which cleaves the polar head groups from cellular phospholipids. The hydrolysis of phosphoinositides catalyzed by phospholipase C has been associated with secretion in a wide variety of cell types, including anterior pituitary cells, endocrine and exocrine pancreas, salivary glands, and adrenal cortex (8, 9). In the present study we have examined whether the effect of arachidonic acid on hPL release is associated with stimulation

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[1] The abbreviations used are: hPL, human placental lactogen; RPMI-1640, Roswell Park Memoral Institute Medium 1640; HEPES, 4-(α-hydroxyethyl-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate; FRIR, fractional rate of inositol release.

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of phosopholipase C activity and phosphoinositide hydrolysis. Since the placenta is a heterogeneous tissue containing numerous cell types, the studies were performed utilizing a trophoblast preparation enriched in cells which synthesize and release hPL prepared by density gradient centrifugation of dispersed placental tissue.

The hydrolysis of phosphoinositide in placental cells was assayed by three methods: 1) the release of ^{3}H from perfused cells prelabeled with [^{3}H]myoinositol; 2) the measurement of inositol phosphate accumulation in response to stimulation; and 3) the loss of ^{32}P from individual phospholipid classes in placental cells prelabeled with [^{32}P]orthophosphate.

MATERIALS AND METHODS

Placental Cell Preparation

Placental tissue was obtained immediately after delivery from women with normal pregnancies of 37 to 40 weeks gestation. Permission to obtain the tissue was granted by the Human Investigation Committee of Duke University. Individual cotyledons were dissected from the placenta and placed in chilled RPMI-1640 (Flow Laboratories) buffered with 12.5 mM HEPES (Sigma) (pH 7.5) for transport to the laboratory.

An enriched fraction of syncytiotrophoblast cells was prepared as previously described (5). Briefly, after removal of decidual tissue from the maternal aspect, placenta was washed and coarsely minced. Minced tissue was dissociated at 37° for 60 min with 0.1% collagenase (type III; Worthington Enzymes) and 0.1% hyaluronidase (Sigma) in RPMI-1640 buffered with bicarbonate (2 mM) and HEPES (12.5 mM), pH 7.5, containing 1% fetal calf serum, 0.01% soybean trypsin inhibitor, and 0.001% deoxyribonuclease I (Worthington Enzymes). The resulting cell suspension was filtered through Nitex cloth (150 μm mesh), and the cells were collected by centrifugation at 100 × g for 6 min. After washing twice in RPMI-1640, cell number and viability were assessed by trypan blue exclusion.

The dissociated cells were separated by isopycnic centrifugation on linear gradients of 40% Percoll (Sigma) (0.8–1.0 × 10^{8} cells/gradient). Previous studies (5) indicate that greater than 90% of the cells which synthesize and release hPL are recovered with a density of 1.02–1.01 g/ml and account for approximately 15% of the total placental DNA. This procedure provides a 10–15-fold enrichment of the population in hPL-producing syncytiotrophoblast.

Assay of Phosphoinositide Hydrolysis

Release of [^{3}H]myoinositol. Freshly prepared cells were washed and resuspended in 20 ml of RPMI-1640 containing 8–10 μCi/ml of [^{3}H]myoinositol (Amersham; 16.4 mCi/μmol). After incubation for 4 hr at 37°, cells were sedimented at 100 × g for 6 min and resuspended in 0.6 ml of RPMI-1640. Two parallel columns (0.15 ml bed volume) of acetylated Sephadex G-10 (Pharmacia) were formed in 1-ml syringes cut to a volume of 0.4 ml and plugged at the tip with acetylated cotton wool. The columns were washed and packed by perfusion with distilled H_2O at a rate of 1 ml/min with a dual-channel peristaltic pump. After extensive washing, the medium was changed to RPMI-1640, pH 7.6, continuously bubbled with 5% CO_2/95% O_2, and maintained at 37° in a water bath. Perfusion with RPMI-1640 was continued for 30 column volumes to ensure equilibration before addition of cells. After removal of 0.05-ml aliquots for protein determination by the method of Bradford (10), 0.2 ml of the cell suspension was layered over each of the two columns and the columns were perfused with RPMI-1640 at a rate of 0.5 ml/min.

The efflux of ^{3}H decreased with time, with a large initial component due to extracellular, unincorporated [^{3}H]myoinositol. However, after approximately 30 min, the release of radioactivity from cells remained nearly constant. When two parallel columns were perfused with control medium, efflux rates for the two columns after the initial 30-min period were essentially identical for extended periods of perfusion (see Fig. 1A). Furthermore, when cells were extracted with chloroform/methanol/2 M KCl (1:2:1) after a 40-min initial perfusion, greater than 95% of the radioactivity was recovered in the organic phase and co-chromatographed with authentic phosphatidylinositol standard (Supelco). Therefore, all experiments involving the effects of variables on the rate of inositol release were performed after an initial 40-min perfusion period.

In each experiment, one cell column was used to examine the effects of arachidonic, palmitic, or oleic acid, while the other column was used to examine the effects of vehicle alone. Arachidonic acid (Calbiochem-Behring), palmitic acid (Sigma), and oleic acid (Sigma) were dissolved in absolute ethanol with a final concentration less than 0.3%. After 40 min of perfusion in control medium, one column was perfused for 30 minutes with medium containing the agent to be examined, while the parallel control column was perfused with medium containing vehicle (ethanol 0.3%) alone. After the 30-min exposure period, both columns were perfused with control medium for an additional 40 min. The effluent was collected in 2-min fractions and an aliquot was counted by liquid scintillation spectroscopy in 10 ml of Aquasol II (New England Nuclear). At the end of each perfusion, the radioactivity remaining in the cells and columns was determined. SDS/5 mM EDTA (5%) was added to each column and the contents of the column were counted by liquid scintillation spectroscopy. In several experiments, the effects of the cyclooxygenase inhibitor indomethacin (Sigma) on arachidonic acid stimulation of hPL and [^{3}H]myoinositol release were examined. In these experiments, both columns were perfused with medium containing the inhibitor for 40 min prior to and during the exposure to arachidonic acid. The effects of indomethacin and BW755c alone on hPL and [^{3}H]myoinositol release were examined in a manner identical to that used to examine the effects of arachidonic acid.

The curve relating the radioactivity to time for each column was integrated according to the method of Uchikawa and Borle (11). The sum of all the radioactivity in each effluent period plus the radioactivity left in the cells at the end of the efflux was taken as the total radioactivity present in the cells at the beginning of the desaturation period. The percentage of the total radioactivity remaining in the cells at each time point was then obtained by sequentially subtracting the radioactivity of each fraction. The FRIR for each fraction was determined as:

$$\text{FRIR} = \text{cpm}_e/[(\text{cpm}_c)_{\text{mean}}t] \times 100$$

where cpm_e is the radioactivity appearing in the effluent during the time interval t and $(\text{cmp}_c)_{\text{mean}}$ is the mean radioactivity remaining in the cells during the time interval.

FRIR values for the experimental fractions were determined as a percentage of the corresponding values for the control column (FRIR ratio). The FRIR ratio for each experiment was then graphed as a function of time on the same scale and the area under each curve during the period of perfusion, representing the total isotope release, was determined by planimetry.

Measurement of inositol phosphate accumulation. Freshly prepared cells were incubated with [^{3}H]myoinositol as above. After incubation, cells washed twice in RPMI-1640 and resuspended at a density of approximately 3 × 10^{6} cells/ml, divided into duplicate flasks, and returned to the incubator for an additional 60 min. At the end of 60 min, 0.8-ml samples were removed from each flask and extracted as described below. The parallel flasks were then exposed to arachidonic acid or vehicle alone and 0.8-ml samples were removed at the appropriate times. Samples were transferred into tubes containing 2.5 ml of chloroform/methanol/HCl (200:100:2), followed by 1.25 ml of chloroform and 1.25 ml of H_2O. After vortexing, the tubes were centrifuged at 2200 × g for 10 min and the upper phase was removed. Samples (1.5 ml) of the aqueous phase were transferred onto columns containing 1 ml of Dowex-1 (Sigma), and the inositol phosphates were eluted sequentially by the method of Berridge (12). The following fractions were collected: inositol and glycerophosphorylinositol (16 ml of 60 mM

ammonium formate/5 mM disodium tetraborate); inositol monophosphate (16 ml of 200 mM ammonium formate/100 mM formic acid); inositol bisphosphate (20 ml of 400 mM ammonium formate/100 mM formic acid); and inositol trisphosphate (12 ml of 1 M ammonium formate/100 mM formic acid). Radioactivity in samples of these fractions was determined by scintillation counting in Aquasol II. Statistical significance of the difference between experimental and control cells was determined by analysis of variance.

Loss of ^{32}P from prelabeled phospholipids. Freshly prepared cells were incubated in suspension for 16 hr at a density of approximately 4×10^6 cells/ml in RPMI-1640 with 0.1 mCi/ml of [^{32}P]orthophosphate (Amersham; carrier-free). Cells were washed, resuspended at a density of 3×10^6 cells/ml, divided into duplicate flasks, and returned to the incubator for an additional 60 min. At the end of the 60 min, the parallel flasks were exposed to arachidonic acid or to vehicle alone. Duplicate 0.75-ml samples were removed at appropriate times and added to 2.5 ml of methanol to terminate the reaction. After vortexing, 1.25 ml of distilled H_2O and 1.25 ml chloroform were added to the methanol, followed by an additional 1.25 ml of chloroform and 1.25 ml of 2 M KCl/5 mM EDTA. The cells were then extracted for 16 hr at 4°. After separation of the phases by centrifugation at $2200 \times g$ for 10 min, the choloroform layer was removed and replaced with 1.25 ml of fresh chloroform. After vortexing and centrifugation, the second chloroform layer was removed and combined with the first. To ensure removal of all non-lipid components, the combined chloroform layers were washed with 10 ml of distilled water. Recoveries of ^3H-phosphatidylcholine and ^3H-phosphatidylinositol (New England Nuclear) by this method were 92% and 87%, respectively.

The chloroform layer was evaporated under a stream of nitrogen at 25° and resuspended in 0.04 ml of chloroform. Samples (0.025 ml) were spotted in the preadsorbent area of a 10 × 10 cm silica gel G thin layer plate (Analtech). Separation of phospholipids was performed by the two-dimensional technique of Yavin and Zutra (13) which offers reproducible separation of phosphatidylinositol and phosphatidylserine. After drying the spot under a stream of warm (55°) air for 3 min, the plates were developed in the first dimension in a solvent system containing chloroform/methanol/40% methylamine (65:35:7.5). Following development, the plates were dried under warm air for 3 min and exposed for 3 min to the fumes of concentrated HCl in order to degrade contaminating plasmalogens. The plates were dried in warm air and ambient air for 3 min and 2 min, respectively. In order to remove neutral lipid contaminants, plates were run in the second direction in diethyl ether/glacial acetic acid (95:5). After development in the second solvent, the plates were dried in ambient air for 3 min and run again in the second direction in chloroform/acetone/methanol/acetic acid/H_2O (50:20:10:13:5). After chromatography, plates were thoroughly dried and exposed for 24 hr to Kodak XR-5 film at −70° in order to locate the radioactivity. Areas of the silica gel corresponding to the radioactivity were scraped, dissolved in 10 ml of Aquasol II and counted by liquid scintillation spectroscopy. The identity of the spots was established by parallel chromatography of pure phospholipid standards (Supelco). Radioactivity present in each phospholipid class was compared between experimental and control cells. Statistical significance was determined by analysis of variance.

Determination of hPL Release from Perfused Cells

Since the amount of hPL released into the media during each time interval was below the detectability of the hPL radioimmunoassay, effluent fractions were pooled and concentrated. Every seven consecutive tubes (15 min) during the perfusion were combined and the proteins precipitated by addition of trichloroacetic acid to a final concentration of 10%. After centrifugation at $3200 \times g$ for 30 min, the supernatants were decanted and the pellets allowed to drain. The pellets were resuspended in 0.4 ml of 0.02 M phosphate-buffered saline, pH 7.4, and assayed by homologous radioimmunoassay (14). Recovery of a known quantity of hPL by this method ranged from 86–92%.

RESULTS

As shown in Fig. 1A, exposure of perfused placental cells for 30 min to 50 μM arachidonic acid stimulated a rapid increase in FRIR which was accompanied by a 2150% increase in the release of hPL into the perfusion medium (Fig. 1B). The increase in FRIR occurred within 5 min after the addition of arachidonic acid, while hPL release increased within 15 min. Both FRIR and hPL release returned toward baseline within 10 min of the removal of the arachidonic acid. In contrast, exposure of perfused cells to control medium alone resulted in no significant changes in either FRIR or hPL release (Fig. 1, A and B). Aqueous [^3H]myoinositol at the beginning of the perfusion period accounted for approximately 5–7% of the total radioactivity. In contrast, [^3H]myoinositol release during the period of exposure to arachidonic acid exceeded the available aqueous pool. Eight to 10% of the total [^3H]myoinositol was released in the 30 min following exposure to 10 μM arachidonic acid, while 18–21% of the total [^3H]myoinositol was released during the 30 min following exposure to 100 μM arachidonic acid. When samples of the effluent were analyzed on Dowex-1 columns as described, radioactivity was recovered only in the free inositol fraction.

The effect of arachidonic acid on FRIR and hPL release was dose-dependent. As shown in Fig. 2, cells exposed for 30 min to 10 μM and 50 μM arachidonic acid

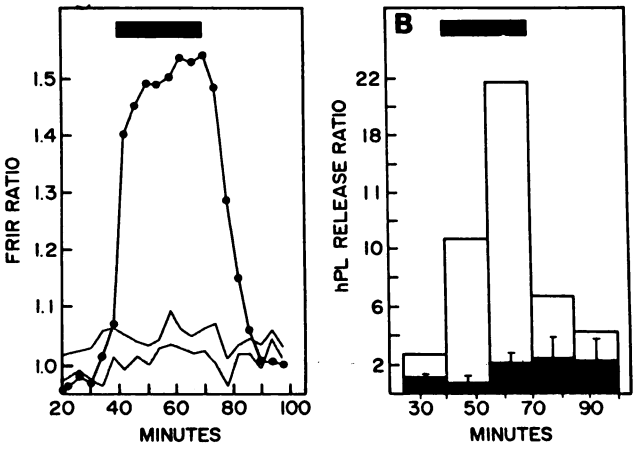

FIG. 1. *The effect of 50 μM arachidonic acid on FRIR and hPL release from perfused placental cells*

After 40 min of perfusion, cells were exposed for 30 min (*bar*) to 50 μM arachidonic acid or to vehicle alone. A, the effect of arachidonic acid on FRIR. The *stippled area* represents the results of control experiments ($N = 6$) in which two parallel columns were perfused with control media. The results are expressed as the ratio of FRIR values for the two columns, mean ± standard error. ●—●, results of a typical experiment in which an experimental column was exposed for 30 min (*bar*) to 50 μM arachidonic acid and a parallel control column was exposed to vehicle alone. The results are expressed as the ratio of experimental to control FRIR values. B, the effect of arachidonic acid on hPL release. The heights of the *solid* bars represent the ratio of hPL release per 15 min by two control columns perfused with media alone, mean ± standard error ($N = 6$ experiments). The heights of the *open bars* represent the ratio of hPL release per 15 min in an experimental column perfused with media containing 50 μM arachidonic acid compared to a parallel column perfused with media containing vehicle alone.

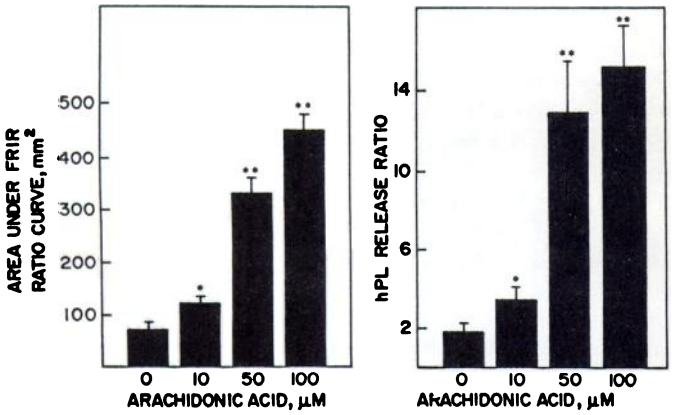

FIG. 2. *The dose-dependent effect of arachidonic acid on FRIR and hPL release from perfused placental cells*

After 40 min of perfusion, cells were exposed for 30 min to the indicated doses of arachidonic acid or to vehicle alone. A, the effect of arachidonic acid on FRIR. For each experiment, the ratio of experimental to control FRIR values was graphed as shown in Fig. 1A. The *bars* represent the relative areas under these curves during the 30-min exposure to arachidonic acid or vehicle as determined by planimetry, mean ± standard error ($N = 6$ for 0 μM arachidonic acid, $N = 3$ for all other doses). B, the effect of arachidonic acid on hPL release. The *bars* represent the ratio of hPL release per 30 min in the experimental columns compared to control columns perfused with media containing vehicle alone, mean ± standard error ($N = 3$). *, $p < 0.05$; ** $p < 0.01$ as determined by analysis of variance.

released 90% and 471% more [^{3}H]myoinositol and 350% and 1287% more hPL, respectively, than did control cells. Incubation of cells with the cyclooxygenase inhibitor indomethacin (20 μM) had no effect on arachidonic acid stimulation of either phosphoinositide hydrolysis or hPL release (Table 1). In contrast, oleic acid had no significant effect on either hPL release or FRIR, while palmitic acid had only minor effects on hPL and inositol release even at 100 μM.

Arachidonic acid stimulated a marked increase in the accumulation of inositol monophosphate. As shown in Fig. 3, exposure to 50 μM arachidonic acid resulted in a 1.5-fold increase in inositol monophosphate after 2 min and a 2.5-fold increase after 5 min. Inositol monophosphate returned to baseline values after 10 min. In contrast, arachidonic acid had no significant effect on the other inositol fractions.

The effects of arachidonic acid on phosphoinositide hydrolysis were relatively specific for phosphatidylinositol and phosphatidylserine. When cells which had been preincubated with [^{32}P]orthophosphate were exposed to arachidonic acid, there was a marked reduction in the radioactivity associated with these two phospholipids. One minute after exposure to 20 μM arachidonic acid, the radioactivity associated with phosphatidylinositol and phosphatidylserine was decreased by 35% ($p < 0.01$) and 28% ($p < 0.05$), respectively (Fig. 4), and returned to control values within 10 min. In contrast, there were no statistically significant changes in radioactivity associated with phosphatidylethanolamine, phosphatidylcholine, or phosphatidylglycerol. As shown in Fig. 5, the effect of arachidonic acid on the loss of ^{32}P-phosphatidylinositol was dose dependent. Arachidonic acid at 20 μM and 100 μM stimulated the loss of radioactivity by 25% and 48%, respectively.

TABLE 1

Effect of palmitic acid, oleic acid, and indomethacin on FRIR and hPL release in perfused placental cells

Cells were prepared and perfused as described in "Materials and Methods." In experiments involving indomethacin and BW755c, cells were perfused with media containing the inhibitors for the entire perifusion period. After 40 min of perfusion, cells were exposed for 30 min to the variable or vehicle alone. For each experiment, the ratios of experimental to control FRIR values were graphed as shown in Fig. 1A. The results are expressed as the relative area under these curves, mean ± standard error ($N = 3$). hPL values represent the ratio of hPL release per 30 min in experimental columns compared to control columns perfused with media containing vehicle alone, mean ± standard error ($N = 3$).

Treatment	Area under FRIR curve	hPL (% change)
Control	63 ± 12	(0.26 ± 0.07 ng/min)
Palmitic acid		
10 μM	57 ± 7	+13.0
100 μM	110 ± 23[a]	+43.7[a]
Oleic acid		
10 μM	41 ± 20	+5.3
100 μM	81 ± 17	+23.7
Indomethacin (20 μM)	76 ± 14	−10.6
Arachidonic acid		
(10 μM)	123 ± 15[b]	+94.4[a]
Indomethacin (20 μM) + arachidonic acid (10 μM)	161 ± 32[a]	+65.6[a]

[a] $p < 0.05$.
[b] $p < 0.01$.

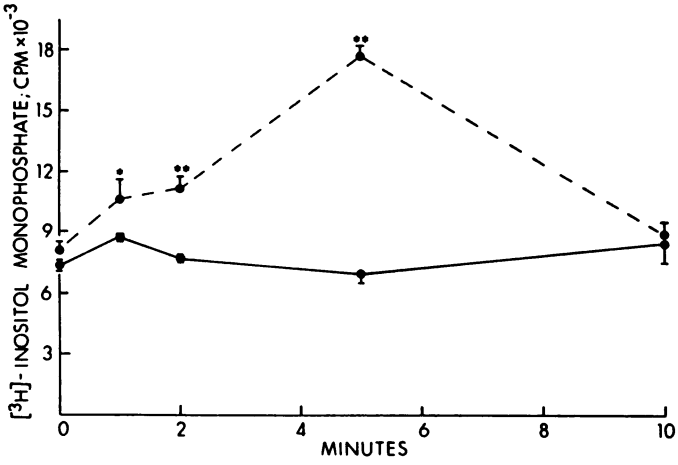

FIG. 3. *The effect of 30 μM arachidonic acid on the accumulation of inositol monophosphate in placental cells*

Cells were prepared and incubated with [^{3}H]myoinositol as described in "Materials and Methods." At time 0, duplicate samples were removed and cells were exposed to 30 μM arachidonic acid or to vehicle alone. At the indicated times, samples were removed and the inositol phosphates were separated on Dowex-1 columns and analyzed by liquid scintillation spectroscopy as described in "Materials and Methods." ●——●, control incubations; ●- - -●, 30 μM arachidonic acid. Results expressed as mean ± standard error ($N = 3$). *, $p < 0.05$; **, $p < 0.01$ as determined by analysis of variance.

DISCUSSION

Several lines of evidence indicate that the efflux of ^{3}H from trophoblast cells demonstrated here reflects the

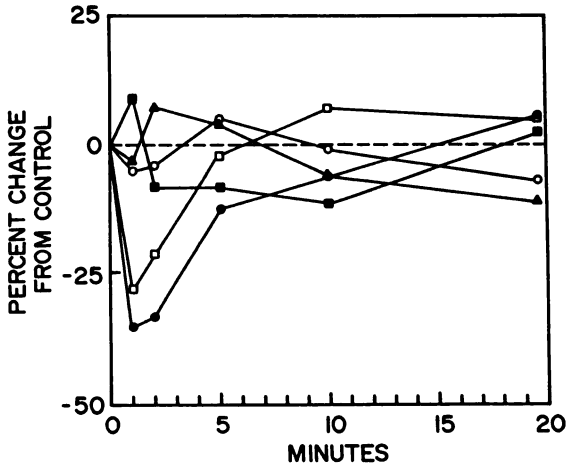

FIG. 4. *The effect of 20 μM arachidonic acid on the loss of ^{32}P radioactivity from placental cells*

Cells were prepared and incubated with ^{32}P as described in "Materials and Methods." At time 0, cells were exposed to 20 μM arachidonic acid or to vehicle alone. At the indicated times, samples were removed and the radioactivity associated with specific phospholipid classes was determined by thin layer chromatography and liquid scintillation spectroscopy. ●—●, phosphatidylinositol; □—□, phosphatidylserine; ■—■, phosphatidylcholine; ▲—▲, phosphatidylethanolamine; ○—○, phosphatidylglycerol. Results are expressed as radioactivity, per cent change from control ($N = 3$). Control values (dpm × 10^{-3} per mg of protein ± standard error, $N = 3$): phosphatidylinositol, 12 ± 2; phosphatidylserine, 24 ± 3; phosphatidylcholine, 1293 ± 136; phosphatidylethanolamine, 742 ± 86; phosphatidylglycerol 59 ± 1. For each *point*, the standard error was less than 15% of the mean.

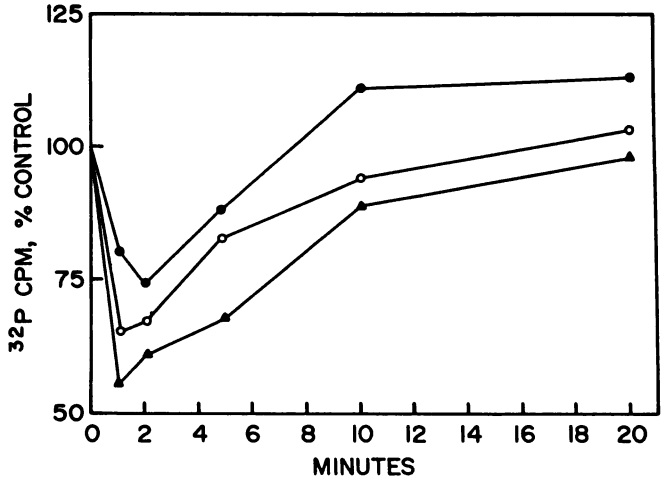

FIG. 5. *The dose-dependent effect of arachidonic acid on the loss of ^{32}P radioactivity from prelabeled placental cells*

Cells were prepared and incubated with ^{32}P as described in "Materials and Methods." At time 0, cells were exposed to arachidonic acid or vehicle. At the indicated times, samples were removed and the radioactivity associated with phosphatidylinositol was determined by thin layer chromatography and liquid scintillation spectroscopy. ●—●, 20 μM arachidonic acid; ○—○, 50 μM arachidonic acid; ▲—▲, 100 μM arachidonic acid. Results are expressed as ^{32}P radioactivity, percentage of control. For each point, the standard error was less than 15% of the mean.

stimulation of phosphoinositide hydrolysis. Essentially all of the radioactivity remaining in the cells at the beginning of the exposure period was incorporated into phosphatidylinositol and polyphosphoinositides. Furthermore, the total ^{3}H efflux in response to arachidonic acid greatly exceeded the unincorporated pool of radioactivity available at the beginning of the exposure period. Thus, the efflux of ^{3}H during the period of exposure to the variable was not due to loss of unincorporated [^{3}H] myoinositol from the cells. The only source that could provide sufficient ^{3}H for the observed increase in efflux during stimulation was ^{3}H-phosphoinositides. Increased release of [^{3}H]myoinositol was also not due to cell lysis since previous experiments (4) have demonstrated that arachidonic acid at concentrations as high as 300 μM have no effect on placental cell integrity. Consequently, the increase in radioactivity released into the media necessarily reflects the increased hydrolysis of labeled phospholipids.

Release of inositol from phosphoinositides may occur by two alternate mechanisms. Phospholipase C cleaves the polar head group of phosphoinositides resulting in diglyceride and inositol phosphates, while phospholipase D cleavage results in phosphatidic acid and free inositol. Since the hydrolysis of phosphoinositides by phospholipase D cannot account for the appearance of inositol phosphates, the demonstration that arachidonic acid results in stimulation of inositol phosphate accumulation in the trophoblast cells indicates that the hydrolysis of phosphoinositides proceeds by the phospholipase C mechanism. The appearance of free inositol in the effluent reflects cleavage of the inositol phosphates by intracellular phosphatases (15). The results of our study are consistent with phospholipase C hydrolysis of either phosphatidylinositol or polyphosphoinositides. However, by analogy with other cell types (16), the immediate substrate may be phosphatidylinositol-4,5-bisphosphate. The inositol triphosphate resulting from hydrolysis is rapidly cleaved by phosphatases to inositol monophosphate. This rapid dephosphorylation may account for the observed lack of accumulation of inositol triphosphate in these experiments.

The stimulation of placental phosphoinositide hydrolysis and phospholipase C activation by arachidonic acid is consistent with previous studies which have demonstrated that arachidonic acid stimulates phospholipase C activity in cell-free preparations isolated from rat brain, lymphocytes, liver, and human fetal membranes (6, 7, 17). The observation that arachidonic acid has a specific effect on the loss of ^{32}P from phosphatidylinositol and, to a lesser extent, phosphatidylserine further supports this suggestion since phosphoinositides are the preferred substrate of phospholipase C (18). The specific effect of arachidonic acid on anionic phospholipids also suggests that the hydrolysis of phosphoinositides does not reflect a generalized degradation of membrane lipids.

The results presented here suggest that arachidonic acid may play a role in the regulation of placental polypeptide hormone release through activation of phosphoinositide hydrolysis. While the physiologic secretagogues for hPL secretion remain unknown, the release of arach-

idonic acid and the formation of metabolites accompanies exposure to secretagogue in other secretory cells. Arachidonic acid released in response to secretagogue may act to promote phosphoinositide hydrolysis. Since arachidonic acid also affects the activity of other cellular enzymes, including guanylate cyclase (19–21), adenylate cyclase (22), and protein kinase C (23), released arachidonic acid could also act to stimulate the coordinated activity of a variety of processes associated with hormone release in the placenta. Further investigation of the potential second messenger role for arachidonic acid in the placenta will require elucidation of the physiologic stimulus for hPL secretion.

The hydrolysis of phosphoinositides has been associated with secretion in a wide variety of cells (8, 9). Although the mechanism by which phosphoinositide hydrolysis evokes secretion is uncertain, a number of suggestions have been advanced. Inositol trisphosphate and phosphatidic acid have been shown to mobilize calcium from internal and extracellular stores (16, 24). Since the concentration of intracellular free calcium appears to be fundamental in the regulation of secretion, phosphoinositide hydrolysis may promote secretion through the generation of one or a variety of intracellular messengers affecting calcium mobilization. In contrast, diglyceride has been reported to be a potent stimulator of the enzyme protein kinase C (25). Protein phosphorylation by this enzyme may be an additional aspect of secretagogue action in endocrine systems.

As noted in the "Introduction," the mechanism of hormone secretion in the trophoblast appears to differ from that found in other secretory cells. While the calcium dependence of secretagogue-induced hPL release is unknown, it has been demonstrated that the removal of extracellular calcium results in an increase in hPL release *in vitro* (2). In contrast, the removal of extracellular calcium in most other secretory systems results in a decrease in release. In addition, hPL, unlike most polypeptide hormones, does not appear to be stored in secretory granules (3). The findings presented here, indicating that arachidonic acid stimulates phosphoinositide hydrolysis and hormone secretion in the placenta despite the lack of clear calcium dependence for secretion, suggests that the formation of diglyceride and the activation of protein kinase C may be an important regulator of hormone secretion in this system. The relevance of these findings for other secretory systems is not clear, although arachidonic acid does stimulate release of prolactin (26), luteinizing hormone (27), somatostatin (28), and histamine (29).

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Send reprint requests to: Dr. Stuart Handwerger, Box 3080, Duke University Medical Center, Durham, NC 27705.