

# Quantification of phosphatidic acid and lysophosphatidic acid by HPLC with evaporative light-scattering detection

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**Abstract** Phosphatidic acid (PA) and lysophosphatidic acid (LPA) are lipids that regulate cellular processes. PA stimulates kinases and may play a role in exocytosis and membrane fusion. LPA can induce cell proliferation, platelet aggregation, and microfilament formation. Due to the growing interest in these lipids, rapid purification and quantification of these lipids is desirable. We now describe a method that utilizes one HPLC run to separate trace amounts of PA and LPA from large amounts of lipids found in cellular extracts. A two-pump HPLC with a solvent system consisting of chloroform, methanol, water, and ammonium hydroxide was employed to produce a reliable, efficient purification of the two lipids. Lipid mass was quantified by a sensitive evaporative light-scattering detector. **Using this new method, insulin addition increased both PA (87%) and LPA (217%) mass in *Xenopus laevis* oocytes.**—Holland, W. L., E. C. Stauter, and B. J. Stith. **Quantification of phosphatidic acid and lysophosphatidic acid by HPLC with evaporative light-scattering detection.** *J. Lipid Res.* 2003. 44: 854–858.

**Supplementary key words** lipid • *Xenopus laevis* • oocyte • method • lipid extraction • lipid signaling • acidic phospholipids • phospholipase D

Phosphatidic acid (PA) is believed to play a critical role in exocytosis, intracellular vesicle formation, membrane fusion (1–3), and insulin action (4). New reports suggest that PA also acts as a regulator in plant cells (5).

Lysophosphatidic acid (LPA) has been identified as an important regulator of platelets and may be used clinically to fight cancer (6–9). LPA binds to G-protein-coupled receptors to stimulate phospholipase C- $\beta$ , induce cell proliferation, and activate platelet aggregation (7).

Thus, there is a desire to rapidly and accurately quantify these bioactive lipids in cellular extracts. However, it has been difficult to separate PA and LPA using HPLC since the trace amounts of these lipids present in cells typically coelute with cellular phospholipids that occur in much greater amounts [e.g., phosphatidylcholine (PC) and sphingomyelin (SM)]. An alternate method, two-dimensional

TLC, has problems associated with the exposure of lipids at the plate surface. Lipid detection on TLC plates, typically with nonspecific stains, is relatively insensitive, and the intensity of the stain is linear only over a small range.

We have modified our HPLC method for separation of major phospholipid groups (10), and with evaporative light-scattering mass detection, we now report an improved method that utilizes one HPLC run to separate PA and LPA from the major phospholipids found in cellular extracts.

For detection of lipids, a spectrophotometer was not used, since lipids show negligible absorbance above about 215 nm. Detection with wavelengths lower than 215 nm prohibits the use of better solvents, such as chloroform (which absorbs in this range). We also did not use radioactive lipid precursors to quantify lipids, since this method is dependent upon labeling of all precursor pools to near-equilibrium; this method rarely produces actual mass values. Instead, we used evaporative light-scattering detection (ELSD) for sensitive quantification of the mass of lipids (10).

## MATERIALS AND METHODS

### Standards and chemicals

Standards (Avanti Polar Lipids; Alabaster, AL) were as follows: PA (1,2-dioleoyl-*sn*-glycero-3-PA); LPA (1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphate); 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine); SM from egg (percentage, fatty acid composition: 78% for 16:0, 7% 18:0, 2% 20:0, 4% 22:0, 4% 24:1, 3% 24:0, 2% 22:6); bovine liver phosphatidylinositol (PI) (2.7% 16:0, 14.5% 18:1, 8.8% 18:2, 9.2% 20:3; 13.4% 20:4); and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine.

To determine recovery efficiency, we utilized radioactive PA and LPA. <sup>14</sup>C-labeled PA (1- $\alpha$ -dipalmitoyl, [glycerol-<sup>14</sup>C(U)]) and tritium-labeled LPA (1-oleoyl[oleoyl-9,10-<sup>3</sup>H(N)]) were purchased from Perkin-Elmer Life Sciences (Boston, MA) (specific activity of 144 mCi/mmol and 57 ci/mmol, respectively). To

Abbreviations: ELSD, evaporative light-scattering detection; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

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Manuscript received 1 November 2002 and in revised form 8 January 2003.

Published, JLR Papers in Press, January 16, 2003.

DOI 10.1194/jlr.D200040-JLR200

make a working solution, we diluted 100  $\mu$ l of labeled lipid solution with 2 ml of chloroform. Some of this labeled lipid solution was quantified by liquid scintillation counting, whereas equivalent samples were mixed with cellular extracts and extracts applied to the HPLC column, and eluent fractions from the HPLC were collected. After evaporating the chloroform and other HPLC solvents, radioactivity was quantified by liquid-scintillation counting.

All solvents (HPLC grade) were from Fisher (Fairlawn, NJ). It is important to obtain solvents with the lowest particulate value (ppm) since the ELSD can detect any molecule less volatile than the solvents.

### Tissue collection

Oocytes were obtained from ovaries of *Xenopus laevis* frogs that were primed with 50 IU of pregnant mare serum gonadotropin (Calbiochem, San Diego, CA) 3–5 days prior to collection. Ovaries were placed in O-R2 [(83 mM NaCl, 0.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM Hepes (pH 7.9)], and oocytes were isolated by manual dissection. Groups of oocytes were placed into 2 ml Dounce homogenizers, and the excess O-R2 was removed before extraction of lipids.

### Extraction of lipids

Nitrogen gas and 2 ml of 1:2 (v/v) chloroform-methanol (–20°C) containing 50 mg/l butylated hydroxytoluene was added to the mortars, and the oocytes were homogenized with 15 strokes. After transferring the extract, the mortar and pestle were rinsed with 1 ml of chloroform and 1 ml of 1 M NaCl (we used NaCl to drive lipids into the organic layer because we found that the use of acidic solutions degraded some lipids).

The extract and washes were combined and centrifuged for 2 min in a clinical centrifuge (800 g). The organic layer was removed and stored at –20°C or –70°C under N<sub>2</sub>.

Just before injection into the HPLC system, samples were dried under N<sub>2</sub> and then reconstituted with 180  $\mu$ l of 2:1 (v/v) chloroform-methanol. Finally, the entire sample was injected into the HPLC.

### HPLC and ELSD equipment

A dual-pump HPXL Varian HPLC (Walnut Creek, CA) was connected to a guard column (#59569; Supelco/Sigma, St. Louis, MO) and a Supelcosil LC-318 diol column (25 cm long, 4.6 ID, 5  $\mu$ m particle size; #58201; Supelco/Sigma) (the column was not heated). A 200  $\mu$ l injection loop and a Rheodine 7725i manual injector were used. Eluate from the column passed through a splitter that sent 52% of the flow to a fraction collector and 48% of the flow to the ELSD.

The Sedex model 55 ELSD (Richard Scientific, Novato, CA) was set to a detector temperature of 40–42°C, N<sub>2</sub> (industrial, 99.9% pure) flow pressure of 1.7–2.2 bar, and a gain of seven. ELSD data were collected, and the peak area was electronically integrated with the Dynamax Method Manager (Rainin-Varian, version 1.4.2). New columns produce very high ELSD response, and washing with about 1 liter of Solvent 1, followed by multiple blank runs helps to reduce the baseline response. In addition, ensuring that the ELSD nebulizer is clean maximizes sensitivity (for cleaning, our nebulizer was sonicated in acetone). Finally, multiple methanol washes (80% to 100% methanol over 5 min, 100% methanol for 15 min or more, then return to 80% methanol over 5 min) may be needed to clean the column between runs so that a blank run produces a flat baseline.

### HPLC gradient

Three solvents at a constant flow rate of 1 ml per min were used to elute the lipids (Table 1). The solvent gradient involved

TABLE 1. HPLC gradient

Time	Solution 1	Solution 2	Solution 3
<i>min</i>		%	
0	100	0	0
5	100	0	0
15	0	100	0
20	0	100	0
30	0	0	100
45	0	0	100
50	0	100	0
55	100	0	0
60	100	0	0

an increase in hydrophilicity: Solvent 1 was 80:19:0.5 chloroform-methanol-30% ammonium hydroxide (v/v/v), Solvent 2 was 60:39:0.5 chloroform-methanol-30% ammonium hydroxide (v/v/v), and Solvent 3 was 60:34.5:5:0.5 chloroform-methanol-water-30% ammonium hydroxide (v/v/v/v). Concentrated ammonium hydroxide (EM Science, Cherry Hill, NJ) was kept at –20°C, and new containers were used as often as possible. A manual switch was used to enable the two-pump HPLC system to produce this gradient of three solvents. In some HPLC runs, 1 ml fractions were collected and analyzed by liquid scintillation counting.

### Data analysis

ELSD peaks were electronically integrated with Dynamax Method Manager 1.4 (Varian). However, the default electronic integration of peaks was always checked, and in many runs with small amounts of material, baselines and droplines were manually set. The ELSD peak area for lipid standards was graphed versus the known amount of standard, and the resulting relationship was analyzed by regression analysis with Sigmaplot 7.1.1 for Windows (SPSS, Chicago, IL). Amounts of lipids were compared by two-sided pooled Student's *t*-test using Sigmaplot. Data are noted as average plus or minus the standard error of the mean, with *n* representing the number of experimental values.

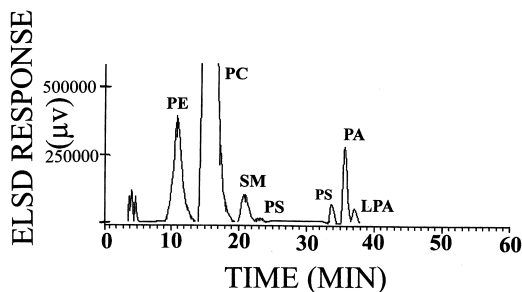
## RESULTS AND DISCUSSION

In a desire to rapidly purify PA and LPA from cellular extracts, we first tested our separation method for major phospholipid classes (10), but found that large amounts of PC and SM coeluted with PA and LPA. Modification of

TABLE 2. Elution time for common phospholipids

Phospholipid Standards	Elution Time
	<i>min</i>
PA (18:1)	36
PA (8:0)	36
LPA	38
PC	14
Phosphatidylinositol	18
Phosphatidylethanolamine	12
Phosphatidylserine	23, 34 dual peaks
SM	21

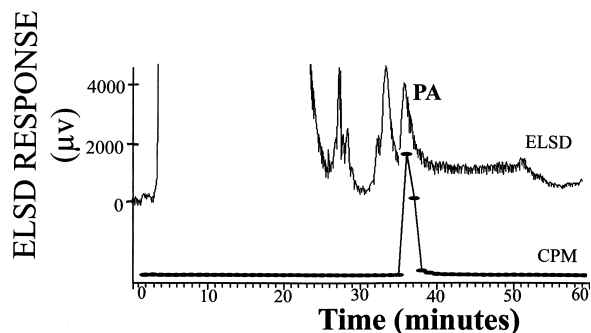
LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; SM, sphingomyelin.



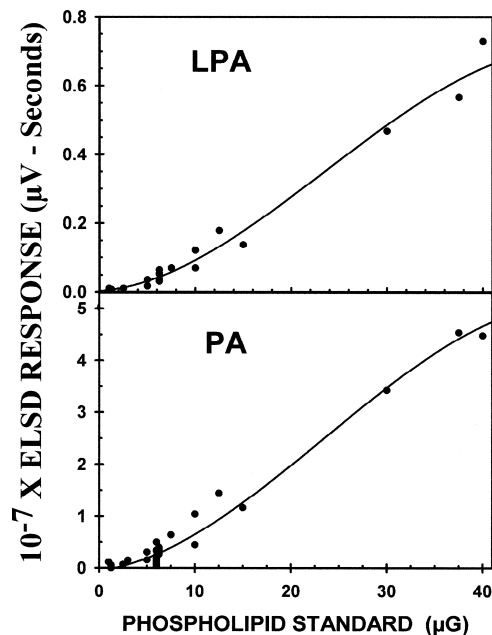
**Fig. 1.** A typical HPLC separation from a mixture of phospholipids standards. Standards were phosphatidic acid (PA), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, phosphatidylcholine (PC), sphingomyelin, and lysophosphatidic acid (LPA) (12.5 µg each). The ordinate presents the evaporative light-scattering detection (ELSD) response (µV), whereas the abscissa is the time of elution from the HPLC column.

the rate of introduction of Solvent 3 (when PA and LPA elute) did not result in enhanced separation from the larger PC or SM peaks. Various modifications of the gradient for the Becart method (11) did not result in isolation of PA from large quantities of cellular lipid. Another method, that of Silversand and Haux (12), did not reliably separate PA from cellular lipids. However, we obtained successful isolation of PA and LPA when we used our earlier method (10) with solvents similar to those used by Becart (wherein the ammonium hydroxide is reduced by half from 1 part to 0.5 parts; Table 1). We also switched from a silica column to a diol HPLC column. Otherwise, the shape and timing of the solvent gradient were the same as that noted in our earlier method (10).

Using this new method, PA eluted at 36 min whereas LPA eluted at 38 min (Table 2). Although this new method is not recommended for other phospholipids, we note that the major classes of phospholipids all elute before PA and LPA (Fig. 1).



**Fig. 2.** Elution of PA standards in a cellular extract. To verify the elution time of PA in the presence of large amounts of lipids,  $^{14}\text{C}$ -labeled PA (18:1) and standard PA (3 µg) were added to an extract of 200 eggs. The ELSD signal (recording mass levels) is the upper irregular line, whereas the radioactivity in each fraction is graphed at the bottom of the figure (oblong circles). In the presence of cellular extract, the unlabeled PA standard (eluted at 36 min) and labeled PA standard (see lower line for radioactivity) eluted at the same time as in the absence of cellular extract.



**Fig. 3.** Analysis of PA and LPA standard lines. Using a wide range of standards, the best fit was obtained with a polynomial third-order cubic regression equation. Apparently due to differences in size between PA and LPA droplets formed by the atomizer of the detector, the ELSD was much more sensitive to PA than to LPA.

As large amounts of lipids could alter the elution time of standards, we wanted to determine whether the elution time of PA and LPA standards changed in the presence of cellular extracts. In the presence or absence of an extract of cellular lipids, the elution time of nonradioactive PA (1,2-dioleoyl-*sn*-glycero-3-PA) or LPA (1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphate) standards did not change (Fig. 2). In addition, the elution time of  $^{14}\text{C}$ PA (16:0) (Fig. 2) or  $^3\text{H}$ LPA (18:1) (data not shown) did not change in the presence of cellular extracts.

Thus, the time of elution was used to identify the PA and LPA peaks in cellular extracts. To confirm the identity of the PA peak in the cellular extracts, the putative PA peak obtained with our new method was rerun with the procedure outlined by Silversand and Haux (12). The putative PA peak eluted at the same time as diolyl PA standard and radioactive PA on the Silversand and Haux gradient.

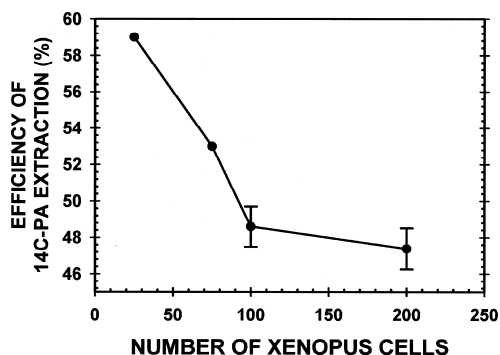
#### Standard lines for PA and LPA quantification

The standard lines for PA and LPA (Fig. 3) were examined by various regression models. The highest regression

TABLE 3. Regression equation constants for PA and LPA standard lines

	$y_0$	a	b	c
LPA	26830	19690	8159	-114.9
PA	-515295	231702	54606	-774

$$\text{Equation: } Y = y_0 + aX + bX^2 + cX^3.$$



**Fig. 4.** The total recovery rate as a function of the number of cells extracted. As the number of cells increased, the total recovery rate (obtained after initial extraction, reconstitution, and HPLC analysis) decreased. Each *Xenopus laevis* cell is about 1 mg of protein.

coefficient ( $r = 0.983$  for PA,  $0.986$  for LPA) was obtained with a polynomial third-order cubic regression equation:

$$Y = y_0 + aX + bX^2 + cX^3 \quad (\text{Eq. 1})$$

This regression equation was also the most accurate for quantification of the major phospholipid classes (10). The constants for the regression lines for LPA and PA are noted in **Table 3**.

A power relationship provides a more accurate regression line for PA standards below about  $7 \mu\text{g}$  (or an ELSD peak area below  $\sim 5,000,000 \mu\text{V}/\text{sec}$ ). With an  $r^2$  value of  $0.91$ , this regression line was used for samples with small amounts of PA:

$$\text{PA } (\mu\text{g}) = (0.001579) (\text{ELSD peak area})^{0.5497} \quad (\text{Eq. 2})$$

For samples with low amounts of LPA (below about  $8 \mu\text{g}$  or  $800,000 \mu\text{V}/\text{sec}$ ), this line was appropriate:

$$\text{LPA } (\mu\text{g}) = (0.002129) (\text{ELSD peak area})^{0.6097} \quad (\text{Eq. 3})$$

#### Efficiency of extraction or recovery rate

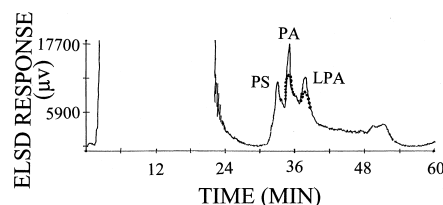
To determine whether PA was lost after injection into the HPLC, a known amount of  $[^{14}\text{C}]\text{PA}$  ( $7014 \pm 348 \text{ SE}$ ,  $n = 4$ ) was injected and run on the HPLC. All cpm ( $102.5\%$  or  $7195\% \pm 588 \text{ SE}$ ,  $n = 3$ ) were recovered in the eluate, so a significant amount of PA did not bind to the HPLC tubing or column.

To concentrate lipid extracts, samples were dried under  $\text{N}_2$  and reconstituted with 2:1 chloroform-methanol (v/v). To determine if we lost PA during this procedure, labeled PA ( $7014 \pm 348 \text{ SE}$ ,  $n = 4$ ) was dried, reconstituted, and injected into the HPLC. The total cpm that eluted from

TABLE 4. Lipids levels in *Xenopus laevis* oocytes

	PA		LPA	
	ng	pmol	ng	pmol
Control	$33.0 \pm 1.2$ (n = 18)	$45.8 \pm 1.7$	$120.3 \pm 8.2$ (n = 11)	$262.7 \pm 17.9$
Insulin-treated	$61.8 \pm 3.0$ (n = 10)	$85.8 \pm 4.2$	$261.0 \pm 21$ (n = 4)	$569.9 \pm 45.9$

Average  $\pm$  SE.



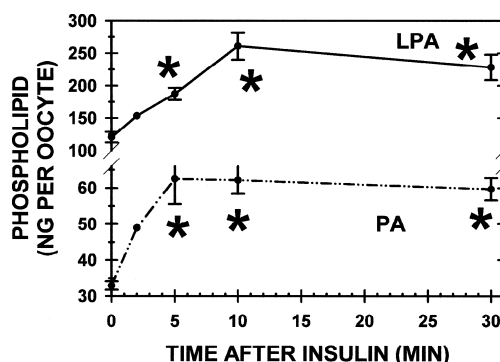
**Fig. 5.** Insulin increased PA levels in *X. laevis* oocytes. Two hundred manually-dissected, stage-VI *X. laevis* oocytes were extracted, and lipids separated and quantified. The control levels of PA and LPA are represented by dotted lines, whereas peaks after hormone treatment ( $1 \mu\text{M}$  insulin for 5 min) are noted by a solid line. After insulin treatment, the mass of phosphatidylserine did not change, whereas the mass of PA and LPA increased.

the HPLC was not decreased by this procedure ( $6740 \pm 904 \text{ SD}$ ;  $n = 3$ ).

To optimize the efficiency of PA extraction from cells, we compared the use of 2:1 versus 1:2 chloroform-methanol (v/v).  $[^{14}\text{C}]\text{PA}$  was added to cellular extracts just after homogenization of the cells with chloroform-methanol, but before the solution was broken into organic and aqueous phases. The use of 1:2 chloroform-methanol (v/v) extracted  $62.1\% \pm 2.4\%$  ( $n = 9$ ) of the labeled PA, but 2:1 chloroform-methanol (v/v) extracted only  $51.12\% \pm 2.7\%$  ( $n = 4$ ). Thus, the use of the extraction solution with the 1:2 ratio was superior ( $P < 0.02$ ).

When extracting over 100 cells (i.e., over 100 mg of protein), some labeled PA was lost in the protein layer ( $11.75\% \pm 0.9\%$ ,  $n = 12$ ), whereas a much smaller amount was lost to the aqueous layer ( $0.5\% \pm 0.1\%$ ,  $n = 10$ ; for 25 to 200 cells).

However, the most important efficiency number is the total recovery rate after all steps in the analysis (extraction from cells, storage, reconstitution, and separation by the HPLC). To determine this total recovery rate, 25 to 200 cells were homogenized in 1:2 chloroform-methanol (v/v), and the labeled lipids were added. For PA in the presence of 100 to 200 *X. Laevis* cells (total protein of about 100 to



**Fig. 6.** Quantification of the increase in the mass of PA and LPA in response to insulin. Insulin ( $1 \mu\text{M}$ ) was added to 200 oocytes, and cellular lipids were extracted, separated, and quantified. To obtain the mass values, appropriate standard lines and correction by the total recovery rate were used. Asterisks denote significance at  $P < 0.0005$  ( $n = 4$ ).

200 mg), we determined a total recovery rate of  $48.0\% \pm 1.0\%$  ( $n = 16$ ). All reported amounts of PA were corrected using this total recovery rate. Perhaps due to the trapping of lipids, extraction efficiency for PA decreased as the amount of cellular extract increased (Fig. 4).

Thus, we can account for 100% of the labeled PA added to 100–200 cells; the major loss occurs at the initial extraction step (loss of  $\sim 38\%$  from the chloroform layer), but there is some trapping of the label in the protein layer ( $\sim 12\%$ ). Only a small amount is in the aqueous layer (0.5%), and when all sums are added, there is a total loss of about 51% (equivalent to the total recovery rate of 48%).

To increase the initial extraction efficiency of PA, an additional 1 ml of chloroform can be added to the aqueous and protein layer left in the test tube after removal of the chloroform layer. A second chloroform extraction increased the initial recovery rate to  $64\% \pm 1.5\%$  ( $n = 5$ ), a third to  $71.5\% \pm 1.0\%$  ( $n = 5$ ), and a fourth extraction to  $74.8\% \pm 0.8\%$  ( $n = 5$ ).

We also examined the efficiency of the initial extraction of [ $^3\text{H}$ ]LPA from 150 oocytes: the use of 1:2 chloroform-methanol (v/v) extracted  $53.4\% \pm 1.0\%$  ( $n = 5$ ) of the labeled LPA. A second extraction of the aqueous and protein layers with chloroform increased the efficiency to 76%, a third to 88%, and a fourth to 89% ( $n = 5$  each). With one chloroform extraction of 150 oocytes, the total recovery rate for [ $^3\text{H}$ ]LPA was  $28.0\% \pm 1.0\%$  ( $n = 5$ ), and this number was used to correct all data.

#### Insulin raises *X. laevis* oocyte PA and LPA levels

With use of a standard line and the total recovery rate, *X. laevis* oocytes were found to contain  $33.0 \pm 1.2$  ng/oocyte of PA ( $n = 18$ ), or, with a molecular weight of 720 for PA, 46 pmol of PA per cell (Table 4). There was  $\sim 3.6$ -fold more LPA than PA; the amount of LPA was  $120.3 \pm 8.2$  ng/oocyte ( $n = 11$ ). Assuming a molecular weight of 458, this amounts to 154 pmol of LPA per cell. These relatively small values can be contrasted with the amount of PC (20.4 nmol/cell with MW 780) or PI (2.7 nmol/cell with MW 909) (10).

Addition of insulin to the *X. laevis* oocyte increased PA and LPA (Figs. 5, 6). As is summarized in Table 4 (for PA, later time points were combined), PA increased by 87% ( $P < 4 \times 10^{-7}$ ), whereas LPA increased by 217% ( $P < 3 \times 10^{-6}$ ) in the presence of insulin.

In addition, PA peaked by 5 min after insulin addition, whereas LPA peaked later (at 10 min). As LPA peaked af-

ter PA, one might suggest that LPA derives from PA; however, the amount of the increase in PA was 7.7 times less than that of LPA (an increase of 40 pmol/oocyte for PA, whereas LPA increased by 307.2 pmol/oocyte).

In summary, we report an improved method of quantification for both lipid messengers PA and LPA in *X. laevis* oocytes. We noted that oocytes contained higher basal levels of LPA than PA, and that insulin increased both of these lipid messengers. ■

This research was supported by a grant from the National Science Foundation (IBN 01106909). The authors thank Jeff Moore and Walt Shaw at Avanti Polar Lipids, Inc. for help with this work and reviewing this manuscript. The authors would also like to thank Ying Chang for help with the efficiency experiments.

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