Separation of phosphatidylinositols and other phospholipids by two-step one-dimensional thin-layer chromatography

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Summary A quick and efficient thin-layer chromatographic procedure is described for the separation of phosphatidylinositol-4,5-bisphosphate, phosphatidylinositol-4-monophosphate, phosphatidylinositol, phosphatidylcholine, phosphatidic acid, and phosphatidylethanolamine. The method involves development of the phospholipids successively in two different solvent systems but in the same direction. The method is simple, reproducible, and gives good resolution of the six different phospholipids tested. About 8–10 ^{32}P-labeled phospholipids isolated from rat hepatocytes were separated by this method; the six mentioned above were identified. Thus, the technique has potential application for both qualitative and quantitative analysis of phospholipid mixtures, such as the phosphoinositides, in cell or tissue extracts. —**Medh, J. D., and P. H. Weigel.** Separation of phosphatidylinositols and other phospholipids by two-step one-dimensional thin-layer chromatography. *J. Lipid Res.* 1989. **30:** 761–764.

Supplementary key words phosphatidic acid • phosphatidylethanolamine • phosphoinositides

Recently a number of reports have implicated an increased rate of phosphatidylinositol lipid metabolism in the receptor-mediated stimulation of cell functions by a variety of hormones, neurotransmitters, and growth factors (for reviews, see 1–3). Most of these investigations have been done by comparing the incorporation of a radiolabel into the three main phosphatidylinositol lipids, namely PIP_2, PIP, and PI. Therefore, in order to make definitive conclusions from such experiments, it is essential to be able to distinguish these three lipids from each other and also from other phospholipids. Different investigators have used different techniques for this purpose. The most popular techniques include chromatography on formaldehyde-treated paper (4) and one-dimensional TLC (5,6) or two-dimensional TLC (7) on silica gel-coated plates. However, these methods have some inherent disadvantages. The formaldehyde-impregnated paper chromatography procedure is very time-consuming. The one-dimensional TLC procedures are not capable of separating all the phospholipids of interest. For example, a solvent system selected to separate the different phosphatidylinositol lipids fails to efficiently resolve PC, PI, PA, and PE (5). If, on the other hand, the solvent is selected to separate these latter major phospholipids, then the different phosphatidylinositol lipids migrate as a single spot (6). For the two-dimensional TLC procedures, a single sample has to be spotted on each plate. As a result, the sample and standards have to be on different plates which then makes comparisons difficult. Multiple samples are also difficult to process since each requires a separate TLC plate. Successive one-dimensional thin-layer chromatographic procedures have been described for the separation of different classes of lipids from one another. However, to our knowledge, such a technique has not yet been successfully applied for the separation of phosphoinositides. Here, we describe a procedure that enables one to separate at least six different phospholipids: PIP_2, PIP, PI, PC, PA, and PE with a minimum of effort and time and with very good resolution and reproducibility.

Abbreviations: PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP, phosphatidylinositol-4-monophosphate; PI, phosphatidylinositol; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; TLC, thin-layer chromatography

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METHODS

Precoated, aluminum-backed thin-layer chromatography plates (20 × 20 cm, No. 5554, Kieselgel 60 F_{254}, 0.2 mm thickness) manufactured by Merck Chemical Company were obtained from EM Science, NJ. [^{8}Arg]-vasopressin (cat. # V-9879) and standard phospholipids, extracted from animal sources (cat. #s: PIP_2, P-9763; PIP, P-9638; PI, P-2517; PC, P-6013; PA, P-9511; PE, P-6386; PS, P-5660) were obtained from Sigma Chemical Company. PIP_2 and PIP were dispersed in methanol–water 1:1 by sonication to a final concentration of 5 μg/μl and PA was dissolved in $CHCl_3$–CH_3OH 1:1 at a final concentration of 10 μg/μl. PC, PE, and PI were obtained as chloroform solutions. Carrier-free $H_3{}^{32}PO_4$ with a specific activity of 285 Ci/mg at 100% isotopic enrichment was obtained from ICN radiochemicals. Rat hepatocytes were isolated by the collagenase perfusion procedure of Seglen (8) with several modifications (9). They were suspended in a Krebs-Ringer-HEPES buffer, pH 7.2, at a density of 2 × 10^6 cells/ml and were radiolabeled with 4 μCi of [^{32}P]PO_4^{-3}/10^6 cells for 120 min at 37°C. Total cellular lipids were then isolated (10) by repeated extraction with $CHCl_3$–CH_3OH–2.4 N HCl 1.5:1:1.5.

The TLC plates were soaked for 90 sec in a 0.054 M oxalic acid solution adjusted to pH 7.2 with KOH. The plates were allowed to dry at room temperature and were then activated by heating at 110°C for 15 min. The standard lipids (50 μg of PIP_2 and PIP; 75 μg of PC and PI; 25 μg of PA and PE) or hepatocyte lipid extracts (obtained from 0.5 × 10^6 cells) in chloroform, mixed with the above standard lipids as carrier, were spotted as 8-mm wide bands on different lanes 1.5 cm from the bottom of the plate. The development was by ascending chromatography successively in two different solvent systems. The first acidic solvent system was composed of $CHCl_3$–CH_3OH–CH_3COOH–H_2O 55:43:3:4. The development was stopped when the solvent front reached about 0.5 cm from the top of the plate. The silica gel was usually scored at this line to prevent the solvent from over-running. The plates were allowed to air dry before the second development in a basic solvent composed of $CHCl_3$–CH_3OH–NH_4OH–H_2O 40:70:10:20. The development was stopped when the solvent front reached 25% the distance of the first solvent front; which was marked on the plate after the first chromatography. The plates were allowed to dry and the lipids were visualized either by exposure of the plates to iodine vapors or by spraying the plates with 10% H_2SO_4 in 50% ethanol and subsequently charring on a hotplate. Identical results were obtained by both these methods. The radiolabeled lipids were visualized by autoradiography as well as with iodine. Kodak diagnostic film (X-OMAT-AR, Cat. No. 165-1454) was used for autoradiography at 25°C for 12–16 h.

RESULTS AND DISCUSSION

A procedure for the successive one-dimensional chromatography of phospholipid mixtures has not yet been applied to the separation of phosphatidylinositol lipids. Using the present procedure, it was possible to separate the six different phospholipids tested by this method of sequential development with two different solvent systems. The rationale was to first separate the less acidic phospholipids in an acidic solvent system and then separate the more acidic lipids in the second alkaline solvent system. The total chromatography time was approximately 3.5 h. A representative chromatogram is shown in **Fig. 1**. The R_f values of each of these lipids with respect to the first and second solvent fronts are given in **Table 1**. The lipids migrated in this system in the order PE > PA > PI > PC > PIP > PIP_2. A seventh phospholipid, phosphatidylserine, was found to migrate slower than PI and faster than PC (**Fig. 2A**). The R_f value of 80 μg of PS was 0.39 ± 0.02 (n = 2). PS was not included in the standard mixture analyzed by this technique or used as a carrier in subsequent experiments.

The R_f values of all the standard phospholipids were found to decrease only slightly as the amount of the lipid

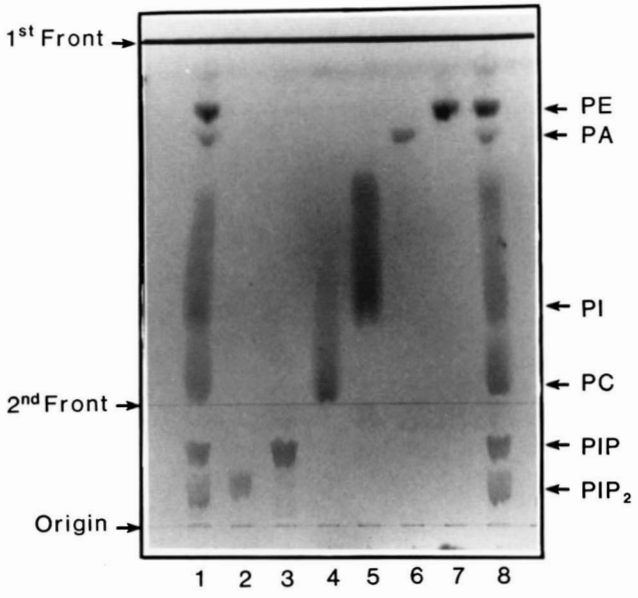

Fig. 1. Phospholipid separation by successive one-dimensional thin-layer chromatography. Standard phospholipids were spotted and chromatograms were developed in first an acidic and then a basic solvent system as described in Methods. Lanes 2 through 7 are, respectively, PIP_2, PIP, PC, PI, PA, and PE. Lanes 1 and 8 are a mixture of these six phospholipids. The fast-migrating spot in the PI standard is an unknown contaminant present in some of the commercial preparations obtained. The phospholipids were visualized by charring after spraying with 10% H_2SO_4 in 50% ethanol. An identical pattern was obtained by exposure to iodine vapors.

TABLE 1. R_f values of phospholipids

Phospholipid	R_f Value Based on Solvent Front	
	First-Solvent System (Acidic)	Second Solvent System (Basic)
PIP_2 (n = 17)	0.09 ± 0.02	0.36 ± 0.07
PIP (n = 18)	0.15 ± 0.01	0.61 ± 0.05
PC (n = 16)	0.30 ± 0.02	
PI (n = 14)	0.48 ± 0.03	
PA (n = 16)	0.79 ± 0.02	
PE (n = 16)	0.85 ± 0.01	

The phospholipids were separated using both solvent systems in succession as described in Methods. Fifty μg of PIP_2 and PIP, 75 μg of PC and PI, and 25 μg of PA and PE were chromatographed. R_f values were calculated with respect to the first solvent front for all the phospholipids and also with respect to the second solvent front for PIP_2 and PIP. The center of each spot was used to calculate the R_f value; n, the number of experiments.

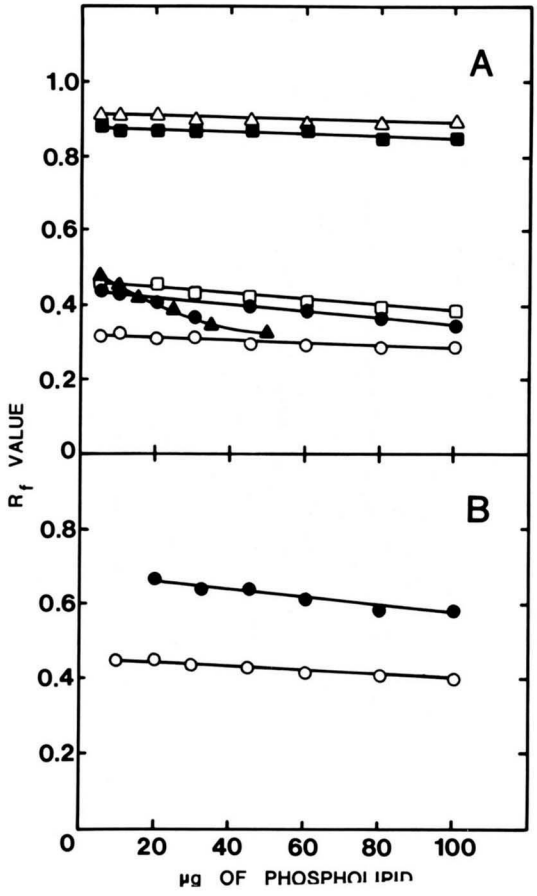

Fig. 2. Effect of the amount of phospholipid chromatogaphed on the R_f value. Different amounts of authentic phospholipids were spotted and separated as described in Methods. A: The R_f values for PC (○), PS (●), PI (□), PA (■), PE (△), and ^{32}P-labeled PC (▲) were calculated with respect to the first (acidic) solvent front. For the ^{32}P-labeled PC (▲) the abscissa represents the volume of the labeled hepatocyte lipid extract spotted. B: The R_f values for PIP_2 (○) and PIP (●) were calculated with respect to the second solvent front.

chromatographed was increased from 5 μg to 100 μg (Fig. 2). Analysis of the total radiolabeled phospholipids extracted from hepatocytes showed that changes in R_f values of most of the ^{32}P -labeled phospholipids were identical to those observed for the standards (not shown). However, the R_f of ^{32}P -labeled PC changed significantly with the amount of extract loaded (Fig. 2A). The reason for this greater sensitivity of the R_f of ^{32}P -labeled PC to the amount of radiolabeled hepatocyte extract spotted is not known. However, this difference could be eliminated by spotting at least 75 to 80 μg of PC. We recommend in using this method that one include the standard phospholipids (PC and others) as carrier lipid during the chromatography. The use of carrier lipid was also desirable because, by themselves, the amounts of individual phospholipids in the cell extract were too low to detect by iodine staining.

As expected, more ^{32}P-labeled lipids were present in the hepatocyte extract than the six standard lipids chosen for this study. The correct identification of the different ^{32}P-labeled lipids was confirmed when a mixture of the six standard phospholipids and the radiolabeled cell extract was co-chromatographed. Autoradiography revealed bands that coincided with the standard phospholipids visualized by exposure to iodine vapors (**Fig. 3**). Thus, of the eight to ten ^{32}P-labeled phospholipids obtained from isolated rat hepatocytes, those corresponding to the six standard phospholipids used here could be identified.

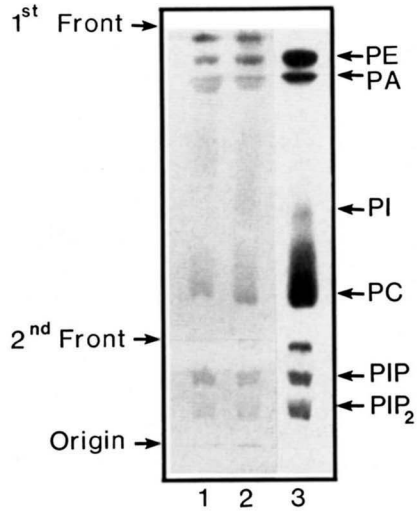

Fig. 3. Chromatography of ^{32}P -radiolabeled phospholipids extracted from rat hepatocytes. Isolated rat hepatocytes were radiolabeled and total lipids were isolated as described in Methods. A mixture of authentic phospholipids was spotted with or without the radiolabeled lipids and developed as described in Methods. Lane 1 is a mixture of the six standard phospholipids (PIP_2, PIP, PC, PI, PA, and PE) as visualized with iodine. Lanes 2 and 3 are the same mixture as in lane 1 along with an aliquot of the radiolabeled lipid extract. Lane 2 was visualized with iodine. Lane 3 was lane 2 visualized by autoradiography.

TABLE 2. Effect of vasopressin on hydrolysis of radiolabeled PIP_2 and PIP in isolated hepatocytes

Phospholipid	Radioactivity (percent of control)	
	Buffer	Vasopressin
PIP_2	110.8 ± 2.3	57.4 ± 0.3
PIP	106.6 ± 10.1	67.0 ± 1.7
PE	97.9 ± 4.5	94.3 ± 3.3

Rat hepatocytes were labeled at 37°C with [^{32}P]phosphate for 120 min. Then at time 0, the cells received Krebs-Ringer-HEPES buffer (pH 7.2) either alone or containing vasopressin (final concentration 10^{-6} M). Phospholipids were extracted, separated, and quantitated as described in Methods at times 0 and 0.5 min. Results for each lipid are expressed as a percentage of the radioactivity in that lipid relative to time zero. Results are the averages of duplicates.

The resolution with the present method is good enough to allow one to scrape the spots, identified by iodine staining or autoradiography., from the silica gel layer and then to quantitate the radioactivity by liquid scintillation spectrometry. An experiment to demonstrate the quantitative utility of the method was performed using the hormone vasopressin. A substantial decrease in the amounts of ^{32}P-labeled PIP_2 and ^{32}P-labeled PIP occurred after stimulation of hepatocytes with 10^{-6}M vasopressin (**Table 2**). Within 30 sec the hormone caused PIP and PIP_2 levels to decrease by 33% and 43%, respectively. No decreases were caused by the addition of buffer alone. As expected, there was no change in ^{32}P-labeled PE, a control phospholipid. These values agree very well with those reported by others and obtained using different techniques (11, 12).

This sequential chromatographic procedure was optimized to give maximum resolution and reproducibility. When the second alkaline solvent front was allowed to migrate further than 25% of the distance of the first solvent, PC and PI were not well resolved. The mobility of the lipids was found to be very sensitive to even slight variations in the solvent composition, for example, as caused by evaporation. The use of a paper wick along one side of the chamber caused a diffused and nonuniform migration in the end lanes giving a convex lipid front. The use of a continuous wick along all sides of the chamber wall, on the other hand, helped to eliminate the curved front. However, in this case, the chamber and TLC plate actually became oversaturated and this caused the R_f values of all the lipids to decrease. Therefore the use of a paper wick was avoided. Instead, each solvent system was added to the chamber at least 2 h in advance of the TLC plate to allow the chamber to saturate. It was essential to soak the plates in oxalic acid in order to prevent streaking of the phospholipids during migration. Oxalic acid has been shown to help in this regard by sequestering the calcium ions present in the silica gel and thereby rendering the plate with a net neutral charge for unhindered mobility of the charged phospholipids (5). Although PC and PI still showed some streaking even after treating the plates with oxalate, both PIP_2 and PIP, the main phospholipids of interest, were resolved as fairly compact bands.

This method should provide a convenient tool for both a qualitative and quantitative analysis of a variety of different phospholipids in cellular or tissue lipid extracts.

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REFERENCES

1. Hokin, L. E. 1985. Receptors and phosphoinositide generated second messengers. *Annu. Rev. Biochem.* **54:** 205–235.
2. Majerus, P. W., T. M. Connolly, H. Deckmyn, T. S. Ross, T. E. Bross, H. Ishii, V. S. Bansal, and D. B. Wilson. 1986. The metabolism of phosphoinositide derived messenger molecules. *Science.* **234:** 1519–1526.
3. Fisher, S. K., L. A. A. Van Rooijen, and B. W. Agranoff. 1984. Renewed interest in the polyphosphoinositides. *Trends Biochem. Sci.* **9:** 53–56.
4. Wagner, H. A., A. Lissau, J. Holzi, and L. Horhammer. 1962. The incorporation of ^{32}P into the inositol phosphatides of rat brain. *J. Lipid Res.* **3:** 177–183.
5. Gonzalez-Sastre, F., and J. Folch-Pi. 1968. Thin-layer chromatography of the phosphoinositides. *J. Lipid Res.* **9:** 532–533.
6. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. Quantitative analysis of phospholipids by thin-layer chromatography. *Biochem. J.* **90:** 374–378.
7. Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two-dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* **5:** 494–496.
8. Seglen, P. O. 1976. Preparation of isolated rat liver cells. *Methods Cell Biol.* **8:** 29–83.
9. Clarke, B. L., J. A. Oka, and P. H. Weigel. 1987. Degradation of asialoglycoproteins mediated by the galactosyl receptor system in isolated hepatocytes. Evidence for two parallel pathways. *J. Biol. Chem* **262:** 17384–17392.
10. Downes, P., and R. H. Michell. 1982. Phosphatidylinositol-4-phosphate and phosphatidylinositol-4, 5-bisphosphate: lipids in search of a function. *Cell Calcium.* **3:** 467–502.
11. Rhodes, D., V. Prpic, J. H. Exton, and P. F. Blackmore. 1983. Stimulation of phosphatidylinositol-4, 5-bisphosphate in hepatocytes by vasopressin. *J. Biol. Chem.* **258:** 2770–2773.
12. Litosch, I., S. Lin, and J. N. Fain. 1983. Rapid changes in hepatocyte phosphoinositides induced by vasopressin. *J. Biol. Chem.* **258:** 13727–13732.