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Research Article

Separation of fluorescent phosphatidyl inositol phosphates by CE

Phosphatidyl inositol 4,5-bisphosphate (PIP_2) and phosphatidyl inositol 3,4,5-trisphosphate (PIP_3) labeled with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid (BODIPY FL) on the acyl chain or a phosphatidyl ethanolamine head group were separated by CE with LIF detection. Several methods and capillary-coating procedures were tested for the separation of these phosphatidyl inositol phosphates (PIPs) at 20°C. Separation of the PIPs in less than 20 min with excellent resolution was achieved using a buffer containing sodium deoxycholate (SDC), 1-propanol, $MgCl_2$ and the polymer coating reagent, EOTrol™ LR. The efficiency of the optimized method was as high as 1.3×10^5 plates. The dependence of the separation on the concentration of 1-propanol, SDC, and $MgCl_2$ was determined. The separation of PIP_2 and PIP_3 was primarily due to differential binding of the lipids to Mg^{2+} rather than to different solubilities in the micellar phase. The role of the SDC was to prevent adsorption of the hydrophobic lipids to the capillary wall and thus enhance the efficiency. The fluorescent PIPs are of value for both *in vitro* and *in vivo* assays of phospholipid metabolism. In particular, the use of these lipids with the optimized capillary-based separation will be of utility for drug screening as well as cell-based assays.

Keywords:
CE / Phosphatidyl inositol phosphates / Phosphatidyl inositol 3,4,5-trisphosphate / Phosphatidyl inositol 4,5-bisphosphate / Phospholipid

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1 Introduction

Phosphatidyl inositides are a minor fraction (typically <10%) of the total membrane lipid content of cells. However, phosphatidyl inositides represent a major class of cell signaling mediators, regulating a broad spectrum of biological functions including cell growth and proliferation, apoptosis, vesicle trafficking, and cytoskeletal changes [1–3]. The disruption of phosphatidyl inositide signaling in cells is a precursor to many disease states, including diabetes, cardiovascular disease, and cancer [4]. As an example the enzyme, phosphotidylinositide 3-kinase (PI 3-K), is a known tumor promoter and converts phosphatidyl inositol 4,5-bisphosphate (PIP_2) to phosphatidyl inositol 3,4,5-trisphosphate (PIP_3). In contrast, the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a known tumor suppressor that converts PIP_3 to PIP_2 [5]. The role of these lipid-modifying enzymes in key cellular signaling pathways makes them therapeutic targets in cancer, cardiovascular diseases, atherosclerosis, inflammation, and other diseases [1–5].

The most common techniques for analyzing phospholipids are TLC [6, 7] and HPLC [8–13]. The drawback of these chromatographic techniques is that they involve time-consuming and labor-intensive solvent extraction and separation. An additional difficulty in the separation of phospholipids is their tendency to aggregate in aqueous media at concentrations above their critical micellar concentrations. Also, phospholipids lack chromophores although UV absorbance detection has been employed at 190–220 nm to detect the unsaturated carbon–carbon bonds on some of the phospholipids. Absorption at these wavelengths is weak and the typical LODs are 50 ppm (~50 μM) [14]. In addition, many solvents, buffer additives, and other analytes absorb in the region of 190–220 nm. These properties of phospholipids present a major challenge for the analysis and quantitation

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Abbreviations: BODIPY FL, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid; **GloPIP$_2$,** PIP_2 attached to BODIPY FL *via* an acyl chain; **GloPIP$_3$,** PIP_3 attached to BODIPY FL *via* an acyl chain; **HyPIP$_2$,** PIP_2 attached to BODIPY FL *via* an accessory phosphatidyl ethanolamine head group; **HyPIP$_3$,** PIP_3 attached to BODIPY FL *via* an accessory phosphatidyl ethanolamine head group; **PEO,** poly(ethylene oxide); **PI 3-K,** phosphotidylinositide 3-kinase; **PIP,** phosphatidyl inositol phosphates; **PIP$_2$,** phosphatidyl inositol 4,5-bisphosphate; **PIP$_3$,** phosphatidyl inositol 3,4,5-trisphosphate; **SDC,** sodium deoxycholate

of phospholipids. In order to improve the sensitivity and selectivity, Haddadian *et al*. [14] developed an MEKC method with an indirect photometric detection which afforded LODs in the range of 2–6 ppm (~2–6 μM) or 0.03–0.1 pmol. Recently, CZE with indirect UV detection was used for the separation of phospholipids with an LOD in 2–5 μg/mL or 2–5 ppm (~2–5 μM) range [15]. This method was used to profile human blood serum and might be useful for the determination of phospholipids in large biological samples. Methods to label aminophospholipids with fluorophores have also been developed for HPLC, with detection limits of 2–20 pmol [16, 17]. However, separation and detection of phospholipids remains difficult. In particular, technologies to measure phospholipids at the level of a single cell will need to achieve detection limits of attomoles to zeptomoles since the concentrations of many of the signaling lipids is nanomoles to micromoles in a 1 pL-cell volume [5].

CE, is a promising technique for the separation of phospholipids due to its high resolution, excellent separation efficiency, minimal sample volume, and short analysis time. Despite its assets, there are few reports that have used CE for the analysis of phospholipids [14, 15, 18–25]. Most phospholipids are quite hydrophobic making them difficult to separate by CZE. In addition, many of the phospholipids possess only small differences in their charge-to-size ratio. NACE has been used for the analysis of phospholipids [24, 25]. One advantage of the NACE methods over other CE-based methods is that the NACE methods can be performed in a buffer appropriate for full dissolution of the lipids. However, many biological samples are dissolved in aqueous solutions that may not be compatible with the organic solvents used in NACE [25].

Most separations of phospholipids by CE employed MEKC [18–22]. With MEKC, highly hydrophobic compounds such as phospholipids are often difficult to separate due to their high solubility in the micellar phase [26, 27]. Short-size *n*-alkyl alcohols, *e.g.*, methanol, ethanol, and 1-propanol, are frequently used in MEKC as a BGE modifier or additive. These modifiers increase the solubility of the more hydrophobic compounds in the aqueous phase permitting the hydrophobic molecules to spend more time in the aqueous phase and decreasing retention times [18, 28]. 1-Propanol is the most frequently used organic modifier for phospholipid separations by MEKC [18–23, 29]. The most common surfactants for the separation of phospholipids are the bile salts, sodium cholate, and SDC. These bile salts are lower in hydrophobicity than detergents such as SDS [18, 30]. Consequently, hydrophobic analytes have lower micellar solubilities in the bile salts relative to those in other detergent micelles. The bile salts are also thought to minimize phospholipid aggregation and enhance phospholipid solubility. A major drawback for most of the MEKC methods developed for the separation of phospholipids is that the separations are carried out in 40–55°C temperature range [18–23]. For many CE applications, it is difficult to maintain this elevated temperature throughout the capillary. An example is the lysis and loading of a cell into a capillary followed by separation of the cellular contents [31]. Thus, the development of a CE method for phospholipid separation at ambient temperature would be of high utility.

Quite recently, Lin *et al*. [29] separated phosphatidylinositol, phosphatidylinositol 3-phosphate, and phosphatidylinositol 3,4-bisphosphate on a microfluidic chip at room temperature. These lipids were linked to 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid (BODIPY FL) *via* their acyl chain. The separation buffer contained 20 mM SDC, 35% 1-propanol, 0.1% coating-3-reagent, 100 mM Tris (pH 8.5), 1 mM EDTA, 1 mM EPES (pH 7.0), 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.4% glycerol. In addition, Lin and co-workers exploited the separation method to develop an assay to monitor enzyme activities of lipid-modifying enzymes. A disadvantage of this method is that it used a complex buffer system. In addition, the biologically important PI 3-K substrate, PIP_2, and the lipid second messenger, PIP_3, were not included in the separation. While microfluidics has many assets, it is not a useful format for many applications, particularly, the analysis of the contents of cells attached to a solid surface. Also, in our hands, this method yielded low quality and irreproducible separation of phospholipids in a capillary-based format.

In this work, several methods were tested for the CE-based separation of PIP_2 and PIP_3 labeled with BODIPY FL on either the acyl chain or a phosphatidyl ethanolamine head group. The names and structures of these phosphatidyl inositol phosphates (PIPs) are shown in Fig. 1. To develop the separation method, the concentration of each of the buffer constituents was optimized. All the separations were performed at 20°C. In addition, the roles of the buffer components were evaluated as well as the influence of the sample matrix. The reproducibility and efficiency of the optimized method was measured. The fluorescent PIPs used as analytes are of value in both *in vitro* and *in vivo* assays of phospholipid metabolism. In particular, the use of these lipids with the optimized capillary-based separation method will be of utility in cell-based assays and may enable measurements of lipid metabolism at the single-cell level.

2 Materials and methods

2.1 Reagents

Poly(ethylene oxide) (PEO), poly(vinyl alcohol), 1-propanol, $MgCl_2$, SDC, Polybrene, Triton X-100, and 1,2-dipalmitoyl PIP_2 (dipalmitoyl PIP_2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the fluorescent PIPs were purchased from Echelon Biosciences (Salt Lake City, UT, USA). Methanol, Tris, and sodium borate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). ACN and chloroform were obtained from EMD Chemicals (Gibbstown, NJ, USA). EOTrol™ LR (Low Reverse), EOTrol HR (High Reverse), and EOTrol LN (Low Normal) were purchased from Target Dis-

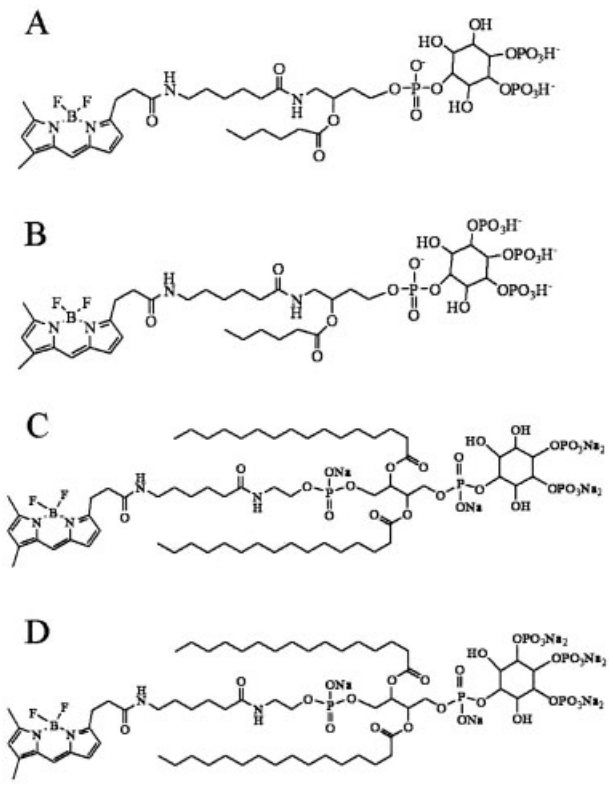

Figure 1. Chemical structures of the phospholipids separated: (A) $GloPIP_2$, (B) $GloPIP_3$, (C) $HyPIP_2$, and (D) $HyPIP_3$.

covery (Palo Alto, CA, USA) while coating-3-reagent was purchased from Caliper Life Sciences (Hopkinton, MA, USA). All the reagents were of analytical reagent grade and used as received.

2.2 Instrumentation

Experiments were performed using a custom-built CE instrument, equipped with a high-voltage power supply (CZE1000R, Spellman, Plainview, NY, USA). Fused-silica capillaries (30 μm and 50 μm id, 360 μm od, Polymicro Technologies, Phoenix, AZ, USA) were used for the lipid separations. For the optimized CE method, the capillary length was 18 cm from the inlet to the optical window with a total length of 41 cm. The inlet reservoir was held at ground potential, while the outlet reservoir was held at a positive voltage of 20 kV unless stated otherwise. Approximately 2 nL of the samples were loaded by hydrodynamic injection. The volume injected was estimated as described previously [32]. All the CE runs were performed at 20°C.

2.3 Fluorescence detection

Unless otherwise stated, an optical window in the polyimide coating of the capillary was created 18 cm from the inlet. The capillary lumen was interrogated by the focused laser beam of a diode-pumped solid-state laser (473 nm; Lasermate, Pomona, CA, USA) coupled to a single-mode optical fiber (Oz Optics, Ottawa, Canada). The capillary was illuminated with 2 mW of light using a lens (focal length of 3 cm) placed at the end of the fiber optic. Fluorescence was collected at a right angle to the capillary and laser beam using a microscope objective (40 ×, 0.75 n.a., Plan Fluor, Nikon, Melville, NY, USA). The light was detected with a photomultiplier tube (PMT, R3896; Hamamatsu, Bridgewater, NJ, USA) after spectral filtering with a band-pass filter (535DF50; Chroma Technology, Rockingham, VT, USA). The PMT current was amplified and converted to a voltage with a preamplifier (PMT-5; Advanced Research Instruments, Golden, CO). A data acquisition board (KPCI-3100; Keithley Metrabyte, Cleveland, OH, USA) in a personal computer (Dell, Round Rock, TX, USA) was used to digitize the signal using custom software (Testpoint, Keithley Metrabyte). The data were plotted and peak areas were calculated using Origin (Microcal, Northhampton, MA, USA).

2.4 Coating reagents and procedures

Prior to use, each new capillary was conditioned with 1 M NaOH for 30 min and rinsed with distilled deionized H_2O for 30 min using a pressurized washing system at 20 psi. Prior to each run, the capillary was flushed with the separation buffer for 3 min. Several coating reagents and procedures were investigated in order to suppress the EOF and minimize adsorption of the phospholipids on the capillary wall. These coating reagents included PEO, Polybrene, dipalmitoyl PIP_2, coating-3-reagent, EOTrol LR, EOTrol HR, EOTrol LN, and poly(vinyl alcohol). Capillaries were coated with PEO by the method of Iki and Yeung [33]. Bare silica capillaries were coated with dipalmitoyl PIP_2 (dissolved in 3:2:1 chloroform/methanol/water) by flushing a solution of dipalmitoyl PIP_2 at 20 psi for 15 min and rinsing with water then buffer each for 5 min. The coating-3-reagent, and EOTrols were independently dynamically coated on the capillary by adding 0.1 and 5% to the run buffer, respectively. Polybrene was placed on the capillary wall based on the procedure of Katayama *et al.* [34] but with modifications. After coating the capillary with the Polybrene layer, dipalmitoyl PIP_2 was flushed through the capillary with a pressurized washing system at 20 psi for 15 min. The capillary was rinsed with water for 5 min and then with the separation buffer for 5 min.

2.5 Preparation of lipid stock solutions

BODIPY FL was attached to PIP_2 or PIP_3 *via* the acyl chain ($GloPIP_2$ or $GloPIP_3$, respectively) or to an accessory phosphatidyl ethanolamine head group ($HyPIP_2$ or $HyPIP_3$, respectively). $GloPIP_2$ and $GloPIP_3$ were dissolved in $CHCl_3$/MeOH (3:1) and dried under argon. The dried phospholipids were dispersed in lipid buffer (0.1% Triton X-100, 5 mM Tris, pH 7.0). Unless otherwise stated, $HyPIP_2$ and $HyPIP_3$ were

dissolved in the lipid buffer. All of the phospholipid samples were diluted to 10–50 nM prior to injection into the capillary. For the separation of the mixtures of $GloPIP_2$ with $GloPIP_3$ or $HyPIP_2$ with $HyPIP_3$, the identity and elution order of the peaks were determined by sequentially adding additional Glo-PIP_3 and $HyPIP_3$ into the solution of the corresponding analyte mixture and repeating the separation.

2.6 Calculations

The number of theoretical plates (N), the resolution (R_s), and the RSD were calculated using equations from [35, 36]. At least nine measurements were made for each experimental condition for which RSD values were calculated.

3 Results and discussion

3.1 Development of a CE-based method for separation of PIPs

The goal of this work was to develop a simple, fast, robust method for the simultaneous separation of $GloPIP_2$, Glo-PIP_3, $HyPIP_2$, and $HyPIP_3$ by CE at 20°C (room temperature). The rationale for the development of a room temperature, separation method was that it could easily be mated to a microscope stage for the laser-based lysis and analysis of single cells [37, 38]. These lipids represent the substrate (PIP_2) and product (PIP_3) of PI 3-K and their separation would enable CE-based assays of the PI 3-K activity. The placement of the fluorophore on PIP_2 can impact the kinetic properties of PI 3-K [5]. For this reason, it is desirable to use both fluorophore-labeled PIP_2 molecules to assess the influence of the fluorophore position on the reaction rate. The initial separation method tested for the separation of $GloPIP_2$, $GloPIP_3$, $HyPIP_2$, and $HyPIP_3$ was modified from the method used for the characterization of lipoprotein by Hu *et al.* [39]. The modified method utilized a bare capillary (30 μm id) with a separation buffer of 25 mM sodium borate (pH 9.3), 20 mM SDC, 20% ACN, and a field strength of 765 V/cm. A negative potential was applied to the capillary outlet. While the method yielded short migration times, only 5 min for $HyPIP_2$ and $HyPIP_3$, the separation of the different lipids was incomplete (Fig. 2). Extensive optimization of the concentration of the buffer constituents failed to improve the separation of the PIPs. For this reason, a second separation method based on that of Lin *et al.* [29] was tested. The separation buffer was composed of 100 mM Tris (pH 8.5), 20 mM SDC, 1 mM $MgCl_2$, 35% 1-propanol. The separation was performed using an uncoated capillary (50 μm id) and a negative potential applied to the outlet of the capillary (field strength of 365 V/cm). Broad and unresolved peaks with pronounced tailing were observed. Furthermore, when the coating-3-reagent of Lin *et al.* was used to suppress the wall interactions, only a slight improvement was noted on the number of theoretical plates. In addition, poor run-to-run reproducibility of the migration times, peak heights, and peak areas was observed (data not shown).

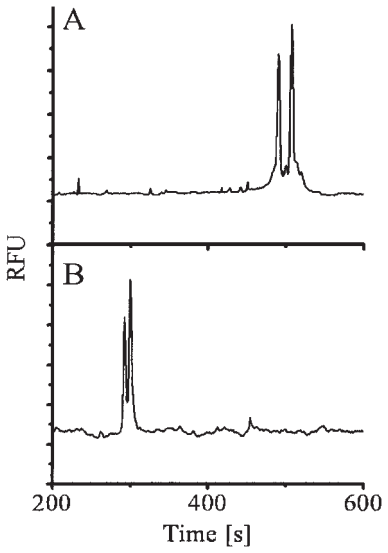

Figure 2. Separation of (A) $GloPIP_2$ and $GloPIP_3$, and (B) $HyPIP_2$ and $HyPIP_3$ at 765 V/cm using 25 mM sodium borate, 20 mM SDC at pH 9.3 and 20% ACN (30 μm id capillary, 30 cm effective length, and 39 cm total length). The *y*-axis is the relative fluorescence units (RFU).

In addition to the coating-3-reagent, many other strategies were tested to suppress wall adsorption. These were PEO, Polybrene, dipalmitoyl PIP_2, EOTrol LR, EOTrol HR, EOTrol LN, and poly(vinyl alcohol). Most of these reagents yielded no or poor separations. EOTrol LR, a dynamic coating based on a substituted polyacrylamide yielded the best results [40–42]. This polymer also reversed and decreased the velocity of the EOF. EOTrol LR was combined with 100 mM Tris (pH 8.5), 20 mM SDC, 1 mM $MgCl_2$, and 35% 1-propanol. When a positive potential was applied to the outlet of the capillary (365 V/cm), these conditions yielded migration times longer (700–1000 s) than that of the borate-based buffer. However, the $GloPIP_2$ was widely separated from the $GloPIP_3$, and the $HyPIP_2$ was well separated from the $HyPIP_3$ (data not shown).

To further decrease the migration time and increase the resolution and efficiency, the concentration of each buffer constituent was independently varied (see details in subsequent sections). The optimal separation buffer at a pH of 8.5 was 100 mM Tris, 5 mM SDC, 1 mM $MgCl_2$, 30% 1-propanol, and 5% EOTrol LR. An electric field strength of 487 V/cm combined with this optimal buffer yielded excellent peak efficiencies without compromising the analyte resolution (Fig. 3, Table 1). This optimized method also permitted the separation of the analytes at reasonable elution times (<900 s). When the four analytes were coinjected and electrophoresed, they were well separated (Fig. 4). Thus, if desired, assays of PI 3-kinase could be performed using both of the fluorescent PIP_2 substrates.

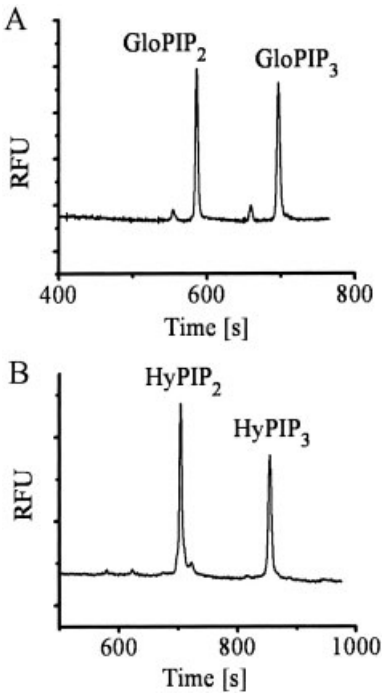

Figure 3. Separation of (A) $GloPIP_2$ and $GloPIP_3$, and (B) $HyPIP_2$ and $HyPIP_3$ at 487 V/cm using 100 mM Tris (pH 8.5), 5 mM SDC, 1 mM $MgCl_2$, 30% 1-propanol, and 5% EOTrol LR.

Table 1. %RSD of the migration times, peak heights, peak areas, and peak efficiency (*N*) for nine sequential runs of BODIPY FL-labeled PIPs separated using the conditions as described in Fig. 4

Analyte	Elution time	Peak height	Peak area	*N*
$GloPIP_2$	1.1	10	9.2	8.3
$GloPIP_3$	1.6	11	8.5	11
$HyPIP_2$	1.4	11	8.0	7.7
$HyPIP_3$	1.5	11	5.8	5.9

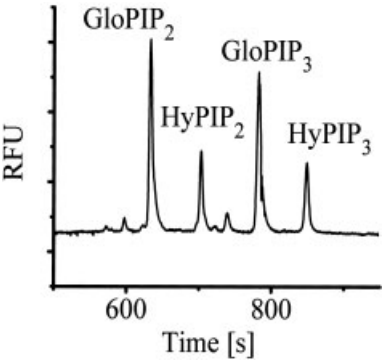

Figure 4. Simultaneous separation of a mixture of $GloPIP_2$, $GloPIP_3$, $HyPIP_2$, and $HyPIP_3$ using the separation conditions described in Fig. 3.

3.2 Optimization of the SDC concentration

The concentration of SDC was varied between 0 and 20 mM in the presence of the other components (100 mM Tris, 1 mM $MgCl_2$, 30% 1-propanol, and 5% EOTrol LR) of the optimal buffer (pH 8.5). The best SDC concentration for the separation of $GloPIP_2$, $GloPIP_3$, $HyPIP_2$, and $HyPIP_3$ was 5 mM (Fig. 3, Table 1). At concentrations of SDC lower than 5 mM, the migration time was not reproducible. Notably, separations were obtained at 0 mM SDC (Fig. 5) and these separations posessed better resolution but poorer efficiency than at 5 mM SDC (Table 2). Since SDC was not required to resolve the PIPs, the separation does not rely on differential partitioning of the analytes into micelles and cannot be described by MEKC. Instead, SDC may block adsorption of the negatively charged PIPs onto the walls of the capillary which are positive in charge due to the dynamic coating.

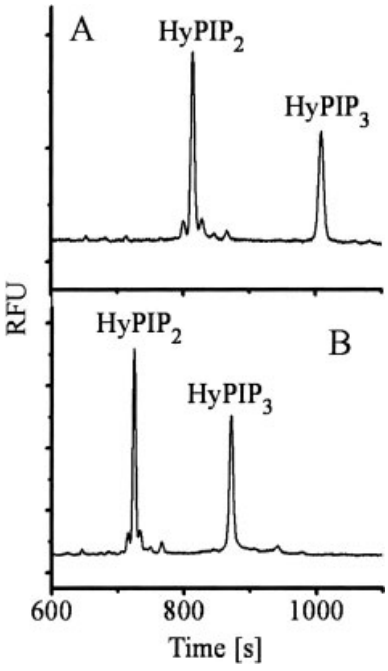

Figure 5. Separation of HyPIP2 and HyPIP3 with 0 mM SDC. Other conditions were as in Fig. 3.

3.3 Optimization of the Mg^{2+} concentration

$MgCl_2$ was found to be an important additive for the phospholipid separations. When $HyPIP_2$ and $HyPIP_3$ were coinjected and then separated in the optimized separation buffer with 0 mM $MgCl_2$, only a single peak was observed with pronounced tailing (Fig. 6A). Two closely migrating peaks were observed when 0.1 mM of $MgCl_2$ was used in the separation buffer (Fig. 6B). Above 1 mM $MgCl_2$ concentration, the separations were similar to those shown in Fig. 4. Previous studies have shown that Mg^{2+} binds to the phosphate groups of phosphatidyl inositides [43–45]. While Mg^{2+}

Table 2. Number of theoretical plates (*N*) and resolution for $HyPIP_2$ and $HyPIP_3$ separated using conditions as in Fig. 4, but by varying different parameters

Varied parameter	*N* ($HyPIP_2$)	*N* ($HyPIP_3$)	Resolution
0 mM SDC [a]	76 000	39 000	7.6
5 mM SDC [a]	130 000	122 000	6.2
20% 1-propanol[b]	129 000	129 000	5.3
30% 1-propanol[b]	128 000	125 000	8.4
Run buffer[c]	51 000	44 000	3.8
Lipid buffer[c]	112 000	121 000	6.5
1-Propanol[c]	50 000	55 000	4.7

a) Comparison between the separation performed using 0 mM SDC with that at 5 mM SDC.
b) Comparison between the separation using 20% 1-propanol with that at 30% 1-propanol.
c) Comparison between the separations performed by dispersing $HyPIP_2$ and $HyPIP_3$ in a sample matrix of run buffer, lipid buffer, and 1-propanol. 365 V/cm was utilized for these separations.

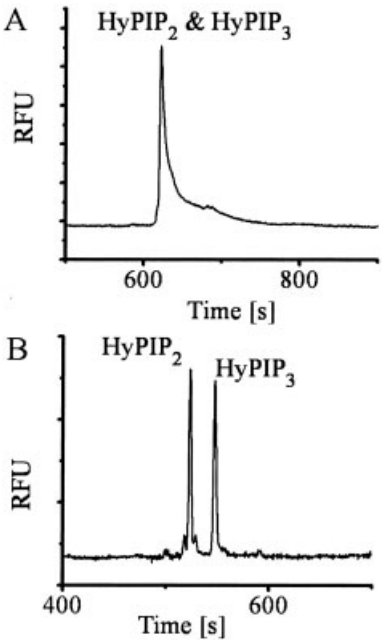

Figure 6. Separation of $HyPIP_2$ and $HyPIP_3$ using separation conditions as described in Fig. 3 except with (A) 0 mM $MgCl_2$ and (B) 0.1 mM $MgCl_2$.

binds to both PIP_2 and PIP_3, it likely binds to the phosphates of PIP_3 tighter and in a higher molar ratio due to the increased charge of the PIP_3 relative to that of PIP_2. This is a reasonable assumption since the binding of counterions to inositol 3,4,5-trisphosphate yields a more hydrophobic molecule than that produced by the binding of counterions to inositol 4,5-bisphosphate during RP-HPLC [46]. The slower migration time of PIP_3 relative to PIP_2 in the presence of Mg^{2+} suggested that the PIP_3 bound to Mg^{2+} possessed a lower charge-to-mass relative to PIP_2 bound to Mg^{2+}.

3.4 Optimization of the 1-propanol concentration

The 1-propanol concentration was varied between 0 and 40% in the presence of the other constituents of the optimal buffer (100 mM Tris (pH 8.5), 5 mM SDC, 1 mM $MgCl_2$, and 5% EOTrol LR). Since the EOTrol LR was insoluble in 1-propanol at concentrations greater than 40% v/v, the 1-propanol concentration was not increased beyond this percentage. 1-Propanol concentrations below 30% diminished the resolution of the analytes although the efficiency was similar to that at higher 1-propanol concentrations (Table 2). The highest quality separations were obtained with 30% 1-propanol. Thus, the 1-propanol was critical for the separation of $HyPIP_2$ and $HyPIP_3$ although not for the sharpness of the peaks. Other organic modifiers such as methanol and ACN were also tested as a replacement for 1-propanol and were added to the aqueous buffer at concentrations of 10, 20, or 30% v/v, but no separation of the PIPs was observed (data not shown). 1-Propanol possesses a lower dielectric constant than either methanol or ACN, and the increased hydrophobicity of 1-propanol may be important in maintaining the solubility of the hydrophobic lipids [47].

3.5 Role of EOTrol LR

To investigate the role of the proprietary polymer coating reagent, EOTrol LR, separations were performed with the optimal separation buffer (pH 8.5), but with and without this dynamic wall coating. When separations were performed without the coating, the polarity of the voltage had to be reversed in order to detect the analytes, confirming that the direction of EOF was indeed reversed. Small broad peaks with slightly faster migration times (compared to the separation in the presence of the coating) were observed (data not shown). When the injection volume was increased, the peak height remained nearly the same but the peak width increased. No separations were observed when SDC and EOTrol LR were both omitted from the buffer. These results suggested that the EOTrol LR played a critical role in minimizing adsorption of the analytes to the capillary wall.

3.6 Effect of the sample matrix on separation of the PIPs

To determine the influence of the sample matrix on the separation of the phospholipids, $HyPIP_2$, and $HyPIP_3$ were dissolved in water, the optimal separation buffer (pH 8.5), the lipid buffer, or 1-propanol. The sample matrix substantially impacted the quality of the separation. When dissolved in water, injected into the capillary, and electrophoresed, neither $HyPIP_2$ nor $HyPIP_3$, could be detected. Most phospholipids are sparingly soluble in water, and con-

sequently form aggregates when placed in water. The most likely explanation for the absence of the lipid peaks on the electropherogram was that the separation buffer was unable to adequately disperse the lipid aggregates that formed in the water. When the separation buffer was used as the sample matrix, the two phospholipids were detected and well separated. However, the efficiency and resolution of the separation were significantly lower than that obtained when the lipid buffer was used as the sample matrix (Table 2). These data also suggest that the separation buffer might not have adequately dissolved the lipids. When the lipids were dissolved in 100% 1-propanol, the efficiency of the separation was improved relative to that with a sample matrix of electrophoretic buffer, but was still inferior to that with the lipid buffer (Table 2). This suggests that efficient dissolution of the PIPs is key to obtain high-quality separations by CE under these conditions.

3.7 Mechanism of separation

When utilizing the optimized separation buffer, both 1-propanol and Mg^{2+} played key roles in providing sufficient resolution to separate PIP_2 and PIP_3. 1-Propanol also acted to adequately disperse the lipids. In contrast, the EOTrol LR was required to block analyte adsorption to the capillary walls as well as stabilize and diminish the rate of EOF. SDC played an important role in preventing adsorption of the negatively charged lipids to the positively charged wall coating since peak efficiency, but not resolution, was dependent on the concentration of SDC. The separation of the lipids was independent of the presence or absence of the SDC micelles. In contrast to prior reports, the mechanism of separation did not depend on differential partitioning of the analytes into micelles, *i.e.*, MEKC. This may be due to the shorter acyl chain (C16) of the lipids used in this work compared to the C18 chains utilized in other reports [18, 22]. In addition, all of the carbons in the acyl chains of the BODIPY FL-labeled lipids were saturated while in other reports some of the carbons possessed double bonds making the lipid more hydrophobic [22]. Lastly, the PIPs have more charge on the head group relative to that of other lipids such as phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidyl choline. In this report, the data are most consistent with a separation mechanism based largely on the differential binding of the lipids to Mg^{2+}. Under the separation conditions employed, the binding of Mg^{2+} to PIP_3 decreases the net charge-to-mass ratio relative to that of PIP_2 bound to Mg^{2+}. Consequently, PIP_3 migrates toward the positively charged outlet with a slower velocity relative to PIP_2.

3.8 Reproducibility and separation efficiency

Reproducibility of the migration time and peak areas using the optimal separation buffer (pH 8.5) was dependent on the pretreatment of the capillary inner surface before each run. The best reproducibility was observed when the capillary was rinsed with NaOH (1 M), water, and the separation buffer sequentially between runs. Table 1 lists the RSD of the migration time, peak heights, peak areas, and peak efficiency for nine sequential runs of BODIPY-labeled PIPs using the optimized conditions of Fig. 4. Variation in the peak heights and areas is most likely due to small variations in the injection volumes since the sample was manually injected into the capillary.

Efficiency of the separation was very good and ranged from 70 000 to 130 000 plates. The optimized method provided sharp and symmetrical peaks for these highly hydrophobic lipids. No peak tailing was observed for these PIP species. High efficiencies obtained are likely due to the coating reagent EOTrol LR as well as the SDC, both of which acted to minimize the adsorption of the PIPs to the capillary wall. In some lots of the fluorescent lipids, small peaks were observed next to the analyte peaks (see Figs. 4 and 5). These peaks were not always present and were attributed to impurities in the analytes.

3.9 Effect of pH on separation

To determine the influence of pH on separation, $HyPIP_2$ and $HyPIP_3$ were separated in the optimal buffer at varying pH (7.0, 8.0, 8.5, 9.0, 10.0, or 11.0). At pH 7.0, 10.0, and 11.0, $HyPIP_2$ and $HyPIP_3$ were not resolved. Separation of the two analytes was observed at pH 8.0, 8.5, and 9.0. The resolution of $HyPIP_2$ and $HyPIP_3$ and efficiency of each analyte at pH 8.0 was poor. At pH 9.0, the efficiencies of $HyPIP_2$ and $HyPIP_3$ were similar to that at pH 8.5. The resolution at pH 9.0 was 9.8 and improved relative that at pH 8.5 (shown in Table 2). At pH 9.0, the migration time of $HyPIP_2$ was nearly identical to that at pH 8.5. However, the migration time of $HyPIP_3$ was significantly slower at pH 9.0 than that at pH 8.5 resulting in the enhanced resolution at pH 9.0. This suggests that at pH 9.0, $HyPIP_3$ is more fully ionized than at pH 8.5 resulting in the tighter binding of magnesium ions, and consequently slower migration time.

4 Concluding remarks

A non-MEKC method was developed for the separation of fluorescently labeled PIPs at 20°C. Several methods and capillary-coating procedures were tested for the separation of PIPs. Separation of two different analogs of PIP_2 and PIP_3 was achieved in less than 15 min and with very good resolution and efficiency. The optimal buffer possessed SDC, 1-propanol, $MgCl_2$, and the polymer coating reagent, EOTrol LR. For good separations, the lipids required adequate dispersion in an aqueous buffer with a detergent or in an organic solvent. The developed method possesses great potential for the analysis of trace PIPs in biological microenvironments such as with purified PI 3-K, cell lysates, or single cells.

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