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Rapid and sensitive anion-exchange high-performance liquid chromatographic determination of radiolabeled inositol phosphates and inositol trisphosphate isomers in cellular systems

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ABSTRACT

A rapid and sensitive high-performance liquid chromatographic method for the determination of multiple inositol phosphates and inositol trisphosphate isomers was developed. The separation of inositol phosphates was optimized by controlling the ionic strength with stepped gradient programs and the pH of mobile phase. Six inositol phosphates were determined within 22 min or the six compounds plus an inositol trisphosphate isomer within 24 min using a single anion-exchange column containing the quaternary ammonium functional group. This technique was successfully applied to the determination of inositol phosphatide turnover by AlF_4^- stimulation in a small amount ($5 \cdot 10^5$–$1 \cdot 10^6$ cells) of cultured retinal capillary pericytes. Because of its efficiency, accuracy and applicability to the separation of inositol phosphates from biological samples, this method may be useful in signal transduction studies in cellular systems.

INTRODUCTION

Agonists activate phospholipase C which triggers the hydrolysis of phosphatidylinositol, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate with the subsequent formation of 1,2-diacylglycerol and inositol phosphates, including inositol 1,4,5-triphosphate [1,2]. The six hydroxyl groups on the myoinositol ring can be substituted by phosphate groups, yielding multiple inositol phosphate species [3]. These inositol phosphates possess various physiological functions [3]. To study the metabolic pathways of inositol phosphates in cellular systems, a rapid and accurate method for the determination of these compounds in biological samples is required.

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However, most data on inositol phosphatide-mediated signal transduction have been generated either by the low-pressure, anion-exchange technique of Downes and Michell [4], or by a time-consuming anion-exchange high-performance liquid chromatographic (HPLC) method [5–7]. Taylor et al. [8] have recently developed a rapid HPLC method for determining radiolabeled inositol phosphates including inositol 1-phosphate (IP), inositol 1,4-bisphosphate (IP_2), inositol trisphosphate (both isomers) and inositol 1,3,4,5-tetrakisphosphate (IP_4) by using a Vydac nucleotide column. To our knowledge, no rapid assay for the determination of IP–inositol hexakisphosphate (IP_6) plus both inositol trisphosphate isomers has yet been reported.

The aim of this study was to develop an HPLC method that is not only rapid but also as comprehensive as the reported time-consuming anion-exchange HPLC method for determining IP–IP_6 plus both inositol triphosphate isomers in biological samples.

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EXPERIMENTAL

Reagents

Radiolabeled [^{3}H]myoinositol, IP, IP_2, inositol 1,3,4-trisphosphate [I(1,3,4)P_3, inositol 1,4,5-trisphosphate [I(1,4,5)P_3] IP_4 and IP_6 were obtained from New England Nuclear (Boston, MA, USA) Nucleotide standards (GMP, GDP and GTP) were obtained from Sigma (St. Louis, MO, USA). Ammonium phosphate, phosphoric acid and other HPLC-grade reagents were purchased from Fisher Scientific (Philadelphia, PA, USA).

Preparation of radiolabeled inositol 1,3,4,5,6-pentakisphosphate (IP_5) standard

The standard of radioactive IP_5 was prepared by the method of Menniti *et al.* [9]. Cultured retinal capillary pericytes (for culture conditions, see below) with a density of 10^7 cells per 100-mm petri dish were labeled with [^{3}H]myoinositol (20 μCi/ml) for 72 h in M199 medium supplemented with 10% dialyzed serum. Supernatants from trichloroacetic acid-stopped reactions were neutralized by the method of Shears *et al.* [10]. Inositol phosphates, including IP_5 in neutralized samples, were separated on an anion-exchange column (Whatman Partisil SAX 10, 5-μm particle size) according to the method of Balla *et al.* [11]. IP_5 was collected and diluted tenfold with H_2O prior to column calibration.

HPLC analysis

The HPLC system (Waters–Millipore) consists of two Model 501 pumps, a Model U6K injector, a Model 484 UV detector and Baseline 810 computer software. For detection of nucleotide standards the detector was set at 254 nm. All separations were performed with Bio-Gel TSK IC-Anion-PW (PW) and Bio-Gel TSK IC-Anion-SW (SW) columns (50 × 4.6 mm I.D.) (Bio-Rad Labs.), a Vydac nucleotide column (50 × 4.6 mm I.D.) (Rainin) and a Whatman Partisil SAX 10 column (250 × 4.6 mm I.D.), with a guard column (Waters Guard-Pak) precolumn module and IC-PAK anion concentrator).

Rapid separation of inositol phosphates with the PW, SW or Vydac column was carried out by using stepped gradients of 0–0.5 *M* ammonium phosphate and by adjusting the pH of the mobile phase with 85% phosphoric acid (optimum pH as indicated in Fig. 1). Elution was carried out at a flow-rate of 3 ml/min with 1.5 ml per fraction. At the designated pH for elution of each inositol phosphate the ionic strength used in the stepped gradient program was first predetermined using a continuous gradient from 0 to 0.5 *M* ammonium phosphate within 60 min (flow-rate 3 ml/min). Based on the values obtained, we then determined the proper ionic strength empirically (exact concentrations of ammonium phosphate as indicated in Fig. 1). The stepped gradients were programmed over the first 3.5-min period to elute myoinositol with H_2O, followed by a sharp increase in the concentration of ammonium phosphate to elute IP within the 3.5-min period during which IP_2 did not elute from the columns. In the subsequent stepped gradient program, for elution of individual inositol phosphates the period was reduced to 3 min instead of 3.5 min (the required ammonium phosphate concentrations for each inositol phosphate with the difference columns are indicated in the figure captions).

The separation of inositol trisphosphate isomers and other inositol phosphates with a single column was carried out with the same stepped gradient program with modification. After the elution of IP, the concentration of ammonium phosphate was sharply increased to a level at which an 8-min isocratic run was used to elute IP_2, I(1,3,4) P_3 and I(1,4,5) P_3. The ionic strength for this isocratic elution was empirically chosen to be slightly lower (3% lower for the Vydac column, 5% lower for the SW column) than the predetermined ionic strength for IP_2 elution (see above). Subsequently, the stepped gradient program was resumed to elute IP_4, IP_5 and IP_6 as described above.

The radioactivity of each fraction was determined with a liquid scintillation counter (Wallac 1410; Pharmacia–LKB), and the counting data were analysed by the Data Capture Program (Beckman software version 2.8503 B) to obtain each radioactive peak area.

Because inositol phosphates lack UV absorbance, the retention times of guanine mono-, bis- and trisphosphate, which are sufficiently close to inositol monophosphate, bisphosphate and trisphosphate, allow the calibration of these columns periodically.

Metabolic labeling, AlF_4^- stimulation and biological sample preparation

Bovine retinal capillary pericytes were isolated and cultured in six-well plates (final cell number $5 \cdot 10^5$ per well) [12] and 1.0 ml of [^{3}H]myoinositol (5 μCi/ml) was introduced into each well. The incubation lasted 48 h in M199 medium supplemented with 10% dialyzed fetal bovine serum; 10 m*M* LiCl (final concentration) was added and incubated for 30 min before 10 μM $AlCl_3$ and 30 m*M* NaF (final concentration) were introduced. The osmolarity was kept constant by adding NaCl to the control. Incubation (30 min) was terminated by taking out the labeling solution and rapidly washing three times with ice-cold Puck's solution, followed by adding 1.5 ml of ice-cold 15% trichloroacetic acid (TCA) to each well. After a 10-min extraction, the cells were scraped off and centrifuged at 16 000 *g* for 5 min. The supernatant solution was washed five times with water-saturated diethyl ether to remove TCA. The whole aqueous fraction (1.5 ml) was neutralized with ammonia solution prior to PW or Vydac column chromatography.

Precision and recovery

The recovery of radioactive inositol phosphates in cellular samples was determined by comparison with aqueous standard solutions [myoinositol 150 000, IP 80 000, IP_2 50 000, I(1,4,5)P_3 50 000, IP_4 30 000, IP_5 10 000, IP_6 30 000 cpm/ml]. The cellular samples were prepared by using scraped unlabeled cells which were extracted with TCA to a final volume of 1.5 ml, and 1.4 ml of this extract were spiked with 0.1 ml of aqueous standard solutions. A 0.1-ml volume of each aqueous standard was diluted with 1.4 ml of water and the standard solution with total volume of 1.5 ml was injected. The recovery was calculated from the peak-area ratios (cellular sample to aqueous standard solution).

The reproducibility was determined by injecting eight times 1.5 ml of water containing 0.1 ml of the aqueous standard of I(1,4,5)P_3 (50 00 cpm/ml).

RESULTS AND DISCUSSION

Chromatographic conditions

To study the cellular signal transduction which is mediated by inositol phosphatide turnover, handling of a large number of samples is usually required. Hence it is desirable to have a rapid and accurate assay to examine multiple inositol phosphates. Low-pressure chromatography with Dowex ion exchangers is rapid, whereas is not adequate for quantitative or accurate separation [8]. The reported anion-exchange HPLC methods are sensitive and comprehensive, but they are time consuming [5–7]. A recently reported HPLC assay is efficient, but it is unable to detect IP_5 and IP_6 [8]. We applied an HPLC method with any one of three quaternary ammonium anion-exchange columns under optimized chromatographic conditions to separate rapidly multiple inositol phosphates. Fig. 1 shows the chromatograms of standards of radioactive myoinositol and six inositol phosphates obtained with these three columns under optimum pH conditions. By using the stepped gradient program, very sharp elution profiles without carryover of radioactivity between two inositol phosphate peaks were achieved. As this program was designed to obtain a suitable ionic strength for eluting a specific inositol phosphate within a few seconds, each compound was completely eluted within 2 min. The total elution time was *ca.* 22 min including IP_5 and IP_6 elution (Fig. 1).

Fig. 2 shows the effect of pH on the ionic strength for eluting individual inositol phosphates. Because of their phosphate ester linkages, inositol phosphates are charged molecules in the pH range 2–7. Over the pH range 2.0–6.0, in view of these charged molecules from IP_2 to IP_6, the more phosphate residues there are on an inositol ring, the higher is the ionic strength required (Fig. 2). In addition, for elution of an individual inositol phosphate, the higher the pH the higher is the ionic strength of the mobile phase needed (Fig. 2). However, each column has a limit of both pH and ionic strength (indicated in the caption of Fig. 2). Under some conditions, the required high salt concentrations may exceed the limit. In a recent report [8], pH 2.7 of the mobile phase was used for the separation of inositol phosphates with a Vydac nucleotide column. The salt concentration for eluting IP_4 already reached the limit of this column. Under such circumstances it was impossible to separate IP_5 and IP_6 further. In contrast, by using the same column and the same range of molar concentration of salt, we selected a higher pH of the mobile phase (pH 4.0 or 6.0). With such a modification, both IP_5 and IP_6 were detected (Fig.

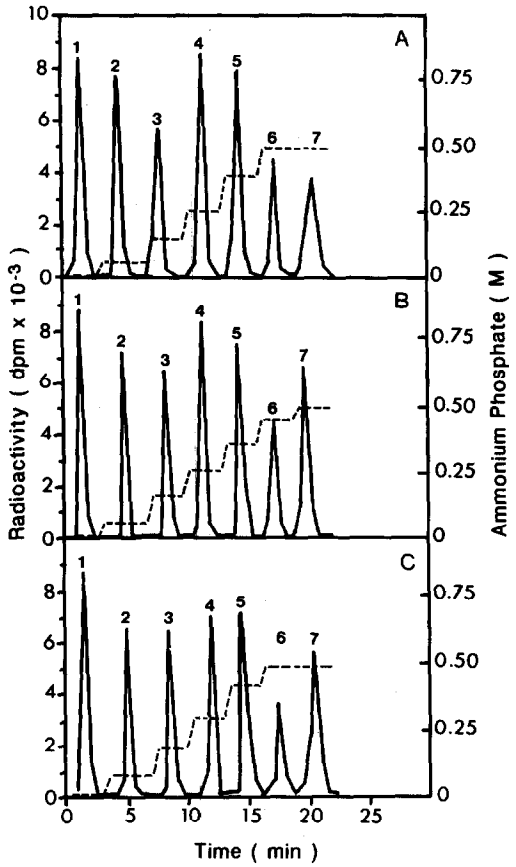

Fig. 1. Chromatogram of aqueous standards of radioactive myoinositol and six inositol phosphates by rapid anion-exchange HPLC. A mixture of labeled (1) myoinositol, (2) IP, (3) IP_2, (4) inositol trisphosphate, (5) IP_4, (6) IP_5 and (7) IP_6 was applied to and resolved by (A) the PW column (50 × 4.6 mm I.D.) (pH 1.0–12.0, maximum salt concentration 0.5 *M*), (B) the SW column (50 × 4.6 mm I.D.) (pH 2.0–8.0, maximum salt concentration 0.5 *M*) and (C) the Vydac column (50 × 4.6 mm I.D.) (pH 2.0–7.0, maximum salt concentration 0.5 *M*). The stepped gradient programs for eluting the six inositol phosphates with column A (pH 2.0), B (pH 2.5) or C (pH 4.0) are as follows: myoinositol, H_2O; IP, 0.060, 0.070, 0.085 *M*; IP_2, 0.150, 0.165, 0.190 *M*; inositol trisphosphate, 0.260, 0.265, 0.300 *M*; IP_4, 0.385, 0.370, 0.415 *M*; IP_5, 0.500, 0.455, 0.500 *M*; IP_6, 0.500, 0.500, 0.500 *M*.

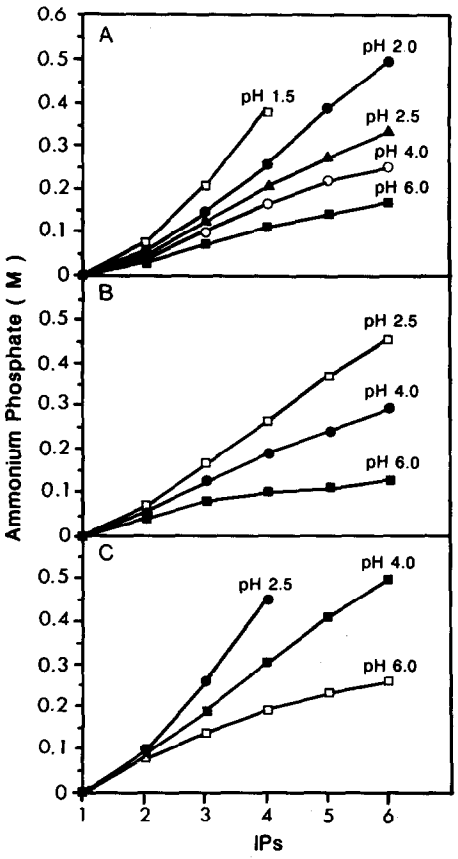

Fig. 2. Effect of pH on ionic strength required for eluting the six inositol phosphates using (A) the PW, (B) the SW and (C) the Vydac column. Compounds numbered on abscissa: 1 = IP; 2 = IP_2; 3 = inositol trisphosphate; 4 = IP_4; 5 = IP_5; 6 = IP_6.

1B). Moreover, because Taylor *et al.*'s study with pH 2.7 buffer [8] failed to elute IP_5 and IP_6, which intrinsically exist in mamalian cells [13,14], these radiolabeled polyphosphates which were retained and degraded in the columns might cause unpredictable radioactive contamination in the subsequent analytical process. Therefore, the use of eluents with pH higher than 2.7 in the Vydac column is recommended in order to avoid this pitfall.

To separate inositol trisphosphate isomers, the chromatographic conditions for the use of the three columns were also examined. Both the SW and Vydac columns were successfully used to separate I[1,3,4]P_3 and I(1,4,5)P_3 (Fig. 3). In order to identify these isomers, a stepped gradient program, with a rapid change of the ionic strength of the mobile phase, is not appropriate. Therefore, an isocratic run (0.165 *M* ammonium phosphate, pH 2.5 for the SW column; 0.175 *M* ammonium phosphate, pH 4.0 for the Vydac column) was inserted in the stepped gradient program after the elution of IP (Fig. 3). With a similar approach, we failed to differentiate I(1,4,5)P_3 and I(1,3,4)P_3 by the PW col-

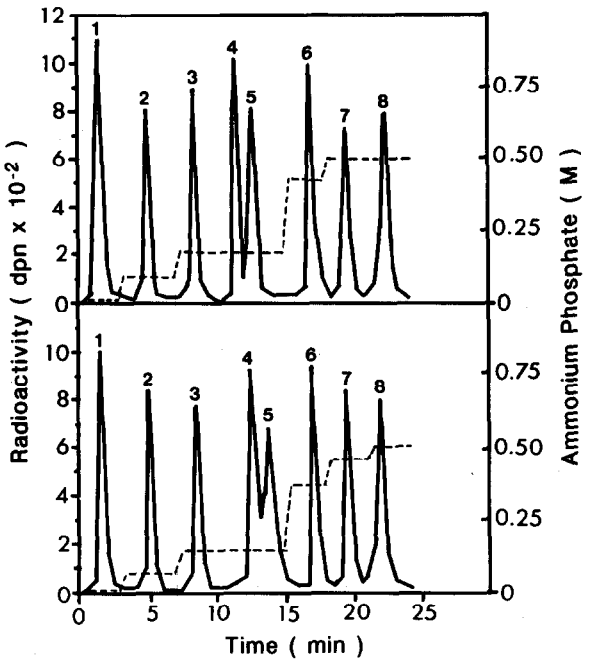

Fig. 3. Separation of radiolabeled I(1,3,4)P_3 and I(1,4,5)P_3 isomers together with myoinositol and five other inositol phosphates using (top) the Vydac column and (bottom) the SW column. Peaks: 1 = myoinositol; 2 = IP; 3 = IP_2; 4 = I(1,3,4)P_3; 5 = I(1,4,5)P_3; 6 = IP_4; 7 = IP_5; 8 = IP_6.

umn. It is possible that because of the much lower theoretical plate (TP) number (TP/column >900) for the PW column in comparison with the Vydac (TP/column >1200) and SW (TP/column >1400) columns, the limited resolution of the PW column was not able to differentiate subtle differences in isomer binding to the exchangers. Although a mobile phase of pH 4.0 was used with the Vydac column in this present study, the separation of I(1,4,5)P_3 and I(1,3,4)P_3 was similar to the results of Taylor *et al.* with an eluent of pH 2.7 [8].

Precision, recovery and detection limit

The reproducibility of the method for the detection of I(1,4,5)P_3 with three different columns resulted in a relative standard deviation of 6% for the PW, 7% for the SW and 9% for the Vydac column.

Recoveries of the six inositol phosphates for cellular media are shown in Table I, indicating high recoveries.

As large volumes (1.5 ml) can be injected on-column, a high sensitivity was achieved with this technique. At an injection volume of 1.5 ml, the method reveals a detection limit of six inositol phosphates and inositol trisphosphate isomers generated from 5 · 10^5 – 1 · 10^6 resting cells.

TABLE I

RECOVERY OF MYOINOSITOL AND SIX INOSITOL PHOSPHATES FROM CULTURED CELLULAR SAMPLES

Results are means of three determinations. The recovery of individual inositol phosphates from cellular samples was calculated as described in the Experimental section.

Column	Recovery of radiolabeled compound (%)						
	MI	IP	IP_2	Inositol trisphosphate	IP_4	IP_5	IP_6
PW	92.5	92.4	95.2	91.3	95.2	93.6	88.4
SW	94.1	97.1	94.0	89.6	95.4	91.7	92.6
Vydac	95.8	91.4	94.4	92.4	91.9	94.3	90.3

Applicability

To confirm the practical utility of the method in the analysis of biological samples, we applied it to the determination of six inositol phosphates in cultured retinal capillary pericytes (10^5 – 10^6 cells) treated with AlF_4^-, a non-specific activator of GTP-binding G proteins [15]. The inositol phosphate profiles of metabolically labeled pericytes treated with or without AlF_4^- are compared in Fig. 4. The results showed a great stimulatory effect of AlF_4^- on IP, IP_2, inositol trisphosphate and IP_4 formation, but little action on IP_5 and IP_6. In comparison, the ratios of stimulated cells to resting cells were 14.8, 14.0, 5.1, 3.9, 1.0 and 0.9, respectively. Because fluoride activates G proteins by mimicking the γ-phosphate of GTP, the increase in the production of IP, IP_2 and inositol trisphosphates indicated that inositol phosphatidylinositol-specific phospholipase C was activated by GTP-binding protein in retinal pericytes [15]. IP_4 was labeled to a steady state after 48 h of incubation with [^{3}H]inositol [9]. The substantial increase in [^{3}H]IP_4 after the stimulation suggests that the inositol trisphosphate–IP_4 pathway was also activated by AlF_4^- (Fig. 4). The levels of both IP_5 and IP_6 in resting or stimulated cells were only slightly changed. Because both IP_5 and IP_6 may not be labeled to a steady state under the present labeling condition [9], the quantitative cor-

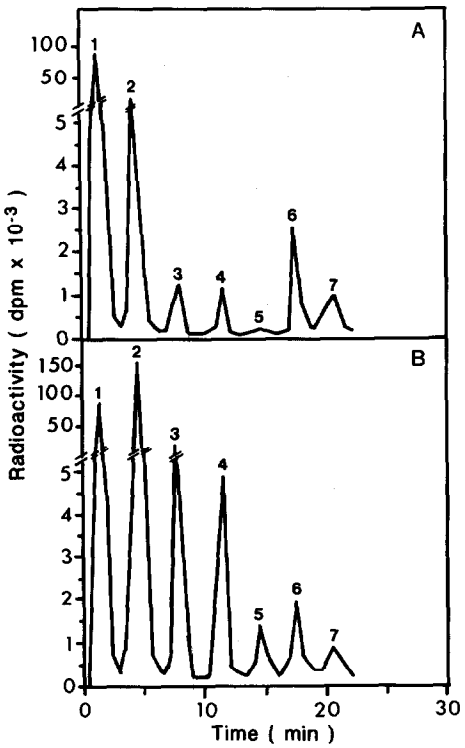

Fig. 4. Comparative chromatograms of inositol phosphate formation in retinal capillary pericytes (A) without and (B) with AlF_4^- treatment. Metabolic labeling, stimulation and TCA extraction are described in the Experimental section. Chromatography with the PW column using a stepped gradient program as described Fig. 1. Peaks: 1 = myoinositol; 2 = IP; 3 = IP_2; 4 = inositol trisphosphate; 5 = IP_4; 6 = IP_5; 7 = IP_6.

relation was not overstated. The above comments are based on assay with the PW column. Similar results were also obtained with the SW and Vydac columns (data not shown).

CONCLUSION

A rapid and sensitive single-column HPLC method has been developed for the determination of radiolabeled inositol phosphates and inositol trisphosphate isomers. Three different anion-exchange columns with the same functional group were tested. Except for the difficulty in detecting inositol trisphosphate isomers with the PW column, the three columns used have similar virtues for examining multiple inositol phosphates. As this method has been applied to evaluate inositol phosphatide turnover of cultured retinal pericytes in the range of $5 \cdot 10^5 - 1 \cdot 10^6$ cells, it is useful for studies of signal transduction in cellular systems.

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