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HPLC Application for Phosphoinositides Analyses of A431 Cells and Human Platelets

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Summary. **A new method for the complete separation of polyphosphoinositides with a single high performance liquid chromatography (HPLC) run was applied to human platelets and cell line A431. The amounts of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4, 5-bisphosphate (PIP_2) were traced with ^{32}P before and after stimulation. With stimulation by thrombin, increased uptakes of ^{32}P to PI, PIP, and PIP_2 were observed in platelets within 3 min. A similar effect was observed in A431 cells with stimulation by epidermal growth factor. Preincubation with platelet aggregation inhibitors such as hirudin and N, N, N-trimethylsphingosine (an artificial sphingosine derivative) did not produce an increase of PI, PIP, or PIP_2 under stimulation with thrombin. Since phosphoinositides play important roles in signal transduction inside cells, our method should be useful for automatic quantitative analysis and large-scale purification of phosphoinositides.**

INTRODUCTION

Phospholipids and polyphosphoinositides play important roles in signal transduction in biological membranes.[1,2] Since structural differences between phosphoinositides (PIs) are minor compared with those of sphingolipids, the separation of PIs with a single high performance liquid chromatography (HPLC) run has been difficult. Thin layer chromatography (TLC) has been generally used for phosphatidylinositol (PI) separations,[3] but this method is usually not suitable for large-scale purification or automatic analysis.

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Recently, we[4] reported an effective method for PI separation by a single HPLC run based on a new solvent system. The method[4] enables us to separate phospholipids completely with a single HPLC run. In this study, we applied it to trace metabolic changes of PIs in human platelets and cancer cell line A431, in order to examine the pharmacological effect of trimethylsphingosine (TMS), a synthetic inhibitor of platelet aggregation.

MATERIALS AND METHODS

Materials

HPLC grade glass-distilled isopropanol, hexane and water were obtained from Mallinckrodt (St. Louis, MO, U. S. A.). PC, PI, PIP, PIP_2, thrombin, hirudin, and phosphate-free Dulbecco's modified Eagle's medium (DMEM) were acquired from Sigma (St. Louis, MO, U. S. A.), with radioactive phosphate ($Na_2H^{32}PO_4$) came from Amersham (UK). The human epithelial cancer cell line A431 came from American Type Culture Collection (ATCC). Epidermal growth factor (EGF) was donated by Earth Pharmaceutical Co. (Tokyo, Japan). Platelets (blood type AB, Rh positive) were obtained from normal human adults via the Red Cross of Oregon (Portland, OR, U. S. A.).

Preparation of biological specimens

A431 cells were cultured to confluency in Falcon 3025 petri dishes. Each dish was washed three times and preincubated with phosphate-free DMEM at 37℃ for 3 h. 200 μ Ci of $Na_2H^{32}PO_4$ was added, followed 2 min later by 1.0 μg of EGF or an equal amount of normal saline (10 μl). Fifteen minutes later, the reaction was

terminated by aspiration of the media and washing with PBS. Cells were scraped off in 4 ml of 0.1 N HCl and homogenized by sonication with 3.75 ml of CM (1 : 2), followed by 1.25 ml of chloroform, 1.25 ml of water and 50 μl of acetone, then shaken well and left to sit overnight. After centrifugation, phospholipids were extracted by taking the lower phase of Folch's partition.[5] Approximately 1×10^5 cpm of total phospholipids were extracted from each plate.

Platelets (1.2×10^9) were suspended in 4.5 ml of calcium-free Mill's buffer[6] (5.5 ml total volume), and incubated at 37℃ for 1 h with 1.0 mCi of $Na_2H^{32}PO_4$. After centrifugation, the radiolabeled platelets were resuspended in 4 ml of the buffer and divided among four tubes: with or without thrombin and TMS. Preincubation took place at 37℃ with 20 μM of TMS or an equal amount of 50% ethanol (10 μl) for 5 min, then 10 nM of thrombin was added. After 15, 30, 60, 180, and 600 sec, the reaction was terminated by adding 3.75 ml of CM (1 : 2), followed by 1.25 ml of 2.0 M HCl and sonication for 10 min. Phospholipids were extracted as described for A431 cells. For the experiment with hirudin, preincubation was performed with or without 4 U of hirudin for 5 min, and cells were stimulated with thrombin as described above.

HPLC procedure

A Varian Liquid Chromatograph Model 5020 with latrobeads 6RS-8010 (Iatron Labs, Tokyo) was washed with isopropanol/hexane (IH) (55 : 45) v/v for 2-3 h at a flow rate of 2.0 ml/min, then equilibrated with the starting solvent isopropanol/hexane/1 M NH_4OH in water (IHW) (55 : 40 : 5). Samples (approximately 5 to 30×10^3 cpm per load) or standard phospholipids (approximately 0.5 mg each) were dissolved in 800 μl of IHW (55 : 40 : 5) and loaded on the column. Isocratic solvent IHW (55 : 40 : 5) was applied for 10 min. Samples were eluted with a linear gradient of IHW (55 : 40 : 5 to 55 : 35 : 10) for 50 min, then switched to another isocratic solvent IHW (55 : 35 : 10) for 50 min. The flow rate was 2.0 ml/min. Fractions were collected in each tube at one-min intervals. The column was washed for the next application with a linear gradient of IHW (55 : 35 : 10 to 55 : 40 : 5) for 30 min at the same flow rate. Quantitative analyses of phosphoinositides were performed by taking aliquots of eluted fractions to count radioactivity of ^{32}P with a Beckman scintillation counter. The eluted peaks were identified by comparison with peaks from authentic phospholipids or phosphoinositides.

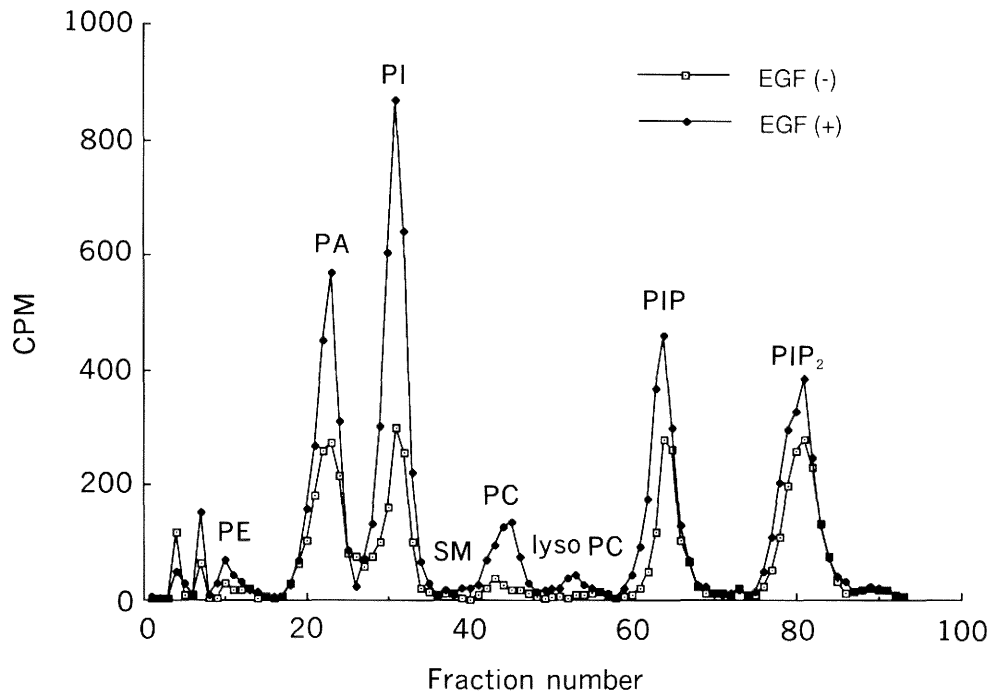

Fig. 1. HPLC separation of phosphoinositides from A431 cells with or without EGF stimulation.

RESULTS

We have previously reported[4] the complete separation method of phospholipids with a single HPLC run. By applying the method, it was possible to separate phospholipids from biological specimens; namely, the A431 cells and platelets. As shown in Figs. 1 and 2, the phospholipids were eluted in the order PA, PI, PC, PIP, PIP_2. EGF stimulation of A431 cells induced an increase of ^{32}P uptake to those phospholipids, especially PI (Fig. 1). The uptake to PA and PC was also increased. Fig. 2 shows the separation of phospholipids extracted from platelets, following 1 min with or without stimulation. Thrombin induced increased amounts of ^{32}P uptake to PI, PIP and PIP_2. TMS inhibited the effect of thrombin. Hirudin, which is known to inhibit the effect of thrombin,[7] also inhibited ^{32}P uptake in a similar manner: levels of PI, PIP, and PIP_2 were not significantly different from those of controls without thrombin stimulation. TMS alone had no stimulatory or inhibitory effect. In the study on time course of PIs, the amount of PI reached its peak at 30 sec; this peak was 3.1 times higher than that of the controls. PIP and PIP_2 reached their peaks at 180 sec after stimulation; these peaks were 3.2 and 5.4 times higher than the controls, respectively.

When the column was washed with a linear gradient of IHW, the column was reusable for at least 50 times after the first application.

DISCUSSION

According to our results, it is possible to separate phospholipids from A431 cells and human platelets with a single HPLC run. Increased amounts of ^{32}P in phospholipids were observed with the stimulation of reagents such as EGF and thrombin. Since PI, PIP and PIP_2 play important roles in signal transduction inside cells,[2] our method could be of value for the evaluation of cell functions in various diseases. For this purpose, the use of HPLC permits automatic and mass screening analysis. HPLC could save time and effort compared to high-performance thin-layer chromatography (HPTLC), as previously reported by Sierra et al.[12] for the analysis of alphatocopherol in erythrocytes.

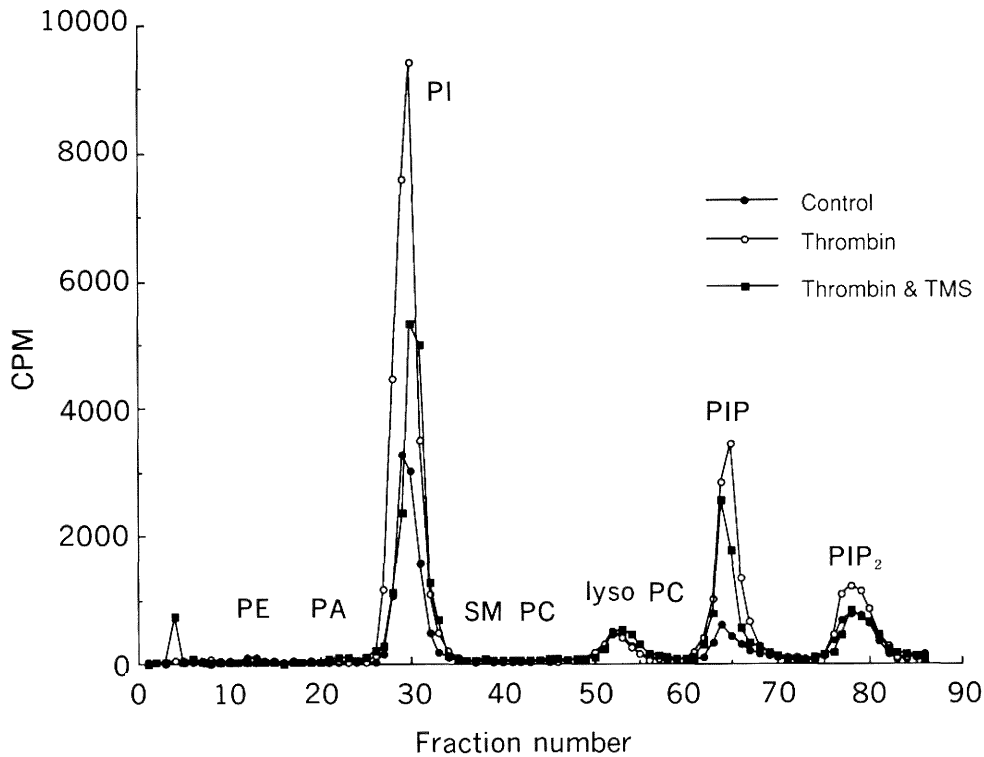

Fig. 2. Effect of thrombin and TMS on phosphoinositides turnover in human platelets. Stimulation period was 1 min.

Another advantage of HPLC is that it can provide more information than HPTLC. With HPLC, not only PI and PIP but also PA, PC, and other phospholipids can be separated. The increased amount of phospholipids (especially PI and PIP) in A431 cells confirms that EGF stimulates the cells through the acceleration of phospholipid metabolism, as reported previously by several authors.[8,9]

The induction of platelet aggregation by thrombin is also related to PI turnover.[10] According to our previous experiment,[4] the metabolic pathway of PI, PIP, and PIP_2 is also stimulated by the platelet activating factor (PAF). The pattern of stimulation of the pathway is essentially similar to that of PAF. HPLC provides an easier way to measure and compare the amount of phosphoinositides. Our time course study indicates that HPLC can be used to investigate the kinetics of PI turnover.

In platelets, thrombin is known to increase IP_3 by degradating PIP_2. The authors consider the increased amount of ^{32}P in PI, PIP and PIP_2 to reflect the acceleration of the turnover rate. As reported,[2] IP_3 degradation occurs quickly within a few min. On the other hand, in our experimental conditions, ^{32}P labelling took place for 15 to 60 min. During this period, the ^{32}P supposed to be recovered by recruiting from IP, IP_2 and IP_3. Thus, to a certain extent, the increased uptake of ^{32}P to PIs reflects the acceleration of PI turnover.

TMS is a strong inhibitor of protein kinase C in neutrophils[13] and cancer cell lines.[14] Our data show that TMS at a concentration of 10 μM inhibits the increase of phospholipids. Okoshi et al.[11] reported an inhibitory effect of TMS on platelet aggregation. Fig. 2 shows the addition of TMS significantly depressed levels of PI and PIP, which indicates that the effect of TMS is closely related to PI turnover inhibition. We are currently studying the effects of TMS on protein phosphorylation in platelets. According to the Okoshi et al.,[11] TMS does not have a toxic effect on platelets at the concentration of 20 μM.

Since HPLC has been used for the automatic analysis and purification of a wide variety of materials, our method should be useful for automatic quantitative analysis or large scale purification of phosphoinositides.

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