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Use of Zn–pyrophosphatase in the high-performance liquid chromatographic analysis of cell extracts containing ^{32}P-labelled inositol phosphates

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ABSTRACT

A simple method is described for eliminating the interference of pyrophosphate and pyrophosphorylated nucleosides in the high-performance liquid chromatographic determination of inositol 1,3,4-triphosphate and inositol 1,4,5-triphosphate of ^{32}P-labelled extracts of cells. Treatment of the extract with pyrophosphatase, but substituting Zn^{2+} for Mg^{2+} as the cofactor, converts all nucleoside triphosphates and pyrophosphate to their di- and monoesters. Such change shifts their position in the elution profile, allowing a clear identification and quantification of the inositol phosphates. Typical overall recoveries near 80% or higher of added markers.

INTRODUCTION

In recent years, receptor-mediated formation of inositol phosphates (Ins Ps) at the expense of inositol-containing phospholipids has been claimed to be a mechanism of membrane signal transduction leading to intracellular calcium release in different cell types [1]. This finding has made imperative the search for methods for the detection of the different Ins Ps that may be generated during cell activation.

The presently used methodologies can be divided into three groups: (1) measurement of the generated Ins Ps using introduced radiolabelled inositol as a core marker [2]; (2) non-radioisotope measurement based on the displacement by Ins Ps or other organic phosphates of the rare metal yttrium from its complex with pyridylazoresorcinol [3]; (3) metabolic incorporation of [^{32}P]phosphate (^{32}Pi) into ATP followed by labelling of the phosphates in the inositol-containing phospholipids and other metabolically derived organic phosphates [4].

The approach using ^{14}C-labelled inositol is the most advantageous owing to the specificity of the label, but it has shown to present problems of penetration of the marker into certain types of cells like platelets. The pyridylazoresorcinol method, although highly sensitive, is highly fastidious on practical grounds because it requires a total absence of stainless steel in the hardware and post-column detection systems. Also, the high absorptivity of the yttrium–pyridylazoresorcinol complex is degraded in practice by the noise generated in the post-column mixing of the eluate with the reagent, especially at the high detection sensitivities required to detect picomole amounts of Ins Ps. This method also presents the problem

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of detecting all phosphorylated compounds. The ^{32}Pi-labelling technique, while being simple and highly sensitive, leads also to the labelling of all ATP-dependent biosynthetic intermediates. This results in the appearance of a large number of radioactive peaks, which superimpose on the Ins Ps of interest, especially the isomers Ins1,4,5P_3 and Ins1,3,4P_3 which are located between the heavily labelled ATP, GTP and pyrophosphate bands [4]. To help solve this problem, cell extracts have been treated with pyrophosphatase (PPase) to hydrolyze the pyrophosphate and with charcoal to reduce the amount of the nucleotides [3–5]. The use of charcoal introduces additional problems due to its binding capacity for the Ins Ps, with the risk of further reducing an already meager recovery yield [3,4].

We propose here a simple alternative to clean the chromatogram in the regions of interest for the Ins P_3s by transforming all the pyrophosphorylated nucleosides into the di- and monophosphates and also pyrophosphate into phosphate by treatment of the extract with inorganic PPase as done previously [4], but using Zn^{2+} instead of Mg^{2+} as cofactor. This is an application of the finding by Schlessinger and Coon [6], who showed that this change widens the substrate selectivity of the enzyme, allowing, apart of the hydrolysis of pyrophosphate, the hydrolysis of all nucleoside pyrophosphate bonds to nucleoside diphosphate and more slowly to the monophosphate esters. Application of this property to extracts containing pyrophosphate, ATP and GTP would therefore shift them from near or over the location peaks of the Ins Ps of interest. As a consequence, the charcoal treatment, reextraction, ion-exchange microcolumn purification and concentration procedures needed previously for purifying the extracts are avoided, further improving reproducibility and decreasing the loss of the Ins P_3s.

Preliminary results of this work have been communicated [7].

EXPERIMENTAL

We will describe the best conditions for nucleotide hydrolysis and present afterwards a prototype protocol which we have followed for the application of the Zn–PPase method to the measurement of changes of Ins Ps in platelet suspensions after thrombin activation.

Acid–citrate–dextrane (ACD) platelet-rich plasma was prepared by centrifugation at 200 *g* for 15 min from human blood taken by venipuncture. After electronic counting, a volume of platelet-rich plasma equivalent to 10^9 platelets was concentrated by centrifugation at 600 *g* for 15 min, resuspended in 0.2 volumes of autologous platelet-poor plasma and incubated for 1 h at 37°C with ^{32}Pi (Amersham, UK) (final concentration of 0.25 mCi/ml). The labeled platelet-rich plasma was gel-filtered at room temperature on Sepharose 2B (Pharmacia LKB Biotechnology, Piscataway, NJ, USA) and resuspended in Ca^{2+}-free Tyrodes saline supplemented with 0.2% albumin, 5 m*M* glucose and 5 m*M* sodium 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate (final concentrations) to a pH of 7.3 (Sigma, St. Louis, MO, USA). After the platelet number was adjusted to a final concentration of $4 \cdot 10^8$ cells per ml, the suspension was incubated at 37°C. Activation was initiated with 5 U/ml thrombin (Thrombostat, Parke-Davis, Morris Plains, NJ, USA) in 1.0 m*M* Ca^{2+} and stopped by adding 1/10 volume of 0.66 *M* perchloric acid after 5, 10, 15, 30 and 60 s. Before further processing, 0.02 μCi of [^{3}H]Ins1,4,5P_3 and Ins1,3,4,5P_4 were added to the perchloric acid extract. The extracts were neutralized with 2.0 *M* K_2CO_3, and aliquots of the neutralized extract (equivalent to approximately $4 \cdot 10^8$ platelets) incubated overnight at 37°C with a mixture of 2.0 m*M* Zn^{2+} acetate in 0.1 *M* Tris–maleic acid buffer (final concentrations), pH 6.4, containing 20 U/ml yeast PPase (Sigma). After chelating the excess Zn^{2+} with EDTA (3 m*M* final concentration) the extract was injected directly into the column and the Ins Ps were separated and analyzed by high-performance liquid chromatography (HPLC) on a strong anion-exchange column (Partisil, 10 μm, 250 mm × 4.6 mm I.D., SAX, Whatman, Hillsboro, OR, USA) which was eluted with a 50-min linear gradient of ammonium formate pH 3.7 starting at 10

m*M* and ending at 1.5 *M*. Aliquots of 0.5 ml of the eluate at a rate of 1.5 ml/min were collected, mixed with scintillation fluid and counted with a dual label program (Beckman LS-7500, Fullerton, CA, USA) [4]. The tritium channel was used to locate and calculate the recovery of the Ins1,4,5P_3 and Ins1,3,4,5P_4, and by analogy the recovery of Ins1,3,4P_3.

The possible contamination of the PPase with acid phosphatase was measured by incubating selected samples with an aliquot of the neutralized extract to which sodium *p*-nitrophenylphosphate (final concentration of 4.5 m*M*) was added [8]. The protocol was the same as for the experimental samples.

RESULTS

The initial conditions for enzymatic treatment were studied by altering the amounts of PPase, Zn^{2+}, pH and incubation time using as substrates solutions of non-labeled ATP and GTP (1 m*M*) and measuring with HPLC the disappearance of the peaks. Our results confirmed the data of Schlessinger and Coon [6] for the existence of a broad hydrolytic optimum pH from 5.6 to 6.4 and for an appreciable increase in the turnover time of the enzyme. We also have found that Zn^{2+} at concentrations higher than 5 m*M* inhibits the reaction. Fig. 1 quantifies such studies. Fig. 2 shows the kinetics of ATP hydrolysis for the first 180 min of incubation using 20 U/ml PPase and 5 m*M* Zn^{2+}. ATP hydrolysis achieved was 98%.

However, in the course of the experiments with platelet extracts we found that there was a significant difference in the amount of ATP hydrolyzed by the PPase depending on whether the platelet suspensions were activated for the first time with thrombin or activated with thrombin under conditions not leading to irreversible aggregation, washed and stimulated again with other agonists [9]. Fig. 3 quantifies the effect and shows it to be dependent on the platelet concentration. The phenomenon suggests that some component of the platelet granules which would appear in the supernatant as a result from secretion is re-

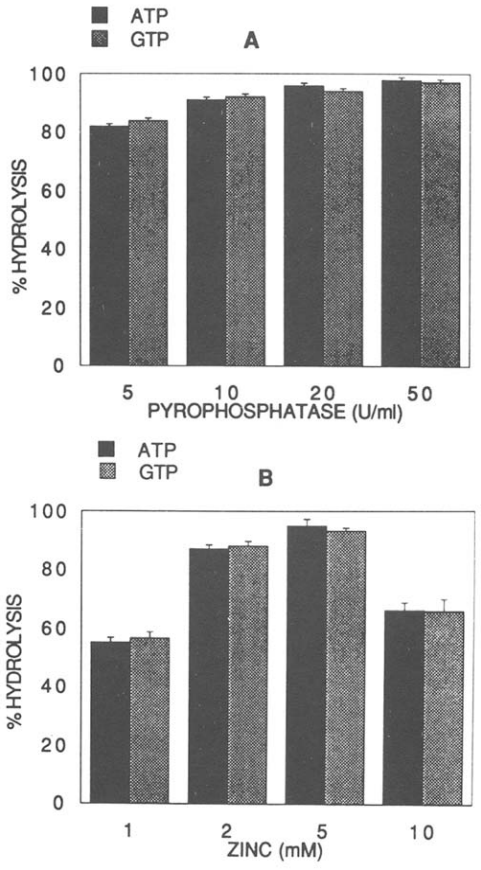

Fig. 1. Effect of pyrophosphatase activity and Zn^{2+} concentration on the hydrolysis of ATP and GTP (initial concentration of the nucleotide is 1.0 m*M*). Conditions are 1 h incubation in a 0.1 *M* Tris–maleic acid buffer, pH 6.4 at 37°C. (A) Zn^{2+} concentration is 5.0 m*M*; (B) pyrophosphatase activity is 20.0 U/ml.

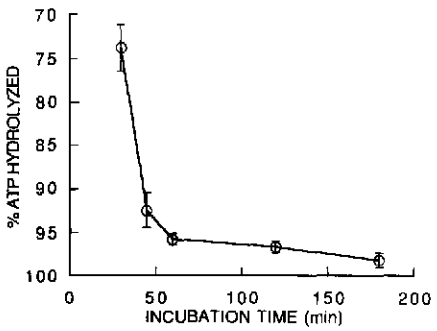

Fig. 2. Kinetics of ATP hydrolysis by Zn–pyrophosphatase. Conditions are as described is the legend to Fig. 1.

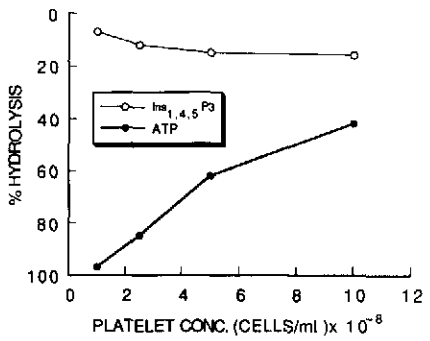

Fig. 3. Effect of the supernatant of thrombin-activated platelets on pyrophosphatase-induced hydrolysis of [^{3}H]ATP. Supernatants were incubated with 20.0 U/ml pyrophosphatase and 2.0 m*M* Zn^{2+} for 3 h at 37°C.

sponsible for inhibiting PPase. Efforts to identify such component among the expected components of a deproteinized granule extract failed. Finally it was decided to solve the problem by increasing the incubation time (Fig. 4). An adequate compromise was attained using 20.0 U/ml enzyme, 2.0 m*M* Zn^{2+} and an overnight incubation period at 37°C. The integrated acid phosphatase activity of the PPase for this period was still quite low ($4.6 \cdot 10^{-6}$ U/h incubation). Efforts to inhibit this residual activity with tartrate or phloretin have been up to now unsuccessful.

Fig. 5 shows the overall changes that this treatment has on the elution region of interest to Ins P_3s. The effect is especially important for elim-

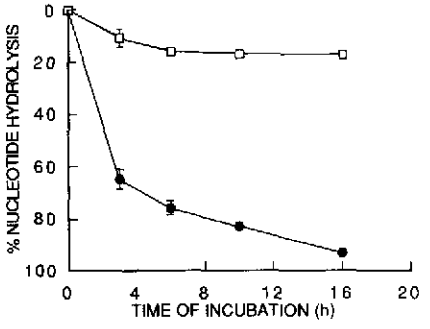

Fig. 4. Hydrolysis of [^{3}H]ATP (10 nCi) and [^{3}H]Ins1,4,5P_3 (10 nCi) added to the supernatant of a platelet suspension as a function of time. $5 \cdot 10^8$ Platelets per ml were activated with 5.0 U/ml thrombin. Experimental conditions were similar to those described in Fig. 3 but the incubation period was 14 h (overnight) at 25°C.

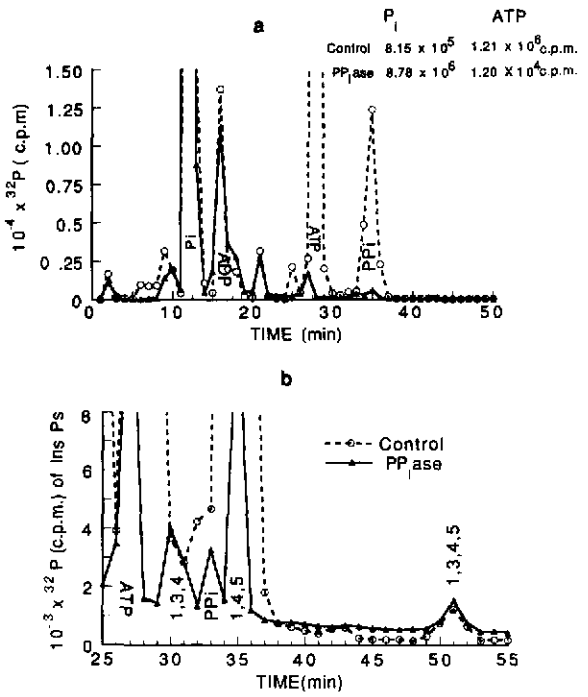

Fig. 5. Comparative HPLC patterns of a ^{32}P-labelled thrombin platelet extract ($5 \cdot 10^8$ platelets per ml) incubated for 16 h at 25°C under the conditions described in Fig. 2. (a) Overall view of the changes in the total chromatogram; (b) magnified detail of the changes in the region of elution of the inositol phosphates.

inating compounds like pyrophosphate, ATP and GTP that are eluted practically at the same time as Ins1,3,4P_3 and Ins1,4,5P_3.

To test the validity of the new experimental approach we show in Fig. 6 the results of a typical experiment where the kinetics of the Ins P changes was measured in an experiment using a ^{32}P-labeled suspension of platelets activated with 5 U/ml thrombin. They are in excellent agreement with the results published by Daniel and co-workers [4,9] from whose work the basic chromatographic procedure described here was extracted.

DISCUSSION

The proposed system appears to resolve some difficult methodological problems which have resulted in low yield and lack of good reproducibility in previously published methods. There is on-

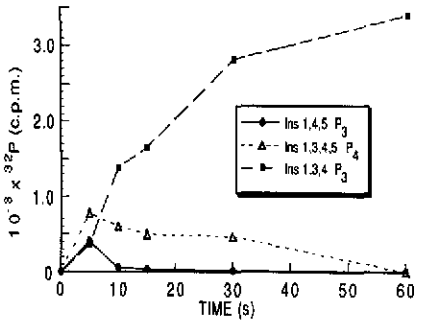

Fig. 6. HPLC-derived pattern of the kinetic changes of the inositol phosphates as a function of time measured in an experiments where ^{32}P-labelled platelets ($5 \cdot 10^8$ per ml) were activated with 5.0 U/ml thrombin. Samples were quenched with perchloric acid at 5, 10, 15, 30 and 60 s after the addition of thrombin and treated after neutralization with Zn–pyrophosphatase as described in the Experimental section. Results are reported as the difference between cpm at time 0 and the time sequence reported.

ly an incubation step after the deproteinization and neutralization, no treatment with activated charcoal, no dilution, no treatment with ion-exchange resins to concentrate the Ins Ps no elution and no concentration before chromatography.

There are, however, two caveats. The first is related to the purity of the PPase used because contamination with phosphatases will decrease the recovery of the Ins Ps. In our case, using the commercially obtained yeast inorganic PPase from Sigma at pH 6.4, recovery of the Ins Ps amounted to 80–88%, depending on the PPase batches. The second caveat is related logically to the amount of pyrophosphorylated nucleosides occurring in the sample, which will determine the incubation time needed to decrease the interference to acceptable levels. In our case the nucleotide concentration contained in $2–4 \cdot 10^8$ platelets appear not only to be a good compromise but helped improve the sensitivity of the method by one order of magnitude with respect to other methods [3].

Other approaches could be used to decrease the presence of pyrophosphorylated nucleosides, such as the one proposed previously by Brown [10] with glucose and hexokinase to convert ATP into ADP, in what she called the "enzyme peak-shift technique", or the method of Nelson [11], which involves the use of fructose 6-phosphate in combination with phosphofructokinase, aldolase, trioseisomerase and glycerophosphate-dehydrogenase, using NADH as the final acceptor to convert all nucleoside triphosphates into their diphosphates. However, in contrast with the Zn^{2+}–PPase method, a large number of specific enzymes have to be used, increasing the risk of introducing contaminating enzymes. Furthermore, pyrophosphate, an important contaminant in the cell extracts, is not hydrolyzed. Therefore, we believe our method to be a better alternative.

Further improvements with respect to overall operational time and yields could be achieved in finding an approach to eliminate the substance(s) that inhibit the PPase activity when extracts containing the platelet granular components are treated and inhibit the contaminating phosphatase activities.

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