Two species of phospholipase C isolated from lymphocytes produce specific ratios of inositol phosphate products

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Chromatography of the soluble porcine lymphocyte phospholipase C on cellulose phosphate resolves the activity into two species. An HPLC method is described for separating the enzyme products, Ins 1,2>P and Ins 1P. Use of these methods reveals that the two iso-enzymes liberate, with a high degree of reproducibility, characteristic ratios of the two products. This suggests that the amount of each product produced is an inherent property of the enzyme mechanism.

Phospholipase C *Phosphoinositide* *Inositol phosphate* *Lymphocyte tranformation*

1. INTRODUCTION

Stimulation of cell surface receptors by growth factors and hormones has, in many cases, been shown to involve an enhanced hydrolysis of the phosphoinositides by the action of a phosphoinositide-specific phosphodiesterase (EC 3.1.4.10) or phospholipase C [1–3]. There is therefore considerable interest in purifying and studying the mechanism of action of enzymes of this type. Purification studies of this enzyme have been confined to the soluble phospholipase C which predominates in all tissues studied to date [4–6], the activity of which has frequently been shown to derive from multiple forms of the enzyme [5–8]. There have been recent reports of a phospholipase C activity resident within purified plasma membranes, closely associated with both the receptor and GTP-binding proteins [9–11]. It is not yet clear whether the species observed in these cases may also exist in a soluble form which associates with the membrane upon cell activation.

In all cases, where investigated, phospholipase C action on PtdIns results in the production of both Ins 1P and the acid-labile Ins 1,2>P [4,5,12]. Recent studies with a purified phospholipase C isolated from sheep seminal vesicles have shown that hydrolysis of PtdIns 4,5P_2 or PtdIns 4P also yields cyclic inositol polyphosphates in addition to the non-cyclic derivatives [13]. Production of Ins(1,2>P)4,5P_3 has not yet been demonstrated to occur in vivo although this compound has been shown to have biological activity in both mobilizing intracellular Ca^{2+} in platelets and inducing changes in conductance in *Limulus* photoreceptor cells [14]. In the latter effect, the cyclic form is more potent than the non-cyclic form. Recently, Ins 1,2>P has been found in vivo upon stimulation of pancreatic tissue [15] and although no physiological role for this product has been identified, it indicates that the cyclic forms of inositol polyphosphates may also be produced in vivo.

We have recently identified in the soluble fraction of porcine lymphocytes a number of species of

Abbreviations: PtdIns, phosphatidylinositol; PtdIns 4P, phosphatidylinositol 4-phosphate; PtdIns 4,5P_2, phosphatidylinositol 4,5-bisphosphate; GroPIns, glycerophosphoinositol; Ins 1P, inositol 1-phosphate; Ins 2P, inositol 2-phosphate; Ins 1,4,5P_3, inositol 1,4,5-trisphosphate; Ins 1,2>P, inositol 1,2-cyclic phosphate; Ins (1,2>P)4,5P_3, inositol 1,2-cyclic 4,5-trisphosphate; PMSF, phenylmethylsulphonyl fluoride; DTE, dithioerythritol

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phospholipase C which may be resolved by gel-filtration chromatography [16]. We report here that further purification by cellulose phosphate chromatography resolves the major peak of activity eluting from such a column into two further species, each of which produces very characteristic and quite distinct proportions of Ins 1,2>P and Ins 1P.

2. MATERIALS AND METHODS

Chromatography media Sephacryl S-200 and cellulose phosphate were supplied by Pharmacia and Bio-Rad Laboratories, respectively. All radiochemicals were obtained from Amersham International and non-radioactive PtdIns from Lipid Products. Ins 2P and Folch fraction I were purchased from Sigma.

2.1. *Purification of phospholipase C*

Phospholipase C was isolated from porcine mesenteric lymph node lymphocytes as described in [16]. The initial step in the purification procedure was that of gel filtration on a Sephacryl S-200 column (90 × 2.5 cm) eluting with 50 mM Tris-HCl (pH 7.2), 1 mM EGTA, 100 μM PMSF, 200 μM DTE, which gave a similar profile to that resulting from chromatography on Sephadex G-200 [16]. The major peak, peak II, was pooled, concentrated and equilibrated with 50 mM citric acid/trisodium citrate (pH 5.8), 1 mM EGTA, 100 μM PMSF, 200 μM DTE. This was loaded on a cellulose phosphate column (10 × 2.5 cm) and eluted with a linear NaCl gradient (0–0.6 M). 4 ml fractions were collected and the protein concentration determined by the absorbance at 280 nm; 50 μl aliquots were assayed for phospholipase C activity using the neutral solvent extraction.

2.2. *Assay for phospholipase C*

Phosphatidyl[2-^{3}H]inositol and unlabelled PtdIns were resuspended in 0.45 ml of 50 mM Tris malcate NaOH (pH 5.5), 1 mM $CaCl_2$ at a final concentration of 3×10^{-4} M and warmed to 37°C. 50 μl of the phospholipase C preparation was added, incubated for 10 min and stopped either by a neutral solvent extraction, 1.8 ml chloroform/methanol (1:2, v/v), followed by 0.6 ml chloroform and 0.6 ml H_2O; or acidic solvent extraction, 2.5 ml chloroform/methanol/conc. HCl (50:50:0.3, by vol.), the phases then being separated by the addition of 0.75 ml of 1 mM EDTA in 0.1 M HCl. The upper aqueous phase was removed, neutralized with NaOH and 100 μl of 50 mM mannitol added before it was freeze-dried. The sample was made up in 1 ml water (acid extraction) or 1 ml of 1 mM EDTA (neutral extraction) and analysed by HPLC.

2.3. *HPLC standards*

[2-^{3}H]GroPIns was prepared by alkaline hydrolysis [17] of [2-^{3}H]PtdIns extracted [18] from [2-^{3}H]inositol-labelled disaggregated liver cells and purified by DE-52 column chromatography using 1 g Folch fraction I as a carrier [19].

A D/L mixture of Ins 1,2>P was prepared using 10 mg D-*myo*-Ins 2P (dicyclohexylamine form) and L-*myo*-[^{14}C]Ins 1P (0.25 μCi) as starting materials. A 3 h reaction time was used, following a preliminary time course which indicated no appreciable increase in formation of product after this time [20]. The Ins 1,2>P product was purified on Partisil SAX-10 by the same gradient system described for analysis of inositol monophosphates, rather than the recrystallization method in [20]. The identity of the product was verified by descending paper chromatography [21] of both acid-treated and untreated standards. Each lane was cut into 2 cm strips and the radioactivity determined. The acid-treated material co-migrated with Ins 1P and Ins 2P while the untreated material ran with similar mobility to GroPIns [21].

2.4. *HPLC analysis*

All HPLC analysis was carried out on a 15 × 0.5 cm column of Partisil SAX-10 preceded by a 5 × 0.5 cm guard column. A flow rate of 1.3 ml/min and a 1 min fraction collection interval was used routinely. The sample was loaded in a final volume of 2 ml, made up to this volume with 1 ml of 1 mM EDTA containing cAMP and AMP (0.05 mg/ml). The routine spiking of the samples with these adenine nucleotides, which co-eluted with Ins 1,2>P and Ins 1P respectively, enabled assessment of the elution profile by following their absorbance at 254 nm. The inositol phosphates were eluted initially for 5 min with water to remove any inositol. This was followed by a 24 min linear gradient, 0–300 mM ammonium

formate buffered to pH 5.0 with orthophosphoric acid. The eluting buffer was maintained at 300 mM ammonium formate (pH 5.0) for a further 6 min before returning to water over a 2 min period.

3. RESULTS

Chromatography of peak II (obtained following gel filtration) on cellulose phosphate (fig.1) provided a rapid and efficient method for the resolution of two further species of phospholipase C from the lymphocyte, designated PLC A and PLC B. Both species displayed approximately equal proportions of activity and each retained its elution characteristics if re-loaded onto a fresh cellulose phosphate column, PLC B having previously been re-equilibrated with the column buffer. Overall, a 47-fold purification of PLC A and 167-fold purification for PLC B were achieved by this two-step purification method. These two species were used in the subsequent experiments to investigate the nature of inositol phosphates produced following their action on PtdIns.

The HPLC elution conditions used here resulted in successful resolution of GroPIns, Ins 1,2>P and Ins 1P, in all cases a baseline separation being achieved. Fig.2a,b shows the production of both Ins 1P and Ins 1,2>P with each species of phospholipase C. PLC A produces predominantly

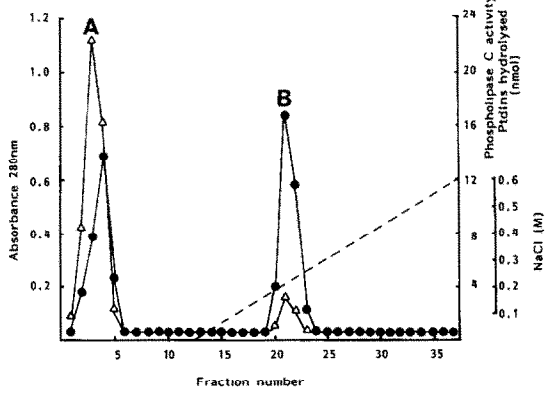

Fig.1. Cellulose phosphate column chromatography of Sephacryl S-200 fractionated phospholipase C. (△) Absorbance at 280 nm. (●) Phospholipase C activity assayed at pH 5.5 as described in section 2. The broken line represents the NaCl eluting concentration. (A) Peak containing PLC A, (B) peak containing PLC B.

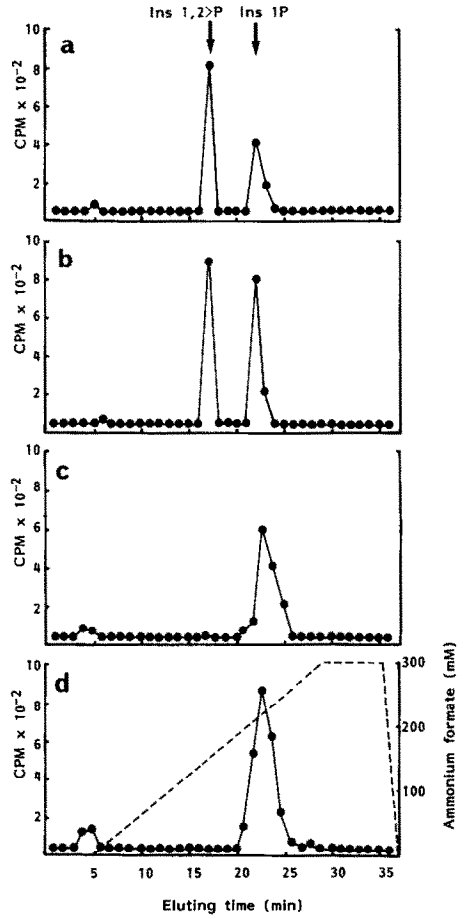

Fig.2. Chromatography on Partisil SAX-10 of inositol phosphate products extracted under neutral and acidic conditions. HPLC profiles of (a) PLC A, neutral extraction; (b) PLC B, neutral extraction; (c) PLC A, acid extraction; (d) PLC B, acid extraction. The broken line indicates the ammonium formate elution gradient.

the cyclic monophosphate while PLC B produces these two isomers in approximately equal amounts. The percentage ratios of these two products showed remarkably little variation between experimental samples (table 1). When the incubation of PtdIns with the two species of phospholipase C was performed with extraction of the products under acidic conditions, in both cases the first peak was abolished and the second peak increased by an amount approximating to the loss of the first peak (fig.2c,d). This confirms that the first peak was indeed Ins 1,2>P and the broader second peak resulting from acidic extraction condi-

Table 1

Water-soluble products released following hydrolysis of phosphatidylinositol catalysed by phospholipase C

Enzyme	% Ins 1,2>P	% Ins 1P
PLC A	64.9 ± 1.35	34.7 ± 1.24
PLC B	51.8 ± 0.55	48.1 ± 0.54

Phospholipase C assays contained 3×10^{-4} M PtdIns in 50 mM maleate-NaOH buffer (pH 5.5) containing 1×10^{-3} M $CaCl_2$ buffered with EDTA. The assay was carried out at 37°C for 10 min and stopped by the neutral solvent extraction. The percentage values expressed here are a means ± SE of the results obtained from a total of three separate experiments, on each occasion using a sample of freshly prepared phospholipase C. The total number of samples for each value was 7

tions represents an isomeric mixture of Ins 1P and Ins 2P.

When Ins 1P and Ins 1,2>P were incubated with samples of phospholipase C species PLC A and PLC B, they maintained their original elution characteristics indicating an absence of any contaminating Ins 1,2>P hydrolase or Ins 1P cyclase type activities. The Ins 1,2>P was purified using the HPLC technique developed here and its identity was confirmed by descending paper chromatography. If a sample was reloaded on the HPLC system it ran as a single peak demonstrating that the elution conditions used (pH 5.0) were not sufficiently acidic to cause any hydrolysis of the acid-labile cyclic compound.

4. DISCUSSION

These results indicate that the production by phospholipase C of two products, Ins 1P and Ins 1,2>P, is an integral property of the enzyme mechanism and that differences in the mechanisms of different iso-enzymes can lead to the formation of specific proportions of each product. It is clear that the varying proportions of the two products liberated by the different iso-enzymes are not due to different degrees of contamination by enzymes which hydrolyse the cyclic compound or cyclise the non-cyclic forms and that this is an enzyme which produces two products from a single substrate. The proportion of the two products is specific for each enzyme species. The two enzymes that have been purified from sheep seminal vesicles also synthesize different proportions of cyclic products [5], the mean for PLC-I being 68% Ins 1,2>P and for PLC-II 45% Ins 1,2>P. However, using a mass spectrometric technique following ^{18}O labelling, these workers found a greater variation in the relative amounts of cyclic and non-cyclic product than we have found using HPLC of the products formed. It is the significant difference in the proportions of Ins 1,2>P and Ins 1P produced by these two enzyme species, resulting from the very small standard deviations obtained from enzymes prepared on different occasions, that prompts us to make the suggestion that this ratio is an intrinsic property of the individual iso-enzyme mechanism. The physiological significance of this finding remains to be determined.

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