Bovine testis and human erythrocytes contain different subtypes of membrane-associated $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphomonoesterases

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1. We have purified membrane-associated $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatases from bovine testis and human erythrocytes by chromatography on several media, including a novel 2,3-bisphosphoglycerate affinity column. 2. The enzymes have apparent molecular masses of 42 kDa (testis) and 70 kDa (erythrocyte), as determined by SDS/PAGE, and affinities for $\text{Ins}(1,4,5)\text{P}_3$ of 14 μM and 22 μM respectively. 3. The two enzymes hydrolyse both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ and are therefore type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatases [nomenclature of Hansen, Johanson, Williamson and Williamson (1987) *J. Biol. Chem.* **262**, 17319–17326]. 4. On chromatofocusing, the partially purified testicular enzyme migrates as two peaks of activity, with

pI values of about 5.8 and 5.5. The erythrocyte enzyme exhibits only the latter peak. 5. The testis 5-phosphatase is labile at 37 °C, but its activity can be maintained in the presence of 50 mM phorbol dibutyrate (PdBu). After PdBu treatment, a third form of the enzyme, with pI about 6.2, appears on chromatofocusing, but without change in its K_m or V_{max} . 6. Consideration of the properties of these enzymes and of the 5-phosphatases from other tissues suggests that type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatases are of two well-defined subtypes. We propose that these be termed type Ia [typified by the testis enzyme: ~40 kDa, higher affinity for $\text{Ins}(1,4,5)\text{P}_3$] and Type Ib [typified by the erythrocyte enzyme: ~70 kDa, lower affinity for $\text{Ins}(1,4,5)\text{P}_3$].

INTRODUCTION

The 5-specific dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ inactivates these intracellular messenger molecules and is essential for the provision of free inositol for inositol lipid re-synthesis (Streb et al., 1983; Irvine et al., 1984; Shears, 1992; Hughes and Michell, 1993). $\text{Ins}(1,3,4)\text{P}_3$, the product of 5-phosphatase attack on $\text{Ins}(1,3,4,5)\text{P}_4$, is also required for the synthesis of the relatively abundant and more highly phosphorylated inositol polyphosphates, $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 , which are themselves precursors of InsP_7 and InsP_8 (Shears et al., 1987b; Stephens et al., 1988, 1993; Pittet et al., 1989; Bunce et al., 1993; Menniti et al., 1993; reviewed by Hughes and Michell, 1993).

Analysis of the 5-phosphatase activity from the soluble fractions of a variety of tissues [e.g. brain (Hansen et al., 1987; Erneux et al., 1989), liver (Shears et al., 1987a; Takimoto et al., 1989), platelets (Connolly et al., 1985, 1986; Mitchell et al., 1989), neutrophils (Kennedy et al., 1990)] has identified two isoforms of the 5-phosphatase activity with different biochemical properties. These were originally termed Type I and Type II by Hansen et al. (1987). Type I 5-phosphatases metabolize both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, and the two substrates act as mutually competitive inhibitors when metabolized by a purified Type I enzyme (Connolly et al., 1987). Type II 5-phosphatase activities are kinetically restrained from metabolizing $\text{Ins}(1,3,4,5)\text{P}_4$ *in vivo* ($K_m > 130 \mu\text{M}$), so they seem likely to act exclusively as $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatases (Hansen et al., 1987; Erneux et al., 1989; Takimoto et al., 1989). The 5-phosphatase isoforms have different pI profiles (Takimoto et al., 1989) and molecular

masses, with Type I 5-phosphatases generally smaller than Type II (Hansen et al., 1987; Erneux et al., 1989; and see Shears, 1992). An antibody to a purified soluble Type I 5-phosphatase precipitated both the soluble and particulate Type I activities from various tissues, but did not cross-react with any Type II enzymes (Verjans et al., 1990).

Most experimental work has been on the soluble 5-phosphatase activities, although the enzyme is predominantly membrane-associated in most cells, particularly at the plasma membrane (Downes et al., 1982; Shears et al., 1988; Rubiera et al., 1990; Estrada-Garcia et al., 1991). There is some evidence that the plasma-membrane 5-phosphatase activity of hepatocytes and enterocytes may be specifically localized to particular membrane domains in these polarized cells (Shears et al., 1988; Rubiera et al., 1990). Studies of the membrane-bound 5-phosphatase activity from liver has identified only a Type I isoform which metabolizes both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (Shears et al., 1987a, 1988; Takimoto et al., 1989).

Changes in the activity of $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase, presumably leading to changed patterns of cell signalling, have been implicated in at least two pathological conditions. Firstly, the 5-phosphatase activity of HIV (human immunodeficiency virus)-infected T-helper cells is dramatically reduced during the progression of AIDS (acquired immunodeficiency syndrome) (Nye et al., 1989, 1991, 1992). $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase and $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase activities were also diminished during the later stages of the disease (Nye et al., 1992). Secondly, Attree et al. (1992) have recently localized the genetic lesion in Lowe's oculocerebrorenal syndrome (OCRL) to a gene which encodes a protein with sequence homology to the

Abbreviations used: PdBu, phorbol dibutyrate; InsP_n (and equivalent abbreviations with added locants), *myo*-inositol phosphates, numbered by reference to *D*-*myo*-inositol 1-phosphate as Ins1P [see Biochem. J. (1989) **258**, 1–2].

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75 kDa soluble 5-phosphatase of platelets (Ross et al., 1991). Ross et al. (1991) have termed this platelet enzyme a 'Type II' activity, although its substrate specificity clearly identifies it as Type I (Hansen et al., 1987). Irvine (1992) has pointed out that the molecular mass of the protein encoded by the OCRL gene (112 kDa) is similar to that of reported Type II enzymes (Hansen et al., 1987; Erneux et al., 1989), thus suggesting a role for the $\text{Ins}(1,4,5)\text{P}_3$ -specific, type II activity in the aetiology of OCRL.

We now describe the purification of membrane-associated 5-phosphatase activities from human erythrocytes, the cell in which the Type I enzyme was first identified (Downes et al., 1982), and from bovine testis, which is a particularly rich source of this and other inositol phosphate-metabolizing enzymes (Hodgkin et al., 1991). A number of mechanisms that might regulate 5-phosphatase activity have previously been suggested. These have included possible roles for protein kinase C (Connolly et al., 1986; King and Rittenhouse, 1989; Verjans et al., 1991), glucose metabolites such as 2,3-bisphosphoglycerate (Downes et al., 1982) and polyamines (Seyfred et al., 1984). We have also examined possible regulatory mechanisms that may influence enzyme activity *in vivo*.

MATERIALS AND METHODS

Materials

$[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (1–2 Ci/mmol; potassium salt) was from Amersham International (Amersham, Bucks., U.K.). $[2\text{-}^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ (17 Ci/mmol; potassium salt) was from New England Nuclear (Boston, MA, U.S.A.). $\text{Ins}(1,4,5)\text{P}_3$ (trilithium salt) and saponin were from Calbiochem/Novabiochem (Nottingham, U.K.). $\text{Ins}(1,3,4,5)\text{P}_4$ (hepta-ammonium salt) was obtained from Boehringer Mannheim (BCL, East Lewes, Sussex, U.K.). Dowex AG1-X8 (200–400 mesh; formate form), protein assay reagent and SDS/PAGE molecular-mass markers were obtained from Bio-Rad (Hemel Hempstead, Herts., U.K.). Partisil 10-SAX columns (4.6 mm $\times$ 250 mm) were from Thames Chromatography (Maidenhead, Berks., U.K.). Pellicular anion-exchange resin was from Whatman (Maidstone, Kent, U.K.).

Cellulose phosphate (fibrous form), Cibacron 3GA Blue-agarose, heparin-agarose (type II), Nonidet P40, phorbol 12,13-dibutyrate (PdBu), Polybuffer 74 and poly(ethylene glycol) (8 kDa) were from Sigma Chemical Co. (Poole, Dorset, U.K.). Thesit and a detergent screening kit were obtained from Boehringer Mannheim. Biochemical-grade SDS was from BDH Chemicals (Poole, Dorset, U.K.). Hydroxyapatite beads (0.08–0.2 mm) were from Fluka (Glossop, Derbyshire, U.K.). A hydroxyapatite column (4.6 mm $\times$ 120 mm; coral form) was from Anachem (Luton, Beds., U.K.). Mono P, Superdex 75 and PD-10 columns were obtained from Pharmacia LKB Biotechnology (Milton Keynes, Bucks., U.K.). A 2,3-bisphosphoglycerate-agarose column was synthesized by standard techniques in collaboration with Pierce Chemicals (Rockford, IL, U.S.A.). Briefly, 200 mg of D-2,3-bisphosphoglycerate was linked to 20 ml of agarose via a spacer of diaminodipropylamine. Ultrafree-MC protein-concentrator units with a nominal molecular-mass cut-off of 10 kDa were from Millipore (Watford, Herts., U.K.). Okadaic acid was generously given by Professor P. Cohen (University of Dundee).

Enzyme assays and analysis of products

$\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase activity was assayed under first-order conditions in column fractions generated during

purification procedures. Incubations contained 10000 d.p.m. of $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ or $[2\text{-}^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ in 500 μl of isotonic assay buffer [20 mM Hepes/KOH (pH 7.2), 103 mM KCl, 10 mM NaCl, 5 mM MgSO_4 , 1 mM EDTA, 0.2 mg/ml saponin and 0.1% (w/v) Nonidet P40]. After incubation at 37 °C, reactions were quenched by addition of ice-cold HClO_4 (final concn. 5%, w/v) and incubated on ice for 15 min. Acid extracts were neutralized, and products and reactants were separated by Dowex chromatography (Estrada-Garcia et al., 1991).

During purification of the $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase, specific radioactivities were determined with a fixed concentration of $\text{Ins}(1,4,5)\text{P}_3$ {50 μM , containing 10000 d.p.m. of $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ }. For the estimation of kinetic parameters for the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$, reactions contained 1–150 μM $\text{Ins}(1,4,5)\text{P}_3$ {containing 10000 d.p.m. of $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ } or 0.1–10 μM $\text{Ins}(1,3,4,5)\text{P}_4$ {containing 10000 d.p.m. of $[2\text{-}^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ }. Incubations were in a final volume of 100 μl , and incubation periods were adjusted such that < 15% of the substrate was metabolized.

When $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ metabolism was to be analysed by h.p.l.c., incubations contained $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$ at the indicated concentration, plus 44000 d.p.m. of the respective tritiated inositol phosphate, in a volume of 100 μl . Reactions were terminated by addition of ice-cold HClO_4 (see above), and the acid extracts were centrifuged at 15000 g for 5 min. The extracts were neutralized with a solution containing 2 M KOH, 0.1 M Mes and 0.25 M EDTA, and the tubes were incubated for 60 min at 4 °C before centrifugation at 15000 g for 5 min. The neutralized supernatants were diluted to 1.015 ml with h.p.l.c.-grade water. Inositol phosphates were separated on a Partisil 10-SAX column (6 mm $\times$ 250 mm) fitted with a pre-column (50 mm) of pellicular anion exchanger. Inositol phosphates were eluted with a non-linear gradient of $(\text{NH}_4)_2\text{HPO}_4$ (pH 3.8) that resolves several InsP_2 and InsP_3 positional isomers (Wong et al., 1992). Eluted radioactivity was quantified by on-line liquid-scintillation spectrometry using an A250 series Radiomatic counter and Flo Scint IV scintillant (Canberra Packard, Pangbourne, Berks., U.K.).

Preparation of tissue extracts

Testis

Bovine testes were obtained from a local slaughterhouse, decapsulated, cut into small sections and either used fresh or rapidly frozen in liquid N_2 . The tissue was homogenized in 4 vol. of ice-cold homogenization buffer containing 250 mM sucrose, 5 mM Tes (pH 7.2 with HCl) 5 $\mu\text{g}/\text{ml}$ antipain, 5 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ chymostatin, 5 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 $\mu\text{g}/\text{ml}$ pepstatin and 17.4 nM phenylmethanesulphonyl fluoride, by using either a top-driven glass/Teflon homogenizer (10 passes, for small amounts of tissue) or a Waring blender (full speed, 15 s, for larger samples). Homogenates were separated into soluble and particulate fractions by centrifugation at 100000 g for 60 min at 4 °C. A detergent extract was prepared from the particulate fraction by re-homogenization of a 25% (w/v) suspension in ice-cold solubilization buffer (20 mM triethanolamine, pH 7.5 with HCl, 1 mM dithiothreitol, 0.1 mM EDTA and 1% Nonidet P40) in a glass/Teflon homogenizer for 5 s, followed by incubation on ice for 30 min. Detergent-insoluble material was removed by centrifugation at 100000 g for 30 min at 4 °C. The supernatant, hereafter referred to as 'membrane extract', could be stored for extended periods at –80 °C with no loss of inositol phosphate phosphatase activities. Solubilized membrane extracts made with other detergents were prepared in the same way.

Erythrocytes

Human erythrocytes, obtained from a local blood bank, were washed twice as a 25% (v/v) suspension in ice-cold buffer containing 154 mM NaCl and 2.5 mM Hepes (pH 7.2 with NaOH) by centrifugation (1000 g, 5 min, 4 °C). The washed cells were lysed by dilution with 8 vol. of ice-cold lysis buffer (20 mM Tris/HCl, pH 7.5) for 5 min at 4 °C, and the membranes were pelleted by centrifugation (20000 g, 40 min). Essentially haemoglobin-free membranes were obtained after 6 washes in 20 mM Tris/HCl (pH 7.5) and were then given a final wash in 20 mM triethanolamine (pH 7.5 with HCl)/0.1 mM EDTA/1 mM dithiothreitol. Membrane proteins were extracted from these erythrocyte ghosts as described for the testis. The erythrocyte membranes could be stored at -20 °C, before or after solubilization, without loss of phosphatase activity.

Purification of membrane-association $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatases from testis and erythrocytes

Testis

Testicular extracts, supplemented with 5 mM MgCl_2 , were incubated with hydroxyapatite beads overnight, and bound material, including 5-phosphatase activity, was recovered by washing the beads in buffer A (20 mM triethanolamine/HCl, pH 7.5, 5 mM MgCl_2 , 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Nonidet P40) supplemented with 0.6 M KH_2PO_4 (pH 7.4 with KOH). Phosphate was removed by overnight dialysis against buffer A, and the extract was concentrated 7-fold by incubation with dry poly(ethylene glycol) (8 kDa) for 6 h at 4 °C. The concentrate was applied to a phosphocellulose column (1.6 cm $\times$ 25 cm) equilibrated in buffer A, which was eluted with a linear gradient of KH_2PO_4 (pH 7.4 with KOH; 0.175–0.5 M over 210 ml) in buffer A. Fractions (6 ml) that contained 5-phosphatase activity were pooled and dialysed as above. The non-diffusible material was applied to a heparin-agarose column (1.0 cm $\times$ 25 cm) equilibrated in buffer A and eluted with a linear gradient of KCl (pH 7.4 with HCl; 0–0.5 M over 56 ml) in buffer A. Fractions (2 ml) that contained 5-phosphatase activity were re-equilibrated with buffer A on PD-10 Sephadex G-25 mini-columns and applied to a Cibacron Blue 3GA-agarose column (1.0 cm $\times$ 3.0 cm), which was eluted with a linear gradient of KCl in buffer A (0–0.75 M over 20 ml). Fractions containing 5-phosphatase activity were re-equilibrated with buffer A, pooled and applied to a Mono P chromatofocusing column (HR5/20) equilibrated in 20 mM imidazole (pH 7.5 with HCl) containing 1 mM dithiothreitol and 0.25% Nonidet P40. Bound material was eluted isocratically with 10% (v/v) Polybuffer 74 (pH 4.0 with HCl) containing 1 mM dithiothreitol, 0.25% Nonidet P40 and 10 mM KCl. This procedure achieves the automatic formation of a gradient of decreasing pH, and proteins bound to the anion-exchange column are eluted at their isoelectric points. Fractions that contained 5-phosphatase activity were pooled and applied to the Cibacron Blue-agarose column which was eluted as described above to remove the polybuffer.

After desalting, 5-phosphatase activity was applied to an h.p.l.c. hydroxyapatite column (0.5 cm $\times$ 12.5 cm) linked to a f.p.l.c. column and equilibrated with buffer B [20 mM triethanolamine/HCl, pH 7.5, 1 mM dithiothreitol, 5 mM MgCl_2 , 0.25% (w/v) Thesit, 10 mM KH_2PO_4]. This column was eluted with a linear gradient of KH_2PO_4 (0–0.49 M over 24 ml) in buffer B: this procedure exchanged Nonidet P40, which offered better properties for the extraction and chromatographic procedures described thus far, for the alternative

detergent Thesit, which does not absorb at 280 nm or interfere with gel-filtration or protein-sequencing techniques.

The fractions containing 5-phosphatase activity were pooled, concentrated to 200 μl with a centrifugal micro-concentrator and applied to Superdex-75 gel-filtration column (1.0 cm $\times$ 30 cm) equilibrated with buffer B containing 0.25 M NaCl. The column was eluted with the same buffer at 0.5 ml/min, and 0.5 ml fractions were collected. Molecular-mass standards for the gel-filtration column were run individually.

SDS/PAGE of samples at various stages during the purification was performed on a 'Mighty Small' apparatus (Hoefer) essentially as described by Laemmli (1970). Small samples of peak fractions were lyophilized and suspended directly in Laemmli sample buffer. Gels were stained using ammoniacal silver.

Erythrocytes

A Nonidet P40-solubilized extract of human erythrocyte membranes (6.5 mg of protein/ml) in buffer C (20 mM triethanolamine/HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Nonidet P40), was incubated overnight with 100 ml of Trisacryl M anion-exchange resin, and 5-phosphatase activity was eluted batchwise with 0.2 M NaCl in buffer C. The fraction containing 5-phosphatase activity was dialysed overnight against buffer C, applied to a Mono Q (HR10/10) anion-exchange f.p.l.c. column, and bound material was eluted in a non-linear gradient of NaCl in buffer C (0–0.2 M over 120 ml, then 0.2–0.5 M over 120 ml). The 5-phosphatase activity was concentrated by dialysis against dry poly(ethylene glycol), desalted into buffer D (20 mM triethanolamine/HCl, pH 7.5, 1 mM dithiothreitol, 0.1% Nonidet P40) on PD-10 Sephadex G-25M pre-packed columns and applied to a hydroxyapatite column (1.6 cm $\times$ 35 cm). Bound material was eluted with a linear gradient of $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (0–0.5 M over 200 ml) in buffer D and desalted as described above. Size-exclusion chromatography of the erythrocyte 5-phosphatase on a Sephacryl S-200 column (1.6 cm $\times$ 56 cm) was performed in buffer C containing 100 mM NaCl. The pooled 5-phosphatase activity was desalted into buffer D containing 1 mM MgCl_2 and applied to a 2,3-bisphosphoglycerate-agarose column (1 cm $\times$ 3 cm). The 5-phosphatase activity was eluted with a linear gradient of EDTA (0–20 mM) in buffer D. Active fractions were desalted and applied to a Mono P HR5/20 column, which was eluted with Polybuffer 74 as described for the testis enzyme. Polybuffer was removed by applying fractions that contained 5-phosphatase activity to a heparin-agarose column (1 cm $\times$ 6 cm), and eluting with a linear gradient of 0–0.5 M NaCl in buffer C.

SDS/PAGE was performed as described for the testis enzyme, except that samples were concentrated by using Millipore Ultrafree-MC micro-concentrators to a final volume of 10 μl before being taken up in Laemmli sample buffer.

RESULTS

Comparison of the characteristics of the membrane-associated 5-phosphatase activities from bovine testis and human erythrocytes

Apparent molecular mass

The apparent molecular masses of the $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatases were determined by size-exclusion chromatography of membrane extracts. Sodium cholate (7.5%, w/v) was used to solubilize 5-phosphatase activity before gel filtration in 1.5% cholate, since this detergent contributed least to the apparent molecular mass of the soluble bovine brain Type 1 5-phosphatase (Erneux et al., 1989). The testicular and erythrocyte

Figure 1 Gel filtration of cholate-solubilized $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase from bovine testis and human erythrocytes

Membrane-associated $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatases from (a) bovine testis or (b) human erythrocytes were solubilized with sodium cholate, applied to a Superose 12 column linked to an f.p.l.c. system, and 5-phosphatase activity was assayed in 0.5 ml fractions as described in the Materials and methods section. Standards of known molecular mass were chromatographed, in the presence or absence of sodium cholate, without effect on their retention times (thyroglobulin, 667 kDa; IgG, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; vitamin B_{12} , 1.35 kDa). Results are from single preparations, typical of three in each case.

enzymes had apparent molecular masses of ~ 45 kDa and ~ 70 kDa respectively (Figure 1).

Detergent solubilization

Both cholate and CHAPS efficiently solubilized 5-phosphatases from testis and erythrocytes, but these detergents were impractical for extended purification procedures: the charged nature of cholate means that it binds to anion-exchange media, and CHAPS has an impractically high critical micellar concentration. Hence the non-ionic detergent Nonidet P40, which also solubilized both of the membrane-bound 5-phosphatase activities, was selected for use.

Isoelectric points

The pI values of the 5-phosphatases were determined by chromatofocusing of Nonidet P40 testicular extracts. The 5-phosphatase activity of these extracts was consistently resolved into two distinct peaks of pI ~ 5.8 and ~ 5.5 (Figure 2a), as was the partially purified testicular 5-phosphatase (after phosphocellulose chromatography) and a Nonidet P40 extract of rat

Figure 2 Chromatofocusing of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase from bovine testis and human erythrocytes

Nonidet P40 extracts of the particulate fractions from (a) bovine testis or (b) human erythrocytes were applied to a Mono P chromatofocusing column and eluted with Polybuffer 74. The pH of the eluates was monitored (∇), and 5-phosphatase activity (testis, $\bullet$; erythrocyte, $\circ$) was assayed in 1 ml fractions. The values given by individual peaks of enzyme activity denote the pH ranges over which they were eluted. Results are from single experiments, and are typical of those observed with 12 (testis) or 5 (erythrocyte) separate preparations.

brain (results not shown). In similar experiments, the erythrocyte 5-phosphatase activity was consistently eluted as a single peak of pI ~ 5.5 (Figure 2b).

The kinetic parameters for the dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (in the presence of Polybuffer 74) by the two peaks of activity resolved by chromatofocusing testicular extracts were identical (results not shown).

Maintenance of 5-phosphatase activity by phorbol esters

When testicular homogenates were incubated at 37°C for 1 h before detergent extraction, 5-phosphatase activity against $\text{Ins}(1,4,5)\text{P}_3$ was decreased as compared with that derived from homogenates which had been maintained on ice (Figure 3). This decrease in enzyme activity was prevented by the inclusion of 50 nM PdBu in the incubation medium: the K_m and V_{max} of the detergent-extracted 5-phosphatase from homogenates which had been PdBu -treated were not significantly different from those of untreated enzyme maintained on ice (Figure 3). Figure 4 shows that phorbol ester treatment did not simply achieve a stabilization of the enzyme, since it resulted in the appearance of a third peak of activity on chromatofocusing, with pI ~ 6.2 .

Analysis of the metabolism of $\text{Ins}(1,3,4,5)\text{P}_4$ by detergent extracts of testis membranes is complicated by the presence of

Figure 3 Influence of temperature and PdBu on the kinetic behaviour of testicular $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase and $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase

Bovine testicular homogenates were maintained for 1 h on ice (○), at 37 °C (●) or at 37 °C in the presence of 50 nM PdBu (▼). Membrane-associated material was sedimented, extracted with Nonidet P40, and enzyme assays were initiated with the addition of 1–150 μM $[\text{}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ containing 10000 d.p.m. as described in the Materials and methods section. The vehicle in which PdBu was dissolved (1% dimethyl sulfoxide) had no effect on 5-phosphatase activity. Results are means ($\pm$ S.E.M.) of triplicate determinations from a single experiment, typical of three. The inset shows the metabolism of $\text{Ins}(1,3,4,5)\text{P}_4$ by 3- and 5-phosphatases (p'tase) in Nonidet P40 extracts of bovine testis membranes which were maintained for 1 h at 37 °C (●, ■) or 0 °C (○, □). The 0 °C samples were rapidly warmed to 37 °C, and 2 μM $[\text{}^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ was added to all incubations. Inositol phosphates were separated by h.p.l.c. on a Partisil 10 SAX column as described in the Materials and methods section. Individual plots show 3-phosphatase products [$\text{Ins}(1,4,5)\text{P}_3 + \text{Ins}(1,4)\text{P}_2$; □, ■] and the 5-phosphatase product [$\text{Ins}(1,3,4)\text{P}_3$; ○, ●]. Results are means ($\pm$ range) of duplicate determinations from one experiment, and are representative of those obtained with three different enzyme preparations.

Figure 4 Influence of PdBu on the pI values of testicular $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity

Nonidet P40 extracts of particulate fractions from bovine testicular homogenates which had been treated with PdBu as described for Figure 3 were subjected to chromatofocusing as described for Figure 2. Eluate pH (▽) and $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity (●) were determined as described for Figure 2. Results are from a single experiment, typical of three.

$\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase, so meaningful kinetic data are hard to obtain. However, Figure 3(a) (inset) shows that the 5-specific dephosphorylation of $\text{Ins}(1,3,4,5)\text{P}_4$, like that of

Table 1 Purification of a membrane-bound $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase from bovine testis

	Total protein (μg)	Specific activity (μmol/min per mg)	Purification factor (fold)	Recovery (%)
Extracted membranes	10 ⁶	0.025	1	100
Hydroxyapatite beads	2.4 × 10 ⁵	0.095	3.8	92
Phosphocellulose	6.5 × 10 ⁴	0.31	12.2	79
Heparin-agarose	1.4 × 10 ⁴	1.1	43	64
Cibacron Blue	5.0 × 10 ³	2.9	116	60
Mono P HR5/20	10 ³	5.5	197	20
Hydroxyapatite	50	15	600	3
Phosphocellulose	2*	175	7000	1.4

* Estimated by direct comparison of silver-stained polypeptides after analytical SDS/PAGE and the A_{280} .

$\text{Ins}(1,4,5)\text{P}_3$, was also diminished when testicular membrane extracts were incubated at 37 °C for 1 h, but that 3-phosphatase activity was unchanged. This result provides further evidence that $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ are metabolized by a single enzyme in these extracts, and also suggests that the diminished 5-phosphatase activity observed after incubation of homogenates or detergent extracts at 37 °C is not a consequence of decreased substrate availability.

Purification of type I 5-phosphatases from the particulate fractions of testis and erythrocytes

The chromatographic steps employed for the purification of the $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase were determined empirically and by reference to other published purifications of inositol phosphate-metabolizing enzymes and binding proteins (e.g. Hansen et al., 1987; Takimoto et al., 1989). There were, however, some interesting differences in the effectiveness of various media for purifying the enzymes from the two tissues studied.

Heparin inhibits both $\text{Ins}(1,4,5)\text{P}_3$ binding to its receptor (Guillemette et al., 1989; Mourey et al., 1990) and $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase activity from bovine brain (Takazawa and Erneux, 1989), but has no effect on $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity in liver membranes (Guillemette et al., 1989). Heparin-agarose has been used in the purification of the $\text{Ins}(1,4,5)\text{P}_3$ receptor, and our experiments revealed that both the erythrocyte and testis 5-phosphatases bind to this medium. However, the binding of the erythrocyte enzyme was severely affected by Polybuffer 74 on a preparative scale.

Blue dyes have previously been used in the purification of $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase and a soluble $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase from bovine brain (Erneux et al., 1989). Although this medium offered excellent characteristics for the purification of the testis 5-phosphatase and binding was unaffected by the presence of Polybuffer, it did not bind the erythrocyte enzyme (results not shown).

2,3-Bisphosphoglycerate-agarose provided a useful step in the purification of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase from erythrocytes, the tissue in which this triose phosphate was first shown to be an effective inhibitor of the enzyme (Downes et al., 1982). However, the binding of the testis enzyme to this medium was not reproducible in our hands (results not shown).

Figure 5 Purification of $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase from bovine testis

(a) Heparin-agarose chromatography. Membrane-associated 5-phosphatase activity was extracted with Nonidet P40 before chromatography on hydroxyapatite and phosphocellulose as described in the Materials and methods section. The pool of activity from the phosphocellulose column was re-equilibrated with buffer A, applied to a heparin-agarose column, and enzyme activity was eluted with a linear gradient of KCl (▽). $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity (●) and total protein (○) were assayed as described in the Materials and methods section. (b) Cibacron Blue dye chromatography. The peak of 5-phosphatase from the heparin-agarose step was equilibrated with buffer A, applied to a Cibacron Blue 3GA column and eluted with a linear gradient of KCl. (c) Preparative gel filtration and SDS/PAGE of the purified enzyme. The peak of activity from (b) was chromatographed on Mono P and hydroxyapatite columns as described in the Materials and methods section, concentrated to 200 μl and applied to a Superdex 75 column in the presence of 0.25% (w/v) Thesit. 5-Phosphatase activity was eluted as described in the text. Standard proteins of known molecular mass were run separately and eluted in the positions shown in the presence or absence of Thesit. Molecular-mass standards were:

Table 2 Purification of a membrane-bound $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase from human erythrocytes

Abbreviation: ND, not determined.

	Total protein (μg)	Specific activity ($\mu\text{mol}/\text{min}$ per mg)	Purification factor (fold)	Recovery (%)
Extracted membranes	8.7×10^5	0.0022	1	100
DEAE Trisacryl M	7.6×10^4	0.019	8.5	79
Mono-Q HR10/10	3.0×10^4	0.037	17	60
Hydroxyapatite	1.2×10^4	0.069	34	46
Sephacryl S-200	3.8×10^3	0.15	68	30
2,3-Bisphosphoglycerate	483	0.62	289	16
Mono-P HR5/20	ND	ND	ND	9
Heparin-agarose	20*	2.7	1260	2.9

* Estimated by direct comparison of silver-stained polypeptides after analytical SDS/PAGE.

Testis

The bovine testis extract was enriched 4-fold in 5-phosphatase activity after batch chromatography of membranes with hydroxyapatite beads (Table 1). After concentration and chromatography on phosphocellulose as described in the Materials and methods section, enzyme activity was eluted as a broad peak around 370 mM KH_2PO_4 . Active fractions were pooled and eluted from heparin-agarose as a single peak at about 300 mM KCl (Figure 5a). Further chromatography on Cibacron Blue-agarose again resulted in the elution of a single peak of activity (Figure 5b). Preparative chromatofocusing then yielded the characteristic two peaks of enzyme activity previously noted for the crude detergent-extracted enzyme. After removal of Polybuffer with Cibacron Blue-agarose, both peaks of activity were further purified on hydroxyapatite and Superdex 75 to yield a single peak of 5-phosphatase activity with an apparent molecular mass of ~ 40 kDa (Figure 5c). SDS/PAGE of column fractions from the Superdex 75 column expressing the highest 5-phosphatase activity showed that they contained a single major protein band at ~ 40 kDa (Figure 5c inset).

Anion-exchange h.p.l.c. of the reaction products generated by the purified testis enzyme revealed that both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were rapidly dephosphorylated in a 5-phosphate-specific manner, thereby defining the enzyme as a Type I 5-phosphatase (results not shown).

Erythrocytes

The erythrocyte 5-phosphatase was purified by the protocol described in the Materials and methods section and Table 2. This pre-dates and differs somewhat from that which we subsequently developed for the testis enzyme. Of particular interest is the use of an affinity column of immobilized D-2,3-bisphosphoglyceric acid (Figure 6a). This metabolic intermediate is a moderately potent inhibitor of several $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatases (Downes et al., 1982; Seyfred et al., 1984; Shears et al., 1987b; Mitchell et

transferrin, 75 kDa; ovalbumin, 44 kDa; carbonic anhydrase, 25 kDa; myoglobin, 17 kDa; antipain, 6 kDa. The inset shows an SDS/PAGE minigel (10% acrylamide), loaded with 10% of fraction nos. 21–23 and stained with ammoniacal silver. The migration of Bio-Rad low-molecular-mass markers is indicated by the arrows. The association of 5-phosphatase activity with a major band at ~ 40 kDa was observed in two purified enzyme preparations.

Figure 6 Purification of $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase from human erythrocyte membranes

(a) 2,3-Bisphosphoglycerate-agarose chromatography. 5-Phosphatase activity was extracted from human erythrocyte membranes with Nonidet P40 before chromatography on anion-exchange, Mono Q, and gel-filtration media as described in the Materials and methods section and Table 2. The peak of activity from the gel filtration column was loaded on a column of immobilized 2,3-bisphosphoglycerate and eluted with a gradient of EDTA. 5-Phosphatase activity (●) and total protein (○) were determined as described in the Materials and methods section. Results are from a single preparation, typical of four. (b) Heparin-agarose chromatography and SDS/PAGE of the purified enzyme. 5-Phosphatase activity eluted from the 2,3-bisphosphoglycerate column was subjected to chromatofocusing on Mono P, and the peak of activity was applied to a heparin-agarose column and eluted as described in the Materials and methods section. The inset shows an SDS/PAGE minigel (7.5% acrylamide), loaded with 5% of fraction nos. 19–23 and stained with ammoniacal silver. The migration of Bio-Rad molecular-mass markers is indicated. The association of 5-phosphatase activity with a major band at ~70 kDa was observed in two purified enzyme preparations.

al., 1989; Cooke et al., 1989). 5-Phosphatase activity bound to the affinity column only in the presence of Mg^{2+} , and it could be efficiently eluted with a linear gradient of EDTA (0–20 mM) or, less efficiently, with D-2,3-bisphosphoglycerate (0–10 mM). Fractions containing erythrocyte 5-phosphatase activity were desalted and subjected to chromatofocusing on a Mono P column. After removal of Polybuffer on heparin-agarose, SDS/PAGE analysis of the active fractions from this final stage revealed a single polypeptide species with a molecular mass of ~70 kDa (Figure 6b). This purification protocol yielded an enzyme that was purified 1260-fold with respect to the erythrocyte membrane extract with a recovery of 2.9%. The specific activity of the purified erythrocyte 5-phosphatase was considerably less than that of the testis enzyme, suggesting that these two enzymes may have different catalytic centre activities.

Analysis of the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$

revealed that the purified erythrocyte 5-phosphatase, like the testicular enzyme, specifically removed the 5-phosphate groups from both substrates (results not shown). The K_m values of the purified 5-phosphatases of erythrocytes and testis for $\text{Ins}(1,4,5)\text{P}_3$ were $22 \pm 2 \mu\text{M}$ and $14 \pm 1 \mu\text{M}$ respectively, and those for $\text{Ins}(1,3,4,5)\text{P}_4$ were $1.9 \pm 0.2 \mu\text{M}$ and $1.1 \pm 0.1 \mu\text{M}$ respectively ($P < 0.01$ by student's *t* test in both cases).

DISCUSSION

Membrane-bound 5-phosphatases appear to be all Type I enzymes

Kinetically distinct Type I and II 5-phosphatase activities have been identified and purified from the cytosol of several cell types (see the Introduction). In contrast, previous studies of the membrane-associated 5-phosphatase activities from rat liver (Shears et al., 1987a; Takimoto et al., 1989) and bovine brain (Erneux et al., 1989) have detected only Type I 5-phosphatases that actively hydrolyse both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$. The present results with purified 5-phosphatases from the particulate fractions of bovine testis and human erythrocytes confirm and extend this pattern. Thus Type II $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatases appear to be exclusively cytosolic.

The molecular mass of the membrane-associated bovine testicular $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase was ~40 kDa (as determined by both gel filtration and SDS/PAGE). This value is similar to those reported for soluble and particulate Type I 5-phosphatases from other tissues, including platelet cytosol (38–45 kDa isoform; Connolly et al., 1986), rat liver membranes (Takimoto et al., 1989), bovine brain (Erneux et al., 1989) and rabbit neutrophil cytosol (Kennedy et al., 1990).

The erythrocyte membrane 5-phosphatase is an example of a new subclass of 'Type I' enzymes

There are now two examples of Type I 5-phosphatases with apparent molecular masses different from 40 kDa. The first was the $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase isolated from platelets and termed 'Type II' by Mitchell et al. (1989). This designation was made because, unlike the platelet Type I phosphatase described by Connolly et al. (1986, 1987), the former enzyme was not a protein kinase C substrate. However, it is clear that this 75 kDa enzyme is a 5-phosphatase that readily attacks both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (Mitchell et al., 1989), and is therefore defined by its substrate-specificity as Type I (Hansen et al., 1987). The second example is the enzyme that we have now purified from human erythrocyte ghosts. This is a Type I activity which dephosphorylates both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, and it has an apparent molecular mass of ~70 kDa (Figure 1), similar to that of the platelet enzyme.

Both of the activities discussed in the preceding paragraph also have unusually low affinities ($> 20 \mu\text{M}$) for $\text{Ins}(1,4,5)\text{P}_3$ compared with the Type I 5-phosphatases of lower molecular mass ($K_m < 20 \mu\text{M}$). We therefore propose that the two higher-molecular-mass $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatases, from platelets and from erythrocytes, should be classified together as the first members of a Type Ib subclass of 5-phosphatases, to distinguish them from the widespread 40–45 kDa Type I enzymes, which we would propose be re-designated as Type Ia. This classification would recognize that Type Ib enzymes rapidly metabolize both $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5)\text{P}_3$, but also that they are kinetically and physically distinct from other previously described Type I 5-phosphatases. Type Ib 5-phosphatases have, thus far, only been described in platelets and erythrocytes,

suggesting that this type of enzyme may be restricted to cells of the haemopoietic lineage.

The existence of three distinct types of 5-phosphatase in mammalian cells is supported by previous results obtained with precipitating antisera against these enzymes. An antiserum raised to the cytosolic Type Ia $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase from bovine brain precipitated cytosolic Type I activities from rat liver and brain, human platelets, and also a partially purified membrane-associated Type I activity from bovine brain. However, this antiserum did not precipitate bovine brain Type II 5-phosphatase (Verjans et al., 1990). These data indicate that cytosolic and particulate Type Ia 5-phosphatase activities contain common antigenic determinants (Verjans et al., 1990). Conversely, an antibody raised to the 75 kDa platelet 5-phosphatase that we would designate as Type Ib (but termed 'Type II' by the authors) did not precipitate the Type Ia 5-phosphatase (45 kDa isoform) of human platelets (Ross et al., 1991). Thus it is likely that Type Ia 5-phosphatases contain conserved sequence motifs that are not shared with Type Ib or Type II enzymes.

It is to be hoped that the nature of the differences in the 5-phosphatase isoforms (Types Ia, Ib and II) will soon be resolved by sequence analysis. The cDNA sequence of the 75 kDa soluble enzyme from platelets (a Type Ib isoform) was recently published (Ross et al., 1991). The reported sequence contained consensus sequences for phosphorylation by several protein kinases, including myosin I heavy-chain kinase, cyclic-GMP-dependent protein kinase and casein kinases I and II, but not for protein kinase C.

Protein kinase C and the activity of 5-phosphatase(s)

It is not clear why the testicular Type Ia 5-phosphatase exists in two differently charged forms, with a third form appearing after treatment with PdBu , but the Type Ib erythrocyte enzyme exists as a single charged species. Takimoto et al. (1989) have analysed the pI values for soluble and particulate rat liver $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatases by sucrose-density-gradient isoelectric focusing. Although the Type I membrane-associated liver enzyme was not resolved into separate isoforms by this technique, the peak of 5-phosphatase activity was clearly non-symmetric towards the acidic side, suggesting that a mixture of differently charged forms might have been present.

In addition to revealing a third charged form of the testis 5-phosphatase, PdBu treatment protected the enzyme against loss of activity at 37°C . This behaviour may be relevant to an unresolved controversy over the role of protein kinase C in the control of 5-phosphatase activity in cells. Connolly et al. (1986) reported that a platelet 5-phosphatase was directly phosphorylated by protein kinase C and that this phosphorylation activated the enzyme. Although these observations have been repeatedly questioned (Tyers et al., 1988; Biden et al., 1988; McIntosh and McIntosh, 1990), others have produced evidence that the sustained activity of 5-phosphatase that is needed for continued hydrolysis of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ may, indeed, depend on the activation of protein kinase C that is a normal component of the inositol-lipid signalling system (King and Rittenhouse, 1989; Verjans et al., 1991).

The mechanism by which phorbol ester treatment of homogenates stabilizes the Type Ia activity at 37°C and generates a new charged form seems unlikely to involve direct phosphorylation by protein kinase C, since the new form is less negatively charged than its progenitors, rather than having the more negative character that would come from addition of one or more phosphate groups (see Figure 4). Rather, protein kinase C may regulate the 5-phosphatase indirectly by promoting a

different form of covalent change, perhaps a dephosphorylation, that is catalysed by another protein kinase C-regulated enzyme. It is worthy of note, however, that treatment of testis homogenates with 50 mM okadaic acid had no effect on the activity profile of this enzyme when analysed by chromatofocusing (results not shown).

During the final stages of preparation of this paper, Laxminarayan et al. (1993) published the purification of a 43 kDa membrane-associated $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase from human placenta with essentially similar characteristics to those of the testis 5-phosphatase which we have described here. Polyclonal antibodies against the placental enzyme immunoprecipitated the platelet Type I 43 kDa soluble 5-phosphatase, but not the 75 kDa soluble enzyme from this tissue. The placental enzyme should clearly be classified as a Type Ia 5-phosphatase according to the scheme which we propose above. Laxminarayan et al. (1993) did not publish any details of the behaviour of the placental enzyme on chromatofocusing, so it remains to be seen if this 5-phosphatase, like the testis enzyme, exists as multiple charged forms.

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