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HUMAN ERYTHROCYTE CYTOSOL PHOSPHATIDYL-INOSITOL-BISPHOSPHATE PHOSPHATASE

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A phosphatidyl-*myo*-inositol-4,5-bisphosphate phosphohydrolase (phosphatidyl-inositol-bisphosphate phosphatase, EC 3.1.3.36) was detected in human erythrocytes and partially purified from the cytosol. Hemoglobin was removed by $(NH_4)_2SO_4$ fractionation and chromatography on CM-Sepharose CL-6B. A 27 000-fold purification was achieved following gel filtration, ion-exchange chromatography and hydrophobic chromatography. Although the preparation was not homogeneous, the molecular mass of the enzyme was estimated to be 105 000 by gel filtration. The activity was stabilized by a non-ionic detergent (Triton X-100). The enzyme was active with PI-P_2 and, to a lesser extent, *myo*-inositol 1,4,5-trisphosphate but not with PI-P nor a variety of other lipid and non-lipid phosphate esters. In the presence of both cationic and non-ionic detergents, the effects of divalent cations were independent of substrate concentration. Mg^{2+} was required ('apparent' K_m = 12 μM). The 'apparent' K_m for the substrate was 0.27 mM and the specific activity was 765 ± 191 (S.D.) nmol/min per mg protein. Inhibition by Ca^{2+} ('apparent' K_i = 50 μM) was competitive with Mg^{2+}. Neomycin was an inhibitor at 10^{-6}–10^{-4} M but only in the absence of Triton X-100. The phosphatase was inhibited by hemoglobin at concentrations higher than 1% (w/v) and by agents which react with sulfhydryl groups, but was unaffected by dithioerythritol and F^-.

Introduction

PI-P and PI-P_2 are metabolically active but quantitatively minor components of the plasma membranes from a variety of mammalian cells and eukaryotic microorganisms [1]. Synthesis by the step-wise phosphorylation of PI is catalysed by specific kinases. In erythrocytes the kinases are located on the cytoplasmic surface of the plasma membrane [2,3]. Degradation is by specific phosphatases and/or phosphodiesterases.

A Ca^{2+}-activated phosphatidyl-inositol phosphate phosphodiesterase was described in rabbit and human erythrocyte membranes [4,5]. However, it seems unlikely that the hydrolysis of PI-P and PI-P_2 by the diesterase would occur in normal erythrocytes. Perturbed phosphoinositide metabolism in ionophore-treated human erythrocytes has been observed at Ca^{2+} concentrations above 10 μM [6], but the intracellular concentration is lower than this [7]. Furthermore, it is generally believed that mature erythrocytes can neither synthesize the precursor PI [8] nor obtain it from plasma lipoproteins [9]. Absence of ^{32}P labelling in the diester phosphate of all the phosphoinositides indicates that resynthesis from phosphatidic acid does not occur [6,10]. Therefore, erythrocytes could not replace PI-P and PI-P_2 once the

Abbreviations: PI, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol; PI-P, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol 4-phosphate; PI-P_2, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol 4,5-bis(phosphate); CTAB, cetyltrimethylammonium bromide; Hepes, *N*-2-hydroxyethylpiperazine-*N'* 2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

meagre supply of precursor in the membrane was depleted. Irreversible loss of PI-P and PI-P_2 may occur in aging [11] or energy depleted erythrocytes which accumulate Ca^{2+} (30–70 μM) or in abnormal erythrocytes which exhibit increased permeability to Ca^{2+} (110–300 μM in homozygous sickle cell disease [7]). Altered phosphoinositide metabolism has been detected in sickle cells and hereditary pyropoikilocytes [6].

It is more likely that a phosphatase acting on PI-P and PI-P_2 and the two kinases are responsible for the turnover of the monoesterified phosphate groups. Specific phosphatases which act on these lipids have been described in some detail from several mammalian tissues and a species of protozoa [12]. The enzyme has been purified to homogeneity from rat brain [13]. In spite of the indirect evidence for its presence [14,15], a similar enzyme has not been reported in erythrocytes. In this paper we describe the properties and partial purification of a phosphatidyl-inositol-bisphosphate phosphatase (phosphatidyl-*myo*-inositol-4,5-bisphosphate phosphohydrolase, EC 3.1.3.36) which is present in the cytosol of human erythrocytes.

Materials and Methods

Materials

PI-P_2 was isolated from bovine brain [16]. PI-P was prepared from PI-P_2 enzymatically [17]. The sodium salts of PI-P and PI-P_2 were stored in chloroform at −20°C. *myo*-Inositol-1,4-bisphosphate and *myo*-inositol-1,4,5-trisphosphate were prepared from PI-P and PI-P_2 respectively, using the *Crithidia fasciculata* triphosphoinositide phosphodiesterase [12]. Yeast PI and other phospholipids were purchased from Serdary Research Laboratories, London, Canada. Cross-linked Sepharose and its derivatives (Pharmacia Fine Chemicals, Uppsala, Sweden) were used for column chromatography. Triton X-100 and CTAB were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.

Enzymic assays

The phosphatidyl-inositol-bisphosphate phosphatase reaction mixture contained 50 mM Hepes (pH 7.2), 0.2% (w/v) Triton X-100, 0.4 mM $MgCl_2$, 0.1 mM EGTA, 1 mM PI-P_2 and 2.5 mM CTAB in a total volume of 0.15 ml. Enzyme preparations were preincubated for 10 min at 37°C with all the constituents except PI-P_2 and CTAB. After the chloroform was evaporated under N_2, the PI-P_2 was suspended in water by vigorous agitation on a vortex mixer and brief sonication. CTAB was added to the clear lipid suspension while agitating on a vortex mixer. Aliquots (0.06 ml) of the PI-P_2/CTAB mixture were used to start the reactions. After incubation at 37°C the reactions were terminated by adding 0.35 ml 5% (w/v) SDS containing 40 mM EDTA, and the inorganic phosphate was measured directly by the automated method of Hegyvary et al. [18].

Non-specific phosphatase activities were measured by observing the hydrolysis of *p*-nitrophenylphosphate at pH 4.8 in 25 mM citrate buffer at pH 7.7 in 25 mM Tris-HCl and at pH 10.5 in 50 mM glycine [19].

Analyses

Aliquots of reaction mixtures and other phospholipid samples were spotted directly on oxalate-treated Silica Gel HR plates and developed as described before [17]. Phospholipids were located with a molybdate-sulfuric acid spray reagent [20] and scraped from the thin-layer plate, and total phosphorus was determined by the method of Bartlett [21] after digestion in perchloric acid. Protein was measured by the method of Bradford [22] using the commercially prepared reagent and globulin standard (Bio-Rad Laboratories, Richmond, CA, USA). Samples containing detergent or having less than 0.2 mg protein/ml were analysed by the fluorescamine method [23] using bovine serum albumin as the standard.

Phosphatidyl-inositol-bisphosphate phosphatase preparation

The packed-cell fraction of outdated human blood (type O^+, stored in acid-citrate-dextrose) was obtained from the Red Cross Transfusion Service. Residual plasma was removed by centrifugation (4 500 × *g*, 10 min) and the cells were washed three times in 3 vol. ice-cold 0.15 M NaCl. All subsequent steps were done at 4°C. The washed cells were lysed in 21 vol. 5 mM phosphate buffer (pH 7.2) containing 10 mM NaCl and 0.05 mM EDTA [24]. The membranes were removed by centrifugation. $(NH_4)_2SO_4$

was added to the membrane-free hemolysate (4–10 l) to give 60% saturation at 0°C (360 g/l). After 1 h, the precipitated protein was recovered by centrifugation (10000 × *g*, 10 min) and redissolved in 10 mM imidazole-HCl buffer (pH 7.0) containing 25 mM NaCl (25 ml buffer per l original hemolysate). Saturated $(NH_4)_2SO_4$ solution was added to give 20% saturation. After 30 min, the precipitate was removed by centrifugation (10000 × *g*, 10 min) and discarded. More saturated $(NH_4)_2SO_4$ solution was added to give a 50%-saturated solution. The precipitate was recovered by centrifugation and resuspended in less than 5 ml 50 mM imidazole buffer (pH 7.0) containing 0.5 mM EGTA, and dialysed against the same buffer.

The dialysed 20–50% saturated $(NH_4)_2SO_4$ fraction (15–20 ml) was applied to a short, wide column (3.5 × 5.5 cm) of CM-Sepharose CL-6B prepared in 50 mM imidazole buffer, pH 7.0, containing 0.5 mM EGTA. Protein passing through the column in this buffer was recovered and concentrated to less than 5 ml by ultrafiltration in Minicon B15 disposable concentrators (Amicon Corp., Lexington, MA, U.S.A.). Adsorbed protein, including residual hemoglobin was stripped from the column with 500 mM NaCl.

A 2.5 × 85 cm column of Sepharose CL-6B was prepared in 50 mM imidazole buffer (pH 7.0) containing 100 mM NaCl, 0.5 mM $MgCl_2$ and 0.5 mM EGTA. The concentrated hemoglobin-free eluate from the CM-Sepharose was applied to the column with an on-line sample applicator SA-50 (Pharmacia, Sweden) and elution continued at 0.8 ml/min using a peristaltic pump. Fractions (4.8 ml) were monitored for protein, phosphatidyl-inositol-bisphosphate phosphatase activity and non-specific phosphatase activities. Those fractions containing the center of the phosphatidyl-inositol-bisphosphate phosphatase peak were combined (50–60 ml) and pumped onto a 1.5 × 30 cm column of DEAE-Sepharose CL-6B prepared in the same buffer. Fractions of 4.3 ml were collected at a flow rate of 0.5 ml/min. Elution with the starting buffer was continued to fraction 20 followed by a linear gradient of NaCl (100–400 mM) in the same buffer (fractions 21–80). Fractions were monitored for protein and enzymic activities.

A 0.9 × 15 cm column of Phenyl-Sepharose CL-4B was prepared in 50 mM imidazole buffer (pH 7.0) containing 0.5 mM $MgCl_2$ and 0.5 mM EGTA. The column was eluted at 0.6 ml/min. The Phenyl-Sepharose was first saturated with detergent by pumping 50 ml 4% (w/v) Triton X-100 in the imidazole buffer through the column followed by a flush (35 ml) of detergent-free buffer. The combined fractions (20–25 ml) from the DEAE-Sepharose (center of the phosphatidyl-inositol-bisphosphate phosphatase peak) were pumped onto the column with the fraction collector set for 4 min (2.3 ml) fractions. The column was then eluted with detergent-free buffer up to fraction 15, followed by 0.5% Triton X-100 in the same buffer (fractions 16–30). The eluate was assayed for phosphatidyl-inositol-bisphosphate phosphatase activity and analysed for protein by the fluorescamine method.

Electrophoresis

A mixture of phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin (Pharmacia) was used for molecular size calibration. Triton X-100 was removed from samples purified on Phenyl-Sepharose by readsorbing the protein onto DEAE-Sepharose CL-6B (0.9 × 1 cm column). The detergent was flushed away with buffer (5 ml) and the protein eluted with 0.5 M NaCl. This product was desalted on a small column (0.9 × 8 cm) of Sephadex G-25, lyophilized and redissolved in 0.1–0.2 ml electrophoresis buffer (100 mM phosphate, pH 7.2, containing 0.2% SDS). All samples were heated at 60°C for 5 min prior to electrophoresis in 6% polyacrylamide gels (0.4 × 8 cm tubes) at 6 mA/tube [25]. The gels were fixed and stained with Coomassie blue [26]. Stained gels were photographed and the negatives scanned with a densitometer.

Results

In preliminary experiments no phosphatidyl-inositol-bisphosphate phosphatase activity was observed with either intact or detergent solubilized membranes from rabbit and human erythrocytes. However, a very low activity was detected in the crude cytosol fraction. This activity was measured reliably only after the cytosol had been dialysed to remove inorganic phosphate and concentrated 25–30-fold in disposable Minicon B-15 concentrators which have an absorbant-backed membrane (Amicon Corp., Lexing-

ton, MA, U.S.A.). Ultrafiltration in pressurized systems was not successful due to the high protein concentration.

Only 1–2% of the protein was precipitated by the initial addition of $(NH_4)_2SO_4$ (60% saturation) to the crude cytosol. The supernatant contained most of the hemoglobin but no phosphatidyl-inositol-bisphosphate phosphatase activity. The redissolved precipitate, now in a much smaller volume, was fractionated again. A small amount of very red material having no phosphatidyl-inositol-bisphosphate phosphatase activity was precipitated at 20% saturated $(NH_4)_2SO_4$. Raising the concentration to 50% saturation precipitated the phosphatase activity and left more hemoglobin in the supernatant. Both EDTA and pH were important factors in limiting the precipitation of hemoglobin by $(NH_4)_2SO_4$. Much greater amounts were precipitated if the cells were hemolysed in either phosphate buffer (pH 7.4) without EDTA [27] or CO_2-saturated water (pH 5.0) [28]. Removal of hemoglobin was the most likely cause of the 2-fold increase in phosphatidyl-inositol-bisphosphate phosphatase activity observed after $(NH_4)_2SO_4$ precipitation (Table I). Hemoglobin that was recovered from the 60%-saturated $(NH_4)_2SO_4$ supernatant by precipitation at 75% saturation and further purified on DEAE-Sepharose CL-6B [29] inhibited the phosphatase. Activity was reduced more than 60% by hemoglobin concentrations comparable to those present in assays of concentrated crude cytosol fractions (6–10 mg/assay). The possibility that another inhibitor was co-purified with the hemoglobin cannot be excluded.

The proteins precipitated by 50% saturated $(NH_4)_2SO_4$ were completely dissolved after brief analysis to yield a red-brown solution. When this material was passed through a column of CM-Sepharose CL-6B most of the phosphatidyl-inositol-bisphosphate phosphatase (70% of applied activity) was not adsorbed. Some of the phosphatase activity (20%) and the residual hemoglobin adhered to the column and were always eluted together in salt gradients. In most experiments a short wide column of CM-Sepharose was used and the adsorbed material was removed with 0.5 M NaCl. The possibility that the adsorbed phosphatase was simply binding to the hemoglobin was excluded. None of the hemoglobin-free phosphatase which initially passed through could be adsorbed onto the CM-Sepharose when mixed with an excess of hemoglobin, and partial removal of hemoglobin from the adsorbed phosphatase by repeated $(NH_4)_2SO_4$ precipitation did not reduce its readsorption to the CM-Sepharose. The unadsorbed fraction was hemoglobin-free and represented a greater than 300-fold purification of the phosphatidyl-inositol-bisphosphate phosphatase activity over the crude cytosol (Table I).

Prior to gel filtration, the preparation was concentrated to a small volume (less than 5 ml) in the disposable Minicon concentrators. Less than 10% of the activity and protein was lost if the concentrator cells were carefully rinsed out. The elution profile

TABLE I

PURIFICATION OF HUMAN ERYTHROCYTE CYTOSOL PHOSPHATIDYL-INOSITOL-BISPHOSPHATE PHOSPHATASE

	% Recovery of activity at each step	Specific activity [a] nmol/min per mg protein	Purification [b] (-fold)
Cytosol	–	0.028 ± 0.006 (5)	–
60% $(NH_4)_2SO_4$ ppt.	166 ± 26 (5)	3.6 ± 0.1 (5)	129
20–50% $(NH_4)_2SO_4$ ppt.	115 ± 18 (6)	9.9 ± 2.0 (6)	354
CM-Sepharose	72 ± 10 (4)	9.1 ± 4.1 (4)	325
Sepharose peak	85 ± 14 (4)	13.1 ± 3.3 (4)	468
DEAE-Sepharose peak	62 ± 8 (5)	118 ± 23 (5)	4 210
Phenyl-Sepharose peak	54 ± 16 (3)	765 ± 191 (3)	27 300

[a] Data are mean ± S.D. (n).

[b] Based on mean specific activities.

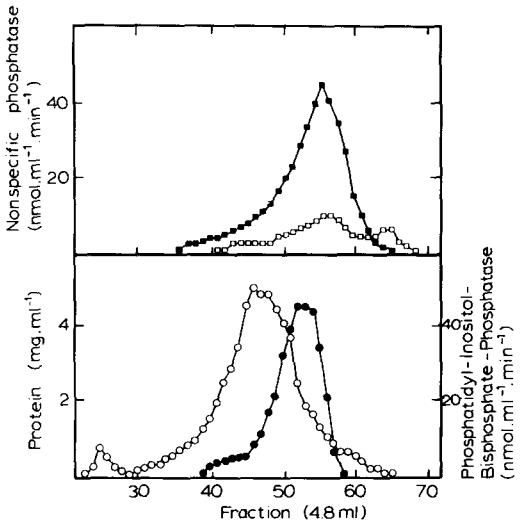


Fig. 1. Gel filtration of the hemoglobin-free concentrate on Sepharose CL-6B. Fractions were assayed for protein (○) by the Bradford method [22], for phosphatidyl-inositol-bisphosphate phosphatase activity (●) and for nonspecific phosphatase activity at pH 4.8 (□) and at pH 7.7 (■).

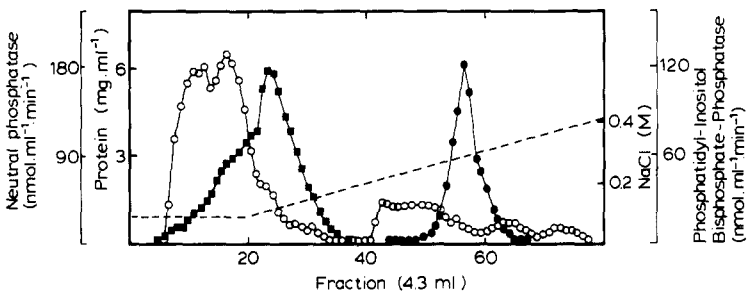
(Fig. 1) revealed a broad peak of protein covering almost the entire fractionation range of the Sepharose CL-6B. A varying quantity of aggregated protein, often appearing as a hazy precipitate, was eluted in the void volumn (Fraction 25–26). The peak of phosphatidyl-inositol-bisphosphate phosphatase activity was displaced from the center of the protein peak. The nonspecific phosphatase activity in fractions 63–67 exhibited the characteristic acid pH optimum and inhibition by copper (over 90% inhibition by 2 mM Cu^{2+}) of the erythrocyte acid phosphatase [30]. Elution at the end of the fractionation range is also consistent with the small molecular mass (44 000) reported for this enzyme [31]. The nonspecific phosphatase activity localized in fractions 52–58 was active over a wide pH range with the optimum at pH 7.5. Apparent alkaline (not shown) and acid phosphatase activities present in these fractions were attributed to the limited activity of this neutral phosphatase at pH 10.5 and 4.8, respectively. This is consistent with reports that normal human erythrocytes are devoid of alkaline phosphatase activity [32]. Up to 85% of the applied phosphatidyl-inositol-bisphosphate phosphatase was recovered from the center of the activity peak (fractions 49–56).

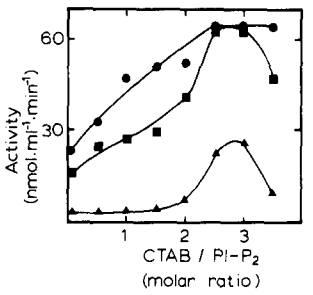
A 7–11-fold purification was achieved by chromatography on DEAE-Sepharose CL-6B. When loaded onto the column in buffered 100 mM NaCl most of the protein in the sample was not adsorbed (Fig. 2). The neutral phosphatase either passed through the column or was eluted at the beginning of the salt gradient. The phosphatidyl-inositol-bisphosphate phosphatase was eluted as a discrete peak later in the gradient by 200–250 mM NaCl. The preparation at this stage was colourless, exhibited no nonspecific phosphatase activity and represented a 4 200-fold purification over the crude cytosol. The activity was very stable when stored in the elution buffer containing NaCl. Very little activity was lost at either 4 or −20°C for up to 3 weeks.

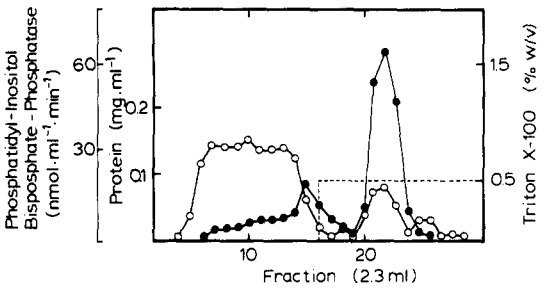
Further purification was obtained by hydrophobic chromatography on columns of detergent-saturated Phenyl-Sepharose CL-6B. Ammonyx LO (dodecyldimethylamine oxide), a detergent used in an earlier study [33] inactivated the erythrocyte phosphatidyl-inositol-bisphosphate phosphatase and was replaced by Triton X-100. When the sample from the DEAE-Sepharose column was applied to the Triton X-100-pretreated hydrophobic gel, a major portion of the protein was not adsorbed (Fig. 3). Although some activity occasionally passed through, most of the phosphatidyl-inositol-bisphosphate phosphatase was adsorbed and could be eluted with low concentrations of Triton X-100 (0.5%, w/v). In Fig. 3, the activity corresponds with a protein peak. However, this was not always the case. The activity was consistently eluted in the same fractions but the protein profile varied considerably. The additional 5–8-fold purification resulted in an overall purification of about 27 000-fold (Table I). The activity was stable only in the presence of 0.5% Triton X-100. After removal of detergent these preparations were completely inactivated by freezing and had a half-life of about 1 day at 4°C.

Molecular size and purity

SDS-polyacrylamide gel electrophoresis of the preparations from the DEAE-Sepharose column indicated the presence of a number of proteins. Samples with higher specific activities obtained after hydrophobic chromatography were less complex mixtures but were not homogeneous. A common feature of both preparations was the presence of two polypeptides having molecular masses of 100 000–105 000 and 50 000–53 000. The smaller polypeptide was







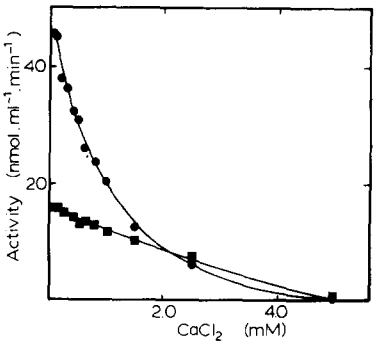


Fig. 2. (Upper left.) Ion-exchange chromatography on DEAE-Sepharose CL-6B. Fractions 49–57 from the Sepharose CL-6B column were combined and applied to the column of DEAE-Sepharose CL-6B in buffer containing 0.1 M NaCl. A linear gradient (0.1–0.4 M NaCl) was begun at fraction 21. Fractions were assayed for protein (○) by the Bradford method [22], for phosphatidyl-inositol-bisphosphate phosphatase (●) and for neutral phosphatase (■).

Fig. 3. (Lower left.) Hydrophobic chromatography on detergent-saturated Phenyl-Sepharose CL-4B. Fractions 53–60 from the DEAE-Sepharose CL-6B column were combined and applied to the prepared column. Unadsorbed protein was eluted with detergent-free buffer. Elution with 0.5% (w/v) Triton X-100 was begun at fraction 16. Fractions were assayed for protein (○) by the fluorescamine method and for phoshatidyl-inositol-bisphosphate phosphatase (●).

Fig. 4. (Upper right.) The effect of non-ionic detergent on phosphatidyl-inositol-bisphosphate phosphatase activity. Reaction mixtures were as described with 1 mM phosphatidyl-inositol-bisphosphate and 0.4 mM $MgCl_2$. CTAB/phosphatidyl-inositol-bisphosphate mixtures were added to the other components to start the reactions. Triton X-100 was absent (▲) or present at either 0.1% (w/v) (■) or 0.2% (w/v) (●).

Fig. 5. (Lower right.) The effect of Ca^{2+} on the phosphatidyl-inositol-bisphosphate phosphatase activity in the presence (●) and absence (■) of 2.5 mM CTAB. EGTA derived from the enzyme preparation was present at 0.1 mM. The reaction was otherwise as described with 1 mM phosphatidyl-inositol-bisphosphate and 0.4 mM $MgCl_2$.

usually present in greater quantity. The molecular size of the phosphatidyl-inositol-bisphosphate phosphatase was also estimated on a calibrated column of Sepharose CL-6B in the absence of SDS. The activity eluted as a single sharp peak with an estimated molecular mass of 104 000.

Reaction characteristics

The preparations obtained after DEAE-Sepharose chromatography were used for all characterization studies. In the absence of non-ionic detergent there was an almost absolute requirement for CTAB (Fig. 4) with the activity occurring over a narrow range of

CTAB/PI-P_2 molar ratios. Triton X-100 stimulated the activity in both the presence and absence of CTAB and reduced the dependence upon a specific CTAB/PI-P_2 ratio. Concentrations of Triton X-100 above 0.2% were not more effective. There was an absolute requirement for added Mg^{2+}. Concentrations above 0.1 mM gave maximum activity. Assay mixtures contained 0.4 mM Mg^{2+}, a concentration which mimics the in vivo environment [34]. When the CTAB/PI-P_2 ratio was held constant at 2.5 the apparent K_m for the substrate was 0.27 mM. Under optimum conditions, the reaction rate was proportional to both protein and time until about 25% of the monoesterified phosphate had been hydrolyzed. The activity exhibited a broad flat pH optimum (pH 6.8–8.0). The enzyme was heat-sensitive and the activity declined sharply at assay temperatures above 37°C.

The phosphatidyl-inositol-bisphosphate phosphatase preparation was inactive with other lipid substrates having a monoesterified phosphate group (Table II), including PI-P, when present alone or as mixtures with CTAB. Thin-layer chromatography of the reaction mixture showed the hydrolysis of PI-P_2 to PI-P to be complete in 2.5 h. Conversion of PI-P to PI was not detected even after 6 h. Human erythrocyte cytosol is known to contain specific phosphatases which act on 2,3-diphosphoglycerate and phosphoglycolate acid [35,36]. Very low activity (near the limit of detection) was observed with phosphoglycolic acid. Some activity was observed with *myo*-inositol 1,4,5-trisphosphate. Other *myo*-inositol phosphates and a variety of other phosphate esters were not hydrolysed.

TABLE II

SPECIFICITY OF THE HUMAN ERYTHROCYTE CYTOSOL PHOSPHATIDYL-INOSITOL-BISPHOSPHATE PHOSPHATASE

	Activity (nmol · ml^{-1} · min^{-1})
Lipid substrates [a]	
PI-P_2	26.9
PI-P	<2 [b]
Phosphatidic acid (dipalmitoyl)	<2 [b]
Phosphatidic acid (egg lecithin)	<2 [b]
Lysophosphatidic acid (palmitoyl)	<2 [b]
Water-soluble phosphate esters	
p-Nitrophenyl phosphate pH 4.8	<1 [c]
p-Nitrophenyl phosphate pH 7.7	<1 [c]
p-Nitrophenyl phosphate pH 10.5	<1 [c]
Inositol 2-phosphate	<2 [d]
Inositol 1,4-phosphate	<2 [d]
Inositol 1,4,5-triphosphate	7.4 [d]
Glucose 1-phosphate	<2 [d]
Glucose 6-phosphate	<2 [d]
Fructose 6-phosphate	<2 [d]
2,3-Diphosphoglycerate	<2 [d]
Phosphoglycolate	4.2 [d]
ATP	<2 [d]
ADP	<2 [d]
AMP	<2 [d]

[a] Substrate concentration was 1 mM. Other conditions were as described in Materials and Methods for the phosphatidyl-inositol-bisphosphate phosphatase assay.

[b] Assayed at CTAB to lipid ratios of 0, 1.0 and 2.5. 2 nmol · ml^{-1} · min^{-1} is the lower limit of activity detection.

[c] Assayed as described in Materials and Methods. 1 nmol · ml^{-1} · min^{-1} is the lower limit of activity detection.

[d] Assayed at substrate concentration of 2 mM in the presence of 50 mM Tris-HCl (pH 7.1) and 0.5 mM $MgCl_2$. 2 nmol · ml^{-1} · min^{-1} is the limit of activity detection.

Inhibition studies

EGTA and *o*-phenanthroline, chelating agents which have a low affinity for Mg^{2+}, had no effect while EDTA was a potent inhibitor. The effect of Cu^{2+}, an inhibitor of the erythrocyte acid phosphatase, could not be evaluated due to precipitation in the assay mixture. F- had no effect. Reagents which react with sulfhydryl groups inactivated the enzyme. *p*-Chloromercuribenzoate and *p*-chloromercuriphenylsulfonate were most effective, even at low concentrations (0.01 mM). Dithioerythritol, a sulfhydryl protecting agent, had no effect on the activity when added either to the assay mixture or to the buffer during storage of the enzyme.

Neomycin did not affect the erythrocyte phosphatidyl-inositol-bisphosphate phosphatase when added to the complete reaction mixtures containing both CTAB and Triton X-100 (Table III). Concentrations above 10^{-3} M caused precipitation of the reaction mixture. If Triton X-100 were omitted, neomycin did cause inhibition without significant precipitation over the range 10^{-6} to 10^{-3} M.

Addition of Ca^{2+} to the assay mixture in the

TABLE III

EFFECT OF NEOMYCIN ON PHOSPHATIDYL-INOSITOL-BISPHOSPHATE PHOSPHATASE ACTIVITY

Neomycin (M)	Activity (nmol · ml^{-1} · min^{-1})	
	0.2% Triton X-100	No Triton X-100
0	48.5	16.7
10^{-10}	48.5	16.7
10^{-8}	48.8	16.1
10^{-6}	48.8	15.2
10^{-4}	48.2	7.6
10^{-3}	5.0 (ppt) [a]	4.0
10^{-2}	0.6 (ppt) [a]	0.3 (ppt) [b]

[a] Obvious precipitation of the assay mixture during incubation.

[b] Complete precipitation of the assay mixture occurred upon addition of 5% SDS/40 mM EDTA solution to stop the reaction.

presence of optimum Mg^{2+} and CTAB resulted in marked inhibition (Fig. 5). In the absence of CTAB, the effect was much less striking. Significant precipitation occurred at Ca^{2+} concentrations above 0.5 and 2 mM in the presence and absence of CTAB, respectively. In both cases the precipitation was very slight at first but became progressively heavier with increasing Ca^{2+} concentrations. However, inhibition occurred at non-precipitating concentrations and there was no break in the inhibition curves at the concentrations where precipitation became apparent. Further studies using the optimum assay system in which the substrate was provided at a constant PI-P_2 : CTAB ratio revealed that the inhibition by Ca^{2+} was non-competitive with respect to substrate (Fig. 6a) but competitive with Mg^{2+} (Fig. 6b). The 'apparent' K_i for Ca^{2+} was calculated to be 50 μM compared to an 'apparent' K_m value of 12 μM for Mg^{2+}.

Discussion

Phosphatidyl-inositol-bisphosphate phosphatase activity is found in both cytosol and membrane fractions from mammalian tissues. The presence and location of this activity in erythrocytes has been less certain. Loss of PI-P_2 and the concomitant rise in PI-P or PI that occurs during storage of mammalian erythrocytes implies phosphatase activity [15,37]. The metabolic turnover of the monoesterified phosphate groups of both PI-P and PI-P_2 in isolated swine erythrocyte membranes incubated with ATP suggests a membrane location for both the phosphoinositide kinases and the phosphatases [14]. However, a similar turnover does not occur in human erythrocyte membranes [38], although both kinases are present [2,3]. Therefore, our finding of phosphatidyl-inositol-bisphosphate phosphatase activity in human erythrocyte cytosol only, is compatible with these earlier studies.

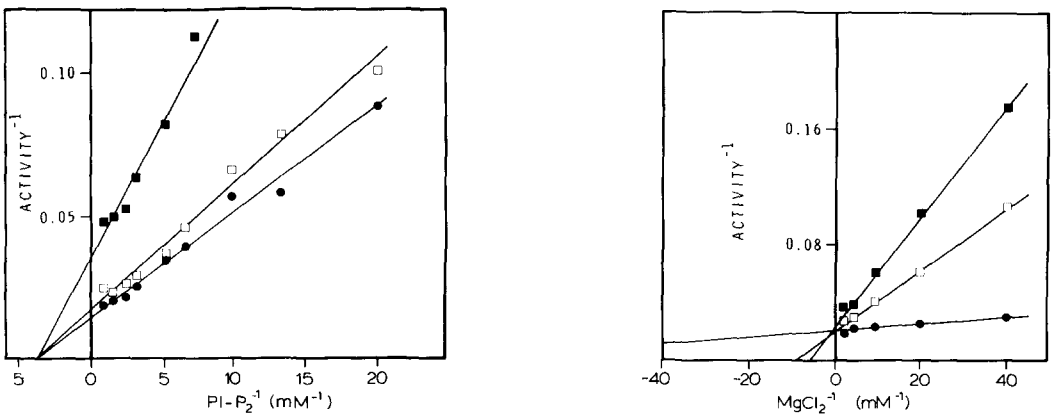


Fig. 6. Double-reciprocal plot of the effect of Ca^{2+} on phosphatidyl-inositol-bisphosphate phosphatase activity as a function of (a) substrate concentration and (b) magnesium concentration. The reaction mixtures were as described with the CTAB/phosphatidyl-inositol-bisphosphate molar ratio constant at 2.5. Saturation curves were determined in the absence (•) and presence of 0.4 mM (□) and 0.75 mM (■) $CaCl_2$. Lines were calculated by unweighted linear regression analysis and the correlation coefficients were greater than 0.98.

Chromatography on CM-Sepharose suggests the possibility of two forms of the phosphatidyl-inositol-bisphosphate phosphatase activity. About 20% of the total activity adhered to CM-Sepharose. However, attempts to separate it from hemoglobin were only partially successful. A substantial purification of the phosphatidyl-inositol-bisphosphate phosphatase in the non-adsorbed fraction was achieved. The highly active acid and neutral phosphatase activities were absent after chromatography on DEAE-Sepharose. The phosphatidyl-inositol-bisphosphate phosphatase at this stage (purified 4 000-fold) was very stable and therefore was used for the characterization studies. After hydrophobic chromatography, an average purification of 27 000-fold was achieved but the specific activity of the product was variable. If the inhibition (60%) by hemoglobin in the assay of the cytosol is taken into account this represents an 11 000-fold purification overall, but only several hundred fold over the non-hemoglobin cytosol proteins. SDS acrylamide gel electrophoresis showed a number of bands, the most consistent and abundant band representing a polypeptide having an estimated molecular mass of 52 000. The molecular size of a minor component was 104 000, the same as the value for the phosphatase activity estimated by gel filtration in the absence of SDS. The purified phosphatidyl-inositol-bisphosphate phosphatase from *C. fasciculata* has a molecular mass of 114 000, which is reduced to 55 000 by SDS [33]. It is likely that both enzymes exist as dimers. The molecular size of the purified rat brain cytosol phosphatase was said to be small but no value was given [13].

Like all phosphatidyl-inositol-bisphosphate phosphatases the erythrocyte enzyme requires moderation of the large negative charge on the substrate. This is accomplished most effectively by a cationic amphipathic compound (CTAB). The optimum CTAB to PI-P_2 ratio varies with the tissue. Non-ionic detergents have been reported to inhibit the kidney enzyme [39]. However, Triton X-100 stimulated the erythrocyte phosphatase in the presence and absence of CTAB and the dependence upon a specific CTAB to substrate ratio was reduced. This may be the result of the formation of mixed micelles in which the substrate molecules are dispersed over the surface, giving a lowered surface charge density. The hydrophobic nature of the enzyme and its stabilization by Triton X-100 suggests that it may also prefer the hydrophobic environment provided by the non-ionic detergent. Similar results were obtained in studies with the *C. fasciculata* enzyme [33]. The apparent K_m of 0.27 mM for PI-P_2 is comparable to values reported for other phosphatidyl-inositol-bisphosphate phosphatases when assayed in the presence of CTAB [12, 33,39].

In the optimum reaction mixture containing both CTAB and Triton X-100, the erythrocyte phosphatase required less Mg^{2+} for full activity ('apparent' $K_m = 12\ \mu M$) than has generally been reported for preparations from other tissues [39,40]. Although not required for charge moderation of the substrate in the presence of CTAB, divalent cations can still interact with the PI-P_2. However, Triton X-100 seems to reduce this interaction. It prevents the inhibition and/or substrate precipitation which occurs at higher Mg^{2+} concentrations [33]. Ca^{2+} has been reported to stimulate the phosphatidyl-inositol-bisphosphate phosphatase if no other provision is made to moderate the charge on the substrate, and to inhibit due to substrate precipitation [13,40]. In the maximally stimulated assay system used here inhibition by Ca^{2+} was observed without an interaction with PI-P_2, as indicated by the absence of substrate precipitation at low concentrations and the lack of competition with the substrate. Under these conditions Ca^{2+} was found to compete with Mg^{2+}. Therefore, it is likely that the observed Mg^{2+} dependence represents the ion requirement of the enzyme.

Neomycin disrupts polyphosphoinositide metabolism in neural tissues [41] and erythrocytes [10]. It is believed to act in vivo by specifically binding to PI-P and PI-P_2 [41], although this interpretation has been questioned on the basis of membrane impermeability [42]. The erythrocyte phosphatidyl-inositol-bisphosphate phosphatase was inhibited by neomycin in vitro at concentrations one or two orders of magnitude below that of the substrate, but only in the absence of Triton X-100. It is not apparent how this non-ionic detergent could interfere with the ionic interaction between this polyamine and the anionic lipids, although the effect is consistent with the reduced interaction with cations.

Phosphatidyl-inositol-bisphosphate phosphatase activities in all mammalian tissues studied so far, either in crude or purified preparations, have also

been able to dephosphorylate PI-P. Both indirect metabolic studies and the direct hydrolysis of exogenous PI-P indicate the presence of phosphatidyl-inositol-phosphate phosphatase activity in swine erythrocyte membranes [14]. In stored erythrocytes from this and other species, PI-P_2 is slowly degraded to PI [15]. The phosphatidyl-inositol-bisphosphate phosphatase isolated from human erythrocyte cytosol was unable to hydrolyse PI-P under a variety of conditions and, in this respect was similar to the protozoan enzyme [12,33]. This observation is consistent with the conversion of PI-P_2 to PI-P in stored human erythrocytes [15]. A preferential hydrolysis of PI-P_2 has been invoked to explain some effects of neomycin on human erythrocyte polyphosphoinositide metabolism [11]. However, there may be a separate phosphatidyl-inositol-phosphate phosphatase located in the membrane as appears to be true in swine erythrocytes [14]. Few preparations, including the highly purified rat brain cytosol enzyme [13], have been extensively tested for other phosphatase activities. The partially purified preparations from both bovine brain [40] and human erythrocyte cytosol exhibit considerable activity with *myo*-inositol-1,4,5-trisphosphate (the hydrophilic end of the substrate) but not with *myo*-inositol 1,4 bisphosphate. The *C. fasciculata* phosphatidyl-inositol-bisphosphate phosphatase is also inactive with *myo*-inositol 1,4-bisphosphate but exhibits only minimal activity with *myo*-inositol 1,4,5-trisphosphate [33]. The latter three preparations have all been inactive with a variety of sugar and nucleotide phosphates and with other phospholipids having a monoesterified phosphate group.

It is difficult to predict in vivo activity from results obtained with the artificial conditions of the assay system. The enzyme itself would be saturated with Mg^{2+} at the in vivo concentration of 0.4 mM [34]. However, the substrate exists in the cell membrane as the Mg^{2+} and, to a limited extent, Ca^{2+} salts [43]. PI-P_2 can also interact with anionic proteins through divalent cation bridges [24] and directly with cationic portions of membrane proteins such as glycophorin [44]. How these interactions might affect the access of the enzyme to its substrate is not known, since the activity of the partially purified and purified phosphatidyl-inositol-bisphosphate phosphatases from erythrocytes and brain [13,40] have not been studied with naturally occurring, membrane-bound substrate. Hemoglobin, inhibited the phosphatase in vitro. It is not known to what extent the very high in vivo concentration of hemoglobin (approximately 35% w/v) might interfere with the enzyme-substrate interaction at the membrane surface. The very low intracellular concentration of free Ca^{2+} (probably less than 1 μM) [7,11] and the 'apparent' K_i value of 50 μM make it unlikely that Ca^{2+} plays a regulatory role in vivo, even in aging erythrocytes when the concentration may rise above 100 mM [6]. In most studies using the ionophore A32178 the calcium influx would be sufficient to block phosphatase activity [10]. When much lower Ca^{2+} concentrations were used [6], a transient increase in PI-P occurred. Presumably this represents the in vivo activity of the phosphatidyl-inositol-bisphosphate phosphatase as does the slow hydrolysis of PI-P_2 to PI-P observed in erythrocytes during storage of human blood [15].

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