Evidence for a specific phosphatidylinositol 4-phosphate phosphatase in human erythrocyte membranes

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Abstract Human erythrocyte membranes exhibit a specific phosphatidylinositol 4-phosphate phosphohydrolase (PtdIns4P phosphatase) activity which hydrolyzes PtdIns4P and lysoPtdIns4P but not phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) or lysoPtdIns(4,5)P_2. Phosphatidic acid, lysophosphatidic acid, glycerophosphoinositol 4-phosphate, glycerophosphoinositol 4,5-bisphosphate, inositol mono, bis, and tris phosphates and several other sugar and nucleoside phosphates are not hydrolyzed. The PtdIns4P phosphatase activity is not affected by Ca^{2+} or Mg^{2+} ions nor inhibited by EDTA. Maximum in vitro activity requires non-ionic (Triton X-100) detergents. The phosphatase is very stable in isolated membranes at low temperatures but is rapidly inactivated above 35°C. This critical inactivation temperature is lowered to 20–25°C by solubilizing the membranes with non-ionic detergents. Arrhenius plots of the activity show an inflection at these critical temperatures, suggesting a temperature-dependent change in the environment or conformation of the enzyme. Sulfhydryl-reacting reagents are potent inhibitors. Dithioerythritol stimulates only when the membranes are solubilized with non-ionic detergent. The location of cation-independent PtdIns4P phosphatase activity in the membrane and of Mg^{2+}-dependent PtdIns(4,5)P_2 phosphatase activity in the cytosol was also observed for monkey, rabbit, rat, and dog erythrocytes. Both activities are located in the cytosol of sheep erythrocytes.—**Mack, S. E., and F. B. St. C. Palmer.** Evidence for a specific phosphatidylinositol 4-phosphate phosphatase in human erythrocyte membranes. *J. Lipid Res.* 1984. **25:** 75–85.

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The monoesterified phosphate groups of phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) exhibit a very rapid metabolic turnover in all tissues and microorganisms that have been studied (1). This occurs via an ATP-dependent phosphorylation-dephosphorylation cycle. Structural analysis of the naturally occurring polyphosphoinositides and of the PtdIns4P produced during both synthesis and dephosphorylation of PtdIns(4,5)P_2 by extracts of bovine brain and protozoa indicates that phosphatidylinositol (PtdIns) is phosphorylated first in the 4 then the 5 position and that PtdIns(4,5)P_2 is sequentially dephosphorylated in the reverse order (2–4). The phosphorylation steps are catalyzed by specific PtdIns and PtdIns4P kinases (1). Most studies have concluded that a single phosphatase removes both phosphates from PtdIns(4,5)P_2. Crude and partially purified preparations from brain, kidney and iris muscle convert PtdIns(4,5)P to PtdIns (5–9). Moreover, PtdIns(4,5)P_2 and PtdIns4P appear to compete for the same phosphatase in kidney extracts (10). Finally, a homogeneous preparation of PtdIns(4,5)P_2 phosphatase from rat brain (11) hydrolyzes PtdIns4P as well as PtdIns(4,5)P_2. However, the possibility of separate PtdIns(4,5)P_2 and PtdIns4P phosphatases must still be considered since highly specific PtdIns(4,5)P_2 phosphatases have now been partially purified from both protozoa and human erythrocytes (12, 13). Neither enzyme hydrolyzes PtdIns4P in vitro. There is a second cyclical pathway which, in some tissues, responds to receptor-mediated influx of Ca^{2+} ions (1). The polyphosphoinositides are hydrolyzed by a calcium-activated phosphodiesterase to diacylglycerol and the corresponding inositol bis and trisphosphates. Replacement occurs by de novo synthesis of PtdIns followed by phosphorylation.

In erythrocytes, the PtdIns and PtdIns4P kinases are present on the cytosolic surface of the membrane (14). With some exceptions, mammalian erythrocyte membranes also contain the calcium-dependent phosphodiesterase (15). A phosphatase in human erythrocyte membranes converts inositol 1,4,5-trisphosphate (16) to inositol

Abbreviations: PtdIns, 1-(3-*sn*-phosphatidyl)-D-myo-inositol; PtdIns4P, 1-(3-*sn*-phosphatidyl)-D-myo-inositol 4-phosphate; GroPIns4P, glycerophosphoryl-D-myo-inositol 4-phosphate; PtdIns(4,5)P_2, 1-(3-*sn*-phosphatidyl)-D-myo-inositol 4,5-bisphosphate; GroPIns(4,5)P_2, glycerophosphoryl-D-myo-inositol 4,5-bisphosphate; Pipes, piperazine-N,N′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)N,N′-tetraacetic acid.

1,4-bisphosphate which is the end product of both PtdIns(4,5)P_2 and PtdIns4P degradation by this route. In some species, the diacylglycerol may be phosphorylated to give phosphatidic acid, but erythrocytes lack the enzymes needed for PtdIns resynthesis. This inability to replace lost phosphoinositide, and an intracellular calcium concentration too low to support diesterase activity, make it unlikely that the diesterase-initiated catabolic pathway is physiologically significant in healthy erythrocytes (17). Therefore, only the phosphorylation-dephosphorylation cycle can account for the rapid labeling of the monoesterified phosphate groups of PtdIns4P and PtdIns(4,5)P_2 in erythrocytes (18). An undocumented report suggests that swine erythrocytes can hydrolyze exogenous PtdIns4P (19). However, of the two phosphatase activities required for this cycle, only the human erythrocyte cytosolic PtdIns-(4,5)P-specific phosphatase has been demonstrated directly (13).

The present report describes the characteristics of a PtdIns4P-specific phosphatase activity in human erythrocyte membranes and provides evidence of a similar activity in erythrocytes from several other mammalian species.

MATERIALS AND METHODS

Lipids and substrates

PtdIns(4,5)P_2 containing less than 5% PtdIns4P was prepared from bovine brain (20) and purified on immobilized neomycin (21). Most PtdIns4P was prepared from the crude PtdIns(4,5)P_2 using the partially purified PtdIns(4,5)P_2 phosphatase from *Crithidia fasciculata* (4). Some naturally occurring PtdIns4P was isolated from extracts of bovine brain by neomycin chromatography (21). Rat liver PtdIns was also isolated by chromatography on immobilized neomycin (21). Other lipids were from Serdary Research Laboratories, London, Canada. Lipids were stored in chloroform at −20°C. The extracellular PtdIns-specific phosphodiesterase of *Bacillus thuringiensis* (22) was used to prepare myoinositol 1,2-cyclic phosphate from the PtdIns. Myoinositol 1,4-bisphosphate and myoinositol 1,4,5-trisphosphate were prepared from the purified PtdIns4P and PtdIns(4,5)P_2 using the polyphosphoinositide phosphodiesterase present in crude extracts of *Crithidia fasciculata* (23). Diesterase reactions were done in 25 mM Pipes (pH 7.2). This contained 0.1% (w/v) sodium deoxycholate for the PtdIns diesterase, and was saturated with diethyl ether in the presence of 0.2 mM $CaCl_2$ and 0.1 M NaCl for the polyphosphoinositide diesterase. Reactions were stopped by adding an equal volume of 10% (w/v) trichloroacetic acid, which precipitated the lipid, protein, and Pipes. The trichloroacetic acid was extracted from the supernatants with diethyl ether. LysoPtdIns4P and lysoPtdIns(4,5)P_2 were prepared from the purified PtdIns4P and PtdIns(4,5)P_2, respectively, using bee venom phospholipase A_2 (Sigma Chemical Co.) in 50 mM triethanolamine at pH 8.5. After acidifying the reaction mixture with a few drops of formic acid and adding 4 volumes of chloroform–methanol 2:1 (v/v), the lysolipids were recovered in the upper aqueous phase. Glycerophosphoinositol 4-phosphate (GroPIns4P) and glycerophosphoinositol 4,5-bisphosphate (GroPIns(4,5)P_2) were prepared by mild alkaline hydrolysis of PtdIns4P and PtdIns(4,5)P_2 (24).

Triton X-100 (*p-tert*-octylphenoxypolyoxyethylene), Tween 60 (polyoxyethylene sorbitan monostearate), and Tween 80 (polyoxyethylene sorbitan monooleate) were from the Sigma Chemical Co. Cutscum (isooctylphenoxypolyoxyethylene) and Brij 35 (polyoxyethylene dodecyl ether) were from Fisher Scientific Ltd., Montreal, P.Q. Ammonyx LO (dodecyldimethylamine oxide) and Miranol H2M (disodium 1-carboxymethyl-1-(2-carboxymethoxylethyl)-2-undecylimidazolinium hydroxide) were gifts of the Onyx Oil and Chemical Co., Jersey City, NJ and the Miranol Chemical Co., Irvington, NJ, respectively.

Erythrocyte preparations

The erythrocyte fraction from human blood, no longer suitable for transfusion, was obtained from the Red Cross Transfusion Service. It had been collected in acid–citrate–dextrose and stored at 4°C for several days. Fresh blood from volunteers was drawn by venipuncture into heparinized containers. Animal blood was collected in heparinized containers by venipuncture (large animals) or cardiac puncture (small animals). Erythrocytes were sedimented at 750 *g* for 10 min and washed twice with ice-cold 150 mM NaCl containing 1 mM EDTA. The supernatant, buffy coat, and the top layer of erythrocytes (approximately 20% of the erythrocytes) were removed each time. Contamination of these preparations was 0.7–1.4 white cells and 7–15 platelets per 10^4 erythrocytes as determined in a Coulter Counter. The washed cells were hemolyzed in 20 volumes of 10 mM Tris (pH 7.4)–1 mM EDTA. The membranes were sedimented at 17,500 *g* for 15 min, washed three times with the hemolysis buffer, and stored at 4°C. Cytosol proteins in the supernatant were obtained free from hemoglobin by repeated ammonium sulfate precipitation at 4°C (13). The precipitated cytosol proteins were suspended in 50 mM imidazole buffer (pH 7.0) containing 0.5 mM EGTA and dialyzed against the same buffer. The dialysate was centrifuged to remove any insoluble material and stored at 4°C.

Analyses

Total phosphorus was measured by the method of Bartlett (25) after digestion of the lipid samples in perchloric

acid. Protein was measured by the Lowry procedure as modified by Peterson (26). Results were calculated using the hyperbolic approximation from two standards (27).

Phosphatase assays

The assay procedures for PtdIns4P and PtdIns(4,5)P_2 phosphatases were adapted from those described before (12, 13). All reaction mixtures contained 50 mM Pipes (pH 7.2), 1.0 mM EGTA, 1 mM substrate, and 0.2% (w/v) Triton X-100 in a total volume of 0.15 ml. The requirements for CTAB and Mg^{2+} varied with the tissue and the substrate. Assays of erythrocyte cytosol PtdIns(4,5)P_2 phosphatase contained 0.4 mM $MgCl_2$ and 2.5 mM CTAB (13). The erythrocyte PtdIns4P phosphatase assays contained 1.5 mM CTAB but no Mg^{2+}. Reaction mixtures were preincubated without enzyme at 37°C for 2 min and the enzymic reactions were started by adding the tissue preparations (0.03 ml). In some later experiments, 0.2% Triton X-100 was added to the tissue preparations at 0°C on an ice bath 10 min before their use in the assay. Reactions were stopped after 5–30 min by adding 0.35 ml of 5% (w/v) SDS-50 mM EDTA and the inorganic phosphate measured by the automated method of Hegyvary, Kang, and Bandi (28). Activity with other phospholipids and with nonlipid phosphate esters was measured in the same way. Nonspecific phosphatase activity was measured spectrophotometrically by observing the hydrolysis of *p*-nitrophenylphosphate at pH 4.8 in 25 mM citrate buffer, at pH 7.7 in 25 mM Tris/chloride, and at pH 10.5 in 50 mM glycine (29).

RESULTS

Hydrolysis of PtdIns4P

Preliminary experiments indicated that the ability to release inorganic phosphate from purified PtdIns4P was localized in the membrane fraction of human erythrocytes. Washed, hemoglobin-free membranes were used to determine optimal assay conditions. Without non-ionic detergent, erythrocyte membrane PtdIns4P phosphatase activity was low (**Fig. 1**) but could be increased almost 3-fold by Triton X-100. Maximum activity was obtained at detergent concentrations of 0.2% (w/v) or higher. Cationic detergent (CTAB) had little effect at low CTAB/PtdIns4P ratios but inhibited the reaction at ratios of 2 or greater. Activity was marginally increased at CTAB/PtdIns4P ratios of 1.0–1.5. Therefore CTAB (1.5 mM) was included in the standard assay.

The assay measured release of inorganic phosphate from PtdIns4P. Thin-layer chromatography (23) of the reaction mixture confirmed that PtdIns was the only other product. The reaction was presumed to be enzymic since PtdIns4P was not hydrolyzed by membranes that had been heated to 100°C for 10 min or exposed to trypsin. The activity was optimal over a broad pH range from 6.5 to 8 and at substrate concentrations above 0.5 mM. The analytical procedure was not sensitive enough to estimate initial reaction rates at very low substrate concentrations but substrate saturation curves suggested a K_m of less than 0.1 mM. Substrate inhibition was not evident at up to 2 mM PtdIns4P. With 1.0 mM PtdIns4P and 1.5 mM CTAB, the release of inorganic phosphate was linear for 5–10 min at 37°C and sufficient inorganic phosphate was released for accurate measurement.

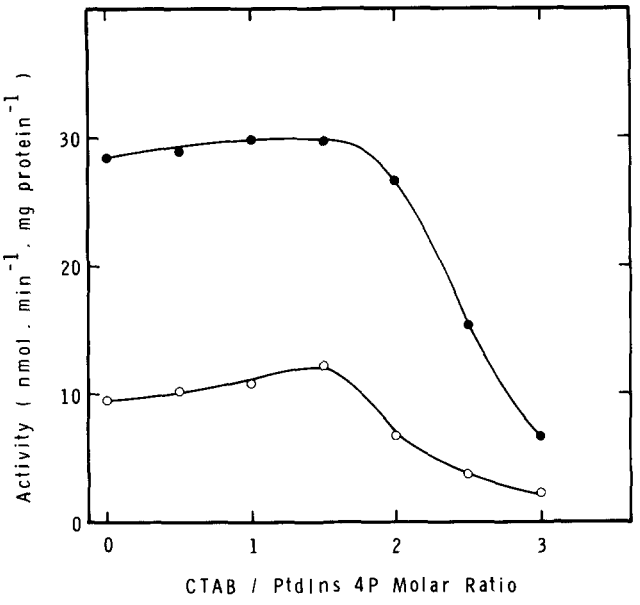

Fig. 1. Dependence of human erythrocyte membrane PtdIns4P phosphatase on the CTAB/PtdIns4P molar ratio. The standard assay procedure was used with (●) and without (○) 0.2% (w/v) Triton X-100 in the reaction mixture which contained 50 mM Pipes (pH 7.2), 1 mM PtdIns4P, CTAB (as indicated), and 0.63 mg·ml^{-1} membrane protein.

A variety of detergents was assessed for the ability to stimulate PtdIns4P phosphatase activity at the optimum CTAB/PtdIns4P ratio of 1.5 (**Table 1.**). Cutscum is structurally very similar to Triton X-100 and gave comparable stimulation. Further increases in activity occurred at concentrations greater than 0.2% (w/v), but sensitivity was reduced by a dramatic increase in the release of inorganic phosphate in the enzyme controls (substrate added after the incubation). The effect was independent of PtdIns4P and CTAB and proportional to the quantity of membranes present. It must represent a detergent-dependent release of phosphate from some membrane component, the nature of which was not investigated further. Octylglucoside stimulated PtdIns4P phosphate activity, but at concentrations above 15 mM it also caused a release of substantial amounts of phosphate from the membranes. For this reason neither Cutscum nor octylglucoside was deemed suitable and no other detergent

TABLE 1. Effect of detergents on human erythrocyte PtdIns4P phosphatase

Detergent[a]	Concentration	Activity[b]
None		10.4
Triton X-100 (control)	0.2%	21.2
Cutscum	0.05%	13.3
	0.1%	19.3
	0.2%	21.0
	0.4%	25.8
	1.0%	28.2
	2.0%	32.2
	3.0%	23.7
Octylglucoside	7.5 mM	2.0
	15.0 mM	13.0
	20.0 mM	34.9
	22.0 mM	30.0
	25.0 mM	24.0
	30.0 mM	16.1
Brij 35	0.05%	13.7
	0.1%	16.7
	0.2%	15.9
	0.4%	11.6
	1.0%	10.5
Miranol H2M	0.2%	15.3
Tween 60	0.2%	10.5
Tween 80	0.2%	14.9
Ammonyx LO	0.2%	12.5
Deoxycholate	0.2%	3.5

[a] Detergents were substituted for Triton X-100 in the standard assay system.
[b] Activities are expressed as nmol · min^{-1} · mg $protein^{-1}$.

was as effective as Triton X-100. Sodium deoxycholate inhibited the reaction.

The erythrocyte membrane PtdIns4P phosphatase was not stimulated by magnesium ions even though the erythrocytes had been prepared in the presence of EDTA (**Table 2.**). Adding 10 mM EDTA to the reaction mixture had no effect, nor did calcium or lithium ions up to 2 mM. The membranes did exhibit a small Mg^{2+}-dependent PtdIns(4,5)P_2 phosphatase activity, usually less than 5% of the activity with PtdIns4P. Some membrane preparations exhibited a very low EDTA-independent PtdIns(4,5)P_2 phosphatase activity which did not exceed 2% of the PtdIns4P phosphatase value.

TABLE 2. Polyphosphoinositide phosphatases of human erythrocyte membranes[a]

Addition	Activity	
	PtdIns4P Phosphatase	PtdIns(4,5)P_2 Phosphatase
	nmol · min^{-1} · mg $protein^{-1}$	
None	23	0.1
$MgCl_2$ (0.5 mM)	25	1.4
EDTA (10 mM)	23	0.1
$CaCl_2$ (2 mM)	23	

[a] Membranes were prepared from blood collected in acid–citrate–dextrose and stored at 4°C for 3 days.

The possibility that a significant proportion of the PtdIns4P phosphatase was contributed by the small number of white blood cells and platelets in the erythrocyte preparations was excluded by analyzing membranes from heavily contaminated preparations. Both whole blood (20 white cells and 425 platelets per 10^4 erythrocytes) and washed blood cells from which the buffy coat had not been removed (13 white cells and 230 platelets per 10^4 erythrocytes) were hemolyzed. In spite of the 10–40-fold greater contamination with white cells and platelets, membranes recovered from these preparations had the same specific activity as membranes from the purified erythrocytes.

The specific activity of human erythrocyte membrane preparations in the standard assay system was 19.4 ± 3.7(10) nmol · min^{-1} · mg $protein^{-1}$ (mean ± SD for ten determinations). The activity was quite stable; membrane suspensions stored at 4°C retained 60–70% of their initial activity after 3 months. The variability of these measurements was a characteristic of the assay rather than the membrane preparations since a similar SD was observed in repeated assays of the same sample of membranes (15.5 ± 3.2 nmol · min^{-1} · mg^{-1}, eight determinations).

Substrate specificity

Among potential lipid substrates (those having monoesterified phosphate groups), appreciable phosphatase activity was observed only with PtdIns4P and lysoPtdIns4P (**Table 3**). Neither phosphatidic acid nor lyso-

TABLE 3. Substrate specificity of human erythrocyte membrane PtdIns4P phosphatase

Substrate	Activity[a]
PtdIns4P (1.5 mM CTAB)	19.7
LysoPtdIns4P (1.0 mM CTAB)	32.0
GroPIns4P	ND[b]
Inositol 1,4-bisphosphate	ND
PtdIns(4,5)P_2 (2.0 mM CTAB, 0.5 mM Mg^{2+})	2.7
LysoPtdIns(4,5)P_2 (2.0 mM CTAB, 0.5 mM Mg^{2+})	1.5
GroPIns(4,5)P_2 (0.5 mM Mg^{2+})	0.3
Inositol 1,4,5-trisphosphate (0.5 mM Mg^{2+})	0.1
Phosphatidic acid	ND
Lysophosphatidic acid	ND

Standard was assay used with 1 mM substrate. Assays were done at several CTAB concentrations with and without 0.5 mM Mg^{2+}. Maximum activity was obtained at the conditions listed.
[a] Activity is expressed as nmol · min^{-1} · mg $protein^{-1}$.
[b] ND is not detectable at 0.05 nmol · min^{-1} · mg $protein^{-1}$.

phosphatidic acid was hydrolyzed under a variety of incubation conditions. The activity with PtdIns(4,5)P_2 and lysoPtdIns(4,5)P_2 was Mg^{2+}-dependent and was at least an order of magnitude smaller than the rate with PtdIns4P. Neither GroPIns4P nor inositol 1,4-bisphosphate was hydrolysed. The very low Mg^{2+}-dependent activity with inositol 1,4,5-trisphosphate and GroPIns(4,5)P_2 was most probably due to the inositol 1,4,5-trisphosphate phosphatase present in human erythrocyte membranes (16).

Other specific or nonspecific phosphatases might be responsible for the observed activity. Alkaline phosphatases of both animal (bovine intestine) and bacterial (*E. coli*) origin and acid phosphatase are able to hydrolyze PtdIns4P and PtdIns(4,5)P_2 although the reaction rate is orders of magnitude smaller than with *p*-nitrophenyl phosphate or glycerol phosphate[1] (5). The erythrocyte membranes were therefore tested for activity with a number of phosphate esters. Each potential substrate was assayed with membranes alone, with 0.2% Triton X-100, with 1.0 mM CTAB, and with both Triton X-100 and CTAB. In each case the assays were done with and without 0.5 mM $MgCl_2$. There was no detectable activity with glucose 6-phosphate, fructose 6-phosphate, glycerol 2-phosphate, glyceraldehyde 3-phosphate, 2,3-bisphosphoglycerate, phosphoglycolate, ATP, ADP, AMP, inositol 1,2-cyclic phosphate, and inositol 2-phosphate. The lower limit of detection in these experiments was 0.1–0.2 nmol · min^{-1} · mg $protein^{-1}$, about 1% of the activity with PtdIns4P. Fructose 1,6-bisphosphate was hydrolyzed very slowly by the membranes (0.5 nmol · min^{-1} · mg $protein^{-1}$). *p*-Nitrophenyl phosphate was also slowly hydrolyzed, the highest rate being at acid pH. The observed activities were 2.2, 0.7, and 0.2 nmol · min^{-1} · mg $protein^{-1}$ at pH 4.8, 7.5, and 10.5, respectively. The hydrolysis of both fructose 1,6-bisphosphate and *p*-nitrophenylphosphate was not inhibited by EDTA.

Inhibitors

Preincubation of human erythrocyte membranes at 37°C for 30 min with fluoride (5 mM), *o*-phenanthroline (5 mM), or dithioerythritol (5 mM) had no effect on the PtdIns4P phosphatase. The sulfhydryl reacting reagents *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonate were potent inhibitors. Inhibition was 70–80% at 0.1 mM and 90–100% at 0.5 mM. Total inhibition by N-ethylmaleimide (10 mM) confirmed the dependence on sulfhydryl groups.

Enzyme stability

Attempts to follow the reaction to completion indicated that the extent to which PtdIns4P was hydrolyzed depended upon the amount of enzyme present. At several membrane concentrations the decline in reaction rate was a function of time rather than of substrate consumed. This suggested that the enzyme was being inactivated during the reaction period although preincubation of the isolated membranes at 37°C for several hours only slightly reduced the activity.

In developing the standard assay, the best activity had been obtained when the reactions were started by adding the membrane suspensions to the mixture of buffer, Triton X-100, substrate, and CTAB. Triton X-100 was found to have several additional effects. Although enough detergent was present in the assay mixture to solubilize the added membranes, prior brief exposure of the membranes to Triton X-100 (2–10 min at 0°C in an ice bath) further increased the PtdIns4P phosphatase activity as measured in the standard assay (**Table 4**). The effect was maximal at 0.2% Triton, the concentration at which the membranes (3–6 mg protein · ml^{-1}) were completely solubilized. Pretreatment alone was insufficient for maximum activity; it was still necessary to have 0.2% detergent in the assay mixture. The increased PtdIns4P phosphatase activity was not EDTA (5 mM)-sensitive. The low level of PtdIns(4,5)P_2 phosphatase was unchanged in these pretreated membranes. Similar pretreatment of the cytosol fraction had no effect on the soluble PtdIns(4,5)P_2 phosphatase activity. Pretreatment stimulated membrane PtdIns4P phosphatase activity from 2- to 4-fold. The greatest stimulation occurred with those membranes having the lowest unstimulated activity. The mean stimulated activity was 35.7 ± 6.7(4) nmol · min^{-1} · mg $protein^{-1}$. Unstimulated activities with membranes isolated from fresh heparinized blood (13.8 ± 5.3(7) nmol · min^{-1} · mg $protein^{-1}$) were lower than those with membranes from stored blood. However, Triton pretreatment increased

TABLE 4. Effect of Triton X-100 pretreatment of human erythrocyte membranes on the PtdIns4P phosphatase

Triton X-100		
Pretreatment	Assay	PtdIns4P Phosphatase
%		*nmol · min^{-1} · mg $protein^{-1}$*
0.0	0.2	16.9
0.1	0.2	29.7
0.2	0.2	31.1
0.3	0.2	31.6
0.2	0.0	13.3
0.2	0.05	23.1
0.2	0.1	34.3
0.2	0.2	36.8
0.2	0.4	35.1

The protein concentration of the membranes was 5.25 mg · ml^{-1} during the pretreatment period at 0°C (16 hr) and 0.53 mg · ml^{-1} in the 10 min assay at 37°C.

[1] Mack, S. E., and F. B. St. C. Palmer. Unpublished data.

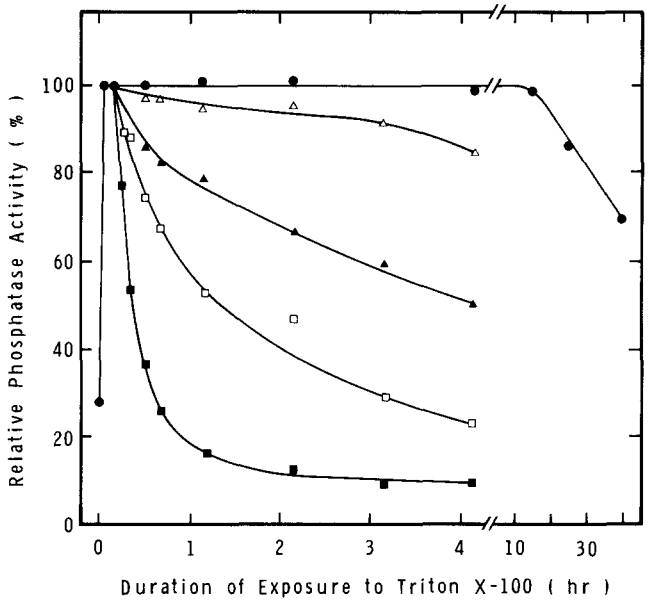

Fig. 2. Effect of temperature on the PtdIns4P phosphatase of Triton X-100 solubilized human erythrocyte membranes. Membranes were solubilized in 0.2% (w/v) Triton X-100 at 0°C. The protein concentration was 4.2 mg·ml^{-1}. After 10 min, aliquots of the membrane solution were transferred to water baths at 20°C (△), 25°C (▲), 30°C (□), and 37°C (■). A control aliquot was maintained at 0°C (●). PtdIns4P phosphatase activity was determined at various times using the standard assay mixture with a 10-min incubation at 37°C. The concentration of membrane protein in the assays was 0.85 mg·ml^{-1}. The relative activity is expressed as a percent of the activity after the initial treatment with detergent at 0°C for 10 min.

this activity to 36.8 ± 6.6(5), the same as the stimulated value for membranes from blood which had been collected and stored in acid–citrate–dextrose. The activities were the same using either the enzymically produced PtdIns4P or the naturally occurring PtdIns4P.

No other detergent was as effective for pretreatment as Triton X-100. The membranes were readily solubilized by most detergents at the concentrations used and there was no detergent-dependent release of inorganic phosphate from the membranes at 0°C. The solubilization detergent was diluted 5-fold when the membranes were added to the assay mixture containing 0.2% Triton X-100. Both Cutscum and octylglucoside were therefore not present in the assay mixtures at concentrations required to cause the release of inorganic phosphate from the membranes at 37°C. Cutscum stimulated PtdIns4P phosphatase activity slightly at 0.5% (w/v) but higher concentrations inhibited. All the other detergents listed in Table 1 were ineffective.

Prior solubilization of the membranes rendered the PtdIns4P phosphatase very temperature-sensitive (**Fig. 2**). The activity was not affected at 0°C for up to 16 hr, and there was little loss of activity over several hours at up to 20°C. At higher temperatures, the loss of activity was increasingly rapid. The data suggest that significant loss would occur during the 10- or 15-min incubation at 37°C in the presence of detergents in the standard assay. Human erythrocyte membranes contain several protease activities, some of which are stimulated by detergents (30, 31). Pretreatment under conditions known to inhibit these protease activities (10 mM phenylmethylsulfonylfluoride, 20 mM EDTA) neither reduced the control activity (pretreatment at 0°C) nor prevented the loss of activity that occurred after pretreatment at 37°C for 1 hr. The same result was obtained with several other protease inhibitors (10 mM aprotinin, 10 mM benzamidine, 0.5 mg/ml pepstatin). The PtdIns4P phosphatase activity of pretreated membranes was abolished by exposure to 5 mM $HgCl_2$, another erythrocyte membrane protease inhibitor. Although effective inhibition of the proteases in such a complex mixture cannot be assured, inactivation of the PtdIns4P phosphatase in the presence of Triton X-100 seems more likely due to heat denaturation than to proteolysis.

Detergent-induced temperature sensitivity was further studied using a 1-hr preincubation of membranes with one of several detergents. **Fig. 3** relates enzymic activity to preincubation temperature. Except with deoxycholate, the activity was stable up to a critical temperature, above

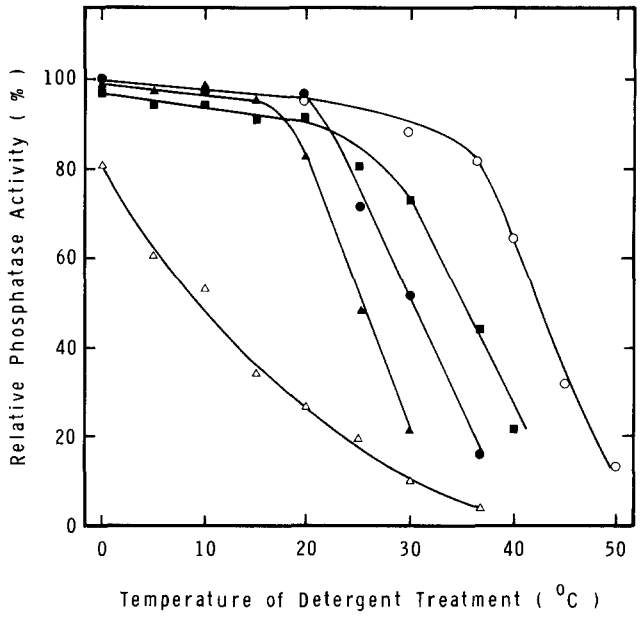

Fig. 3. Effect of detergents on the temperature sensitivity of human erythrocyte membrane PtdIns4P phosphatase. Membrane suspensions were mixed at 0°C for 10 min with detergent: 0.2% (w/v) Triton X-100 (●), 25 mM octylglucoside (▲), 0.2% (w/v) Ammonyx LO (■), or 5 mM deoxycholate (△). Detergent was omitted from the control (○). Aliquots of each treated suspension were incubated for 1 hr at several temperatures as indicated. The membrane protein concentration during detergent treatment was 4.2–4.6 mg·ml^{-1} except for samples treated with deoxycholate where it was 3.5 ml·ml^{-1}. PtdIns4P phosphatase activity was measured using the standard assay procedure with a 10-min incubation period at 37°C and 0.7–1.0 mg·ml^{-1} of membrane protein. Relative activity was calculated as described for Fig. 2.

which it became very unstable. For untreated membranes that temperature was about 35°C. Solubilization of the membranes in Triton X-100 lowered it to 20°C. Similar shifts in temperature sensitivity were observed with Amonyx LO (25°C) and octylglucoside (18°C), detergents that did not stimulate the PtdIns4P phosphatase activity. The data suggest that some abrupt change in the environment or conformation of the enzyme occurs at the critical temperature, most likely a fluidity or phase change in the membranes or the mixed micelles of membrane protein, lipid, and detergent. For experiments described in Fig. 3, the membrane-detergent mixtures contained approximately equal masses of detergent, membrane lipid, and membrane protein. With deoxycholate (0.2% w/v) the membranes were only partially solubilized; nevertheless the PtdIns4P phosphatase was very unstable with some inactivation occurring even at 0°C. The shape of the curve was different from those observed with either untreated membranes or membranes solubilized with the other detergents, and suggests that the deoxycholate-induced temperature sensitivity results from a progressive rather than an abrupt change.

An inflection in the Arrhenius plot, both in the presence and absence of Triton X-100, coincided with the temperature at which the enzyme became temperature labile (**Fig. 4**). Inactivation of the enzyme during the assay could contribute to the lowering of the activity observed above the critical temperature. To minimize this effect the incubation times were reduced. Assays in the Triton X-100-solubilized system were shortened from 15 min at 0–20°C, to 5 min at 25° and 30°C, and to 2 min at 35° and 40°C. From the data in Fig. 2 it can be determined that there would be less than 10% inactivation occurring under these conditions. Since this is not sufficient to account for the observed inflection, the data suggest that a temperature-dependent transition in the detergent-membrane mixed micelles at 20°C alters the activation energy as well as increases the sensitivity of the enzyme to inactivation. A similar transition occurs in the Triton X-100-free membranes, although at a higher temperature, producing a similar change in the activation energy and increased susceptibility to inactivation above the critical temperature.

Effect of dithioerythritol

Inhibition by sulfhydryl-reacting reagents suggests that one or more sulfhydryl group(s) are necessary for enzymic activity. Dithioerythritol had no effect when added to the reaction mixture in the assay of unsolubilized membranes (**Table 5**). It did retard the slow loss of activity during prolonged preincubation of membranes at 0°C. It also stimulated the activity of Triton-solubilized membranes when added either during the initial pretreatment period at 0°C or later in the assay. This result suggests

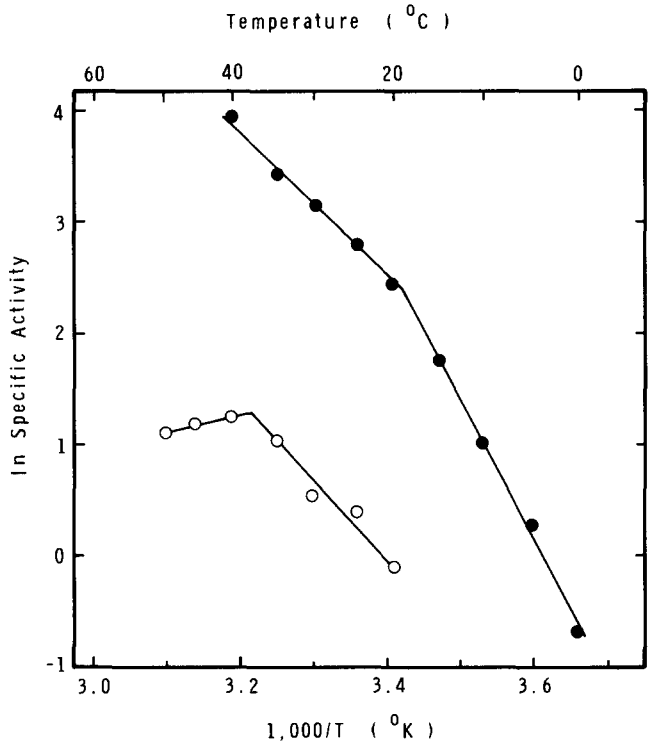

Fig. 4. Arrhenius plot of the temperature dependence of the human erythrocyte PtdIns4P phosphatase. Triton X-100-pretreated membranes were assayed in the standard assay mixture containing Triton X-100 (●). Untreated membranes were assayed in the absence (○) of Triton X-100 in the assay mixture. Incubation times were shorter at the higher temperatures (see text).

that the initial solubilization in pure detergent permits dithioerythritol access to the enzyme that cannot be achieved when membranes are solubilized by the detergent–CTAB–substrate mixture in the course of the assay.

TABLE 5. Effect of dithioerythritol and Triton X-100 pretreatment on human erythrocyte membrane PtdIns4P phosphatase

Pretreatment Conditions	Dithioerythritol (5 mM) Pre-treatment	Dithioerythritol (5 mM) Assay	Activity
None		−	20.9
None		+	19.5
30 min, 37°C	−	+	16.6
30 min, 37°C	+	−	18.1
10 min, 0°C, 0.2% Triton X-100	−	−	31.9
10 min, 0°C, 0.2% Triton X-100	+	−	55.5
10 min, 0°C, 0.2% Triton X-100	−	+	53.3
60 min, 37°C, 0.2% Triton X-100	−	−	4.3
60 min, 37°C, 0.2% Triton X-100	+	−	10.5
60 min, 37°C, 0.2% Triton X-100	−	+	7.9

PtdIns4P phosphatase activity was measured in the standard assay mixture containing 0.2% Triton X-100 (10-min incubation at 37°C).

Dithioerythritol did not, however, prevent the inactivation of the Triton-solubilized enzyme at 37°C. This further suggests that inactivation is the result of thermal denaturation rather than, for instance, oxidation of an essential sulfhydryl group.

Phosphoinositide phosphatases in other mammalian erythrocytes

The results of a survey of PtdIns4P and PtdIns(4,5)P_2 phosphatase activities in the cytosol and membrane fractions of erythrocytes from several mammalian species are given in **Table 6.** Sheep erythrocytes were atypical, having both PtdIns4P and PtdIns(4,5)P_2 phosphatase activities located in the cytosol fraction. However, two different enzymes were present since the PtdIns(4,5)P_2 phosphatase but not the PtdIns4P phosphatase was inhibited by EDTA. Preferential location of an EDTA-insensitive PtdIns4P phosphatase in the membranes and of a Mg^{2+}-dependent PtdIns(4,5)P_2 phosphatase in the cytosol was observed for all other species. Separation of activities was clearest in human erythrocytes. Membranes from rabbit, rat, and dog erythrocytes all had more PtdIns(4,5)P_2 phosphatase activity than human erythrocyte membranes (10–25% of the activity with PtdIns4P), but since this activity was inhibited by EDTA it could not be attributed to the PtdIns4P phosphatase. The difference may be due to the procedure for isolating and washing the erythrocyte membranes, which had been optimized for human erythrocytes. Membranes prepared from all animal erythrocytes except sheep were pink, indicating incomplete removal of hemoglobin. PtdIns(4,5)P_2 phosphatase may also have been less effectively removed from these membranes. There was no similarity in the relative specific activities of the two enzymes among the different species. Human erythrocytes had the highest specific activity for the membrane PtdIns4P phosphatase and a low activity for the cytosol PtdIns(4,5)P_2 phosphatase, while the converse was true for erythrocytes from the other species. Hemoglobin contamination may contribute to the lower specific activity of PtdIns4P phosphatase in nonhuman erythrocyte membranes.

DISCUSSION

We have previously reported on the PtdIns(4,5)P_2 phosphatases of human erythrocytes and protozoa (12, 13). Both are highly specific and do not hydrolyze PtdIns4P under a variety of assay conditions. Whether or not these enzymes can degrade PtdIns4P in vivo, the rapid metabolic turnover of both monoesterified phosphates of the polyphosphoinositides requires such an activity. Previously reported PtdIns4P phosphatase activity was associated either with crude preparations (6, 7, 9, 10) or purified preparations isolated for their PtdIns(4,5)P_2 phosphatase activity (5, 8, 11), which completely degraded PtdIns(4,5)P_2 to PtdIns with the transient appearance of PtdIns4P. Few studies have used purified PtdIns4P and PtdIns(4,5)P_2 to assess both activities. Phosphomonoesterase activity of rabbit iris smooth muscle homogenates hydrolyzes PtdIns4P at 80% of the rate for PtdIns(4,5)P_2 (9). The highly purified polyphosphoinositide phosphatase from rat brain cytosol is equally active with PtdIns4P and PtdIns(4,5)P_2 (11), but its specificity with respect to other possible substrates remains to be established. PtdIns4P in chromaffin vesicle membranes

TABLE 6. Polyphosphoinositide phosphatases of other mammalian erythrocytes

Species (n)	EDTA (10 mM)	PtdIns4P Phosphatase		PtdIns(4,5)P_2 Phosphatase	
		Cytosol	Membrane	Cytosol	Membrane
Monkey[a] (1)	−	1.0	14.5	54.2	
	+		13.8		
Rabbit (3)	−	0.8 ± 0.3	9.1 ± 1.5	15.4 ± 1.8	4.6 ± 0.6
	+		7.6 ± 0.9	0.8 ± 1.1	0.4 ± 0.2
Rat (3)	−	1.0 ± 0.1	14.7 ± 6.4	49.6 ± 21	10.2 ± 1.9
	+		11.7 ± 3.5	2.3 ± 2.1	1.8 ± 0.6
Dog (3)	−	1.7 ± 0.2	4.4 ± 2.5	44.7 ± 2.0	2.5 ± 1.6
	+		1.7 ± 1.3	3.4	1.4
Sheep (3)	−	10.6 ± 0.6	2.8 ± 0.1	4.0 ± 0.4	0.8 ± 0.2
	+	10.0 ± 0.5	3.3± 1.0	1.1 ± 0.3	0.7 ± 0.1

Each phosphatase was assayed under optimum conditions as determined with human erythrocyte preparations. All preparations were from blood freshly collected in heparin. Activity is expressed as nmol · min^{-1} · mg $protein^{-1}$ ± standard deviation for n determinations when n is 3 or more.

[a] *Macaca fascicularis.*

is hydrolyzed by both phosphatase and phosphodiesterase activities in fractions of rat adrenal medulla homogenates (32). The vesicle membranes do not contain PtdIns(4,5)P_2 and the ability of these preparations to hydrolyze PtdIns(4,5)P_2 was not determined.

The apparent similarity in the characteristics and subcellular distribution of PtdIns4P and PtdIns(4,5)P_2 phosphatase activities of several tissues (6,10) led to the idea that one phosphatase removes both monoester phosphates from PtdIns(4,5)P_2. However, the EDTA-insensitive PtdIns4P phosphatase activity of human erythrocyte membranes reported here is clearly distinct from the PtdIns(4,5)P_2 phosphatase described earlier (13). That enzyme is localized in the cytosol and exhibits an absolute requirement for Mg^{2+} ions. The differential location and cation requirement of the two phosphatase activities was confirmed in erythrocytes from several other mammalian species. Human erythrocyte membranes have phosphatidic acid phosphatase activity (33). Lack of phosphatidic acid phosphatase activity in our preparations is consistent with the reported low specific activity (<1 nmol · min^{-1} · mg $protein^{-1}$) which is at the limit of detection in our assay system. Furthermore, the Mg^{2+}-dependence of this enzyme excludes it as a source of PtdIns4P phosphatase activity. The absence of activity with 2,3-bisphosphoglycerate or phosphoglycolate (34, 35) argues against the possible contamination with cytosol phosphatases, which might otherwise be implied by the low but consistent PtdIns(4,5)P_2 phosphatase activity present in the membranes. Lack of PtdIns4P phosphatase activity in the cytosol also excludes this enzyme as the source of the activity. Membrane-bound nucleotidases and both specific and nonspecific phosphatase were also excluded by negative results with a variety of possible substrates.

The erythrocyte membrane PtdIns4P phosphatase is quite specific. Only PtdIns4P and lysoPtdIns4P, but not PtdIns(4,5)P_2 or phosphate monoesters derived from it, were hydrolyzed in the absence of divalent cations. Lack of activity with either glycerophosphoinositol 4-phosphate or myoinositol 1,4-bisphosphate indicates that this phosphatase requires a lipid substrate. It is also consistent with the reported inability of the membrane inositol trisphosphate phosphatase to hydrolyze these compounds (16).

Enzymes acting on polyphosphoinositides are frequently assayed in complex reaction mixtures and there is considerable variety in the reported requirements of PtdIns(4,5)P_2 phosphatases. In general, maximum activity is obtained when requirements for Mg^{2+}, charge reduction of the substrate, and detergent solubilization are all met. Mg^{2+}-dependence has been shown for partially purified PtdIns(4,5)P_2 phosphatase from bovine brain, human erythrocyte cytosol, and protozoa (5, 12, 13) but not for any other system studied, including the highly purified enzyme from rat brain (11). Charge reduction is most readily achieved by forming mixed micelles of the substrate and a cationic detergent such as CTAB, although increasing the divalent cation concentration can achieve similar results (5). Prior studies of PtdIns4P hydrolysis have simply substituted PtdIns4P for PtdIns(4,5)P_2 in assay systems optimized for PtdIns(4,5)P_2. There is some evidence that PtdIns4P hydrolysis requires Mg^{2+} in kidney homogenates (10). Activity was stimulated by Mg^{2+} and inhibited by EDTA but there was a large residual EDTA-resistant activity. Comparison of PtdIns4P and PtdIns(4,5)P_2 hydrolysis by the purified rat brain PtdIns(4,5)P_2 phosphatase was limited to determining saturation curves in the absence of detergent but at the high Mg^{2+} concentration that is optimal for PtdIns(4,5)P_2 (11). Mg^{2+} was not required for the erythrocyte PtdIns4P phosphatase, the activity of which was not affected by either Mg^{2+} or EDTA. Our reaction conditions were optimized for PtdIns4P phosphatase activity and were different from the optimum PtdIns(4,5)P_2 phosphatase conditions. PtdIns4P is negatively charged and might be expected to require some charge moderation for maximum enzymic activity, although less than the more highly charged PtdIns(4,5)P_2. However, there is no evidence of such a requirement. CTAB had no effect on this activity in kidney homogenates when premixed with PtdIns4P and presented as mixed micelles (10). Our experience with erythrocyte membranes was similar but high CTAB concentrations, as are used in PtdIns(4,5)P_2 phosphatase assays, were inhibitory.

The effect of non-ionic detergents is complex. With soluble enzymes such as the PtdIns(4,5)P_2 phosphatases of *C. fasciculata* and human erythrocyte cytosol, the major advantage is reduced dependence on cationic compounds with little or no effect on maximum activity or the optimum CTAB/substrate ratio (12, 13). This suggests that dispersal of the substrate over the surface of large mixed micelles reduces the charge density encountered by the enzyme at the micelle surface. The major effect on the membrane-bound erythrocyte PtdIns4P phosphatase was to increase the accessibility of the enzyme by solubilizing the membranes. This view is supported by increased sensitivity to thermal inactivation and dithioerythritol stimulation following solubilization of the membranes with Triton X-100. Furthermore, the higher activity of untreated membranes with lysoPtdIns4P was comparable to that of solubilized membranes with PtdIns4P. This is probably due to the greater ability of lysolipids to penetrate and solubilize membranes. However, this may be an oversimplification, since other detergents, which dissolved the membranes, increased the temperature sensitivity but not the enzymic activity. This enzyme is clearly

quite sensitive to its environment, a conclusion that is further supported by the distinct transition in the Arrhenius plots of the activity which coincided with the onset of temperature sensitivity in both whole and solubilized membranes.

Under the most favorable assay conditions, the PtdIns4P phosphatase activity of human erythrocyte membranes was 40–50 nmol · min^{-1} · mg $protein^{-1}$. This is an order of magnitude greater than the inositol trisphosphate phosphatase activity measured in vitro with exogenous substrate (16) and approximately two orders of magnitude greater than the polyphosphoinositide phosphodiesterase activity measured with endogenous membrane-bound substrate (15). In view of the large effects of non-ionic detergents and of temperature on the PtdIns4P phosphatase in vitro, it is very likely that the environment of the enzyme in the membrane will greatly influence the in vivo activity and activities measured in the optimized assay may be much greater than can occur in vivo.

Although no specifically identified PtdIns4P phosphatase activity has been detected with endogenous membrane-bound substrate either in vitro or in vivo, there is some indirect evidence of such activity. Incubation of human erythrocytes with inorganic ^{32}P labels only the monoesterified phosphate groups of phosphatidic acid, PtdIns4P and PtdIns(4,5)P_2. Following lysis of the cells there is a cation-independent, EDTA-insensitive loss of both phosphatidic acid and PtdIns4P from the membrane (36). Ca^{2+}-activation of the polyphosphoinositide phosphodiesterase increases PtdIns4P loss and initiates PtdIns(4,5)P_2 loss. The cation-independent PtdIns4P phosphatase seems the most likely cause of the EDTA-insensitive component of PtdIns4P breakdown in these membranes. In more recent experiments (15) a small cation-independent release of inorganic phosphate from similarly labeled membranes was observed. Activation of the polyphosphoinositide phosphodiesterase caused release of inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate from PtdIns4P and PtdIns(4,5)P_2, but inorganic phosphate release was unchanged. The amount of radioactivity released as inorganic phosphate was approximately the same as that in the inositol 1,4-bisphosphate. If PtdIns4P phosphatase activity is truly the source of the inorganic phosphate, the in vivo activity of this enzyme is comparable to that of the polyphosphoinositide phosphodiesterase and much lower than that observed with the optimized in vitro assay.

The possibility that the PtdIns4P phosphatase is more widely distributed remains to be explored. Complete dephosphorylation of PtdIns(4,5)P_2 by a single phosphatase would require either an enzyme having dual activities or some other mechanism to explain the highly specific, sequential hydrolysis of the phosphate from the 5 and then the 4 position. Since neither phosphate is sterically hindered, it has been suggested that PtdIns(4,5)P_2 must be oriented in a membrane lipoprotein complex in such a way that only the 5-phosphate is initially available to a single phosphatase (2). Sequential action of two highly specific phosphatases, as now appears to be the case in erythrocytes, provides a simpler explanation for the specificity of the system.

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