The inositol trisphosphate phosphomonoesterase of the human erythrocyte membrane

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Human erythrocyte ghosts exhibit an inositol trisphosphate phosphomonoesterase activity that rapidly converts inositol 1,4,5-trisphosphate into inositol 1,4-bisphosphate and P_i . Degradation of the released inositol 1,4-bisphosphate is not observed. This activity is dependent on Mg^{2+} (or Mn^{2+}) and it is not activated by Ca^{2+} . Optimum activity is around pH 7 and activity is abolished by heat denaturation. The K_m for inositol trisphosphate is approx. $25\ \mu M$. 2,3-Bisphosphoglycerate is a competitive inhibitor, with a K_i of approx. $0.35\ mM$. Glycerophosphoinositol 4,5-bisphosphate is attacked at about one-eighth of the rate for inositol trisphosphate, but glycerophosphoinositol 4-phosphate is not a substrate. Incubation of ^{32}P -labelled erythrocyte membranes with Mg^{2+} causes little breakdown of phosphatidylinositol 4,5-bisphosphate, the parent compound from which both glycerophosphoinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate are derived. On the basis of its substrate specificity and the inhibition by 2,3-bisphosphoglycerate, we suggest that this enzyme is selective for the 5-phosphate in those water-soluble phosphate esters of inositol that possess the vicinal pair of 4,5-phosphates but that it may also interact less strongly with other water-soluble compounds that have pairs of vicinal phosphates.

Three inositol phospholipids, namely phosphatidylinositol, phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) P_2] appear likely to be constituents of all eukaryote cells (Michell, 1975; Hawthorne & White, 1975). In addition, a ubiquitous effect of hormones and neurotransmitters that use Ca^{2+} as their second messenger is to cause the breakdown of phosphatidylinositol, probably by a phosphodiesterase that releases a mixture of inositol 1-phosphate and inositol 1:2-cyclic phosphate (see Michell, 1975; Michell & Kirk, 1981). PtdIns4P and PtdIns(4,5) P_2 are also susceptible to degradation by phosphodiesterases, yielding Ins(1,4) P_2 and Ins(1,4,5) P_3 respectively. Recent studies have revealed situations in which such phosphodiesterases become activated by Ca^{2+} in erythrocytes (Allan & Michell, 1978; Downes &

Michell, 1981a,b; Allan & Thomas, 1981a,b) or synaptosomes (Griffin & Hawthorne, 1978), or through activation of certain cell-surface receptors in iris smooth muscle (see Akhtar & Abdel-Latif, 1980) and probably also in hepatocytes (Michell *et al.*, 1982; Kirk *et al.*, 1981). Although it seems unlikely that the stimulatory effects of Ca^{2+} on the polyphosphoinositide phosphodiesterase are ever expressed at the cytosolic $[Ca^{2+}]$ prevalent in healthy cells (Downes & Michell, 1982), it seems probable that the activation caused by hormones and neurotransmitters is a normal occurrence that would continuously liberate small quantities of Ins(1,4) P_2 and Ins(1,4,5) P_3 in cells. If this were so, then there would be a need for enzymic mechanisms to degrade these inositol polyphosphates. Breakdown of inositol trisphosphate has recently been observed both in iris smooth muscle (Akhtar & Abdel-Latif, 1980) and in human erythrocyte membranes (Downes & Michell, 1981a). The present paper describes in more detail the characteristics of the Ins(1,4,5) P_3 phosphomonoesterase of the erythrocyte membrane.

Abbreviations used: PtdIns4P, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4-phosphate; PtdIns(4,5) P_2 , 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4,5-bisphosphate; Ins(1,4) P_2 , L-*myo*-inositol 1,4-bisphosphate; Ins(1,4,5) P_3 , L-*myo*-inositol 1,4,5-trisphosphate.

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Materials and methods

Human blood was obtained from the Blood

Transfusion Centre within 7 days of donation. 2,3-Bisphosphoglycerate was from Sigma (London) Chemical Company, and other reagents were from sources specified previously (Downes & Michell, 1981a,b, 1982).

Preparation of ^{32}P -labelled $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$

^{32}P -labelled erythrocyte ghosts were prepared as described previously (Downes & Michell, 1981a). The ghosts were incubated in the presence of 1 mM-Ca^{2+} at 37°C for 30 min to activate the membrane-bound polyphosphoinositide phosphodiesterase and thus liberate radioactive $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$ from the polyphosphoinositides (Allan & Michell, 1978; Downes & Michell, 1981a). The incubations were diluted with ice-cold water and the membranes sedimented by centrifugation at $15000g$ for 15 min at 4°C . The membrane-free supernatant, which contained the inositol phosphates, was applied to a column containing 1 ml of Dowex-1 (formate form; 100–200 mesh). $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$ were sequentially eluted from the column by using ammonium formate and formic acid solutions as described previously (Downes & Michell, 1981a). Column eluates were freed of ammonium ions by treatment with Dowex-50 (H^+ form) and were freeze-dried. The dried samples were dissolved in water and stored frozen.

Preparation of ^{32}P -labelled glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate

These were prepared from the same samples of labelled erythrocyte ghosts as those used for the preparation of inositol phosphates. Phospholipids were extracted from the membranes by the method of Allan & Michell (1978). They were deacylated and the products separated on Dowex-1 (formate form) as described previously (Downes & Michell, 1981a): this procedure gave a quantitative conversion of lipid-soluble ^{32}P into water-soluble ^{32}P , and the only radioactive products detected by high-voltage electrophoresis were glycerophosphate, glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate. To obtain the latter two esters at convenient specific radioactivities, an appropriate sample of Folch fraction I/II (Folch, 1949), a lipid mixture enriched in $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$, was sometimes added before deacylation and ion-exchange chromatography. Simple treatment of these column eluates with Dowex-50 (to yield cation-free samples of these two esters) generated relatively concentrated solutions of formic acid, which caused considerable hydrolysis of the phosphodiester bond, thus leading to contamination of the samples with inositol

phosphates during subsequent handling. To avoid this problem, the eluates were diluted 3-fold with water and applied to 0.2 ml columns of Dowex-1 (formate form). The phosphate esters were then eluted with 3 ml of 2 M-LiCl . After freeze-drying, LiCl was removed by extraction with $4 \times 4\text{ ml}$ of ethanol. The salt-free samples were dried under vacuum, taken up in a small volume of water and stored frozen.

Preparation of unlabelled glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate

These were prepared exactly as described for the labelled compounds, the starting material being a sample of Folch fraction I/II from ox brain.

Analysis of phosphate ester preparations

The identities of the isolated phosphate esters were confirmed by high-voltage electrophoresis on Whatman no. 1 paper in a pyridine/acetic acid buffer ($\text{pH } 3.5$) at 50 V/cm for 75 min as described previously (Dawson *et al.*, 1962; Dawson & Clarke, 1972; Allan & Michell, 1978). Radioactive compounds were detected by autoradiography and phosphate-containing compounds by using the Hanes and Isherwood reagent as modified by Dawson (1960). Each radioactive sample migrated as a single component with the expected electrophoretic mobility (relative to P_i). The unlabelled glycerophosphoinositol 4-phosphate and 4,5-bisphosphate showed the expected electrophoretic mobilities and were uncontaminated by other phosphate-containing compounds. The concentrations of the final phosphate ester preparations were determined by phosphate analysis by the method of Bartlett (1959).

In a typical preparation starting from 30 ml of packed erythrocytes that had been labelled for 2 h at 37°C with 1.0 mCi of $[^{32}\text{P}]\text{P}_i$ (see Downes & Michell, 1981a, for details), we obtained $0.11\text{ }\mu\text{mol}$ of $\text{Ins}(1,4)\text{P}_2$ (sp. radioactivity $1.9 \times 10^6\text{ d.p.m./}\mu\text{mol}$) and $0.34\text{ }\mu\text{mol}$ of $\text{Ins}(1,4,5)\text{P}_3$ (sp. radioactivity $2.6 \times 10^6\text{ d.p.m./}\mu\text{mol}$).

The radioactive phosphate esters produced by these techniques are labelled only in their monoester phosphates (i.e. those at the 4- and 5-positions of the inositol ring). The 1-phosphate remains unlabelled since mammalian erythrocytes cannot synthesize phospholipids *de novo*. The monoesterified phosphate groups of $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ are labelled directly from ATP as a result of the reactions shown below.

Isolation of erythrocyte ghosts

Permeable erythrocyte ghosts were prepared by hypo-osmotic lysis of packed cells as described previously (Downes & Michell, 1981b). After the usual isolation in 2.5 mM-Hepes/1 mM-EGTA, the membranes were given a final wash in 10 vol of 1.5 mM-Hepes, pH 7.2, containing 0.154 M-NaCl. This procedure removed contaminating haemoglobin and some Mg^{2+} -independent $Ins(1,4,5)P_3$ phosphomonoesterase activity; the latter could account for up to 10% of the total membrane-associated activity in ghosts that had not been washed with an 'isotonic' salt solution.

The protein content of ghost preparations was determined by the method of Lowry *et al.* (1951).

$Ins(1,4,5)P_3$ phosphomonoesterase assay

For analysis of the products of membrane-catalysed breakdown, $Ins(1,4,5)P_3$ (10–100 μM) was incubated with 30 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.0, containing 2 mM- Mg^{2+} and 0.4–1 mg of membrane protein in 1 ml at 37°C for various times. Assays were stopped by the addition of an equal volume of 20% (w/v) trichloroacetic acid, kept on ice for 10 min and the precipitated proteins sedimented by centrifugation. Trichloroacetic acid was extracted from the supernatant with diethyl ether (4 $\times$ 4 volumes) and the aqueous samples were neutralized with NaOH. These were then applied to columns containing 1 ml of Dowex-1 (formate form). The columns were eluted sequentially with ammonium formate/formic acid solutions to separate P_i , inositol bisphosphate and $Ins(1,4,5)P_3$ (Downes & Michell, 1981a). Radioactivity in fractions obtained from columns was determined by Čerenkov counting.

As shown in the Results section, the $Ins(1,4,5)P_3$ phosphomonoesterase of the erythrocyte membrane only removes the 5-phosphate. This presented a problem when we came to devise a simplified enzyme assay that was based on the release of [^{32}P] P_i ; only the 60–80% of the ^{32}P of any individual preparation of $Ins(1,4,5)P_3$ that was in the 5-phosphate would be released as P_i , even when all of the $Ins(1,4,5)P_3$ was hydrolysed. For each preparation of substrate, therefore, a sample was incubated with erythrocyte ghosts until about half was hydrolysed, and the proportions of the total radioactivity that were in P_i (i.e. the 5-phosphate) and in $Ins(1,4)P_2$ (i.e. the 4-phosphate) determined. In presentation of kinetic experiments, [^{32}P] P_i release was then expressed in terms of only the release of that fraction of the radioactivity that was initially in the 5-phosphate.

For the simplified assay, $Ins(1,4,5)P_3$ (or another phosphate ester) was incubated in a final volume of 30–200 μl (this was varied, depending on the final concentration of substrate and the amount of

radioactive substrate needed) containing 30 mM-Hepes and, generally, 2 mM- Mg^{2+} . The reaction was started by the addition of erythrocyte membranes (0.4–0.7 mg of protein per ml of assay mixture). Incubations were stopped after 15 min by addition of 1M- $HClO_4$ to give a final volume of 1 ml. After centrifugation, a sample of the supernatant was removed and made 1% (w/v) with respect to ammonium molybdate (the precipitated protein was removed because addition of ammonium molybdate in the presence of denatured membranes resulted in substantial binding of P_i to the pellet). Isobutyl alcohol/toluene (1:1, v/v; 2 vol.) was then added and the sample mixed vigorously. A sample of the upper, organic phase, containing only P_i , was removed and mixed with one-third of its volume of methanol and 3 vol. of toluene scintillant, and its radioactivity was determined by scintillation counting.

Analysis of the products of $Ins(1,4,5)P_3$ breakdown

In addition to P_i , a smaller amount of ^{32}P was found in the inositol bisphosphate fraction after incubation of membranes with inositol trisphosphate and the separation of the products on Dowex-1 (see the Results section). For further analysis, this fraction was desalted and freeze-dried (see above). The inositol bisphosphate was dissolved in water containing a slight molar excess of cyclohexylamine and subjected to paper chromatography for 24 h on Whatman no. 1 paper in propan-2-ol-conc. NH_3 (sp.gr. 0.88)-water (5:4:1, by vol.) (Desjobert & Petek, 1956). Radioactive compounds were located by autoradiography using X-ray film.

In addition, the liberated inositol bisphosphate was subjected to periodate oxidation (Chang & Ballou, 1967; Prottey *et al.*, 1968). A sample containing approx. 0.1 μmol of inositol bisphosphate was incubated in 1 ml of 50 mM-sodium periodate, pH 4.0–5.0, in the dark at 37°C. Samples were removed at intervals and the radioactivity present as P_i and as organic phosphate was determined by solvent partition of the molybdate complex of P_i (see above). A sample of radiochemically pure $Ins(1,4)P_2$ produced by phosphodiesterase cleavage of erythrocyte $PtdIns4P$ (Downes & Michell, 1981a) was simultaneously oxidized under identical conditions.

Results

In our original experiments, ^{32}P -labelled erythrocyte ghosts were incubated with Ca^{2+} to activate a membrane-bound polyphosphoinositide phosphodiesterase. The $Ins(1,4)P_2$ and $Ins(1,4,5)P_3$ released by this activity were then separated by ion-exchange chromatography. In the presence of Mg^{2+} , the

released $\text{Ins}(1,4,5)\text{P}_3$ was further degraded to a compound that eluted with $\text{Ins}(1,4)\text{P}_2$, and this appeared to be relatively stable in the presence of erythrocyte membranes and Mg^{2+} . We therefore thought that erythrocyte membranes possessed an $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase (Downes & Michell, 1981a). We have now characterized this activity further by using purified radioactive inositol phosphates and related compounds as substrates.

The products of inositol trisphosphate breakdown

The results from the fractionation on Dowex-1 (formate form) of the trichloroacetic acid-soluble products generated during incubation of $\text{Ins}(1,4,5)\text{P}_3$ with erythrocyte membranes and Mg^{2+} are shown in Table 1. After incubation for 30 min at 37°C in the presence of Mg^{2+} , almost two-thirds of the radioactivity from $\text{Ins}(1,4,5)\text{P}_3$ was found in the fractions containing P_i and inositol bisphosphate, with about three times as much radioactivity in P_i as in inositol bisphosphate. Solvent partition of the molybdate complex (see above) confirmed that all of the radioactivity in the first fraction was P_i . There was no appreciable activity in the absence of Mg^{2+} . Since only the 4- and 5-phosphate groups will be labelled in the $\text{Ins}(1,4,5)\text{P}_3$ that is derived from erythrocyte $\text{PtdIns}(4,5)\text{P}_2$, the obvious interpretation for this selective release of highly labelled P_i was that one of these phosphomonoesters was being selectively removed by phosphomonoesterase activity.

The identity of the compound(s) eluting in the 'inositol bisphosphate' fraction was examined in more detail, using samples obtained from experiments in which about $0.1\ \mu\text{mol}$ of labelled inositol bisphosphate was degraded by incubation with erythrocyte membranes. A single radioactive compound was detected by paper chromatography and this co-chromatographed precisely with $\text{Ins}(1,4)\text{P}_2$ derived from $\text{PtdIns}4\text{P}$.

Periodate oxidation has been used previously to positively identify the 1,4-isomer of inositol bisphosphate (Chang & Ballou, 1967; Prottey *et al.*, 1968). Only *para*-isomers of inositol bisphosphate yield P_i on periodate treatment, and $\text{Ins}(1,4)\text{P}_2$ is the

only such isomer that can arise from $\text{Ins}(1,4,5)\text{P}_3$. Fig. 1 shows the liberation of radioactive P_i during prolonged periodate oxidation of a sample of the inositol bisphosphate derived from $\text{Ins}(1,4,5)\text{P}_3$, together with that from a parallel oxidation of the $\text{Ins}(1,4)\text{P}_2$ derived from $\text{PtdIns}4\text{P}$. In both cases, the oxidation displayed identical kinetics; at least 90% of the ^{32}P was ultimately released as P_i .

Thus the inositol bisphosphate released by the $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase of the erythrocyte membrane has the chemical and chromatographic properties of $\text{Ins}(1,4)\text{P}_2$, and the route of inositol bisphosphate breakdown is identified as the selective loss of the 5-phosphate.

Substrate specificity

Mammalian tissues possess enzymes that catalyse

Fig. 1. Periodate oxidation of the inositol bisphosphate produced from $\text{Ins}(1,4,5)\text{P}_3$ during incubation with erythrocyte membranes and Mg^{2+}

Separate incubations contained authentic ^{32}P -labelled $\text{Ins}(1,4)\text{P}_2$ obtained after treating erythrocyte membranes with Ca^{2+} (O) and ^{32}P -labelled inositol bisphosphate produced during incubation of $\text{Ins}(1,4,5)\text{P}_3$ with erythrocyte membranes and Mg^{2+} (●). At each time a sample was removed and the radioactivity present as organic phosphate and that present as P_i was determined by solvent partition (see the Materials and methods section). The results are expressed as the percentage of the total radioactivity that was in the form of radioactive P_i .

Table 1. Fractionation on Dowex-1 (formate form) of the products of inositol trisphosphate hydrolysis
Results are means $\pm$ S.E.M. of quadruplicate incubations in one experiment typical of three that gave similar results.

Period of incubation (min)	Radioactivity (d.p.m.) eluted in:		
	0.2 M-Ammonium formate/ 0.1 M-formic acid (P_i)	0.4 M-Ammonium formate/ 0.1 M-formic acid [$\text{Ins}(1,4)\text{P}_2$]	1.0 M-Ammonium formate/ 0.1 M-formic acid [$\text{Ins}(1,4,5)\text{P}_3$]
0	0	90 $\pm$ 27	3360 $\pm$ 87
30 (no Mg^{2+})	52 $\pm$ 31	93 $\pm$ 11	3200 $\pm$ 103
30 (+2 mM- Mg^{2+})	1420 $\pm$ 93	413 $\pm$ 13	1210 $\pm$ 18

the stepwise degradation of $\text{PtdIns}(4,5)\text{P}_2$ to $\text{PtdIns}4\text{P}$, and thence to phosphatidylinositol (Hawthorne, 1964; Thompson & Dawson, 1964*a,b*; Hawthorne & White, 1975; Michell, 1975). $\text{PtdIns}(4,5)\text{P}_2$ phosphomonoesterase specifically removes the 5-phosphate to give the naturally occurring $\text{PtdIns}4\text{P}$ (Chang & Ballou, 1967; Prottey *et al.*, 1968). Although it has been reported that erythrocyte membranes do not show $\text{PtdIns}(4,5)\text{P}_2$ phosphomonoesterase activity (Hokin & Hokin, 1964), it seemed essential, in view of the observed $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase activity, to determine whether our membrane preparations could degrade either the parent lipid (present in the same membranes) or glycerophosphoinositol 4,5-bisphosphate derived from this lipid by alkaline deacylation. When ^{32}P -labelled erythrocyte ghosts containing labelled $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ were incubated with 2 mM- Mg^{2+} for 30 min at 37°C there was a very slight loss of radioactivity from $\text{PtdIns}(4,5)\text{P}_2$, compared with control samples incubated with EDTA (Table 2). This was paralleled by a slight increase in the labelling of $\text{PtdIns}4\text{P}$, suggesting that the membranes displayed sufficient $\text{PtdIns}(4,5)\text{P}_2$ phosphomonoesterase activity to degrade approx. 5% of the total membrane $\text{PtdIns}(4,5)\text{P}_2$ in 30 min. By contrast, incubation of ghosts with Ca^{2+} rather than Mg^{2+} activated the polyphosphoinositide phosphodiesterase, and the radioactivity released from $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ was all accounted for by the accumulation of equivalent amounts of $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$. Further incubation of such samples that had been incubated with Ca^{2+} , this time for 15 min with 2 mM- Mg^{2+} , resulted in 90% breakdown of the accumulated $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,4)\text{P}_2$. Such assays do not yield initial rates, but there is no doubt that free $\text{Ins}(1,4,5)\text{P}_3$

was hydrolysed far more rapidly by the membrane-bound phosphomonoesterase than was $\text{PtdIns}(4,5)\text{P}_2$, its parent lipid in the membranes.

The substrate specificity of the inositol trisphosphate phosphomonoesterase was further assessed by using other ^{32}P -labelled phosphate esters as putative substrates in our routine assay procedures. $\text{Ins}(1,4,5)\text{P}_3$ and glycerophosphoinositol 4,5-bisphosphate were both substrates, with the former being attacked at about eight times the rate of the latter: at 60 μM substrate, the rates of attack were 2.5 nmol of $\text{Ins}(1,4,5)\text{P}_3$ /mg of membrane protein per min and 0.32 nmol of glycerophosphoinositol 4,5-bisphosphate/mg of protein per min. Neither $\text{Ins}(1,4)\text{P}_2$ nor glycerophosphoinositol 4-phosphate was hydrolysed at a detectable rate.

The positional specificity of glycerophosphoinositol 4,5-bisphosphate breakdown was then examined by comparing the distributions of radioactivity in P_i and organic phosphate after its hydrolysis and after the simultaneous hydrolysis of $\text{Ins}(1,4,5)\text{P}_3$ derived from the same samples of $\text{PtdIns}(4,5)\text{P}_2$. After $\text{Ins}(1,4,5)\text{P}_3$ breakdown the released P_i (i.e. the 5-phosphate) possessed 2.42 times the radioactivity of $\text{Ins}(1,4)\text{P}_2$ (i.e. the radioactivity of the 4-phosphate). The P_i released during glycerophosphoinositol 4,5-bisphosphate breakdown had 2.74 times the activity of glycerophosphoinositol 4-phosphate, i.e. an almost identical distribution of ^{32}P to that during the breakdown of $\text{Ins}(1,4,5)\text{P}_3$. This demonstrates that the glycerophosphoinositol 4,5-bisphosphate phosphomonoesterase activity also proceeded by the specific liberation of the 5-phosphate.

Properties of the $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase

The following results were all obtained using the

Table 2. *The phosphomonoesterase is much more active against $\text{Ins}(1,4,5)\text{P}_3$ than $\text{PtdIns}(4,5)\text{P}_2$*

^{32}P -labelled erythrocyte ghosts (0.5 ml) were incubated in a final volume of 1.0 ml containing 1 mM-EDTA, 2 mM- Mg^{2+} or 1 mM- Ca^{2+} . Samples containing 1 mM- Ca^{2+} were stopped after 30 min or incubated for a further 15 min after adding appropriate concentrations of EGTA (to bring the final concentration to 2 mM) or of EGTA and Mg^{2+} (to bring the final concentration of each to 2 mM). Reactions were stopped by the addition of 1 ml of 10% trichloroacetic acid and the radioactive polyphosphoinositides and/or inositol phosphates analysed (Downes & Michell, 1981*a*). The results are means $\pm$ s.e.m. of triplicate incubations from one of three experiments that gave similar results. Abbreviation: n.a., not assayed.

Incubation conditions	Radioactivity in various components of assay mixture (d.p.m.)			
	$\text{PtdIns}(4,5)\text{P}_2$	$\text{PtdIns}4\text{P}$	$\text{Ins}(1,4,5)\text{P}_3$	$\text{Ins}(1,4)\text{P}_2$
1 mM-EGTA for 30 min	54 200 $\pm$ 960	5730 $\pm$ 180	n.a.	n.a.
2 mM- Mg^{2+} for 30 min	51 200 $\pm$ 2500	6720 $\pm$ 170	n.a.	n.a.
1 mM- Ca^{2+} for 30 min	33 800 $\pm$ 1270	2060 $\pm$ 230	19 300 $\pm$ 1520	4520 $\pm$ 150
1 mM- Ca^{2+} for 30 min, then 2 mM-EGTA for 15 min	n.a.	n.a.	19 800 $\pm$ 730	4850 $\pm$ 370
1 mM- Ca^{2+} for 30 min, then 2 mM-EGTA and 2 mM- Mg^{2+} for 15 min	n.a.	n.a.	2380 $\pm$ 680	9540 $\pm$ 430

simplified phase-partition assay procedure. Owing to the limited amount of $\text{Ins}(1,4,5)\text{P}_3$ that was available, most of the assays were done using substrate concentrations of 30–60 μM , i.e. at approx. one to two times the K_m (see Fig. 4). Membrane concentrations and periods of incubation were usually chosen so as to ensure that $\leq 25\%$ of the substrate was degraded and the experiments thus yielded approximate initial reaction rates that were between 50% and 70% of $V_{\max}$.

The $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase activity of erythrocyte ghosts was completely inactivated by incubation of the membranes in a boiling-water bath for 2 min. There was no detectable activity in the presence of 1 mM-EDTA. Negligible hydrolysis of $\text{Ins}(1,4,5)\text{P}_3$ occurred in the absence of membranes.

The effects of pH on $\text{Ins}(1,4,5)\text{P}_3$ hydrolysis are shown in Fig. 2; 20 mM-Hepes/20 mM-cacodylate buffers were used (cacodylate had no effect on activity at pH 7.0). Greatest activity was exhibited around pH 7.0.

Bivalent cations were required for enzymic attack on $\text{Ins}(1,4,5)\text{P}_3$. Mg^{2+} appears likely to be the activating species *in vivo*, but Mn^{2+} could substitute partially, giving up to 50% of the activity obtained with Mg^{2+} (Fig. 3). Maximum activity required about 1–5 mM- Mg^{2+} and substantially higher concentrations (12 mM) caused slight inhibition (result not shown). There was no detectable activity in the presence of 5 mM- Ca^{2+} , and Ca^{2+} inhibited the Mg^{2+} -stimulated reaction (result not shown).

The hydrolysis of $\text{Ins}(1,4,5)\text{P}_3$ by the membrane-bound enzyme displayed normal Michaelis–

Menten kinetics with a K_m of $25 \pm 8 \mu\text{M}$ (mean $\pm$ S.E.M., obtained from three experiments with different erythrocyte ghost preparations) and $V_{\max}$ of 2.8 nmol/min per mg of membrane protein; the data in Fig. 4 are for one such experiment.

Fig. 3. Activation of the $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase by Mg^{2+} and Mn^{2+} .

$\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase activity was assayed as described in the Materials and methods section, but in the presence of various concentrations of MgCl_2 (O) or MnCl_2 (Δ). Each point is the mean of duplicate incubations in one of three experiments that gave similar results.

Fig. 2. pH dependence of the $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase

pH was varied using Hepes/cacodylate buffers and $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase was assayed as described in the Materials and methods section. Each point is the mean of duplicate incubations, and two experiments using ghosts prepared from different blood samples gave similar results.

Fig. 4. Determination of K_m and $V_{\max}$ for $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase

$\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase activity was assayed at various substrate concentrations as described in the Materials and methods section. The units of rate are $\text{nmol} \cdot \text{min}^{-1} (\text{mg of membrane protein})^{-1}$. The data were derived from duplicate incubations in a single experiment, one of three experiments using erythrocyte ghosts prepared from different blood samples that gave similar results (see the text).

Inhibitors

Several phosphate esters were tested to see whether they would inhibit the $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase. Unlabelled glycerophosphoinositol 4,5-bisphosphate (0.35 mM), a known substrate (see above), inhibited the hydrolysis of $60\mu\text{M}$ - $\text{Ins}(1,4,5)\text{P}_3$ by 76%. Unexpectedly, 0.4 mM- $\text{Ins}(1,4)\text{P}_2$, which is produced during $\text{Ins}(1,4,5)\text{P}_3$ breakdown, did not inhibit, but 0.35 mM-glycerophosphoinositol 4-phosphate produced 40% inhibition.

A variety of nucleotides and sugar phosphates were tested, of which the only relatively potent inhibitor was 2,3-bisphosphoglycerate. This, like $\text{Ins}(1,4,5)\text{P}_3$ and glycerophosphoinositol 4,5-bisphosphate, possesses a pair of vicinal phosphate groups. Inhibition by 2,3-bisphosphoglycerate of the hydrolysis of $\text{Ins}(1,4,5)\text{P}_3$ was therefore examined more closely, using substrate concentrations from 0.5 to 5 times the K_m , and the data were analysed by the method of Dixon (see Cornish-Bowden, 1979). The results, shown in Fig. 5, demonstrate that 2,3-bisphosphoglycerate competitively inhibited $\text{Ins}(1,4,5)\text{P}_3$ breakdown with a K_i of approx. 0.35 mM. Preliminary experiments have suggested that 2,3-bisphosphoglycerate may in fact be a slowly hydrolysed substrate, since a very slow release of P_i was observed after prolonged treatment of 2,3-bisphosphoglycerate with erythrocyte membranes (C. P. Downes, M. C. Mussat & R. H. Michell, unpublished work).

Fig. 5. Determination of the K_i for the competitive component of inhibition by 2,3-bisphosphoglycerate. $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase activity was assayed at three different substrate concentrations (O, $30\mu\text{M}$; □, $60\mu\text{M}$; Δ, $120\mu\text{M}$). The concentration of 2,3-bisphosphoglycerate was varied as indicated. $-K_i$ is given by the point of intersection of the lines obtained at different substrate concentrations. This is one of two experiments that gave similar results. Units of r are $\text{nmol} \cdot \text{min}^{-1} \cdot (\text{mg of membrane protein})^{-1}$.

Subcellular distribution

The protein content of erythrocyte ghost preparations varies substantially, depending on the ionic composition of the lysis medium (Bramley *et al.*, 1971; Bramley & Coleman, 1972; Schwach & Passow, 1973). To ensure that the observation of a membrane-bound enzyme was not an artefact of the preparation procedure, experiments were done using samples of an isoionic preparation of erythrocyte membranes (Billah *et al.*, 1976, 1977) that had been made permeable with Triton X-100. The membrane associated activity remained constant throughout the isolation of these haemoglobin-free ghosts. In addition, there was a readily detectable soluble activity that also degraded $\text{Ins}(1,4,5)\text{P}_2$ in preference to $\text{Ins}(1,4)\text{P}_2$ and required Mg^{2+} (C. P. Downes, M. C. Mussat & R. H. Michell, unpublished work). Thus it seems possible that erythrocytes possess two enzymes capable of degrading $\text{Ins}(1,4,5)\text{P}_3$ in a specific manner, with one firmly bound to the membrane and the other in the cytosol. Roach & Palmer (1981) have recently described a cytosolic $\text{PtdIns}(4,5)\text{P}_2$ phosphomonoesterase in human erythrocytes that also attacks $\text{Ins}(1,4,5)\text{P}_3$; we suspect that this is responsible for the $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase activity that we observed in erythrocyte cytosol.

Discussion

$\text{Ins}(1,4,5)\text{P}_3$ phosphatase in the erythrocyte

We previously showed that human erythrocyte membranes display phosphomonoesterase activity towards $\text{Ins}(1,4,5)\text{P}_3$ (Downes & Michell, 1981a), and the results presented here demonstrate that such membranes possess an Mg^{2+} -dependent phosphomonoesterase that selectively attacks only the 5-phosphate of $\text{Ins}(1,4,5)\text{P}_3$ and of glycerophosphoinositol 4,5-bisphosphate [and maybe, very slowly, of $\text{PtdIns}(4,5)\text{P}_2$]. Inhibition by 2,3-bisphosphoglycerate suggested that the enzyme has a particular affinity for pairs of vicinal monoester phosphates, and also raised the possibility that we might have been observing degradation of $\text{Ins}(1,4,5)\text{P}_3$ by the previously described 2,3-bisphosphoglycerate phosphomonoesterase activity of the erythrocyte (Rapoport & Luebering, 1951); this activity represents a composite of the reversal of 2,3-bisphosphoglycerate synthase and a side-reaction catalysed by 2,3-bisphosphoglycerate mutase (Rose, 1980). The 'apparent K_m ' for 2,3-bisphosphoglycerate of this composite activity is close to the K_i (0.35 mM) for inhibition of $\text{Ins}(1,4,5)\text{P}_3$ breakdown by 2,3-bisphosphoglycerate. For several reasons, we consider it unlikely that the $\text{Ins}(1,4,5)\text{P}_3$ activity that we have observed is a further facet of the activities of these 2,3-bisphosphoglycerate-metabolizing enzymes. The

Ins(1,4,5) P_3 phosphomonoesterase that we studied was tightly bound to the membrane, persisting in an isoionic ghost preparation and in low-ionic-strength ghosts that were washed with iso-osmotic saline; 2,3-bisphosphoglycerate phosphomonoesterase activity is mainly cytosolic (Rose, 1980). Further, it has been reported that the 2,3-bisphosphoglycerate phosphatase activity is activated by 0.2 mM- Ag^+ and strongly inhibited by 0.2 mM- Cu^{2+} ; 0.2 mM- Ag^+ almost abolished the activity of the Ins(1,4,5) P_3 phosphomonoesterase and 0.2 mM- Cu^{2+} only weakly inhibited it (results not shown).

Human erythrocytes have a Ca^{2+} -dependent membrane-bound polyphosphoinositide phosphodiesterase that releases Ins(1,4,5) P_3 from PtdIns(4,5) P_2 (Allan & Michell, 1978; Downes & Michell, 1981*a,b*), and the Ins(1,4,5) P_3 phosphatase of the erythrocyte was first noted by virtue of its ability to hydrolyse the Ins(1,4,5) P_3 released by this enzyme (Downes & Michell, 1981*a*). However, there are cogent reasons for thinking that the polyphosphoinositide phosphodiesterase of the erythrocyte is never activated during the lifespan of the circulating healthy erythrocyte (Downes & Michell, 1982) and that maybe it is a relic that remains from an earlier stage in cellular development (e.g. from erythropoiesis). If this is correct, then the same arguments apply to the Ins(1,4,5) P_3 phosphomonoesterase; if no PtdIns(4,5) P_2 ever breaks down by the phosphodiesterase route in the healthy mature erythrocyte then there can never be any substrate for the Ins(1,4,5) P_3 phosphomonoesterase. We therefore wonder, as with the polyphosphoinositide phosphodiesterase (Downes & Michell, 1982), whether this Ins(1,4,5) P_3 phosphomonoesterase may simply serve as an easily studied exemplar, but with a need for the function of Ins(1,4,5) P_3 phosphomonoesterase to be defined by studies using other cells.

A general role for Ins(1,4,5) P_3 phosphatases?

This raises the obvious question as to whether Ins(1,4,5) P_3 is ever produced within cells other than the erythrocyte, and hence whether there is a need for an enzyme to degrade it. There are hints that suggest that such situations may be widespread. Abdel-Latif and his colleagues have defined a phosphodiesterase-mediated breakdown of PtdIns(4,5) P_2 in iris smooth muscle when stimulated by muscarinic cholinergic or α -adrenergic ligands (Abdel-Latif *et al.*, 1978), but they find that the majority of the released lipid head-groups accumulate in the tissue as inositol monophosphate rather than Ins(1,4,5) P_3 (Akhtar & Abdel-Latif, 1980). Indeed, they claim to have detected Ins(1,4,5) P_3 phosphatase in iris smooth muscle (Akhtar & Abdel-Latif, 1980). In previous experiments, Griffin & Hawthorne (1978) had seen a Ca^{2+} -activated

breakdown of PtdIns(4,5) P_2 in synaptosomes that led to accumulation of inositol bisphosphate rather than Ins(1,4,5) P_3 . They interpreted this as the result of the sequential action of PtdIns(4,5) P_2 phosphomonoesterase and PtdIns4*P* phosphodiesterase, but they also acknowledged the alternative possibility of phosphodiesterase action followed by an Ins(1,4,5) P_3 phosphomonoesterase-catalysed production of inositol bisphosphate. The latter alternative now seems more likely. Most recently, we have found very rapid breakdown of PtdIns4*P* and PtdIns(4,5) P_2 in rat hepatocytes stimulated with Ca^{2+} -mobilizing stimuli such as vasopressin (Kirk *et al.*, 1981; Michell *et al.*, 1982). This response is very rapid [approx. 30% breakdown of PtdIns(4,5) P_2 in 1 min], it has appropriate characteristics for it to be a candidate for a role in the coupling between receptor activation and Ca^{2+} mobilization and it seems likely to be catalysed by a phosphodiesterase.

It seems likely that Ins(1,4,5) P_3 will be released from membrane-bound PtdIns(4,5) P_2 into the cytosol in each of these situations, and that cells will therefore have a need to further metabolize this product. The importance of this degradative reaction may be accentuated by the fact that the high affinity of PtdIns(4,5) P_2 for bivalent cations resides mainly in its vicinal pair of phosphates (Hendrickson & Reinertsen, 1969); this grouping is preserved during the release of Ins(1,4,5) P_3 but destroyed when the phosphomonoesterase removes the 5-phosphate. It is possible that Ins(1,4,5) P_3 liberated by hormone action might have some function within a stimulated cell, in which case the presence in high activity of an enzyme capable of its degradation (and hence inactivation) might be expected. We would also anticipate that, in order for inositol and inositol lipid metabolism to continue freely, cells should possess additional phosphomonoesterase activities, either specific or non-specific, that are ultimately capable of returning the inositol of inositol trisphosphate to the free inositol pool of the cell.

A useful analytical tool?

Not only does the newly-discovered Ins(1,4,5) P_3 phosphomonoesterase seem likely to provide the enzymic mechanism for a metabolic step that might have been anticipated, but it also promises to be a remarkably valuable analytical tool. One of the technical deficiencies that has prevented detailed study of the metabolism of the polyphosphoinositides has been the lack of readily available and simple tools with which to analyse the metabolic behaviour of the three phosphates in the headgroup of PtdIns(4,5) P_2 ; for example, we do not even know the relative turnover rates of the two monoesterified phosphates. The ability of the Ins(1,4,5) P_3 phosphomonoesterase of easily prepared erythrocyte ghosts to attack glycerophosphoinositol 4,5-bis-

phosphate, the water-soluble phosphate ester produced by treatment of $\text{PtdIns}(4,5)\text{P}_2$ with mild alkali, opens up the possibility of using sequential attack on glycerophosphoinositol 4,5-bisphosphate, first by $\text{Ins}(1,4,5)\text{P}_3$ phosphatase to remove the 5-phosphate and then by an unspecific phosphatase to remove the 4-phosphate, to analyse the detailed metabolism of each of these three phosphates in $\text{PtdIns}(4,5)\text{P}_2$.

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