Subcellular localization of the enzymes that dephosphorylate *myo*-inositol polyphosphates in human platelets

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The phosphatase-induced hydrolysis of [^3H]inositol 1,4-bisphosphate [$\text{Ins}(1,4)\text{P}_2$] and [^3H]inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] was studied in platelet subcellular fractions. The activity that hydrolyses $\text{Ins}(1,4)\text{P}_2$ is cytosolic, whereas the activity that hydrolyses $\text{Ins}(1,4,5)\text{P}_3$ is present in both particulate and cytosolic fractions. The cytosolic $\text{Ins}(1,4)\text{P}_2$ phosphatase hydrolyses the 1-phosphate of $\text{Ins}(1,4)\text{P}_2$, whereas the cytosolic and membrane-bound $\text{Ins}(1,4,5)\text{P}_3$ phosphatases hydrolyse the 5-phosphate of $\text{Ins}(1,4,5)\text{P}_3$. In the presence of ATP, it is possible to observe a cytosolic $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase that phosphorylates $\text{Ins}(1,4,5)\text{P}_3$ to inositol 1,3,4,5-tetrakisphosphate. Apparent K_m values for the particulate and the cytosolic $\text{Ins}(1,4,5)\text{P}_3$ phosphatases are $100\ \mu\text{M}$ and $40\ \mu\text{M}$ respectively. A large proportion of the membrane-associated $\text{Ins}(1,4,5)\text{P}_3$ phosphatase can be extracted with $1\ \text{M-NaCl}$, and the M_r of this enzyme, as determined by hydrodynamic studies, is 49 000, whereas that of the cytosolic enzyme is 59 000. The K_m values for the cytosolic $\text{Ins}(1,4)\text{P}_2$ phosphatase is $40\ \mu\text{M}$; this enzyme has an M_r of 49 000. The highest specific activity of the $\text{Ins}(1,4,5)\text{P}_3$ phosphatase is present in a highly purified plasma-membrane fraction.

INTRODUCTION

Inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] releases Ca^{2+} from the endoplasmic reticulum to the cytosol [1–5]. The persistence of this action depends on the 5-phosphatase that hydrolyses $\text{Ins}(1,4,5)\text{P}_3$ to inositol 1,4-bisphosphate [$\text{Ins}(1,4)\text{P}_2$]. $\text{Ins}(1,4)\text{P}_2$ can be further dephosphorylated to inositol 4-monophosphate [$\text{Ins}(4)\text{P}$] or inositol 1-monophosphate [$\text{Ins}(1)\text{P}$] and inositol [6]. $\text{Ins}(1,4,5)\text{P}_3$ phosphatase is present in the membranes and cytosol of various tissues [7–13]. In platelets, however, the enzyme has been reported to be almost exclusively in the soluble fraction, with an M_r of 45 000, hydrolysing the 5-phosphate from $\text{Ins}(1,4,5)\text{P}_3$, cyclic $\text{Ins}(1,2,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ [10,14]. We have now found that a considerable percentage of the activity of $\text{Ins}(1,4,5)\text{P}_3$ phosphatase is membrane-bound, and it is extracted by high concentrations of NaCl, whereas the enzyme that degrades $\text{Ins}(1,4)\text{P}_2$ is soluble. As in other tissues [14], platelet cytosol catalyses the phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,3,4,5)\text{P}_4$ in the presence of ATP.

EXPERIMENTAL

Preparation of platelet subcellular fractions

Human platelets were washed, lysed and fractionated by a method previously published [15]. Platelets from fresh human blood or outdated platelets from the American Red Cross were washed twice with a phosphate buffer, pH 6.5, containing 113 mM-NaCl, 4.3 mM- K_2HPO_4 , 4.3 mM- Na_2HPO_4 , 24.4 mM- NaH_2PO_4 , 5.5 mM-glucose and 1 mM-EGTA. Platelets were then resuspended in a hypo-osmotic buffer (11 mM-Tris, 14 mM-Mes, 3 mM- MgCl_2 , 20 mM- β -mercaptoethanol, pH 6.5) and lysed by one cycle of freezing and thawing, followed by nitrogen cavitation at 1000 lb/in 2 (6.9 MPa) for 1 h.

The homogenate was centrifuged at 100 000 g for 1 h to obtain the cytosolic and particulate fractions. The particulate fraction was washed twice with the hypo-osmotic buffer described above and resuspended in 1 ml of the same buffer. The particulate fraction was further fractionated to obtain a platelet plasma-membrane fraction as described previously [15,16]. Briefly, the resuspended particulate fraction (1 ml) was layered on top of 6.5 ml of 27% (w/v) sucrose (in the above Tris/Mes buffer, pH 6.5) and centrifuged at 100 000 g for 3 h. Membranes, which were observed as a band at the top of the sucrose layer, were carefully aspirated with a Pasteur pipette and diluted with the same buffer. The membrane fraction was centrifuged at 100 000 g for 1 h and resuspended in the same buffer. Particulate fraction or purified membranes were extracted with 1 M-NaCl in the presence of 3 mM- MgCl_2 , 14 mM-Mes and 11 mM-Tris for 10 min at 4 $^{\circ}\text{C}$ and then pelleted at 170 000 g for 10 min in a Beckman Airfuge centrifuge. This method extracted membrane-associated $\text{Ins}(1,4,5)\text{P}_3$ phosphatase.

Determination of molecular and hydrodynamic parameters

Molecular and hydrodynamic parameters were determined as previously reported [17,18], except that gel filtration was performed on a fast-performance-liquid-chromatography Superose 12 column (Pharmacia) equilibrated in the Tris/Mes buffer described above containing 100 mM-NaCl. This buffer with NaCl was also used for the preparation of the sucrose gradients with H_2O or $^2\text{H}_2\text{O}$. Calibrating proteins (catalase, lactate dehydrogenase, malate dehydrogenase and cytochrome c) were added to the samples (gradient centrifugation or gel filtration) at the concentrations previously described

Abbreviations used: Ins, inositol; $\text{Ins}(1)\text{P}$, inositol 1-monophosphate; $\text{Ins}(4)\text{P}$, inositol 4-monophosphate; $\text{Ins}(1,3)\text{P}_2$, inositol 1,3-bisphosphate; $\text{Ins}(1,3,4)\text{P}_3$, inositol 1,3,4-trisphosphate; $\text{Ins}(1,4)\text{P}_2$, inositol 1,4-bisphosphate; $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; cyclic $\text{Ins}(1,2,4,5)\text{P}_3$, inositol 1,2(cyclic),4,5-trisphosphate; $\text{Ins}(1,3,4,5)\text{P}_4$, inositol 1,3,4,5-tetrakisphosphate.

[18] and were assayed by previously published methods [17,18]. Sedimentation coefficients ($s_{20,w}$) were calculated as described by Martin & Ames [19]. The 5'-nucleotidase was determined as previously described [20].

Determination of inositol polyphosphate hydrolysis by platelet subcellular fractions

Inositol polyphosphate hydrolysis was determined by measuring the activity of phosphatases converting [^3H]Ins(1,4,5) P_3 into [^3H]Ins(1,4) P_2 or [^3H]Ins(1,4) P_2 into [^3H]Ins(4) P . The activity of the various inositol phosphatases was determined in 50 μl assay mixtures containing 10 μM of non-radioactive Ins(1,4) P_2 or Ins(1,4,5) P_3 plus 15000 c.p.m. of [^3H]Ins(1,4) P_2 or [^3H]Ins(1,4,5) P_3 , respectively, 50 mM-Mes, 3 mM-MgCl₂ and 5–20 μg of protein from the different platelet subcellular fractions. In some experiments 5 mM-ATP was included. Incubations were carried out at 37 °C for 1–5 min, and reactions were stopped by addition of 8 ml of water. The mixture was applied to Dowex AG1-X8 columns, and the inositol phosphates were eluted with increasing concentrations of ammonium formate as previously described [21–23]. In some experiments, the reaction products were characterized by h.p.l.c.

Samples for h.p.l.c. were incubated as above, in a final volume of 100 μl , and contained 50000 cpm of [^3H]Ins(1,4,5) P_3 or [^3H]Ins(1,4) P_2 . The reaction was stopped with 450 μl of boiling water containing 1 % SDS and 30 mM-EDTA, and the mixture was boiled for a further 5 min.

Inositol polyphosphates were separated by h.p.l.c. on a Whatman Partisil 10 SAX column with ammonium phosphate, pH 3.8, flow rate 1 ml/min, in accordance with a method previously described [24]. The gradients used were: 0–0.1 M over 35 min for inositol, Ins(1) P and Ins(4) P ; 0.2–0.28 M over 30 min for Ins(1,3) P_2 , Ins(3,4) P_2 and Ins(1,4) P_2 ; 0.5–0.52 M over 30 min for Ins(1,3,4) P_3 and Ins(1,4,5) P_3 ; and 2 M over 20 min for Ins(1,3,4,5) P_4 . Radioactivity was monitored with a Flo-one/Beta (model BD) flow detector (Radiomatic

Instruments, Tampa, FL, U.S.A. Inositol polyphosphates and monophosphates were identified on the basis of their co-elution with authentic radiolabelled standards.

[^3H]Ins(1,3,4) P_3 standard was prepared by incubating [^3H]Ins(1,3,4,5) P_4 with platelet cytosol, yielding a product that was eluted immediately before Ins(1,4,5) P_3 . This product has been shown to be Ins(1,3,4) P_3 [25]. Another peak from this incubation eluted before Ins(1,4) P_3 was either [^3H]Ins(1,3) P_2 or [^3H]Ins(3,4) P_2 [26].

Materials

D-[2- ^3H (n)]Inositol 1-phosphate, D-[2- ^3H (n)]inositol 4-phosphate, D-[2- ^3H (n)]inositol 1,4-bisphosphate and D-[2- ^3H (n)]inositol 1,4,5-trisphosphate were from New England Nuclear Research Products, Boston, MA, U.S.A. Ins(1) P and Ins(1,4) P_2 were from Amersham, Arlington Heights, IL, U.S.A. Ins(1,4,5) P_3 , catalase (bovine liver), lactate dehydrogenase (rabbit muscle), malate dehydrogenase (pig heart) and cytochrome *c* (horse heart) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Dowex AG1-X8 anion resin (100–200 mesh; formate form) was from Bio-Rad, Richmond, CA, U.S.A. $^3\text{H}_2\text{O}$ was from Aldrich Chemical Co., Milwaukee, WI, U.S.A.

RESULTS

Inositol phosphatases that hydrolyse Ins(1,4,5) P_3 and Ins(1,4) P_2 were assayed in platelet homogenate, cytosol, total particulate fraction and a purified plasma-membrane fraction. Ins(1,4) P_2 phosphatase was almost exclusively confined to the cytosolic fraction. This localization paralleled the activity of lactate dehydrogenase (Table 1), which is a cytosolic marker. Ins(1,4,5) P_3 phosphatase was present in both cytosol and particulate fractions in about equal amounts (Table 1), the particulate fraction being enriched in 5'-nucleotidase. The specific activity of the Ins(1,4,5) P_3 phosphatase was much higher in the particulate fraction than in the cytosolic fraction (Table 1). Further fractionation of the

Table 1. Subcellular localization of the phosphatases degrading Ins(1,4) P_2 and Ins(1,4,5) P_3 of human platelets

The degradation of [^3H]Ins(1,4) P_2 and [^3H]Ins(1,4,5) P_3 was measured in various subcellular fractions of human platelets as detailed in the Experimental section. Concentration of the various inositol phosphates was 10 μM . Recoveries are expressed as % of the activity in the cavitated sample. Lactate dehydrogenase units are $\mu\text{mol}/\text{min}$ and 5'-nucleotidase units are mmol/min .

	Cavitated sample		Cytosol			Particulate		
	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Recovery (%)	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Recovery (%)
Ins(1,4) P_2 phosphatase	0.307	120.20	0.455	116.20	96.8	0.077	4	3.3
Ins(1,4,5) P_3 phosphatase	2.094	818.90	0.604	259.70	31.8	4.420	233	28.5
	(units/mg)	(units)	(units/mg)	(units)	(%)	(units/mg)	(units)	(%)
Lactate dehydrogenase	0.876	342	1.580	404	118	—	—	—
5'-Nucleotidase	2.270	888	—	—	—	5.790	612	68

Fig. 1. Filtration through Superose 12 of inositol polyphosphate phosphatases

The designated markers are: 1, cytochrome *c*; 2, malate dehydrogenase; 3, lactate dehydrogenase; 4, catalase.

particulate with a 27 %-sucrose cushion yielded a purified plasma-membrane fraction that was highly enriched in Ins(1,4,5) P_3 phosphatase activity, with a specific activity of 39.7 nmol/min per mg of protein. This was the highest specific activity for Ins(1,4,5) P_3 phosphatase among all the platelet fractions studied.

About 40 % of the Ins(1,4,5) P_3 phosphatase activity associated with the purified membrane fraction was extracted with 1 M-NaCl and subjected to gel chromatography and ultracentrifugation to determine the

Table 2. Molecular parameters for enzymes that dephosphorylate inositol phosphates

	Ins(1,4) P_2 phosphatase	Ins(1,4,5) P_3 phosphatase (cytosolic)	Ins(1,4,5) P_3 phosphatase (membrane-associated)
Stokes radius (nm)	3	3.2	3
Partial specific volume (ml/g)	0.74	0.74	0.74
Sedimentation coefficient (S)	3.8	4.3	3.8
M_r	49000	59000	49000
Frictional ratio	1.2	1.2	1.2

molecular parameters of the enzyme. Molecular parameters were also determined for the cytosolic Ins(1,4) P_2 phosphatase and Ins(1,4,5) P_3 phosphatase. Fig. 1 shows the standard curve of K_{av} (partition coefficient) versus Stokes radius for the calibrating enzymes and the various phosphatases. With these data and those obtained from ultracentrifugation of 5–20 % sucrose gradients in H_2O and 2H_2O , the molecular parameters presented in Table 2 were calculated. Ins(1,4) P_2 phosphatase and membrane-bound and cytosolic Ins(1,4,5) P_3 phosphatases are all globular proteins, with M_r values of 49000, 49000 and 59000 respectively.

It was previously reported that the K_m value for the platelet cytosolic Ins(1,4,5) P_3 phosphatase was 30 μM [27] and 17 μM [28]. Our study indicates a K_m of 40 μM for the cytosolic enzyme, whereas the K_m for the particulate Ins(1,4,5) P_3 phosphatase is 100 μM . The K_m for the NaCl-extracted enzyme from the particulate fraction is

Fig. 2. H.p.l.c. profile of metabolites of $[^3H]$ Ins(1,4,5) P_3 incubated with platelet cytosol

Preparation of cytosol, incubation for 3 min in the presence of 5 mM-ATP, and chromatography were carried out as described in the Experimental section.

Fig. 3. H.p.l.c. profile of metabolites of $[^3\text{H}]\text{Ins}(1,4,5)P_3$ incubated with extract of particulate fraction

Preparation of the NaCl extract of platelet particulate fraction, incubation for 3 min in the presence of 5 mM-ATP, and chromatography were carried out as described in the Experimental section.

100 μM . The K_m for the cytosolic $\text{Ins}(1,4)P_2$ phosphatase is 40 μM .

Fig. 2 shows the h.p.l.c. profile of radioactive products obtained by incubation of $[^3\text{H}]\text{Ins}(1,4,5)P_3$ with platelet cytosolic fraction in the presence of ATP. The peak of radioactivity associated with $[^3\text{H}]\text{Ins}(1,4,5)P_3$ is greatly diminished after incubation for 3 min. The major products are co-eluted with $[^3\text{H}]\text{Ins}(1,4)P_2$ and $[^3\text{H}]\text{Ins}(4)P$ standards. The peak co-eluted with $\text{Ins}(1)P$ is relatively insignificant.

Two further peaks are evident after a 3 min incubation and exhibit the chromatographic mobility of $[^3\text{H}]\text{Ins}(1,3,4)P_3$ (90 min) and $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ (112 min). The formation of these latter peaks is dependent on the presence of ATP. In the absence of the nucleotide, $[^3\text{H}]\text{Ins}(1,4,5)P_3$ yields only $[^3\text{H}]\text{Ins}(1,4)P_2$ and $[^3\text{H}]\text{Ins}(4)P$ (results not shown). The small peak at 5 min has the mobility of $[^3\text{H}]\text{inositol}$. Interestingly, there is also a peak of radioactivity (peak A) eluted at 68 min in the region of the inositol bisphosphates.

When $[^3\text{H}]\text{Ins}(1,4,5)P_3$ was incubated with a NaCl extract of the platelet particulate fraction for 3 min, in the presence of ATP (Fig. 3) or in its absence (results not shown), $[^3\text{H}]\text{Ins}(1,4)P_2$ is formed. Peak A is present again at 68 min. Because $[^3\text{H}]\text{Ins}(1,4,5)P_3$ is the only possible precursor in this case, peak A might be $[^3\text{H}]\text{Ins}(4,5)P_2$, which is eluted several minutes after $[^3\text{H}]\text{Ins}(1,4)P_2$ [24].

In the incubation with the saline extract of platelet particulate fraction there is no evidence of $[^3\text{H}]\text{Ins}(4)P$, nor is there any indication of $\text{Ins}(1,4,5)P_3$ 3-kinase activity, as evidenced by peaks co-eluting with $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ and $[^3\text{H}]\text{Ins}(1,3,4)P_3$ (Fig. 3).

To characterize further the products of the metabolism of $[^3\text{H}]\text{Ins}(1,4)P_2$, this substrate was incubated for 3 min with platelet cytosolic fraction. The metabolic profile is

shown in Fig. 4. Again, the product is co-eluted with $[^3\text{H}]\text{Ins}(4)P$.

DISCUSSION

A considerable percentage of the total activity of platelet $\text{Ins}(1,4,5)P_3$ phosphatase is associated with the particulate fraction: the highest specific enzymic activity is in a purified plasma-membrane fraction. The percentage of membrane-bound activity that we found is higher than the value reported by Connolly *et al.* [27]; they found that only about 15% of the activity was present in the particulate fraction. Our data are consistent with data from other tissues, in which a considerable amount of the total enzymic activity is associated with the membranes [14]. Some 40% of the membrane-bound $\text{Ins}(1,4,5)P_3$ phosphatase can be extracted by a high concentration of NaCl, which indicates that this activity is not intrinsically associated with the membrane. It also seems to be different from the cytosolic activity, because the M_r values as determined by hydrodynamic and molecular parameters are different, and they also differ in their respective K_m values.

Connolly *et al.* [27] were unable to detect activity that would dephosphorylate $\text{Ins}(1,4)P_2$ in platelet homogenate, and they further indicated that $\text{Ins}(1,4)P_2$ inhibits $\text{Ins}(1,4,5)P_3$ phosphatase. Conversely, we find $\text{Ins}(1,4)P_2$ phosphatase activity with a subcellular distribution different from that of $\text{Ins}(1,4,5)P_3$ phosphatase. $\text{Ins}(1,4)P_2$ phosphatase is predominantly cytosolic, whereas $\text{Ins}(1,4,5)P_3$ phosphatase is evenly distributed between the cytosolic and particulate fractions.

The reported M_r of the soluble $\text{Ins}(1,4,5)P_3$ 5-phosphatase in human platelets is 45000 [14], but this value was obtained after gel electrophoresis in the presence of SDS. We have found an M_r of 59000 by

Fig. 4. H.p.l.c. profile of the metabolites of $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ incubated with platelet cytosol

Preparation of cytosol, incubation for 3 min in the absence of ATP, and chromatography were carried out as described in the Experimental section.

using molecular sieves and sucrose gradients for the determinations of molecular parameters. Therefore, the different methods used could explain the different values of M_r for the same activity.

Shears *et al.* [29] have recently shown in the rat liver soluble fraction that the M_r of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase in its native form is 77000, indicating the possibility that the enzyme exists in a multimeric form.

We have also determined the molecular parameters of a membrane-associated $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase, with an M_r of 49000. The difference in M_r and K_m values ($100\ \mu\text{M}$ for the membrane-associated and $40\ \mu\text{M}$ for the cytosolic) might indicate two isoforms of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase in platelets.

The $\text{Ins}(1,4)\text{P}_2$ 1-phosphatase that we studied in platelets shows an M_r of 49000 and a K_m of $40\ \mu\text{M}$, which are values different from the activity reported in rat liver soluble fraction (M_r 58000, K_m $0.9\ \mu\text{M}$) [30], suggesting that both phosphatases of these two cell types may be different entities.

To characterize the products of the enzymic reactions involved in inositol polyphosphate degradation in platelets, we used a chromatographic system that allows separation of different isomers [24]. The major metabolite of $\text{Ins}(1,4,5)\text{P}_3$ in platelet cytosol and membranes was found to be the product of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase, $\text{Ins}(1,4)\text{P}_2$. This is a predominant pathway of degradation of $\text{Ins}(1,4,5)\text{P}_3$ in other cell types also [14,31], but the platelets are unusual in that half of the activity is soluble and half is associated with the particulate fraction. In addition to $\text{Ins}(1,4)\text{P}_2$, we found another peak (peak A, Figs. 2 and 3) with chromatographic mobility similar to that of $\text{Ins}(4,5)\text{P}_2$ [24]. This may indicate the presence of an inositol polyphosphate 1-phosphatase in platelets that hydrolyses $\text{Ins}(1,4,5)\text{P}_3$. An inositol polyphosphate 1-phosphatase was described previously [29,32,33], but

this activity hydrolyses $\text{Ins}(1,3,4)\text{P}_3$ and produces $\text{Ins}(3,4)\text{P}_2$.

When $\text{Ins}(1,4)\text{P}_2$ was incubated with platelet cytosol, $\text{Ins}(4)\text{P}$ was the predominant product. This agrees with previous reports in which $\text{Ins}(4)\text{P}$ was shown to be the predominant inositol monophosphate formed [33–35]. However, there are also reports of the production of $\text{Ins}(1)\text{P}$ or mixtures of $\text{Ins}(1)\text{P}$ and $\text{Ins}(4)\text{P}$ in which $\text{Ins}(1)\text{P}$ is the predominant form [36,37]. However, this could be explained by the fact that Siess [37] used whole platelets stimulated with thrombin or vasopressin, whereas in the other study inositol monophosphates were measured in a different tissue, calf brain.

After incubation of platelet cytosol with ATP, formation of $\text{Ins}(1,3,4,5)\text{P}_4$ was detected, indicating the presence of soluble $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase. This kinase has been demonstrated in platelet homogenate [38] and in the soluble fraction of homogenates from several tissues [14]. No such activity was seen in the saline extract of platelet membranes.

In summary, we have described the molecular parameters and the distribution of the enzymes that metabolize inositol polyphosphates in human platelets, and we have also characterized the products of these enzymes. The finding that a considerable percentage of the platelet $\text{Ins}(1,4,5)\text{P}_3$ phosphatase is associated with the membranes could be relevant to platelet physiology. This is of particular interest in light of the finding that $\text{Ins}(1,4,5)\text{P}_3$ -binding sites are enriched in a fraction that co-purified with the plasma membrane of hepatocytes [39]. This enzyme is then adjacent to the site at which $\text{Ins}(1,4,5)\text{P}_3$ is produced. It is well known that in the initial stages of platelet activation there is a translocation of protein kinase C from the cytosol to the membrane. This translocation could influence phosphorylation and activation of the membrane-bound $\text{Ins}(1,4,5)\text{P}_3$ phosphatase.

tase, as it has previously been shown for the cytosolic Ins(1,4,5) P_3 phosphatase [28] and for permeabilized platelets [40].

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