Identification of Inorganic Pyrophosphate in Human Platelets and Its Release on Stimulation with Thrombin

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ABSTRACT Serum inorganic pyrophosphate (PPi) levels were consistently two- to threefold higher than plasma PPi prepared from the same blood. PPi was found in platelets in amounts ranging from 1.4 to 3 nmol/10^8 cells, using three different techniques for quantification. These levels are approximately 800 times higher than the mean PPi concentration in normal plasma and approximate the levels of ADP found in platelets by other workers.

About 50% of platelet PPi was specifically released extracellularly after stimulation with thrombin. Timed release experiments showed a pattern of release that resembled that described for ADP and ATP. This pattern was clearly different from that shown by platelet calcium, serotonin, or β-glucuronidase. Platelet inorganic pyrophosphatase was not released into the supernate in detectable amounts.

Platelets from patients with nucleotide storage pool deficiency showed greatly reduced levels of PPi as compared with control. There was no detectable release of PPi into extracellular medium after thrombin addition to a suspension of these platelets.

INTRODUCTION

We recently developed an isotopic dilution method for direct measurement of inorganic pyrophosphate (PPi) in biological fluids and tissues (1). This was done to study pyrophosphate metabolism in calcium pyrophosphate crystal deposition disease (pseudogout syndrome; articular chondrocalcinosis) (2, 3). During development of this technique it became obvious that serum contained two to three times as much PPi as did platelet-poor plasma prepared from the same blood. That platelets contain PPi was first noticed by Russell and co-workers, who recently described a column chromatographic technique for PPi measurement also using isotope dilution, and accounts for their choice of plasma rather than serum for quantification of this substance (4).[1]

Analysis of the various cellular elements of blood showed that platelets contained sufficient PPi to account for the serum-plasma difference. Data relating to the identification of PPi in platelets, to its possible intracellular localization, and to its release on thrombin stimulation are presented herein.

METHODS

General

Glassware washed in acid and disposable plastic ware was used throughout. All solutions were prepared using doubly distilled, deionized water. Phosphate salts used to prepare standards and other solutions were stored over anhydrous $CaCl_2$ in a vacuum desiccator.

Reagents and abbreviations. The following reagents and enzymes were obtained from the Sigma Chemical Co., St. Louis, Mo.: disodium adenosine triphosphate (ATP); uridine diphosphoglucose (UDPG); nicotinamide adenine dinucleotide phosphate, reduced form (NADPH); nicotinamide adenine dinucleotide phosphate, oxidized form (NADP); *d*-glucose 1-6-diphosphate (G1-6DP); uridine diphosphoglucose pyrophosphorylase; phosphoglucomutase; glucose-6-phosphate dehydrogenase; yeast inorganic pyrophosphatase, 2 × crystallized (PPiase).

Other materials used included: disodium ethylene diamine tetra-acetate (Na_2 EDTA); thrombin NF, bovine, Upjohn Co., Kalamazoo, Mich.; sodium heparin, liquaemin disodium "10," Organon Inc., West Orange, N. J.; radioactive ATP (gamma labeled with ^{32}P) 2–6 mCi/μmol, and radioactive pyrophosphate ($[^{32}P]^{32}Pi$) 770–6,200 μCi/μmol, both from New England Nuclear Corp., Boston, Mass.

Procedure

PPi in serum and plasma from normal blood and from blood with abnormal platelet counts, and in samples obtained traumatically. The yeast PPiase method was used

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[1] Russell, R. G. G. Personal communication.

for all of these samples. PPi values were obtained in serum and platelet-poor plasma prepared from identical blood of healthy donors, from two patients with very low platelet counts, and from one patient with thrombocytosis. PPi levels were also obtained in plasma prepared from blood obtained at uncomplicated venipuncture and in that obtained in a different syringe after trauma to the vein had occurred inadvertently.

Platelet preparations. Washed platelets suspended in 3 mM EDTA, 0.12 M NaCl, 30 mM tris Cl buffer pH 7.4 were prepared according to methods described by Holmsen and Day (5). Platelet counts were performed by the method of Schweizer and Gerarde (6). Suspensions containing 6–19.7 $\times 10^8$ platelets were sonicated with a model W 185 Sonifier cell disruptor, Ultrasonic Inc., Farmingdale, N. Y., at a setting of 7, 4 s $\times$ 4 at 4°C in the presence of tracer [^{32}P]^{32}Pi. PPi levels were determined on one or more occasions by the yeast PPiase method on platelets obtained from normal donors D. M., D. S., S. J., and D. P., and from N. B. and J. E., patients with osteoarthritis; PPi was also determined in platelets of subject D. S. by the UDPG pyrophosphorylase method and in D. M. and D. S. after chromatographic separation of PPi. In the last instance, and in one sample analyzed by the yeast PPiase method the platelets were sonicated directly in ice-cold 0.5 N TCA after addition of [^{32}P]^{32}Pi tracer.

Release reaction. Thrombin in buffer or buffer alone was added to suspensions of 10.7–19.7 $\times 10^8$ platelets. Final concentration of thrombin was 5 U/ml. The method was performed exactly as described by Holmsen and Day (5).

The platelet release reaction induced with thrombin was monitored by measurement of protein, serotonin, and β-glucuronidase in the supernatant and pellet of thrombin-treated and control samples. PPi was measured in soluble and sedimentable phases by the yeast PPiase method. Inorganic pyrophosphatase was also measured in both phases.

The time course of the release reaction stimulated by thrombin was followed in three experiments using platelets from normal donors. The entire procedure was performed exactly as described by Holmsen and Day (5); PPi, serotonin, calcium, β-glucuronidase, protein and inorganic pyrophosphatase were each measured sequentially. The serotonin measurements were made on the same day, all other determinations were performed on samples stored at -20°C.

Studies on platelets with nucleotide storage defect. The PPi content of platelets from two patients with nucleotide storage pool defect was compared with that in platelets from a normal subject. (These platelets were kindly supplied by Dr. Harvey Weiss of the Roosevelt Hospital, New York.) Platelets were pelleted from platelet-rich plasma and stored in the frozen state. They were transported by hand in the frozen state to our laboratory in Chicago, thawed after the addition of 2 ml of ice-cold 0.9% NaCl containing [^{32}P]^{32}Pi tracer, sonicated, and refrozen at -20°C.

Platelets from an additional patient with this defect and control platelets were each treated with 5 U of thrombin per milliliter in the presence of tracer [^{32}P]^{32}Pi in the laboratory of Dr. H. James Day at Temple University in Philadelphia; the supernatant and sedimentable phases were separated and the samples transported by hand in the frozen state to our laboratory. The yeast PPiase method was used for all of these determinations.

Other experiments. Cold ATP was added to tracer ^{32}P gamma-labeled ATP to a final concentration of 0.7 mM and applied to a Dowex-1 column as described for ^{32}PPi (7). Elution with 200 ml of 0.25 M KCl-0.08 M NH_4OH, was followed by 100 ml of 0.2 N HCl.

The possibility of generation of PPi from ATP or other nucleotides by acid hydrolysis during the preparation of the sample for analysis was considered. Thrombin was added to 60×10^8 washed platelets suspended in buffer in the presence of tracer [^{32}P]^{32}Pi. After centrifugation, the supernatant was ultrafiltered through Amicon PM 10 membranes (Amicon Corp., Lexington, Mass.), as described previously (7). PPi was separated chromatographically and analyzed by the UDPG pyrophosphorylase method (*vide infra*). The PPi content of an ATP solution acidified with TCA (final acid normality = 0.5) was determined by UDPG pyrophosphorylase method after coprecipitation of tracer [^{32}P]^{32}Pi with calcium phosphate as described (*vide infra*), and compared with that in an identical ATP solution not treated with acid. All solutions were maintained at 4°C. The PPi content of the Pi solution used for coprecipitation was determined by the UDPG pyrophosphorylase method.

Methods of PPi measurement

All procedures were performed at 4°C (in crushed ice) except as indicated; all centrifugation steps were carried out in a Sorvall RC-2 centrifuge, Ivan Sorvall, Inc., Norwalk, Conn., unless otherwise specified. [^{32}P]^{32}Pi was counted in 0.5 N HCl as Cerenkov radiation in a Packard tricarb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) with correction for background, and for quenching if indicated. All spectrophotometric measurements were made in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a Gilford model 210 absorbance indicator and a model 222 automatic cuvette changer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Yeast PPiase method. Details of this procedure will be published elsewhere (1). Briefly, the material to be analyzed was collected into a plastic container in ice containing a tracer amount of [^{32}P]^{32}Pi. Inorganic orthophosphate and protein were removed using a modification of the technique of Sugino and Miyoshi (8); the residual Pi and water sufficient to concentrate the sample approximately 10-fold were extracted with isobutanol. After adjustment of the pH to 7.2, aliquots of sample were then incubated at room temperature with either yeast PPiase in buffer or with buffer alone. The Pi in both samples was then determined using a modification of the Chen method (9). Corrections were made for incomplete enzyme action and for loss of PPi due to spontaneous hydrolysis in the buffer-treated sample. The PPi concentration in the original sample was then calculated by isotopic dilution. The standard deviation of 17 plasma samples measured in duplicate was ± 0.18 or 10% of the mean value of 1.8 μmol/liter.

UDPG pyrophosphorylase method. A modification of the method described by Johnson et al. (11) as an assay for RNA polymerase was used to independently confirm data obtained with the yeast PPiase technique.[2] $CaCl_2$ and Pi were added to the supernate obtained after centrifugation of the platelets sonicated as described below at 28,000 *g* for 10 min to achieve final concentrations of 2.5 mM and 1 mM, respectively. Aliquots were taken for [^{32}P]^{32}Pi counts and then the pH was raised to 9.5–10.5 by addition of

[2] This modification was developed by one of us (McCarty) in the laboratory of Dr. S. Bisaz and Prof. H. Fleisch in Berne, Switzerland, and will be published in detail elsewhere.

TABLE I

Inorganic Pyrophosphate Levels in Serum and Plasma Prepared from Identical Blood

Platelets $cm^{-1}(\times 10^{-3})$	N*	N	N	N	N	1	2	1,100
Plasma PPi (μM)	2.9	1.7	2.9	2.5	2.5	2.4	1.4	5.1
Serum PPi (μM)	9.2	3.9	7.3	4.1	7.4	1.7	2.1	7.0

* N = normal blood.

NaOH to coprecipitate PPi with calcium phosphate (4). After centrifugation at 1,500 *g* for 15 min in an International PR-2 centrifuge (International Scientific Instruments, Inc., Palo Alto, Calif), the precipitate was dissolved with 1 or 2 drops or 0.1 N HCl, the volume adjusted to 4 ml with water, and precipitation again effected with NaOH. The precipitate was either stored at −20° until analysis or processed directly.

After dissolution in a few drops of 0.1 N HCl, the solution was passed over 100–200 mg of Dowex 50 × 4, 200–400 mesh (Na form) packed into the neck of a Pasteur pipette, both ends of which had been filed off 1 in above and below the shoulder. Approximately 400 μl of effluent was collected into 100 μl of 1 M tris Cl buffer, pH 7.8, containing 0.5 mM EDTA and 5 mM $MgCl_2$; after checking the pH to make certain that it was 7.8±0.1 U, 200-μl samples were placed in quartz cuvettes of 10 mm light path, and 20 μl of a solution containing UDPG 0.4 mM, NADP 0.4 mM, cysteine 2 mM, tris Cl buffer pH 7.8 100 mM, 5 U of phosphoglucomutase and 0.1 U of glucose-6-phosphate dehydrogenase were added (10). The resultant OD_{340} was recorded and 10 μl of UDPG pyrophosphorylase (E_1) (0.5 U) was added and mixed with a plastic rod. The reaction went to completion in 10–50 min as judged by the stability of the optical density. Then 10 μl of a 60 μM PPi solution was added and mixed. The observed optical density change in the sample was corrected by subtraction of the E_1 blank value and by the percentage of completeness of enzyme action calculated by comparing the observed optical density change after addition of internal standard to that expected on theoretical grounds using a molar extinction coefficient of 6.02×10^3. The calculated OD in the final solution was corrected for possible hydrolysis as the $[^{32}P]^{32}Pi/^{32}Pi$ was determined in 50 μl of final sample by using the isobutanol-petroleum ether extraction method described by Hall (11) as adapted by Russell and co-workers (4). The specificity of the reaction was confirmed by incubation of an aliquot of the final sample with yeast PPiase.

Column chromatographic method. This was performed using anion exchange resin as previously described (7) as an additional independent check on the specificity of identification. 10-ml fractions were collected and the PPi peak identified by ^{32}P counts. PPi was determined by a modified Chen method after hydrolysis by boiling aliquots from each fraction in screw top tubes for 30 min after acidification with HCl (final concentration 1.1 N). In addition, the PPi in 5 ml of each of the fractions in the PPi peak was coprecipitated by addition of Ca and Pi. After a second coprecipitation, the calcium was exchanged for sodium and PPi measured by the UDPG pyrophosphorylase method as described above. The specific activity of $[^{32}P]^{32}Pi$ was calculated for each fraction, and the PPi concentration was determined in the original sample by isotopic dilution.

Other methods. 5 hydroxytryptamine (Serotonin) was measured spectrofluorometrically by the method of Crosti and Lucchelli (12). One drop of 3% H_2O_2 was added to each sample before rescanning as suggested by Contractor (13). This treatment reduced each sample to base line on rescanning (blank value). *β-glucuronidase* was determined by a modification of the method of Baehner, Karnovsky, and Karnovsky (14). *Protein* was measured by the method of Lowry, Rosebrough, Farr, and Randall (15). *Calcium* was measured by atomic absorption spectrophotometry.

Inorganic pyrophosphatase was determined in samples dialyzed at 9°C against three changes of 10 mM barbital buffer, pH 7.7, containing 1 mM $MgCl_2$, 0.2 mM EDTA, 1 mM mercaptoethanol, penicillin 100 U/ml, and streptomycin 100 μg/ml. Protein was determined and dilutions made in barbital buffer to give protein concentrations of 250–500 μg/ml sample. The reaction mixture contained 40 μM sodium pyrophosphate, 50 μM $MgCl_2$, tracer $[^{32}P]^{32}Pi$ in 10 mM barbital buffer pH 7.7; 100 μl of dialysate was added to 0.9 ml reaction mixture and incubated for 0, 3, 5 min at 37°C. At the end of incubation the reaction was stopped by placing the tube in ice and adding the acid reagent described by Hall (11). After all reactions were complete, Pi was extracted with butanol-petroleum ether (11); the ^{32}Pi in the butanol phase and the $[^{32}P]^{32}Pi$ remaining in the aqueous phase were counted. Reaction mixture run with buffer alone served as control.

RESULTS

Plasma vs. serum PPi. Serum PPi concentrations were two- to threefold higher than those found in plasma prepared from identical blood (Table I). Plasma and serum PPi from two patients with thrombocytopenia were similar whereas plasma PPi from a patient with thrombocytosis was high but showed the expected increase in the serum. The PPi level in plasma prepared from blood obtained at uncomplicated venipuncture was lower than that in a sample prepared from blood obtained after trauma to the vein had occurred (2.7 vs. 3.9 μM).

Platelet PPi concentrations. PPi was found in human platelets by each of the three analytical methods used, in concentrations of 1.4–3.1 nmol/10^8 cells (Table II). All three methods yielded values of the same order of magnitude. The specific activities of $[^{32}P]^{32}Pi$ in the three major fractions of the PPi peak obtained on column chromatography were 4,500, 4,200, and 3,800 cpm/nmol PPi. The identity of the material eluted with $[^{32}P]^{32}Pi$ was confirmed by a duplicate analysis using the UDPG pyrophosphorylase method (Table II). Incubation of an aliquot of eluate with yeast inorganic pyrophosphatase abolished the reaction on subsequent analysis by the UDPG pyrophosphorylase method.

TABLE II

Inorganic Pyrophosphate Levels in Human Platelets*

Donor:	D. M.	D. S.	S. J.	D. P.	N. B.	J. E.
Yeast PPiase method (1)	1.5 (8.2)	2.1 (7.3)	1.5 (12.0)	3.0 (6.6)	3.1 (7.2)	3.1 (6.2)
		1.9 (5.8)	1.4‡			
		2.8 (7.0)				
UDPG pyrophosphorylase method (10)		1.4				
Chromatographic method (7)	2.4§	2.8				
	2.6‖					

* All PPi values are given as nanomoles PPi/10^8 platelets; numbers in parentheses indicate nanomoles PPi/mg protein.
‡ Sonication directly in 0.5 N TCA.
§ Colorimetric analysis.
‖ UDPG pyrophosphorylase method.

PPi release from platelets after thrombin stimulation. Approximately 50–60% of platelet PPi was released into the supernate after thrombin stimulation; serotonin and β-glucuronidase were released with respect to control, confirming that the release reaction had occurred (Table III). The "specific activity" of the released substances, calculated with respect to supernate protein as suggested by Holmsen and Day (5), indicated secretion of these substances by platelets and not nonspecific cellular disruption.

A neutrally active, Mg^{++}-dependent, inorganic pyrophosphatase in platelets was originally described by Datta and Zajicek (16). This enzyme was not released in detectable amounts after thrombin stimulation (Table III and Fig. 1).

Time course of PPi release after thrombin stimulation. In time course experiments, about 45% of platelet PPi was released extracellularly within 3 s after the addition of thrombin (Fig. 1). There was a suggestion of a modest secondary rise of 5–8%. Over 80% of serotonin and calcium were released within the first 3 s and no further rise occurred. In contrast, only about 25% of β-glucuronidase was released even after 5 min, and only about one-third of this occurred in the first 3 s. No release of inorganic pyrophosphatase was detected in any sample.

Studies on platelets with nucleotide storage defect. The platelets from two patients with this syndrome contained very low to undetectable amounts of PPi compared with control platelets (Table IV). The PPi content of the control was also somewhat reduced, possibly due to hydrolysis accompanying thawing. The platelets of another such patient failed to release PPi after thrombin stimulation (0.73 nmol in control supernate vs. 0.75 nmol in thrombin supernate) compared with control platelets processed identically (0.34 nmol in control supernate vs. 1.4 nmol in thrombin supernatant).

TABLE III

Inorganic Pyrophosphate, Protein, β-Glucuronidase, Serotonin, and Inorganic Pyrophosphatase (PPiase) in Pellets and Supernate from Platelet Suspensions Exposed to Thrombin vs. Control

		PPi		Protein		β-glucuronidase		Serotonin		PPiase	
Donor		T	C	T	C	T	C	T	C	T	C
		nmol		*μg**		*U/h‡*		*μg*		*nmol Pi/min*	
S. J.	Supernate	0.8	0.2	69	46	3.5	0.3	0.1	0.0	0.0	0.0
	Pellet	0.8	1.5	142	148	5.9	7.8	0.0	0.1	0.4	0.4
D. M.	Supernate	1.2	0.3	72	48	1.6	0.0	0.2	0.0	—	—
	Pellet	0.5	1.1	181	181	2.3	3.9	0.0	0.2	—	—
D. M.	Supernate	1.8	0.6	122	136	1.1	0.3	—	—	—	—
	Pellet	0.9	2.7	275	261	3.8	4.7	—	—	—	—
D. S.	Supernate	2.5	0.6	99	93	1.3	0.1	—	—	—	—
	Pellet	0.7	3.0	281	277	4.5	6.6	—	—	—	—

T, thrombin; C, control. The protein added with the thrombin was subtracted from the thrombin supernate; this amounted to 43 μg/U of thrombin.
* All values expressed/10^8 platelets.
‡ U = 1 μg *p*-nitrophenol.

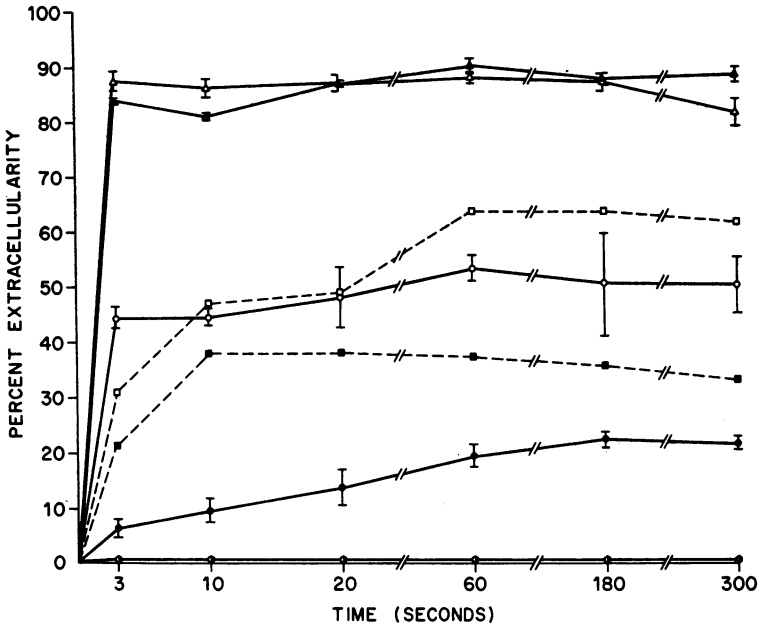

FIGURE 1 The release of serotonin (△), calcium (▲), inorganic pyrophosphate (○), and β-glucuronidase (●) and inorganic pyrophosphatase (◑) expressed as percent extracellularity (mean ±SEM) is shown plotted against time. The timed release of ADP (□) and ATP (■) found by Holmsen and Day using identical conditions (5) are plotted for comparison. See text for details.

Specificity of chromatographic separation of [^{32}P]^{32}Pi. ^{32}P gamma-labeled ATP eluted only with 0.2 N HCl; only 2% of radioactivity eluted in the PPi region (as a peak); an additional 6% eluted in the fractions of the Pi region (as a peak). The nuclide eluting in the Pi and PPi regions presumably represented contamination of [^{32}P]ATP with these anions.

Possible generation of PPi by acid hydrolysis of ATP or other compounds. The PPi content of platelet supernatant prepared without exposure to acid until after column chromatography was 1.3 nmol/10^8 cells, an amount entirely consistent with the results obtained with the other methods of preparation. The PPi content of commercially obtained equine ATP was 11.2%; acid treatment of this ATP yielded no increase in PPi.

PPi contamination of Pi solution. 0.56 μM PPi was found in the 100 mM Pi solution added for coprecipitation. This amount is insignificant in relation to the amounts of Pi used and the PPi content of platelets.

DISCUSSION

Data have been presented to document the presence of inorganic pyrophosphate (PPi) in human platelets. PPi concentrations ranged betwen 1.4 and 3.1 nmol/10^8 platelets, values approximating their ADP concentrations and about one-half that of their ATP content as reported by others (6, 17, 20). Assuming a platelet mass of 0.5% of the blood volume (21), this represents a PPi concentration of over 800 times that found in normal plasma by the pyrophosphatase method (mean = 1.8 μM). About 50–60% of platelet PPi appeared in the supernate after stimulation of platelets with thrombin, an amount proportional to reported release of ADP after such stimulation (5). Platelets contain a potent inorganic pyrophosphatase which is not released on thrombin stimulation, suggesting the existence of a pool of intracellular PPi normally inaccessible to enzyme action. As PPi is liberated extracellularly during the release reaction, it is probably stored in an intracellular granule, as has been postulated for other substances participating in the release reaction (5). The amount and rate of PPi release resembled that reported for ADP by Holmsen and

TABLE IV
*Inorganic Pyrophosphate Levels in Platelets from Patients with Nucleotide Storage Defect**

	Control J. R.	Patient R. C.‡	Patient L. G.§
PPi (nmol)	0.86	0.1	0.0
Calcium (nmol)	11.0	12.6	4.4
Protein (μg)	256	533	180

* All values expressed in nanomoles/10^8 platelets.
‡ Platelets from this patient are described in reference 17.
§ Platelets from this patient are described in references 18 and 19.

Day. Time course experiments clearly showed a pattern that differed from that of the lysosomal enzyme β-glucuronidase and from calcium or serotonin (Fig. 1). The finding of low to absent PPi in platelets from patients with known deficiency in nucleotide storage pool suggests that PPi is stored in the same granule as "nonmetabolic" ATP and ADP. Further study of the stoichiometric relationships of storage pool nucleotides, PPi, and calcium are indicated. The practical importance of these observations is that blood or other fluids and tissues to be analyzed for PPi must be obtained with minimal trauma and platelets effectively separated before analysis or falsely high values may result. The high values for serum PPi reported by Solomons and Styner for example, probably reflect platelet PPi, at least in part (22). Whether platelet PPi has a significant physiological role as do ADP and ATP, remains to be determined. The present study suggests the existence of an intracellular sequestered pool of PPi in mammalian tissue, although PPi has been found complexed with calcium and magnesium in the intracellular ("volutin") granules of Tetrahymena pyriformis (23).

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