Running title: Polyphosphate in platelet dense granules

Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes

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Inorganic polyphosphate (polyP) has been identified and measured in human platelets. Millimolar levels (in terms of P\textsubscript{i} residues) of short chain polyP were found. The presence of polyP of about 70-75 phosphate units was identified by \textsuperscript{31}P NMR and by urea-polyacrylamide gel electrophoresis of platelet extracts. Analysis of human platelet dense granules, purified using metrizamide gradient centrifugation, indicated that polyP was preferentially located in these organelles. This was confirmed by visualization of polyP in the dense granules using 4',6-diamidino-2-phenylindole and by its release together with pyrophosphate (PP\textsubscript{i}), and serotonin upon thrombin stimulation of intact platelets. Dense granules were also shown to contain large amounts of calcium, and potassium, and both bafilomycin A\textsubscript{1}-sensitive ATPase, and pyrophosphatase activities. In agreement with these results, when human platelets were loaded with the fluorescent calcium indicator fura-2 acetoxybenzylester to measure their intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) they were shown to possess a significant amount of Ca\textsuperscript{2+} stored in an acidic compartment. This was indicated by: (1) the increase in [Ca\textsuperscript{2+}]\textsubscript{i}, induced by nigericin, monensin, or the weak base, NH\textsubscript{4}Cl, in the nominal absence of extracellular Ca\textsuperscript{2+}, and (2) the effect of ionomycin, which cannot take Ca\textsuperscript{2+} out of acidic organelles and was more effective after alkalinization of this compartment by the previous addition of nigericin, monensin, or NH\textsubscript{4}Cl. All these characteristics of the platelet dense granules, together with their known acidity and high density (both by weight and by electron microscopy), are similar to those of acidocalcisomes (volutin granules, polyP bodies) of bacteria and unicellular eukaryotes. The results suggest that acidocalcisomes have been conserved during evolution from bacteria to humans.
Inorganic polyphosphate (polyP) is a ubiquitous polymer formed by phosphate (P_i) residues linked by high-energy phosphoanhydride bonds. Although the presence of polyP in bacteria, fungi, algae, and protozoa has been widely noted, the distribution and abundance of polyP in more complex eukaryotic forms has remained uncertain for many years (1). The very low levels of polyP in animal cells and subcellular compartments and the lack of sensitive methods have left its metabolic and functional roles entirely obscure (1). The recent introduction of novel quantitative enzymatic analytical methods (2) has permitted the detection of levels of 20 to 120 µM (in terms of P_i residues), in chains 50 to 800 residues long in rodent tissues (brain, heart, kidneys, liver, and lungs) and subcellular fractions (nuclei, mitochondria, plasma membrane, and microsomes) (3) but no studies have been reported with human tissues.

In bacteria (4) as well as in several unicellular eukaryotes, like trypanosomatid and apicomplexan parasites (5), the green algae *Chlamydomonas reinhardtii* (6), and the slime mold *Dictyostelium discoideum* (7), polyP is accumulated in acidic granules known as acidocalcisomes where it can reach millimolar or molar levels. Acidocalcisomes have been shown to possess calcium- and proton-accumulating activities in their limiting membranes, have very high density (both in weight and by electron microscopy), and are also rich in pyrophosphate (PP_i), calcium, and other cations (5).

Human platelets possess an organelle, the dense granule, with morphological and biochemical similarities to acidocalcisomes. Platelet dense granules store extremely high concentrations of calcium, together with ATP, ADP, serotonin, and PP_i (8). Holmsen and Weiss (9) calculated the intragranular calcium concentration to be 2.2 M. The internal matrix pH of the dense granules is about 5.4 (10), and this acidic pH is maintained by the function of an H^+-ATPase, which is different from F_{i}F_{o}^- and P-type ATPases (11). Since acridine orange uptake in dense granules was inhibited by bafilomycin A_1 (12), which is a
specific inhibitor of vacuolar H⁺-ATPases (13), it was suggested that this proton pump is a V-H⁺-ATPase. In addition, calcium release from intracellular acidic stores by the K⁺/H⁺ ionophore nigericin has been observed in a number of unicellular eukaryotes that posses acidocacisomes (5) as well as from platelets (14, 15).

Because acidocalcisomes in bacteria and unicellular eukaryotes are morphologically very similar to platelet dense granules, we investigated whether these granules have similar characteristics to acidocalcisomes, also known as volutin granules or polyP bodies (5). In this report, we describe the isolation and biochemical characterization of dense granules from human platelets and demonstrate that, as the acidocalcisomes, they are rich in polyP, which is secreted upon thrombin stimulation. In addition, we report experiments using intact fura 2-loaded platelets that demonstrate that a considerable portion of the releasable Ca²⁺ is located in these organelles. Our results suggest that acidocalcisome-like organelles are the only ones described to date that are present from bacteria to humans.

EXPERIMENTAL PROCEDURES

Chemicals-Leupeptin, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64), N-p-tosyl-L-lysine chloromethyl ketone, ATP, ionophores, metrizamide, reagents for marker enzyme assays, apyrase, anti-human von Willebrand factor IgG fraction antibodies, apyrase and polyPs were purchased from Sigma Chemicals Co. (St. Louis, MO). 4-(2-aminoethyl)benzenesulfonyl fluoride and ionomycin (free acid) were from Calbiochem (La Jolla, CA). Pepstatin came from Roche Molecular Biochemicals (Indianapolis, IN). Coomassie Blue protein assay reagent was from Bio-Rad (Hercules, CA). Escherichia coli strain CA38 pTrcPPX1 was kindly provided by Prof. Arthur Kornberg, Stanford University School of Medicine (Stanford, CA). Fresh Human platelets were obtained from the
Community Blood Services of Illinois, (Urbana, IL). Aminomethylenediphosphonate was synthesized by Michael Martin, Department of Chemistry, University of Illinois at Urbana-Champaign. All other reagents were analytical grade.

**Platelet Dense Granule Isolation**—Dense granules were isolated using metrizamide gradient centrifugation by a method previously described (16) with minor modifications. A unit of platelet rich plasma was centrifuged twice at 200 x g for 10 min, to eliminate contaminant red blood cells and leukocytes. Then, the supernatant was centrifuged at 1,000 x g for 15 min, and the resulting pellet was resuspended at 2 x 10^9 platelet/ml in lysis buffer (25 mM Hepes, 0.25 M sucrose, 12 mM sodium citrate, 1 mM EDTA, 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 10 μM pepstatin, 10 μM leupeptin, 10 μM trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane and 10 μM N-α-tosyl-L-lysine chloromethyl ketone, pH. 6.5). Platelets were sonicated twice (5 sec at 20% intensity in a Branson sonifier, model 102c) and unbroken cells were separated by centrifugation (1,000 x g for 15 min). Sonication and centrifugation were repeated twice with the pellets resuspended in a similar volume of lysis buffer. The three supernatants were combined and centrifuged for 20 min at 19,000 x g at 4 °C. The pellet was resuspended with lysis buffer up to 1.5 ml and mixed with 1.5 ml of metrizamide solution (40% w/v in nanopure water). This mixture was applied at the top of a discontinuous gradient of metrizamide, with 3-ml steps of 35, 38, and 40% metrizamide (prepared by dilution of the 40% solution in lysis buffer). The gradient was centrifuged at 100,000 x g using a Beckman SW 41 rotor for 60 min. The dense granule rich fraction pelleted at the bottom of the tube and was resuspended in lysis buffer.

To determine serotonin incorporation into gradient fractions, platelet rich plasma lacking red blood cells and leukocytes, were incubated for 15 min at 37 °C with 1 μCi/ml of
1 mM 5-[2-14C]-hydroxytryptamine binoxalate (PerkinElmer Life Sciences) and the fractionation procedure was performed as described above. Von Willebrand factor levels in the gradient fractions were measured by ELISA using Anti-human von Willebrand factor IgG fraction antibodies. Gradient fractions were assayed also for succinate-cytochrome C reductase (mitochondrial marker) and acid phosphatase (lysosome marker) (17), short and long chain chain polyP and PPi (18), bafilomycin A1(0.5 µM)-sensitive ATPase (V-H+-ATPase), and pyrophosphatase (7). The construction of normalized density distribution histograms was carried out as described before (19).

**Electrophoretic analysis of polyP**—Urea-polyacrylamide gels were prepared and stained with toluidine blue as previously described (18). Marker polyPs were obtained by electrophoresis of phosphate glass in 1.5% agarose gels. Four mm-long gel slices were eluted by centrifugation trough Millipore Ultrafree-MC columns to obtain polyPs of different sizes. The markers were localized by toluidine blue staining and their size was calculated by calibration with commercial polyPs.

**Electron Microscopy and X-ray Microanalysis**—For imaging whole cells, platelets were washed first in Tris citrate buffer (63 mM Tris, 95 mM NaCl, 5 mM KCl, 12 mM citric acid, pH 6.5) and then with 0.25 M sucrose. Dense granule fractions were washed with 0.25 M sucrose. Samples were placed on a Formvar-coated 200-mesh copper grid, allowed to adhere for 10 min at room temperature, blotted dry, and observed directly with a Hitachi 600 transmission electron microscope operating at 100 kV (20). Energy-dispersive x-ray analysis was done at the Electron Microscopy Center, Southern Illinois University (Carbondale, IL). Specimen grids were examined in a Hitachi H-7100FA transmission electron microscope at an accelerating voltage of 50 kV. Fine probe sizes were adjusted to cover the dense granules (or a similar area of the background), and x-rays were collected for 100 s by utilizing a thin window (Norvar) detector. Analysis was performed by using a
Noran Voyager III analyzer with a standardless analysis identification program. Conventional electron microscopy of the dense granule fraction was done as described before (20).

**Fluorescence Microscopy**-Platelets (~10^8) were washed with New Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 1 mM MgCl₂, 5.5 mM glucose, 0.35% BSA, pH 7.35). The pellet was resuspended in 2 ml of the same buffer, and 45 µl of this suspension was incubated at room temperature with 10 µg of 4',6-diamidino-2-phenylindole (DAPI)/ml. After 10 min, the samples were mounted on a slide and observed with an Olympus model BX-60 epifluorescence microscope. An Olympus WU filter (excitation: 380-385 nm; emission: > 420) was used. The images were recorded with a charge-coupled device camera (model CH250; Photometrics Ltd., Tucson, AZ) and IPLab software (Signal Analytics, Vienna, VA) as described previously (21).

**Intracellular Calcium Measurements**-Platelet rich plasma was centrifuged at 700 x g for 5 min and 100 µM aspirin, and 40µg/ml apyrase were added to the supernatant. Platelets were incubated at 37 °C with 1 µM fura-2/AM for 1h. Labeled platelets were centrifuged at 1,000 x g 10 min and washed in New Tyrode’s buffer. Then, cells were resuspended in the same buffer at 2 x 10^8 platelets/ml and kept at 37 °C. For fluorescence measurements, 1.25 x 10^8 cells/ml (final density) were suspended in a cuvette containing New Tyrode's buffer and 1 mM EGTA. Fluorescence emission at 510 nm was measured with 340 and 380nm excitation in a thermostated Hitachi F-2000 spectrofluorometer at 37 °C with agitation. Calibration was performed as described before (22).

**Perchloric acid extracts**-For NMR, platelets were washed twice with New Tyrode’s buffer, then extracted with ice-cold 0.5 M HClO₄ (2 mL/g wet weight cells). After 30 min incubation on ice, the extracts were centrifuged at 3,000 x g for 5 min. The supernatants
were neutralized by the addition of 0.72 M KOH/0.6 M KHCO₃ (18). Precipitated KClO₄ was removed by centrifugation at 12,000 x g for 10 min., the supernatant separated and EDTA added to a final concentration of 400 µM prior to adjusting to pH 7. All extracts contained 10% D₂O (v/v) to provide a field-frequency lock.

**NMR spectroscopy**—Phosphorus NMR spectra were acquired at 303.6 MHz using a Varian INOVA NMR spectrometer equipped with a 17.6 Tesla Oxford Instruments magnet. For perchloric acid extracts, 8,192 transients were collected at room temperature using 25 µs (90°) pulse excitation, a 20 kHz spectral width, 32,768 data points, and a 5 sec recycle time. Inverse gate proton decoupling was used to remove NOE and J-coupling effects. The chemical shifts of all ³¹P spectra were referenced to 0 ppm using an 85% phosphoric acid external standard (23). The specific assignments of individual resonances were based on published chemical shifts and ³¹P-³¹P scalar couplings (24). NMR spectra were processed using the VNMR 6.1B software package (Varian Inc., Palo Alto, CA) running on a Sun Ultra 5 (Sun Microsystems, Santa Clara, CA) workstation, and included baseline correction, zero-filling, and a 3 Hz exponential line broadening prior to Fourier transformation.

**Analysis of Platelet Released Material**—Platelet rich plasma lacking red blood cells and leukocytes, were incubated for 15 min at 37 °C with 1 µCi/ml of 1 µM 5-[2-¹⁴C]-hydroxytryptamine binoxalate. Platelets were washed and resuspended in New Tyrode's buffer at 10⁸ cell/ml. The platelet suspension was placed in a cuvette with agitation. Thrombin was added at a final concentration of 1.2 U/ml and aliquots were taken at different times. Samples were directly filtered through a 0.2 µm filter to separate the secreted material from the suspension. Content of PP₃, short chain polyP, and serotonin in the samples were analyzed as described above.
H+ Transport Assays—H+ uptake into platelet dense granules was assayed using acridine orange as described before (6) using a standard buffer containing 120 mM KCl, 2 mM MgCl2, 50 mM K-Hepes, 50 μM EGTA, pH 7.2.

RESULTS

Elemental Analysis and Isolation of Human Platelet Dense Granules—Human platelet dense granules are known to be acidic (10) and to accumulate calcium (9). They are characterized, as the acidocalcisomes (5), by their high electron-density. They were first described as electron-opaque organelles in air-dried whole mounts by Bull (25), White (26) and Costa et al. (27). Several dense granules with high electron density of varying diameter (average 161 ± 51 nm) were seen when whole human platelets were observed by transmission electron microscopy without fixation and staining (Fig. 1A). 5 to 20 granules of various shapes and sizes were observed per platelet (n = 20). This is in agreement with previous reports of the presence a variable number of dense bodies per platelet ranging from none to more than 20 (28). X-ray microanalysis was performed on these granules (Fig. 1B). All ten spectra taken from different platelets were qualitatively similar, the P/Ca ratio being 1.76 as it has been reported before (29). Potassium and oxygen were also detected. Carbon, silicon and copper arose from the formvar-coated grids, and were also detected in spectra taken from the background.

To purify the dense granules and investigate their chemical and enzymatic content, we adapted the purification procedure used by Rendu et al. (16). Examination of the densest fraction 12 (see below) by transmission electron microscopy without fixation and staining showed round electron-dense granules of variable size, up to 200 nm diameter (Fig. 2A). When submitted to the electron beam, changes in their internal structure led to
the appearance of a sponge-like structure (Fig. 2A, inset), which has been described before in acidocalcisomes of T. cruzi (30), T. brucei (31), C. reinhardtii (6) and Agrobacterium tumefaciens (4). Examination of the fraction using conventional transmission electron microscopy with fixation, dehydration and staining procedures showed membrane-bound organelles of similar size that appeared empty (Fig. 2B).

Marker enzymes were used to characterize the different fractions (Fig. 3). Serotonin incorporation (26) was used as a marker for dense granules. Its yield in the dense granule fraction (fraction 12) was 25%, whereas the yield of protein in the same fraction was only 6%, a 4.2-fold purification. Although the incorporation of serotonin was greater in fraction 3, which consisted of whole platelets and large fragments, the purest dense granule fraction was the densest fraction (fraction 12). Mitochondria (marked by succinate cytochrome c reductase), lysosomes (marked by acid phosphatase), and alpha granules (marked by the presence of von Willebrand factor) were not enriched in the densest fraction. The dense granule fraction contained about 20% of the total PP\textsubscript{i} and short chain polyphosphate (polyP; see below) (Fig. 3).

**PolyP in human platelets**-Platelet extracts yielded levels of polyP of 0.74 ± 0.08 nmol per 10\textsuperscript{8} platelets (n = 8) and no detectable long chain polyP (data not shown). If we assume that the platelet volume is 6.8 mm\textsuperscript{3} (9), the concentration of polyP in platelets is around 1.1 mM. Thus, platelet polyP concentration is 10 to 20 times higher than that measured in rodent tissues such as brain, heart, kidney, lung, and liver (3). Interestingly, in contrast to other cell types studied, all the polyP detected was less than 100 residues long. PolyP levels were in the same order of magnitude as the PP\textsubscript{i} (0.92 ± 0.19 nmol per 10\textsuperscript{8} platelets; n = 8) measured by its hydrolysis with yeast pyrophosphatase. Excess of pyrophosphatase in our assay degraded less than 2% of the polyP, demonstrating the reliability of the obtained values for the different populations of phosphate polymers. We can estimate the
concentration of polyP in the dense granule from the total amount of polyP in the whole platelet (see above). If we assume that the polyP in the dense granule is about 60% of the total (as occurs with calcium, ADP, ATP and PPi), the volume of each granule is 0.1% of the platelet volume (9), and there are an average of 5 dense granules per platelet (8, 28), the intra-granular concentration of polyP is about 130 mM.

Further evidence for the localization of polyP in the dense granules-We also investigated the location of polyP using DAPI (Fig. 4). DAPI has been shown to shift its emission fluorescence to a maximum wavelength of 525 nm in the presence of polyP, this change being specific for polyP and not produced by PPi or other anions (18, 32). Human platelets incubated in solutions of DAPI (0.2 mg/ml) were mounted on slides and examined by confocal fluorescence microscopy. We detected staining of numerous intracellular vacuoles. No staining was detected when DAPI was omitted (data not shown).

Identification of polyP by 31P NMR and urea-PAGE analysis-Fig. 5 shows the 303.6 MHz (1H decoupled) 31P NMR spectra of perchloric acid extracts of human platelets. Resonance assignments for these spectra are given in Table 1. The predominant peak in the spectrum is inorganic phosphate (A). The region between –6 and –8 ppm is shown in inset magnified 2.5X respective to the overall spectra and contains peaks for the terminal phosphates of adenosine di- (B) and triphosphate (C) in addition to peaks for the terminal phosphates of polyPs (D) and a large peak for PPi (E). The region from –10 to –12 ppm contains peaks for the –phosphates of adenosine di- (F) and triphosphate (G). The region from –20 to –23 ppm is again shown in inset magnified 2.5X respective to the overall spectra and contains peaks for the central phosphate of adenosine triphosphate (H) and the –phosphates (central) of polyPs (I).
PolyPs extracted from human platelets were electrophoresed by 6% urea-PAGE to determine their size distribution (Fig. 6). Only one size class of polyP was detected in the three samples analyzed: short-chain polyP of about 70-75 residues.

*PolyP release after stimulation of platelets with thrombin*- Since 80% of the total PPi is secreted by platelets treated with thrombin with a time course similar to secretion of ATP, ADP and serotonin from the platelet dense granules (33) we investigated whether thrombin had a similar effect on the release of polyP. Fig. 7 shows that this was the case, confirming that polyP is in the same compartment containing PPi and serotonin.

*Intracellular Ca²⁺ Concentration [Ca²⁺], in Human Platelets: Effect of Thapsigargin and Nigericin*- The concentration of cytosolic calcium in human platelets was 22 ± 2.8 nM (n = 6) in the absence of extracellular Ca²⁺ (1 mM EGTA added). This concentration is in the range observed in previous studies (34). Addition of 1 µM thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca²⁺-ATPase when used at low concentrations (35), increased the [Ca²⁺]i (Fig. 8A, upper trace). Subsequent addition of nigericin (a K⁺/H⁺ ionophore; 2 µM) caused a second rise in [Ca²⁺]i to a level that was significantly higher to that in the absence of nigericin. Similar results were observed when the order of additions was reversed (Fig. 8A, lower trace), suggesting the presence of a thapsigargin-sensitive, and an acidic thapsigargin-insensitive Ca²⁺ compartment.

*Synergistic Effect of Ionomycin and Ammonium Chloride on Ca²⁺ Release from Intracellular Compartments*- To provide more evidence for the presence of an acidic Ca²⁺ store in human platelets, we performed experiments using sequential additions of ionomycin and ammonium chloride. It has been demonstrated (36) that ionomycin can only mobilize Ca²⁺ from neutral or alkaline compartments, but releases more Ca²⁺ after alkalinizing agents have elevated the pH of acidic compartments. Accordingly, adding ionomycin to human platelets previously exposed to NH₄Cl (10 mM) caused an increase in [Ca²⁺], to a level
which was significantly higher to that in the absence of NH$_4$Cl (Fig. 8B, *upper trace*). Similar results were obtained when the order of additions was reversed (Fig. 8B, *upper trace*) and when nigericin (2 µM) or monensin (1 µM) were used as alkalinizing agents (data not shown).

Taken together these results indicate the presence of an acidic compartment, sensitive to a K$^+$/H$^+$ ionophore (nigericin), and a Na$^+$/H$^+$ ionophore (monensin), and insensitive to thapsigargin, that contains a significant amount of Ca$^{2+}$ in human platelets and which is physiologically similar to that described as the acidocalcisome in trypanosomatids and apicomplexan parasites (4).

*V-H$^+$-ATPase and Pyrophosphatase Activities in the Isolated Dense Granules-
Bafilomycin A$_1$-sensitive ATPase activity (measured by P$_i$ release from ATP) was enriched in the dense granule fraction (Fig. 9A). Although isolated dense granules could take up acridine orange, demonstrating that they were still intact and acidic, neither ATP nor PP$_i$ were able to stimulate this process. Alkalinization of dense granules by addition of NH$_4$Cl resulted in release of the acridine orange accumulated (Fig. 9B). A pyrophosphatase activity was also enriched in the dense granule fraction (Fig. 9A). Interestingly, when this activity was measured in the presence of aminomethylenediphosphonate (AMDP), an inhibitor of vacuolar proton translocating pyrophosphatases (V-H$^+$-PPases) (37), its yield in the dense granule fraction was higher than in any other fraction (30%).

**DISCUSSION**

We report here that human platelets are rich in polyP. PolyP was detected in platelet extracts by biochemical methods and $^{31}$P NMR and shown to be present at millimolar
levels, 10 to 20 times higher than in rodent tissues previously investigated (3). The presence of polyP of about 70-75 phosphate units was identified by urea-polyacrylamide gel electrophoresis of platelet extracts. Most polyP was found in the dense granules, purified using metrizamide gradient centrifugation. This was confirmed by visualization of polyP in the dense granules using 4′,6-diamidino-2-phenylindole and by its release together with pyrophosphate (PPᵢ), and serotonin upon thrombin stimulation of intact platelets. Dense granules were also shown to contain large amounts of calcium, and potassium, and both bafilomycin A₁-sensitive ATPase, and pyrophosphatase activities. In agreement with these results, when human platelets were loaded with the fluorescent calcium indicator fura-2 acetoxyethyl ester to measure their intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) they were shown to possess a significant amount of Ca²⁺ stored in an acidic compartment.

Numerous studies have defined the role of dense granules in platelets. Activation of platelets leads to the secretion of dense granule components such as ATP, ADP, PPᵢ, calcium, and serotonin. ATP could act on P₂Y receptors on endothelial cells to release prostacyclin and nitric oxide, which in turn cause vasodilation (8). ADP acts as a platelet agonist, and is important for the activation of additional platelets and their recruitment to the site of injury (8). Serotonin acts to activate additional platelets and thus recruit them into the aggregate and also has a vasoconstrictive action, which reduces flow at the site of injury and thereby limits blood loss (8). Calcium could be important for the binding of adhesive proteins to their platelet receptors (8). The functions of PPᵢ and polyP are unknown. However, polyP, as heparin, is a negatively charged polymer that has been postulated to stabilize fibroblast growth factor (FGF) and facilitate FGF-2 binding to its receptor tyrosine kinase promoting signaling via the formation of receptor dimers, and to prevent FGF degradation in tissues that have been injured (38). Since polyP is released
from platelets after thrombin stimulation (Fig. 7), it could contribute to the homeostatic functions of platelets and further work will be needed to investigate its role.

Calcium is important for many physiological processes leading to platelet activation, e.g. shape change, eicosanoid formation, adhesion, aggregation and granule secretion. Platelet agonist such as ADP and thrombin increase cytosolic Ca\(^{2+}\) by initially releasing it from one or more internal storage sites. Two different Ca\(^{2+}\) stores had been reported in human platelets (14, 39). Thapsigargin, a sarcoplasmic-endoplasmic reticulum (SERCA) Ca\(^{2+}\)-ATPase inhibitor (35), was shown to release Ca\(^{2+}\) from an inositol 1,4,5-trisphosphate (InsP\(_3\))-sensitive intracellular store (14) (possibly the dense tubular system, a structure derived from the megakaryocyte endoplasmic reticulum; ref. 40). Addition of thrombin to thapsigargin-treated platelets resulted in further Ca\(^{2+}\) release, and this Ca\(^{2+}\) release was diminished by previous treatment with nigericin (14), suggesting that Ca\(^{2+}\) was being released from an acidic compartment. The addition of a combination of ionomycin with alkalinizing agents, such as nigericin, monensin, or NH\(_4\)Cl, which has been previously used to identify acidocalcisomes in trypanosomatids and apicomplexan parasites (5), confirmed the presence of an acidic Ca\(^{2+}\) pool in human platelets (Fig. 8). In this regard, it has been shown that platelets express both SERCA\(_{2b}\) and SERCA\(_{3}\) Ca\(^{2+}\)-ATPase isoforms suggesting that the two isoforms may be located in functionally distinct Ca\(^{2+}\) storage pools of the cells (41, 42).

Platelet dense granules have, therefore, many characteristics in common to acidocalcisomes: [1] they are acidic due to the operation of a bafilomycin A\(_1\)-sensitive proton ATPase (Fig. 9A; and ref. 12), and are able to accumulate acidophilic dyes such as acridine orange (Fig. 9B, and refs. 12, and 43) or mepacrine (quinacrine) (44); [2] they can store extremely high concentrations of calcium, and other cations, such as potassium (Fig. 1), or lithium (45), and can release Ca\(^{2+}\) in the presence of ionophores such as nigericin or
monensin (Fig. 8); [3] they contain very high concentrations of phosphorus in the form of PP_1 (33) and polyP (Figs. 3-5); [4] they have very high density both by electron microscopy (Fig. 1), and by weight (Fig. 3); [5] they have a diameter of about 200 nm, and are predominantly spherical, although they can also show elongated or irregular shapes.

Acidocalcisomes described in bacteria and unicellular eukaryotes have some peculiarities not found in dense granules such as the presence of a vacuolar-type H^+-pyrophosphatase in their surrounding membrane (5). As occurs with acidocalcisomes of *Trypanosoma brucei* (46) a pyrophosphatase activity was enriched in the dense granule fraction of human platelets, and this activity was inhibited by AMDP, an inhibitor of V-H^+-PPases (37) (Fig. 9A). Although one study reported PP_1-induced acidification of trans cisternal elements of rat liver Golgi membranes (47), there has been no reports of the presence of genes with similarity to those encoding V-H^+-PPases of bacteria, plants, and protists. Since no PP_1-dependent H^+-transport could be measured in dense granule fractions (Fig. 9B), and AMDP has also been shown to inhibit soluble pyrophosphatases at micromolar levels (37), we can rule out that the activity detected is due to a V-H^+-PPase. Although a bafilomycin A_1-sensitive ATPase activity could be measured by P_1 release from ATP (Fig. 9A), H^+-transport in isolated dense granules could not be detected (Fig. 9B). This has been observed with other acidocalcisomes that are known to posses the V-H^+-ATPase and it has been proposed that the V-H^+-ATPase complex dissociates, losing its peripheral subunits, or otherwise becomes inactive in H^+ transport during purification (48), as has been observed in other cases (49, 50).

On the other hand, platelet dense granules accumulate high concentrations of ATP, ADP, and serotonin, which have not been reported to be present in acidocalcisomes. However, acidocalcisomes of trypanosomatids have been found to contain the biogenic amine γ-aminobutyric acid (GABA; Rohloff and Docampo, unpublished results), which has
been shown to accumulate in platelets (51), and porcine dense granules possess histamine (8, 9).

Having a homolog enzyme (H⁺-ATPase) and similar chemical composition (PP₁, polyP, Ca, K, etc) in a similar organelle is a strong argument of the common origin of the two organelles. Acidocalcisomes have been shown previously (5) to be similar to volutin granules or metachromatic granules found in bacteria and in a number of unicellular eukaryotes (5). Volutin granules were discovered 100 years ago (52), and as dense granules, were easily identified in different microorganisms because of their density in whole mount electron microscopy (53). They were originally thought to be devoid of a limiting membrane. In recent years they have been found to be surrounded by a membrane containing a number of pumps and exchangers and were named acidocalcisomes (5).

In conclusion, our results suggest that human platelet dense granules belong to the same class of organelles as volutin granules or acidocalcisomes and are therefore the only known organelle group that has been conserved during evolution from bacteria to humans.

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Table 1. $^{31}$P NMR resonance assignments for perchloric acid extracts of human platelets.

| Peak | Assignment                      | Chemical shift (ppm) |
|------|---------------------------------|----------------------|
| A    | Inorganic phosphate             | 1.22                 |
| B    | $\square$P of ATP               | -7.06                |
| C    | $\square$-P ADP                 | -7.23                |
| D    | $\square$-P of polyphosphates   | -7.52                |
| E    | Pyrophosphate                   | -7.76                |
| F    | $\square$-P of ADP              | -10.42               |
| G    | $\square$-P of ATP              | -10.76               |
| H    | $\square$-P of ATP              | -20.38               |
| I    | Central P of polyphosphates     | -22.02               |
FIGURE LEGENDS

FIG. 1. **Electron microscopy and X-ray microanalysis of whole platelets.** **A,** Transmission electron microscopy of an unfixed and unstained platelet preparation. Dense granules are identified by arrows. Bar, 1 µm. **B,** Typical X-ray microanalysis spectrum of dense granules in whole platelets. **C,** X-ray microanalysis spectrum of the background of whole platelets.

FIG. 2. **Electron microscopy of the dense granule fraction.** **A,** direct observation of unfixed and unstained dense granules air-dried directly onto microscope grids. The inset shows, at higher magnification, the sponge-like structure of the dense granules after submission to the electron beam. **B,** Fixed and sectioned dense granule fraction. Bars: 0.25 µm

FIG. 3. **Distribution of different markers from human platelets on metrizamide gradients.** Serotonin, PPi, and short chain polyP are all present in the dense granule fraction (fraction 12). This distribution was compared with that of established platelet organelle markers, acid phosphatase (lysosomes), von Willebrand factor (alpha granules) and cytochrome c reductase (mitochondria).

FIG. 4. **Fluorescence microscopy showing the localization of polyP using DAPI.** **A,** Platelets were treated with DAPI as described under Experimental Procedures. Note the accumulation of DAPI in small organelles. Arrow in **B** (bright field microscopy) shows an activated platelet. Bar: 10 µm.

FIG. 5. **303.6 MHz (1H decoupled) 31P NMR spectra of perchloric acid extracts of human platelets.** Resonance assignments are given in Table 1.
FIG. 6. Urea-PAGE analysis of polyP from human platelets from three different donors. PolyP extracted from platelets was electrophoresed by 6% urea-PAGE. Chain lengths of standards are on the left. An arrowhead on the right shows the position of migration of samples from three different donors.

FIG. 7. Time course of thrombin-induced serotonin release compared to PPi and polyP. Release of serotonin (triangles), PPi (squares), and short chain polyP (circles) in platelets after the addition of 1.2 U/ml thrombin as described under Experimental Procedures. Results are expressed as percentage of the material released at 300 sec (representative of 3 experiments).

FIG. 8. Effect of ionophores and NH4Cl on human platelets [Ca2+]. Platelets were loaded with fura-2/AM as described under Experimental Procedures and resuspended in New Tyrode’s buffer containing 1 mM EGTA. Thapsigargin (TG, 1 µM), nigericin (NIG, 2 µM), ionomycin (ION, 1 µM), or NH4Cl (20 mM) were added where indicated.

FIG. 9. Distribution of bafilomycin A1-sensitive ATPase and pyrophosphatase activities on metrizamide gradients and acridine orange uptake by isolated dense granule fraction. A, bafilomycin A1 (1 µM)-sensitive ATPase, pyrophosphatase, and AMDP (20 µM)-sensitive pyrophosphatase activities are all enriched in the dense granule fraction (Fraction 12). Other conditions were as described in the legend to Fig. 3, and under Experimental Proceures. B, Dense granule fraction (45 µg protein) was added to 2.5 ml of a reaction medium described under Experimental Procedures. Acridine orange (3 µM), ATP (1 mM),
PP$_i$ (0.1 mM), of NH$_4$Cl (20 mM) were added where indicated. Changes in absorbance were followed at 493-530 nm.
Ruiz et al., Fig. 1
Ruiz et al., Fig. 2
Ruiz et al., Fig. 3
Ruiz et al., Fig. 5
Ruiz et al., Fig. 6
Ruiz et al., Fig. 7
Intracellular Ca\(^{2+}\) concentrations as a function of time (s).

A

B

Ruiz et al., Fig. 8
A

% Total Pyrophosphatase activity

% PPase (AMDP-sensitive) activity

% ATPase (BAF-sensitive) activity

Fraction

1 2 3 4 5 6 7 8 9 10 11 12

B

Ruiz et al., Fig. 9