THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 43, Issue of October 22, pp. 44250–44257, 2004
Printed in U.S.A.
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

Human Platelet Dense Granules Contain Polyphosphate and Are Similar to Acidocalcisomes of Bacteria and Unicellular Eukaryotes*

Received for publication, June 4, 2004, and in revised form, July 14, 2004
Published, JBC Papers in Press, August 11, 2004, DOI 10.1074/jbc.M406261200

Felix A. Ruiz‡§, Christopher R. Lea¶, Eric Oldfield¶, and Roberto Docampo‡‖

From the ‡Laboratory of Molecular Parasitology, Department of Pathobiology and Center for Zoonoses Research, University of Illinois at Urbana-Champaign, Urbana, Illinois 61802 and the ¶Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Inorganic polyphosphate (polyP) has been identified and measured in human platelets. Millimolar levels (in terms of P_i residues) of short chain polyP were found. The presence of polyP of ~70–75 phosphate units was identified by ^{31}P NMR and by urea-polyacrylamide gel electrophoresis of platelet extracts. An 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 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_1-sensitive ATPase and pyrophosphatase activities. In agreement with these results, when human platelets were loaded with the fluorescent calcium indicator Fura-2 acetoxymethyl ester to measure their intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), they were shown to possess a significant amount of Ca^{2+} stored in an acidic compartment. This was indicated by the following: 1) the increase in $[Ca^{2+}]_i$ induced by nigericin, monensin, or the weak base, NH_4Cl, in the nominal absence of extracellular Ca^2 and 2) the effect of ionomycin, which could not take Ca^{2+} out of acidic organelles and was more effective after alkalinization of this compartment by the previous addition of nigericin, monensin, or NH_4Cl. All of 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)[1] 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 have 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 20–120 μM (in terms of P_i residues) in chains that are 50–800 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, similar to 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 ~5.4 (10), and this acidic pH is maintained by the function of an H^+-ATPase, which is different from F_1F_0- 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 possess acidocalcisomes (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 Ca^{2+} is located in these organelles. Our re-

* This work was supported in part by the National Institutes of Health Grants AI23259 (to R. D.) and GM50694 (to E. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Unidad de Investigacion, Hospital Universitario "Puerta del Mar," Ave. Ana de Viya 21, 11009 Cadiz, Spain.

‖ To whom correspondence should be addressed at: Dept. of Pathobiology and Center for Zoonoses Research, Laboratory of Molecular Parasitology, College of Veterinary Medicine, University of Illinois at Urbana-Champaign, 2001 S. Lincoln Avenue, Urbana, IL 61802. Tel.: 217-333-3845; Fax: 217-244-7421; E-mail: rodoc@uiuc.edu.

[1] The abbreviations used are: polyP, inorganic polyphosphate; PPi, pyrophosphate; DAPI, 4′,6-diamidino-2-phenylindole; AMDP, aminomethylenediphosphonate.

This paper is available on line at http://www.jbc.org

Downloaded from www.jbc.org at University of Illinois - Urbana on December 3, 2007

sults 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*-α-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. 4-(2-Aminoethyl)benzenesulfonyl fluoride and ionomycin (free acid) were from Calbiochem. Pepstatin came from Roche Applied Science. Coomassie Blue protein assay reagent was from Bio-Rad. *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 of the other reagents were of 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 × *g* for 10 min to eliminate contaminant red blood cells and leukocytes. The supernatant then was centrifuged at 1,000 × *g* for 15 min, and the resulting pellet was resuspended at 2 × 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 s at 20% intensity in a Branson sonifier, model 102c), and unbroken cells were separated by centrifugation (1,000 × *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 × *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 × *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 1 μM 5-[2-^{14}C]-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 enzyme-linked immunosorbent assay 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 PP_i (18), bafilomycin A_1(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. 4-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 previously (20).

Fluorescence Microscopy—Platelets (~10^8) were washed with New Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.5 mM glucose, 0.35% bovine serum albumin, 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 × *g* for 5 min and 100 μM aspirin, and 40 μg/ml apyrase were added to the supernatant. Platelets were incubated at 37 °C with 1 μM Fura-2/AM for 1 h. Labeled platelets were centrifuged at 1,000 × *g* 10 min and washed in New Tyrode's buffer. Cells then were resuspended in the same buffer at 2 × 10^8 platelets/ml and kept at 37 °C. For fluorescence measurements, 1.25 × 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 380 nm excitation in a thermostated Hitachi F-2000 spectrofluorometer at 37 °C with agitation. Calibration was performed as described previously (22).

Perchloric Acid Extracts—For NMR, platelets were washed twice with New Tyrode's buffer and then extracted with ice-cold 0.5 M $HClO_4$ (2 ml/g wet weight cells). After a 30-min incubation on ice, the extracts were centrifuged at 3,000 × *g* for 5 min. The supernatants were neutralized by the addition of 0.72 M KOH, 0.6 M $KHCO_3$ (18). Precipitated $KClO_4$ was removed by centrifugation at 12,000 × *g* for 10 min, the supernatant was separated, and EDTA was added to a final concentration of 400 μM prior to adjusting to pH 7. All of the extracts contained 10% D_2O (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-s recycle time. Inverse gated proton decoupling was used to remove NOE and J-coupling effects. The chemical shifts of all of the ^{31}P 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 ^{31}P-^{31}P 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) work station and included base-line 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 1 μM 5-[2-^{14}C]hydroxytryptamine binoxalate. Platelets were washed and resuspended in New Tyrode's buffer at 10^8 cell/ml. The platelet suspension was placed in a cuvette with agitation. Thrombin was added at a final concentration of 1.2 units/ml, and aliquots were taken at different times. Samples were directly filtered trough a 0.2-μm filter to separate the secreted material from the suspension. The contents of PP_i, 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 previously (6) using a standard buffer containing 120 mM KCl, 2 mM $MgCl_2$, 50 mM K-Hepes, and 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 mi-

Downloaded from www.jbc.org at University of Illinois - Urbana on December 3, 2007

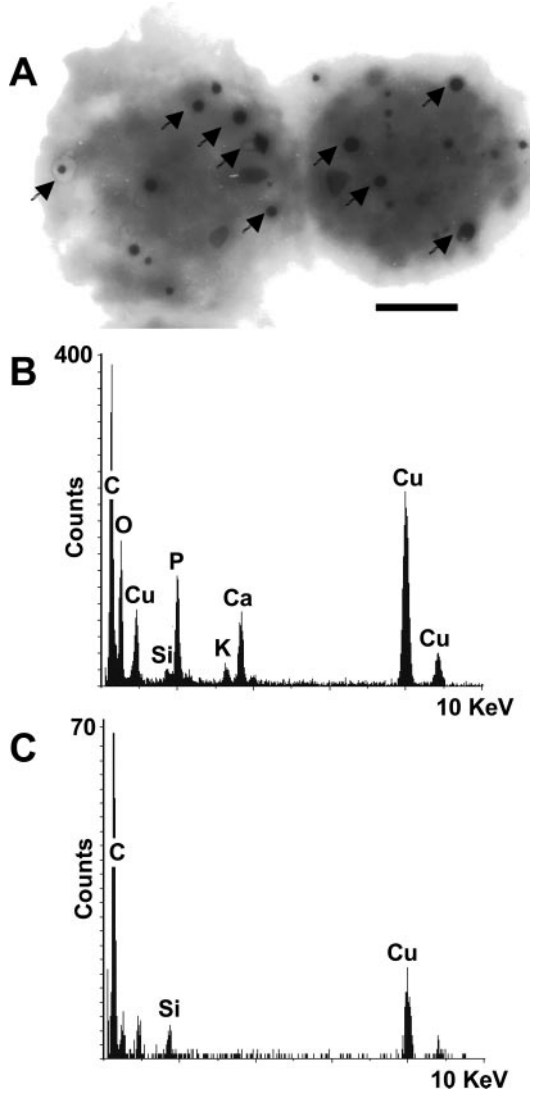

FIG. 1. **Electron microscopy and x-ray microanalysis of whole platelets.** *A*, Transmission electron microscopy of an unfixed and unstained platelet preparation. Dense granules are identified by *arrows*. Bar, 1 μm. *B*, Typical x-ray microanalysis spectrum of dense granules in whole platelets. *C*,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

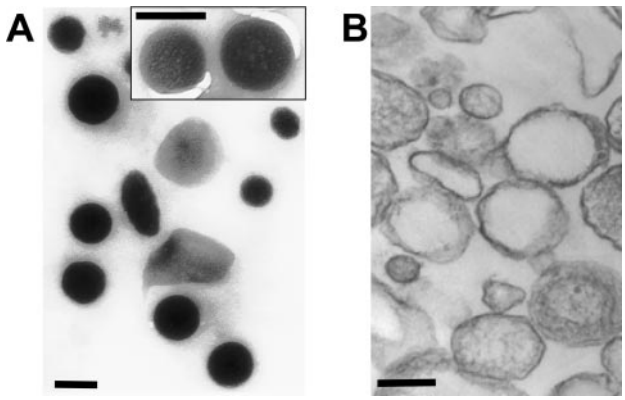

FIG. 2. **Electron microscopy of the dense granule fraction.** *A*, direct observation of unfixed and unstained dense granules air-dried directly onto microscope grids. The *inset* shows at higher magnification the spongelike structure of the dense granules after exposure to the electron beam. *B*, Fixed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

croscopy without fixation and staining (Fig. 1*A*). 5–20 granules of various shapes and sizes were observed per platelet (n = 20). This finding is in agreement with previous reports of the presence a variable number of dense bodies per platelet ranging from none to >20 (28). X-ray microanalysis was performed on these granules (Fig. 1*B*). All of the ten spectra taken from different platelets were qualitatively similar, the phosphorus/calcium ratio being 1.76 as it has been reported previously (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). An 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 in diameter (Fig. 2*A*). When submitted to the electron beam, changes in their internal structure led to the appearance of a spongelike structure (Fig. 2*A*, *inset*), which has been described before in the acidocalcisomes of *Trypanosoma cruzi* (30), *Trypanosoma brucei* (31), *C. reinhardtii* (6), and *Agrobacterium tumefaciens* (4). An 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. 2*B*).

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 α-granules (marked by the presence of von Willebrand factor) were not enriched in the densest fraction. The dense granule fraction contained ~20% total PP_i and short chain polyP (see below) (Fig. 3).

PolyP in Human Platelets—Platelet extracts yielded levels of polyP of 0.74 ± 0.08 nmol/10^8 platelets (n = 8) and no detectable long chain polyP (data not shown). If we assume that the platelet volume is 6.8 mm^3 (9), the concentration of polyP in platelets is around 1.1 mM. Thus, platelet polyP concentration is 10–20 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 of the polyP detected was <100 residues long. PolyP levels were in the same order of magnitude as the PP_i (0.92 ± 0.19 nmol/10^8 platelets; n = 8) measured by its hydrolysis with yeast pyrophosphatase. An excess of pyrophosphatase in our assay degraded <2% 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 ~60% of the total (as it occurs with calcium, ADP, ATP, and PP_i), the volume of each granule is 0.1% platelet volume (9) and there is an average of 5 dense granules/platelet (8, 28), the intragranular concentration of polyP is ~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 PP_i or other anions (18, 32). Human platelets

Downloaded from www.jbc.org at University of Illinois - Urbana on December 3, 2007

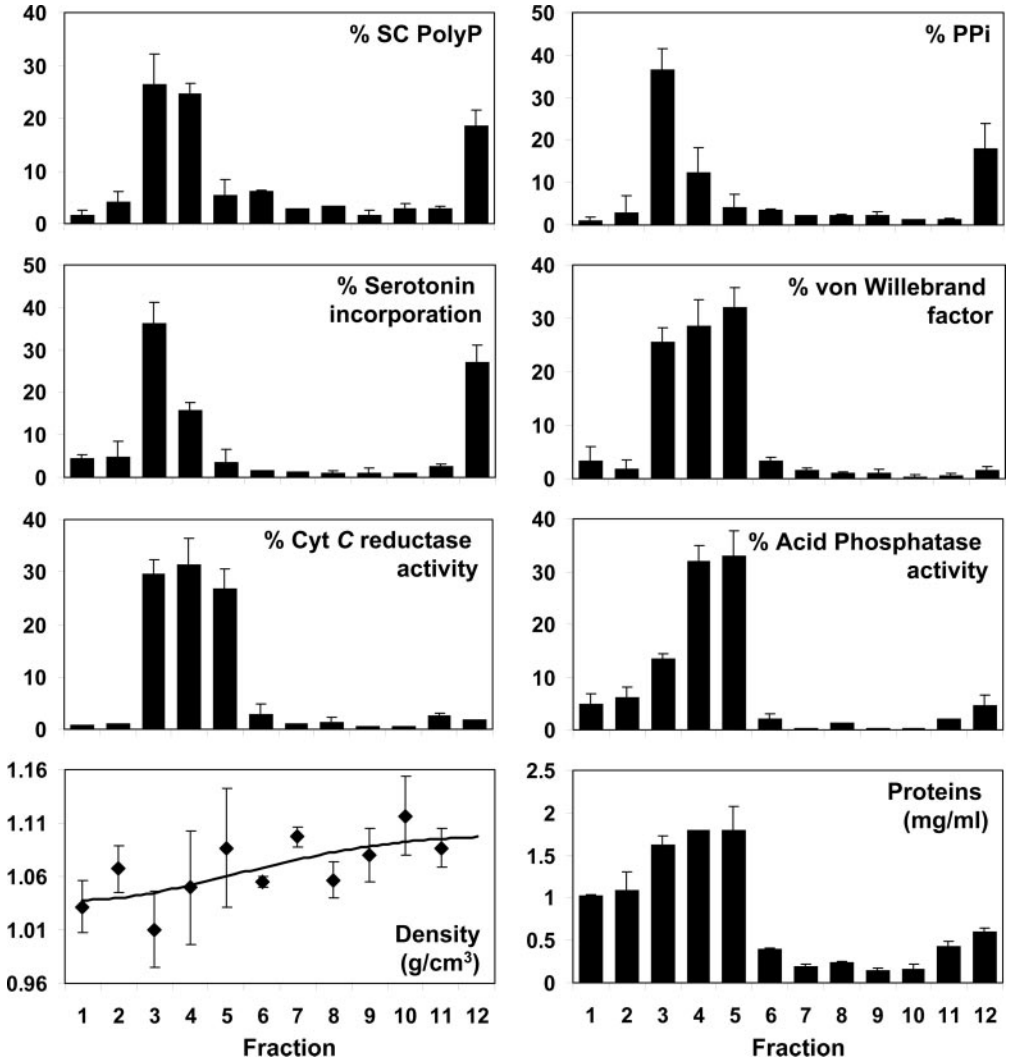

FIG. 3. **Distribution of different markers from human platelets on metrizamide gradients.** Serotonin, PP_i, and short chain polyP are all present in the dense granule fraction (*fraction 12*). This distribution was compared with that of established platelet organelle markers, acid phosphatase (lysosomes),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

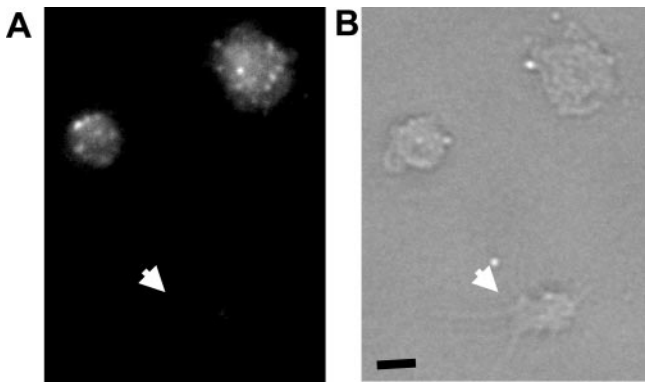

FIG. 4. **Fluorescence microscopy showing the localization of polyP using DAPI.** *A*, Platelets were treated with DAPI as described under "Experimental Procedures." Note the accumulation of DAPI in small organelles. *Arrow* in *B* (bright-field microscopy) shows an activated platelet. *Bar*: 10 μm.

incubated in solutions of DAPI (0.2 mg/ml) were mounted on slides and examined by confocal fluorescence microscopy. We detected the staining of numerous intracellular vacuoles. No staining was detected when DAPI was omitted (data not shown).

Identification of PolyP by ^{31}P NMR and Urea-PAGE Analysis—Fig. 5 shows the 303.6-MHz (^1H decoupled) ^{31}P NMR spectra of perchloric acid extracts of human platelets. Resonance assignments for these spectra are given in Table I. The predominant peak in the spectrum is inorganic phosphate (*peak A*). The region between −6 and −8 ppm is shown in left inset magnified 2.5× with respect to the overall spectra and contains peaks for the terminal phosphates of adenosine diphosphate (*peak B*) and triphosphate (*peak C*) in addition to peaks for the terminal phosphates of polyPs (*peak D*) and a large peak for PP_i (*peak E*). The region from −10 to −12 ppm contains peaks for the α-phosphates of adenosine diphosphate (F) and triphosphate (G). The region from −20 to −23 ppm is shown in the right inset magnified 2.5× with respect to the overall spectra and contains peaks for the central phosphate of adenosine triphosphate (*peak H*) and the γ-phosphates (*Central P*) of polyPs (*peak 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, the short chain polyP of ~70–75 residues.

PolyP Release after Stimulation of Platelets with Thrombin—Because 80% total PP_i is secreted by platelets treated with thrombin with a time course similar to the 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

Downloaded from www.jbc.org at University of Illinois - Urbana on December 3, 2007

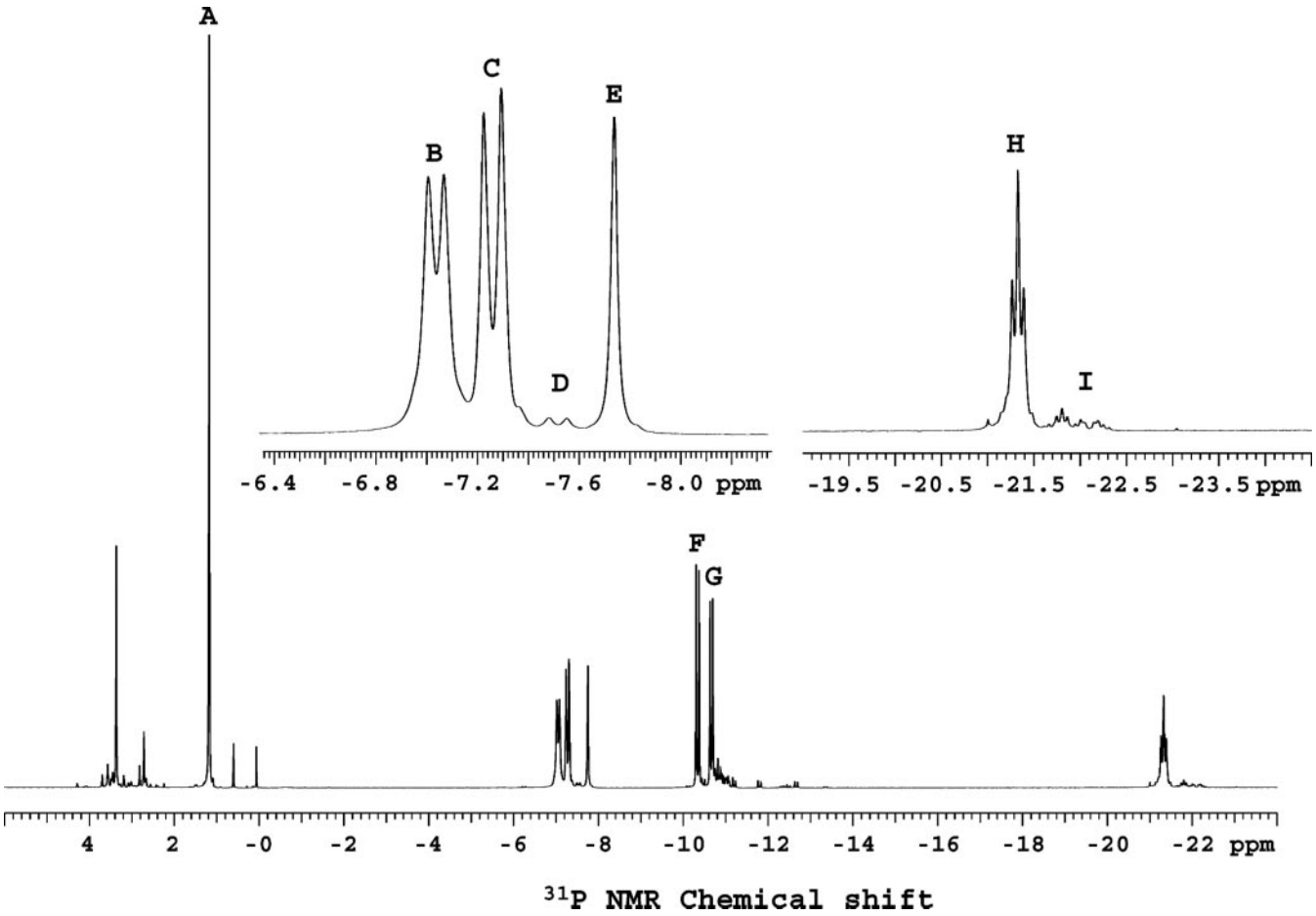

FIG. 5. **303.6 MHz (^{1}H decoupled) ^{31}P NMR spectra of perchloric acid extracts of human platelets.** Resonance assignments are given in Table I.

TABLE I
^{31}P NMR resonance assignments for perchloric acid extracts of human platelets

Peak	Assignment	Chemical shift
		ppm
A	Inorganic phosphate	1.22
B	γ-P of ATP	−7.06
C	β-P ADP	−7.23
D	α-P of polyphosphates	−7.52
E	Pyrophosphate	−7.76
F	α-P of ADP	−10.42
G	α-P of ATP	−10.76
H	β-P of ATP	−20.38
I	Central P of polyphosphates	−22.02

that polyP is in the same compartment containing PP_i and serotonin.

Intracellular Ca^{2+} Concentration $[Ca^{2+}]_i$ 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 Ca^{2+} (1 mM EGTA added). This concentration is in the range observed in previous studies (34). The addition of 1 μM thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase when used at low concentrations (35), increased the $[Ca^{2+}]_i$ (Fig. 8*A*, *upper trace*). The subsequent addition of nigericin (a K^+/H^+ ionophore; 2 μM) caused a second rise in $[Ca^{2+}]_i$ to a level that was significantly higher than that in the absence of nigericin. Similar results were observed when the order of additions was reversed (Fig. 8*A*, *lower trace*), suggesting the presence of a thapsigargin-sensitive and an acidic thapsigargin-insensitive Ca^{2+} compartment.

Synergistic Effect of Ionomycin and Ammonium Chloride on Ca^{2+} Release from Intracellular Compartments—To provide more evidence for the presence of an acidic Ca^{2+} 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 Ca^{2+} from neutral or alkaline compartments but releases more Ca^{2+} after alkalinizing agents have elevated the pH of acidic compartments. Accordingly, adding ionomycin to human platelets previously exposed to NH_4Cl (10 mM) caused an increase in $[Ca^{2+}]_i$ to a level that was significantly higher than that in the absence of NH_4Cl (Fig. 8*B*, *upper trace*). Similar results were obtained when the order of additions was reversed (Fig. 8*B*, *upper trace*) and when nigericin (2 μM) or monensin (1 μM) was 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) insensitive to thapsigargin, which 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. 9*A*). Although isolated dense granules could take up acridine orange, demonstrating that they were still intact and acidic, neither ATP nor PP_i was able to stimulate this process. Alkalinization of dense granules by the addition of NH_4Cl resulted in the release of the acridine orange

Downloaded from www.jbc.org at University of Illinois - Urbana on December 3, 2007

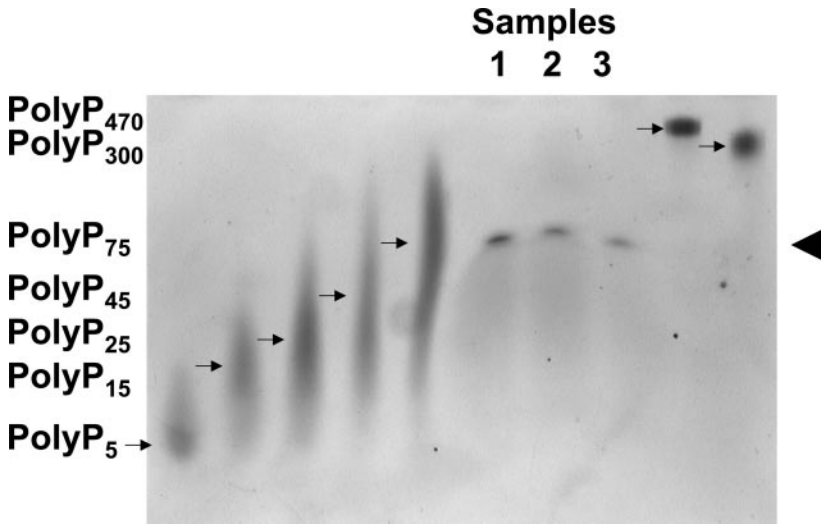

FIG. 6. **Urea-PAGE analysis of polyP from human platelets from three different donors.** PolyP extracted from platelets was electrophoresed by 6% urea-PAGE. Chain lengths of standards are on the *left*. An *arrowhead* on the *right* shows the position of migration of samples from three different donors.

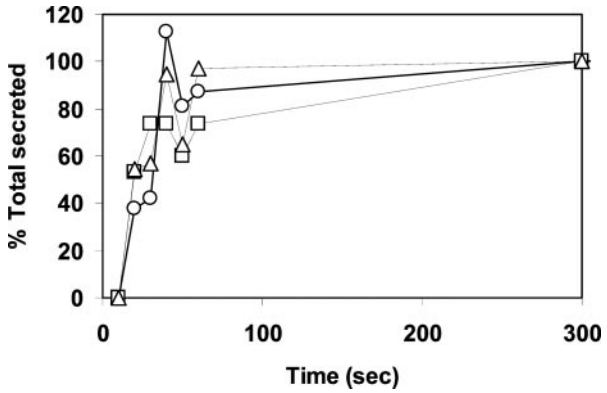

FIG. 7. **Time course of thrombin-induced serotonin release compared with PP_i and polyP.** Release of serotonin (*triangles*), PP_i (*squares*), and short chain polyP (*circles*) in platelets after the addition of 1.2 units/ml thrombin as described under "Experimental Procedures." Results are expressed as percentage of the material released at 300 s (representative of three experiments).

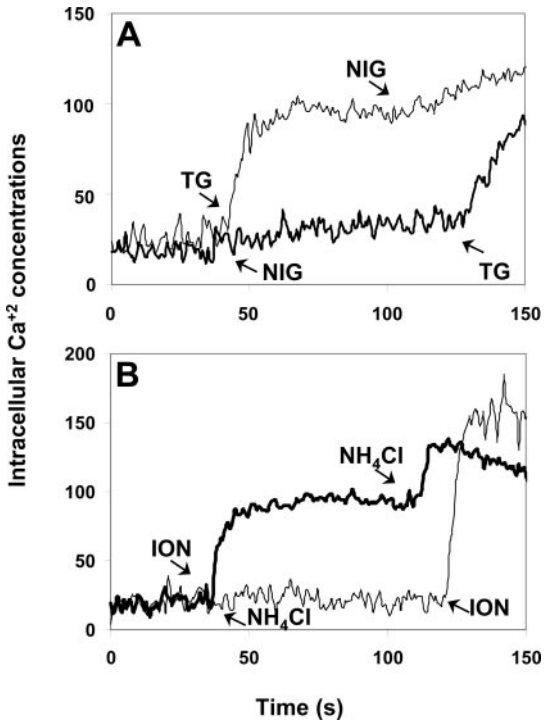

FIG. 8. **Effect of ionophores and NH_4Cl on human platelets $[Ca^{2+}]_i$.** Platelets were loaded with Fura-2/acetoxymethyl ester as described under "Experimental Procedures" and resuspended in New Tyrode's buffer containing 1 mM EGTA. Thapsigargin (*TG*, 1 μM), nigericin (*NIG*, 2 μM), ionomycin (*ION*, 1 μM), or NH_4Cl (20 mM) were added where indicated.

accumulated (Fig. 9*B*). A pyrophosphatase activity was also enriched in the dense granule fraction (Fig. 9*A*). Interestingly, when this activity was measured in the presence of aminomethylenediphosphonate (AMDP), an inhibitor of vacuolar proton-translocating pyrophosphatases ($V\text{-}H^+$-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 was shown to be present at millimolar levels, 10–20 times higher than in rodent tissues investigated previously (3). The presence of polyP of ~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 PP_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_1-sensitive ATPase and pyrophosphatase activities. In agreement with these results, when human platelets were loaded with the fluorescent calcium indicator Fura-2/acetoxymethyl ester to measure their $[Ca^{2+}]_i$, they were shown to possess a significant amount of Ca^{2+} stored in an acidic compartment.

Numerous studies have defined the role of dense granules in platelets. The activation of platelets leads to the secretion of dense granule components such as ATP, ADP, PP_i, 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 PP_i and polyP are unknown. However, polyP, similar to heparin, is a negatively charged polymer that has been postulated to stabilize fibro-

Downloaded from www.jbc.org at University of Illinois - Urbana on December 3, 2007

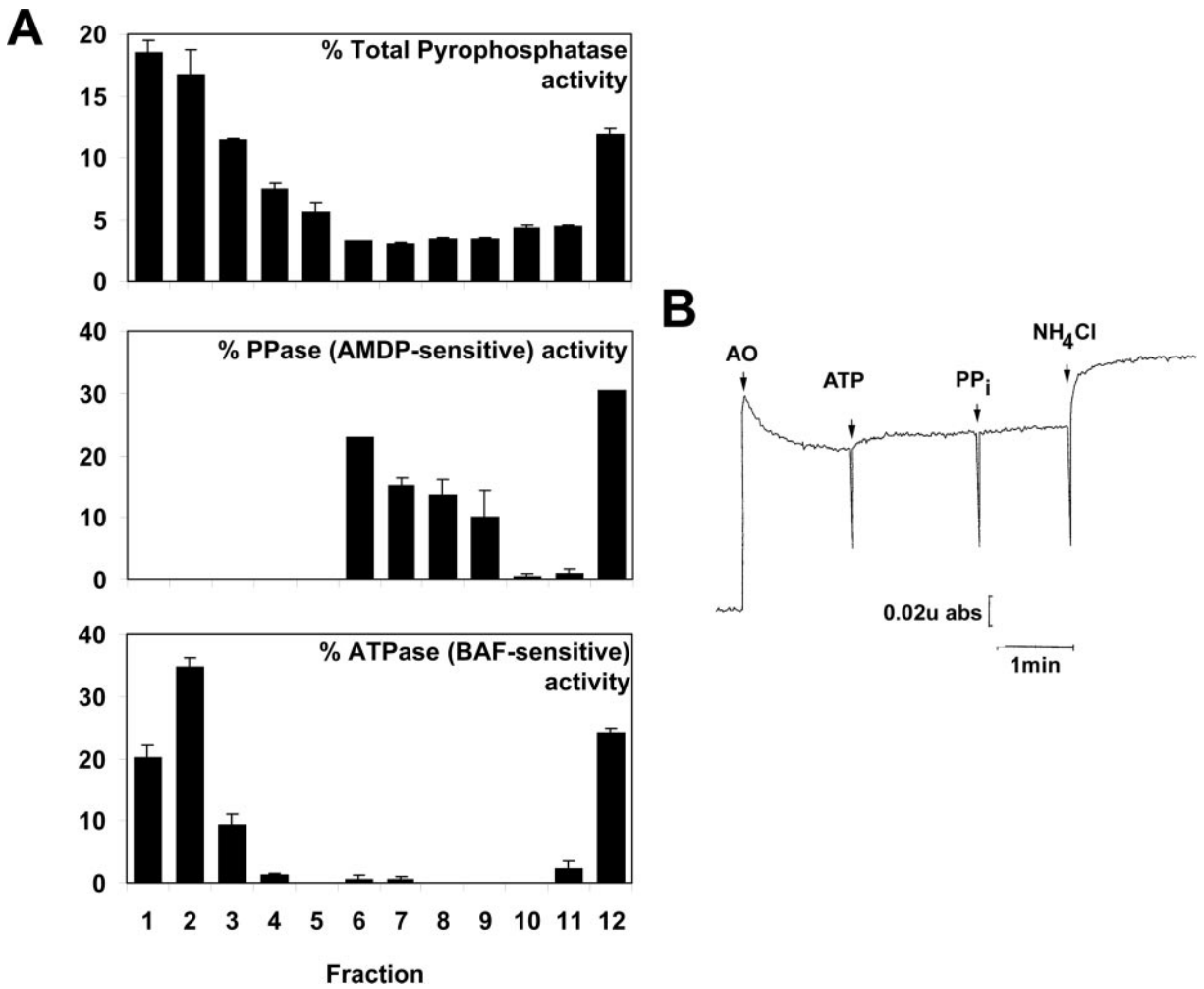

FIG. 9. **Distribution of bafilomycin A_1-sensitive ATPase and pyrophosphatase activities on metrizamide gradients and acridine orange uptake by isolated dense granule fraction.** *A*, bafilomycin A_1 (1 μM)-sensitive ATPase, pyrophosphatase, and AMDP (20 μM)-sensitive pyrophosphatase activities are all enriched in the dense granule fraction (*fraction 12*). Other conditions were as described in the legend to Fig. 3 and under "Experimental Procedures." *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), and PP_i (0.1 mM) of NH_4Cl (20 mM) were added where indicated. Changes in absorbance were followed at 493–530 nm.

blast growth factor and facilitate fibroblast growth factor 2 binding to its receptor tyrosine kinase promoting signaling via the formation of receptor dimers and to prevent fibroblast growth factor degradation in tissues that have been injured (38). Because polyP is released from platelets after thrombin stimulation (Fig. 7), it could contribute to the homeostatic functions of platelets; thus, 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 Ca^{2+}-ATPase inhibitor (35), was shown to release Ca^{2+} from an inositol 1,4,5-trisphosphate-sensitive intracellular store (14) (possibly the dense tubular system, a structure derived from the megakaryocyte endoplasmic reticulum) (40). The 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_4Cl, 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 sarcoplasmic endoplasmic reticulum-2b and sarcoplasmic endoplasmic reticulum-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).

Therefore, platelet dense granules have many characteristics in common with acidocalcisomes. 1) They are acidic due to the operation of a bafilomycin A_1-sensitive proton ATPase (Fig. 9*A*) (12) and are able to accumulate acidophilic dyes such as acridine orange (Fig. 9*B*) (see 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_i (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 ~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

Downloaded from www.jbc.org at University of Illinois - Urbana on December 3, 2007

their surrounding membrane (5). As it occurs with acidocalcisomes of *T. brucei* (46), 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 (Fig. 9*A*) (37). Although one study reported PP_i-induced acidification of *trans*-cisternal elements of rat liver Golgi membranes (47), there have been no reports on the presence of genes with similarity to those encoding V-H^+-PPases of bacteria, plants, and protists. Because no PP_i-dependent H^+ transport could be measured in dense granule fractions (Fig. 9*B*) 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 bafilomycin A_1-sensitive ATPase activity could be measured by P_i release from ATP (Fig. 9*A*), H^+ transport in isolated dense granules could not be detected (Fig. 9*B*). This has been observed with other acidocalcisomes that are known to possess 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,[2] 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 (PP_i, polyP, calcium, potassium, and so forth) 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.

Acknowledgments—We thank Arthur Kornberg for *E. coli* CA38 pTrcPPX1, Michael Martin for the synthesis of AMDP, and John Bozzola and Steve Schmitt for help with the x-ray microanalysis.

REFERENCES

1. Kornberg, A. (1995) *J. Bacteriol.* **177,** 491–496
2. Kornberg, A., Rao, N. N., and Ault-Riche, D. (1999) *Annu. Rev. Biochem.* **68,** 89–125
3. Kumble, K. D., and Kornberg, A. (1995) *J. Biol. Chem.* **270,** 5818–5822
4. Seufferheld, M., Vieira, M. C. F., Ruiz, F. A., Rodrigues, C. O., Moreno, S. N. J., and Docampo, R. (2003) *J. Biol. Chem.* **278,** 29971–29978
5. Docampo, R., and Moreno, S. N. J. (2001) *Mol. Biochem. Parasitol.* **114,** 151–159
6. Ruiz, F. A., Marchesini, N., Seufferheld, M., Govindjee, and Docampo, R. (2001) *J. Biol. Chem.* **276,** 46196–46203
7. Marchesini, N., Ruiz, F. A., Vieira, M., and Docampo, R. (2002) *J. Biol. Chem.* **277,** 8146–8153
8. McNicol, A., and Israels, S. J. (1999) *Thromb. Res.* **95,** 1–18
9. Holmsen, H., and Weiss, H. J. (1979) *Annu. Rev. Med.* **30,** 119–134
10. Carty, S. E., Johnson, R. G., and Scarpa, A. (1981) *J. Biol. Chem.* **256,** 11244–11250
11. Dean, G. E., Fishkes, H., Nelson, P. J., and Rudnick, G. (1984) *J. Biol. Chem.* **259,** 9569–9574
12. Turetta, L., Bazzan, E., Bertagno, K., Musacchio, E., and Deana, R. (2002) *Cell Calcium* **31,** 235–244
13. Bowman, E. J., Siebers, A., and Altendorf, K. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7972–7976
14. Brüne, B., and Ulrich, V. (1991) *J. Biol. Chem.* **266,** 19232–19237
15. Tsunoda, Y., Matsuno, K., and Tashiro, Y. (1991) *Exp. Cell Res.* **193,** 356–363
16. Rendu, F., Lebret, M., Nurden, A. T., and Caen, J. P. (1982) *Br. J. Haematol.* **52,** 241–251
17. Fukami, M. H., Bauer, J. S., Stewart, G. J., and Salganicoff, L. (1978) *J. Cell Biol.* **77,** 389–399
18. Ruiz, F. A., Rodrigues, C. O., and Docampo, R. (2001) *J. Biol. Chem.* **276,** 26114–26121
19. Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S., and De Duve, C. (1968) *J. Cell Biol.* **37,** 482–513
20. Scott, D. A., de Souza, W., Benchimol, M., Zhong, L., Lu, H.-G., Moreno, S. N. J., and Docampo, R. (1998) *J. Biol. Chem.* **273,** 22151–22158
21. Lu, H.-G., Zhong, L., Chang, K.-P., and Docampo, R. (1997) *J. Biol. Chem.* **272,** 9464–9473
22. Docampo, R., Scott, D. A., Vercesi, A. E., and Moreno, S. N. J. (1995) *Biochem. J.* **310,** 1005–1012
23. Van Wazer, J. R., and Ditchfield, R. (1987) in *Phosphorus NMR in Biology* (Burt, C. T., ed) pp. 1–23, CRC Press, Inc., Boca Raton, FL
24. Moreno, B., Urbina, J. A., Oldfield, E., Bailey, B. N., Rodrigues, C. O., and Docampo, R. (2000) *J. Biol. Chem.* **275,** 28356–28362
25. Bull, B. S. (1966) *Blood* **28,** 901–912
26. White, J. G. (1969) *Blood* **33,** 598–606
27. Costa, J. L., Reese, T. S., and Murphy, D. L. (1974) *Science* **183,** 537–538
28. Costa, J. L., Detwiler, T. C., Feinman, R. D., Murphy, D. L., Patlak, D. S., and Pettigrew, K. D. (1977) *J. Physiol. (Lond.)* **264,** 297–306
29. Skaer, R. J., Emmines, J. P., and Peters, P. D. (1976) *J. Cell Sci.* **20,** 441–457
30. Lu, H.-G., Zhong, L., de Souza, W., Benchimol, M., Moreno, S. N. J., and Docampo, R. (1998) *Mol. Cell. Biol.* **18,** 2309–2323
31. Rodrigues, C. O., Scott, D. A., and Docampo, R. (1999) *Mol. Cell. Biol.* **19,** 7712–7723
32. Allan, R. A., and Miller, J. J. (1980) *Can. J. Microbiol.* **26,** 912–920
33. Fukami, M. H., Dangelmaier, C. A., Bauer, J. S., and Holmsen, H. (1980) *Biochem. J.* **192,** 99–105
34. Sun, B., and Kambayashi, J. (2000) *J. Biomed. Sci.* **7,** 35–41
35. Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R., and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 2466–2470
36. Liu, C., and Hermann, T. E. (1978) *J. Biol. Chem.* **253,** 5892–5894
37. Zhen, R.-G., Baykov, A. A., Bakuleva, N. P., and Rea, P. A. (1994) *Plant Physiol.* **104,** 153–159
38. Shiba, T., Nishimura, D., Kawazoe, Y., Onodera, Y., Tsutsumi, K., Nakamura, R., and Ohshiro, M. (2003) *J. Biol. Chem.* **278,** 26788–26792
39. Authi, K. S., Bokkala, S., Patel, Y., Kakkar, V.V., and Munkonge, F. (1993) *Biochem. J.* **294,** 119–126
40. Brass, L. F., and Joseph, S. K. (1985) *J. Biol. Chem.* **260,** 15172–15179
41. Papp, B., Enyedi, A., Paszty, K., Kovacs, T., Sarkadi, B., Gardos, G., Magnier, C., Wuytack, F., and Enouf, J. (1992) *Biochem. J.* **288,** 297–302
42. Engelender, S., Wolosker, H., and de Meis, L. (1995) *J. Biol. Chem.* **270,** 21050–21055
43. Popov, E. G., Gabbasov, Z. A., Gavrilov, I. Y., Pozin, E. Y., Mejlumian, A. G. (1987) *Thromb. Res.* **47,** 639–645
44. Skaer, R. J., Flemans, R. J., and McQuilkan, S. (1981) *Br. J. Haematol.* **49,** 435–438
45. Costa, J. L., Fay, D. D., Nurnberger, J. I., and Murphy, D. L. (1982) *Biochem. Pharmacol.* **31,** 3215–3218
46. Lemercier, G., Espiau, B., Ruiz, F. A., Vieira, M., Luo, S., Baltz, T., Docampo, R., and Bakalara, N. (2004) *J. Biol. Chem.* **279,** 3420–3425
47. Brightman, A. O., Navas, P., Minnifield, N. M., and Morré, D. J. (1992) *Biochim. Biophys. Acta* **1104,** 188–194
48. Scott, D. A., and Docampo, R. (2000) *J. Biol. Chem.* **275,** 24215–24221
49. Merzendorfer, H., Gräf, R., Huss, M., Harvey, W. R., and Wieczorek, H. (1997) *J. Exp. Biol.* **200,** 225–235
50. Moriyama, Y., and Nelson, N. (1989) *J. Biol. Chem.* **264,** 3577–3582
51. Boullin, D. J., and Green, A. R. (1972) *Br. J. Pharmacol.* **45,** 83–94
52. Meyer, A. (1904) *Bot. Zeit.* **62,** 113–152
53. Jensen, T. E. (1993) in *Ultrastructure of Microalgae* (Berner, T., ed) pp. 7–50, CRC Press, Inc., Boca Raton, FL

[2] P. Rohloff and R. Docampo, unpublished results.

Downloaded from www.jbc.org at University of Illinois - Urbana on December 3, 2007