Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes*

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Background: Polyphosphate has pro-coagulant and pro-inflammatory activities.

Results: Mast cells polyphosphate was found in their acidocalcisome-like granules and stimulated bradikinin formation.

Conclusion: Polyphosphate is a novel component of mast cell granules.

Significance: Results suggest that the pro-coagulant and pro-inflammatory activities of mast cells could in part be attributed to polyphosphate.

Polyphosphate (polyP) is a pro-inflammatory agent and a potent modulator of the human blood-clotting system. The presence of polyP of 60 phosphate units was identified in rat basophilic leukemia (RBL-2H3) mast cells using specific enzymatic assays, urea-polyacrylamide gel electrophoresis of cell extracts, and staining of cells with 4,6-diamidino-2-phenylindole (DAPI), and the polyP-binding domain of Escherichia coli exopolyphosphatase (PPX). PolyP co-localizes with serotonin- but not with histamine-containing granules. PolyP levels greatly decreased in mast cells stimulated to degranulate by IgE. Mast cell granules were isolated and found to be acidic and decrease their polyP content upon alkalinization. In agreement with these results, when RBL-2H3 mast cells were loaded with the fluorescent calcium indicator fura-2 acetoxymethyl ester to measure their intracellular Ca2+ concentration ([Ca2+]i), they were shown to possess a significant amount of Ca2+ stored in an acidic compartment different from lysosomes. PolyP derived from RBL-2H3 mast cells stimulated bradykinin formation and it was also detected in human basophils. All these characteristics of mast cell granules, together with their known elemental composition, and high density, are similar to those of acidocalcisomes. The results suggest that mast cells polyP could be an important mediator of their pro-inflammatory and pro-coagulant activities.

Inorganic polyphosphate (polyP) has been recently described as a modulator of human hemostasis and inflammation (1). PolyP is a linear anionic polymer of orthophosphate (P_i) residues linked by high-energy phosphoanhydride bonds present in all cells (2). In both prokaryotes and eukaryotes, polyP is packaged, along with calcium and other cations, in acidic organelles termed acidocalcisomes (3). The discovery of acidocalcisome-like organelles in human platelets (dense granules) (4) led to the suggestion that acidocalcisomes have been conserved over evolutionary time or have appeared more than one time by convergent evolution (3). PolyP is secreted from platelets upon activation (4,5) and has considerable pro-coagulant and antifibrinolytic activities (6). Recent studies have demonstrated that polyP acts at four points in the blood-clotting cascade (reviewed by (1)). PolyP of the size range that accumulates in bacteria and other microorganisms (long-chain polyP) potently triggers the contact pathway, accelerates Factor V activation, and enhances fibrin polymerization (7). In contrast, short-chain polyP polymers, of the size secreted by human platelets, are less potent than long-chain polyP in triggering contact activation or in enhancing fibrin polymerization, while retaining full ability to promote Factor V activation (8,9). Both short- and long-chain polymers potently stimulate the activation of Factor XI by thrombin as well as Factor XI autoactivation (1). PolyP initiate the contact pathway by activating Factor XII to Factor XIIa, leading to thrombin and fibrin
generation via the intrinsic pathway of coagulation, and also to bradykinin formation by kallikrein-mediated high molecular weight kininogen cleavage (5). Bradykinin is the ligand of kinin B2 receptor, which activates various intracellular signaling pathways that lead to inflammatory reactions (reviewed in (10)).

Like acidocalcisomes, human platelet dense granules are spherical, acidic, electron dense, and have high concentrations of cations and polyP (4). Platelet dense granules and acidocalcisomes also share the system for targeting of their membrane proteins via adaptor protein-3 (AP-3) complex (11). Both organelles are considered lysosome-related organelles (LROs) (3), defined as cell type-specific modifications of the post-Golgi endomembrane system that have a variety of functions and share some characteristics with lysosomes (12). LROs include melanosomes, lytic granules in lymphocytes, platelet dense-granules, basophil and mast cell granules, neutrophil azurophil granules and others (13).

Mast cell granules are also similar to acidocalcisomes. They are spherical, electron-dense (14), have high concentrations of cations (calcium, magnesium, potassium, sodium, and zinc) and phosphorus (14,15) and, as acidocalcisomes, have been proposed to possess a proton pump (vacuolar ATPase) (16) and a calcium pump (Ca$^{2+}$-Mg$^{2+}$-ATPase) (17) to maintain their acidity and high calcium levels, respectively. Mast cells and blood basophils are associated with pathological conditions such as asthma, allergy and anaphylaxis (18). Basophils are a population of circulating white blood cells that are functionally similar to mast cells. Both cell types share many common features, such as high-affinity IgE receptor expression, metachromatic staining, and histamine release, among others (18). Mast cells also have key roles in the modulation of inflammation and the enhancement of adaptive immune responses, where they contribute to pathogen clearance (19) and regulation of angiogenesis (20). The most striking feature of mast cells is that their cytoplasm is filled with dense metachromatic granules. When activated, mast cells secrete their cytoplasmic granules to the media, releasing a wide selection of cell modulators such as heparin, histamine, serotonin, neutral proteases, growth factors, and proinflammatory cytokines (21). These secretory granules are distributed in different subsets wherein histamine and serotonin are localized separately (22,23). Interestingly, mast cell lysates have been shown to have a pro-coagulant effect (24), and chronic urticaria, which is an autoimmune disease associated with the presence of histamine releasing autoantibodies against the high affinity IgE receptor of mast cells and basophils, is associated with activation of blood coagulation (25). Furthermore, mast cell-derived heparin, as polyP (5), is also known to initiate the contact pathway-mediated bradykinin formation (26).

The similarities between acidocalcisomes and mast cell granules and the roles of mast cells in inflammation and hemostasis suggested to us that polyP might be present in these organelles. In this report, we demonstrate that, as acidocalcisomes, mast cell granules are rich in polyP. PolyP co-localizes with serotonin- but not with histamine-containing granules. Mast cell polyP greatly decreased when cells were stimulated to degranulate by IgE and was able to stimulate bradykinin formation, suggesting that mast cell polyP is an important mediator of mast cell pro-inflammatory and pro-coagulant activities.

**EXPERIMENTAL PROCEDURES**

**Materials**-PolyP of mean polymer length 25, 45 and 65 (PolyP$_{25}$, PolyP$_{45}$, PolyP$_{65}$), 4',6-diamidino-2-phenylindole (DAPI), Heparinase III, monoclonal anti-Dinitrophenol (DNP)-IgE antibody, p-nitrophenyl-N-acetyl-β-D-glucosamine, digitonin, and nigericin were purchased from Sigma Chemicals Co. (St. Louis, MO). The anti-Xpress epitope mouse monoclonal antibody, Alexa Fluor-488 Donkey anti-rabbit IgG, Alexa Fluor-647 donkey anti-Goat, Alexa Fluor-488 goat anti-mouse IgG, and DNA ladders were from Invitrogen (Carlsbad, CA). Cy3 goat anti-mouse IgG was from Jackson ImmunoResearch Europe Ltd. (Suffolk, UK). Rabbit polyclonal antibody anti-histamine and goat polyclonal antibody anti-serotonin were purchased from Abcam (Cambridge, UK). Bovine DNP-albumin conjugate (DNP-BSA) was form Calbiochem (La Jolla, CA). Formvar/carbon coated copper grids were form Ted Pella, Inc. (Redding, CA). *E. coli* strain CA38 pTrcPPX1 was kindly provided by late Prof. Arthur Kornberg, Stanford University School of Medicine (Stanford, CA). *E. coli* strain CA38 pTrcPPX1 was kindly provided by late Prof. Arthur Kornberg, Stanford University School of Medicine (Stanford, CA).
coli strain DH5α pTrc-PPBD was kindly provided by Dr. Katsuharu Saito (Shinshu University, Nagano-Ken, Japan). All other reagents were of analytical grade.

Cell Culture—The rat mast cell line RBL-2H3 was purchased from ATCC (Rockville, MD) and cultured at 37 °C and 5% CO₂, in minimum essential medium (MEM) supplemented with 15% heat-inactivated fetal bovine serum, 100 i.u./ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate and 10 mM glutamine.

Blood samples—Blood samples were obtained from healthy volunteers. Approval for this study was obtained from the institutional review board (Ethics Committee). Informed consent was provided according to the Declaration of Helsinki.

PolyP Localization Using DAPI—Detection of polyP by DAPI-labeling and epifluorescence or fluorescence confocal microscopy was performed as described before (27). Briefly, washed cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Then, cells were washed twice and resuspended in PBS, and 1 mg/ml of DAPI was added. Samples were mounted on slides and observed using an Olympus BX-40 epifluorescence microscope with U-MNUA2 filters for DNA detection (excitation 360-370 nm, emission: 420-460 nm), or U-MWIBA filters for polyP detection (excitation 460-490 nm, emission: 515-550 nm). Localization of polyP in the samples was also done with a laser TCS-SL confocal imaging system (Leica Microsystems) (excitation: 458 nm, emission: 530-570 nm).

PolyP Localization Using the PolyP-Binding Domain of E. coli Exopolyphosphatase—Additional experiments of polyP localization were performed using the recombinant PPBD (polyP-binding domain) of E. coli exopolyphosphatase linked to an Xpress epitope tag (28), with some modifications. Briefly, RBL-2H3 mast cells were seeded on 10-mm-diameter glass coverslips 16 h before analysis. Peripheral blood mononuclear cells (PBMC) were obtained after Ficoll/Hypaque density-gradient centrifugation of peripheral blood from volunteers as previously described (29). Cells were fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 for 5 min, and coverslips were blocked with 3% BSA for 60 min. PolyP staining was done by sequential incubations with: a) PPBD (8 µg/ml) and anti-Xpress epitope monoclonal antibody (10 µg/ml) for 2 h; and b) 1:500 diluted Alexa488 Cy3-labeled or 1:100 diluted Cy3-labeled anti-mouse IgGs as a secondary antibody for 45 min. In general, for co-localization with other proteins, a) 1:500 dilutions of primary-specific antibodies; and b) 1:500 dilutions of labeled secondary antibodies (Alexa488 anti-rabbit or Alexa647 anti-goat IgGs) were included in the incubations. For specific co-localization with IgE, a goat FITC-labeled polyclonal antibody against human IgE (Biosource, Camarillo, CA, USA) was included in the secondary incubation (1:100 dilution). All dilutions were done with Tris-buffered saline (100 mM Tris/HCl, pH 7.2, and 150 mM NaCl), and 1% BSA was added to the media in both incubations with antibodies. Negative controls were prepared by a similar procedure but without PPBD or primary-specific antibodies. Coverslips were mounted with DABCO antifading agent onto slides for imaging using a laser TCS-SL confocal imaging system (Leica Microsystems).

Flow Cytometry Analysis—FACS analysis was performed on a FACScalibur cytometer (Becton Dickinson) as described before (27), with modifications. For analysis of DAPI-polyP fluorescence, fixed cells were resuspended with 0.9% NaCl. Then, 1 mg/ml of DAPI, or a similar volume of water, was added. A final concentration of 160 µg/ml of DAPI was used. Fluorescence was collected with a 670 long-pass filter (FL3). Data from 10,000 cells per sample were collected. For detection of IgE expression on human blood cells, we used the BASOTEST kit (Glycopte Biotechnology, Heidelberg, Germany) that contains a phycoerythrin-labeled monoclonal antibody against human IgE. Red/orange fluorescence was collected with a 585/42 nm bandpass filter (FL2). Cell analysis was performed with CELLQUEST software (Becton Dickinson).

PolyP Isolation, and Measurement, and Urea-Polyacrylamide Gel Analysis—Total polyP isolation was performed as described (30). Final concentration of polyP was measured after incubation with an excess of purified recombinant yeast exopolyphosphatase (ScPPX), as described before (31). Extracted total polyP was analyzed in a 6% urea polyacrylamide gel (4), and DAPI-negative staining was used to visualize polyP as described by Smith and Morrisey (32).
Mast Cell Activation-RBL-2H3 mast cells were stimulated to degranulate as described (33) with modifications. Briefly, washed cells were sensitized for 30 min by incubation with anti-DNP mouse monoclonal IgE (1 µg/ml) and then stimulated with 100 ng/ml DNP-BSA for 30 min at 37 °C, in Heps-BSA buffer (140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1 mM CaCl₂, 0.1% glucose, 0.1% BSA, 10 mM Heps, pH 7.4). Aliquots were separated before stimulation and they were used as control (Basal). Basal and activated cell supernatants were separated by centrifugation, and cell pellets were lysed with 10% Triton-X-100. Evaluation of mast cell activation was done by the analysis of β-hexosaminidase activity in supernatants and cell pellets.

Measurement of β-hexosaminidase-Aliquots (23µl) of mast cell lysates, supernatants from activated and basal mast cells, and isolated mast cell granules were incubated with 57 µl of 1.3 mg/ml p-nitrophenyl-N-acetyl-β-glucosamine (enzyme substrate), dissolved in 0.1 M sodium citrate (pH 4.3) for 90 min at 37ºC. Then, 170 µl of 0.2 M glycine, pH 10.5, was added, and absorbance was measured at 405 nm.

Electron Microscopy-Whole mount electron microscopy was performed as described previously (4) but using RBL-2H3 mast cells.

Isolation of Mast Cell Granules-The isolation was performed as previously described Lindstedt and Kovanen (34) with some modifications. Washed RBL-2H3 mast cells were resuspended in buffer B (PBS with magnesium and calcium from GIBCO, 1.75 mg/ml bovine serum albumin, 5.6 mM glucose) at 5 x 10⁶ cells/ml and subjected to mild sonication in a water bath sonicator for 30 sec at 4 ºC. Samples were centrifuged 10 min at 150 g and 4 ºC. Pellets were resuspended in a similar volume of buffer B and sonication and centrifugation steps were repeated twice. Supernatants containing the membrane-covered granules were checked at the microscope to verify the absence of cellular debris. Analysis of β-hexosaminidase activity in isolated granules and cell debris determined that recovery was higher than 80% in all cases.

Fluorimetry-Changes in acridine orange fluorescence and Ca²⁺ determinations were monitored in a thermostated Hitachi F4500 fluorometer at 37 °C with agitation. Changes in Acridine Orange fluorescence were determined by following changes in Acridine Orange (6 µM) fluorescence (excitation at 495 nm, emission at 530 nm) in digitonin permeabilized RBL-2H3 mast cells. Washed cells were incubated with 2 µg/ml oligomycin, 2 µg/ml antimycin A, and 0.2% digitonin at 37 °C for 10 min in assay buffer (25 mM sucrose, 65 mM KCl, 2.5 mM KH₂PO₄, 1 mM MgCl₂, 10 mM Heps, pH 7.2). Acridine Orange signal was allowed to equilibrate for 5–10 min and then, 5 µM nigericin and 20 mM NH₄Cl were added as indicated. For intracellular calcium measurements, RBL-2H3 mast cells were incubated at 37 ºC with 2 µM fura-2-AM for 30 min. Labeled cells were washed 3 times with PBS, resuspended at 2 x 10⁶ cell/ml in assay buffer, and kept at 37 ºC. For fluorescence measurements cells were suspended in a cuvette containing 100 µM EGTA. Fluorescence emission at 510 nm was measured with 340 and 380 nm excitation. Calibration was performed as described previously (35).

Staining with Acridine Orange-Isolated mast cell granules were bound to polylysine-coated coverslips and stained with 6 µM Acridine Orange for 10–20 min at room temperature in the dark. Coverslips were washed three times with PBS. A drop of DABCO antifading agent was added to the slides and they were observed using a laser TCS-SL confocal imaging system (Leica Microsystems) (excitation: 450-490nm, emission: >500nm).

Bradykinin Generation in Human Plasma-Determination of activation of Bradykinin generation was performed as described previously (5). Briefly, human plasma was incubated with synthetic- or RBL-2H3 extracted- polyP for 30 min at 37 ºC, and plasma bradykinin concentrations were quantified with MARKIT-M-Bradykinin ELISA (Dainippon Pharmaceutical, Osaka, Japan).

RESULTS

Detection of PolyP in Mast Cells-We used several techniques to investigate the localization of polyP in mast cell granules. DAPI is known to change its emission fluorescence to a maximum wavelength of 525 nm in the presence of polyP and this change is specific for polyP and not produced by other anions (31,36). RBL-2H3 cells, a mast cell-derived cell line, were fixed and incubated in solutions of DAPI (1.0 mg/ml), mounted on slides, and examined by epifluorescence microscopy (Fig.
1A, red), or confocal fluorescence microscopy (Fig. 1B, green). We detected staining of numerous intracellular vesicles, while nuclear staining was detected when emission due to DAPI-DNA complex formation was measured (Figs. 1A and 1B, blue). Fig. 1C shows the flow cytometry analysis, with settings for DAPI-PolyP detection, of RBL-2H3 cells in the absence (blue) and presence (green) of DAPI.

PolyP was also localized using the polyP binding domain (PPBD) of Escherichia coli exopolyphosphatase (28) in small vesicles randomly distributed in the cytosol of RBL-2H3 cells (Fig. 1D). There was no polyP signal in the negative controls (i.e. in the absence of PPDB). Note that the different staining pattern with DAPI (Figs. 1A and 1B) and PPDB (Fig. 1C) could be due to the more specific staining of polyP longer than 30 P, using PPDB (28) and the use of permeabilization that could eliminate some soluble staining. In addition, we cannot rule out the possibility that DAPI is also staining other polyP pools present in these cells, as polyP has been reported to be present in other compartments in mammalian cells (2).

PolyP was also extracted from RBL-2H3 cells and electrophoresed by 6% urea-PAGE to determine its size distribution (Fig. 2A). With specific staining for polyP, an intense band appeared with an electrophoretic mobility similar to that of polyP of an estimated length of 60 phosphate units (Fig. 2A, arrow). Pretreatment of the samples with active recombinant yeast exopolyphosphatase (ScPPX), or heparinase III did not result in decreased staining of these bands but their use in combination almost completely abolished staining, as seen in the densitometry analysis of the labeled gel (Fig. 2B). These results suggest that the presence of some heparin in the cell extracts prevents the enzymatic activity of PPX.

Co-localization of PolyP with Serotonin-containing Granules-It has been reported that mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms (22). Antizyme inhibitor 2 (AZIN2), an activator of polyamine biosynthesis localizes to Vamp-8 positive, serotonin-containing subsets of mast cells granules (23), while histamine is present is distinct granules (22). PolyP, as detected using the PPBD of E. coli exopolyphosphatase, co-localized in small vesicles with antibodies against serotonin (Fig. 3A) but not with antibodies against histamine (Fig. 3B). There was only partial co-localization of histamine and serotonin antibodies (Fig. 3C).

Changes in PolyP Levels After Mast Cell Activation-To investigate whether mast cell activation resulted in mobilization of polyP, we sensitized RBL-2H3 cells with anti-DNP IgE and then treated them with dinitrophenyl (DNP)-BSA antigen (33). Mast cell activation resulted in loss of DAPI (Fig. 4A), PPBD-Xpress antibody (Fig. S1A and S1B), and serotonin antibody staining (Fig. S1A and S1C) but not in histamine antibody staining (Fig. S1B and S1C). These effects were accompanied by β-hexosaminidase release (Fig. 4C) and decreased content in total mast cell polyP (Fig. 4B).

Electron Density, Calcium Content and Acidity of Mast Cell Granules-Hundreds of granules with high electron density are seen in the cytoplasm of RBL-2H3 cells (data not shown). Fig. 5A shows some of the intracellular granules as observed by transmission electron microscopy without fixation and staining. The granules have a diameter of about 1 µm and five to twelve intragranular rounded areas of high electron density.

The intracellular calcium concentration ([Ca^{2+}]_i) of RBL-2H3 cells was about 100 nM in the absence of extracellular Ca^{2+} (1 mM EGTA was added), a concentration in the range of previous studies (37,38) (Fig. 5B). Mammalian lysosomes are an important acidic calcium store (13) and to test whether calcium was present in lysosomes or in the secretory granules we used glycyl-L-phenylalanine-naphthylamide (GPN), which is specifically hydrolyzed in the lysosome of a variety of cell types by a cathepsin C protease. This results in an increase in osmolarity within the lysosome leading to its swelling and release of stored calcium into the cytosol (39,40). Addition of GPN to RBL-2H3 cells in the nominal absence of extracellular calcium resulted in a slow calcium release to the cytosol. In contrast addition of nigericin (a K^+/H^+ ionophore) released a higher amount of calcium, which was not further increase by addition of the alkalinizing agent NH_4Cl and was independent from the calcium release induced by thapsigargin, a specific inhibitor of the sarcoplasmic-endoplasmic reticulum (SERCA) Ca^{2+}-ATPase (41) (Figs. 5B and 5C). Taking together, these results suggest that RBL-2H3...
cells possess two independent acidic calcium stores, the lysosome, which is sensitive to GPN, and the secretory granules, which respond to K⁺ ionophores and alkalinizing agents. This is in agreement with the detection by X-ray microanalysis of high calcium levels in mast cells granules of diverse origin (14,15).

Acridine Orange has been used to label acidic secretory granules of RBL-2H3 cells (16). When cells were permeabilized with digitonin and incubated with Acridine Orange and mitochondrial inhibitors, mast cells granules were labeled and addition of either nigericin or NH₄Cl resulted in alkalinization and release of the dye (Figs. 5D and 5E), in agreement with their acidic content.

Isolation and characterization of mast cell granules-To isolate intact mast cell granules and investigate their chemical composition, we used the method of Lindstedt and Kovanen (34). Short-chain and long-chain polyP was enriched 4.7- and 30-fold, respectively, in the granule fraction as compared to intact cells (Fig. 6A). Fig. 2C shows that polyP extracted from isolated mast cell granules and electrophoresed by 6% urea-PAGE had a lower estimated length than that extracted for intact cells (Fig. 2A), suggesting its hydrolysis during the isolation procedure. As occurred with polyP extracted from intact cells (Fig. 2B), combination of recombinant yeast exopolyphosphatase (ScPPX) and heparinase III treatment was necessary to produce almost complete hydrolysis (Fig. 2D).

Isolated mast cell granules also conserved their acidity as suggested by their labeling with Acridine Orange after depositing them in polylysine–coated slides (Fig. 6B).

It has been shown that Ca²⁺ release from acidicalciosomes occurs in parallel with polyP hydrolysis (31). We therefore investigated whether the same agents that induced Ca²⁺ release from mast cell acidic compartments (Fig. 5B) were also able to initiate polyP hydrolysis in isolated granules. Addition of nigericin or NH₄Cl, to isolated granules resulted in a time-dependent decrease in the levels of short-chain and long-chain polyP confirming the link between alkalinization, Ca²⁺ release, and polyP hydrolysis (Fig. 6C).

Presence of PolyP in Basophils-Basophils are a small population of peripheral blood leukocytes that have several similarities to mast cells, including the general composition of their secretory granules and the presence of IgE bound to their specific membrane receptors (18). Human peripheral blood mononuclear cells (PBMC) were labeled with an anti-IgE antibody to differentiate basophils (Figs. 7A, upper panel, and 7B). Co-labeling with the PPBD of E. coli exopolyphosphatase, revealed that polyP granules are present in these cells (Fig 7A, center). In addition, we also analyzed whole human blood cells by flow cytometry, in the absence and presence of DAPI, and settings for specific DAPI-polyP detection (Figs. 7C and 7D), confirming the presence of high polyP levels in basophils in comparison with IgE-negative cells (IgE⁻).

Mast Cells PolyP Activate Bradykinin Generation-It has recently been demonstrated that synthetic, and platelet–derived polyP, stimulates bradykinin release in human plasma (5). We then determined whether RBL-2H3 mast cell-derived polyP could also activate bradykinin generation at similar levels than synthetic polyP and the results are shown in Fig. 7E. A dose-response increase in bradikynin generation was detected.

**DISCUSSION**

We report here that the secretory granules of mast cells are acidic, rich in polyP and cations, and have similarities with platelet dense granules (4) and acidocalcisomes (3). The presence of polyP of 60 phosphate units was identified by urea-polyacrylamide gel electrophoresis of mast cell extracts. PolyP co-localizes predominantly with a particular subset of mast cell granules, which also contain serotonin, as demonstrated using the specific polyP-binding domain (PPBD) of E. coli exopolyphosphatase (PPX). PolyP levels decreased after mast cells stimulation by IgE. Isolated mast cell granules were acidic, enriched in polyP as compared to whole lysates, and lost their polyP after their alkalinization. These results were correlated with the loss of acidity and Ca²⁺ release in permeabilized or intact cells, respectively, upon addition of the alkalinizing agents nigericin and NH₄Cl but not of lysosome permeabilizing agents (GPN). The results agree with work reporting high levels of calcium and phosphorus in mast cells secretory granules (14,15), and with previous results of similar behavior of acidocalcisomes in a variety of cells (31). PolyP was also present in human basophils and, when derived from mast cells, was
able to stimulate bradykinin generation in human plasma.

Acidocalcisomes were first recognized in bacteria (42) and named metachromatic (42) or volutin (43) granules. Their name derived from their property to stain red when treated with basic blue dyes, a property shared with mast cell granules, and for their detection in the bacterium *Spirillum volutans*, respectively. These volutin granules were then found in algae, yeast, coccidian and trypanosomes and when it was found that there was a correlation between the amount of polyP in yeast and the number of these granules, they were called polyP granules (44). Although they were thought to lack a membrane, more recent work in bacteria (45), and early branching eukaryotes (reviewed in (3)) determined the presence of a membrane rich in proton pumps, and other transporters and were called acidocalcisomes. The identification of polyP in similar organelles from human platelets (4) led to the discovery of the potent pro-coagulant, anti-fibrinolytic (6) and pro-inflammatory (5) actions of polyP. Our results further support the concept that acidocalcisomes are lysosome-related organelles and indicate that acidocalcisomes and serotonin-containing mast cell granules share several properties: they are acidic due to the operation of proton pumps and able to accumulate acidophilic dyes such as Acridine Orange, they can store large amounts of calcium and other cations such as potassium, sodium, magnesium and zinc, they can release Ca$^{2+}$ in the presence of alkalinizing agents that also promote the hydrolysis of polyP, they contain large amounts of phosphorus in the form of polyP, and they have very high density when observed by direct electron microscopy. Other secretory granules, such as those in pancreatic islets of Langerhans (46), atrial specific granules (47), and secretory chromaffin granules (48) are known to possess large amounts of phosphorus and cations, as shown by X-ray microanalysis, and it would be interesting to investigate whether they are also rich in polyP.

Since DAPI (as well as DNA and RNA) can also bind to heparin (32) we used the PPBD of *E. coli* PPX to confirm the presence of polyP in granules and investigate its co-localization with serotonin-containing granules. This technique was used before to localize polyP in *Saccharomyces cerevisiae* vacuoles (28), and in acidocalcisomes of sea urchin eggs (49) and *Rhodnius prolixus* (50). The negative staining by DAPI used for the urea-polyacrylamide gel electrophoresis analysis allows the discrimination between DNA, RNA and glycosaminoglycans which do not photobleach in the time frame necessary for complete photobleaching of even nanomolar amounts of polyP, and heparin, which photobleach very slowly on exposure to UV light (32).

The size of the polyP detected in mast cells granules using urea-polyacrylamide gel electrophoresis is very close to the size of polyP present in human platelet dense granules (4), and it might contribute to the inflammatory and procoagulant activities of mast cells. The presence of polyP in mast cell granules possessing serotonin might suggest some similarity with human platelet dense granules and further characterization of these granules might help identify common functions of platelets and mast cells.

Acknowledgments—We thank the late Professor Arthur Kornberg for *E. coli* CA38 pTrcPPX1, and Dr. Katsuharu Saito, Shinshu University, Japan, for *E. coli* DH5a pTrc-PPBD.
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FOOTNOTES

*This work was supported by grants from EU-FEDER and the Spanish Ministry of Economy and Competitiveness (Grant FIS/P110/01222) and Junta de Andalucia (Grant P07-CTS-02765 and C.S.0257/09) to F.A. Ruiz and by the Barbara and Sanford Orkin/Georgia Research Alliance Endowment to R. Docampo.

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The abbreviations used are: polyP, polyphosphate; PPX: exopolyphosphatase; PPBD: polyP binding domain; SC polyP, short-chain polyP; LC, long-chain polyP; GPN, glycyl-L-phenylalanine-naphthylamide; ScPPX, yeast exopolyphosphatase.
FIGURE LEGENDS

FIGURE 1. Detection of polyP in mast cells. (A) Epifluorescence microscopy with specific filters for localization of polyP (red) and DNA (blue). (B) Confocal fluorescence microscopy of fixed RBL-2H3 cells labeled with DAPI, showing localization of DNA (blue) and polyP (green). Fluorescent (left) and merged bright field (BF, right) images are shown. (C) Flow cytometry analysis of RBL-2H3 cells in the absence (Control) and presence of DAPI (+DAPI). The geometric Mean Fluorescence Intensities (MFIs) for DAPI-polyP complexes (in the FL3 channel of the flow cytometer) are indicated. (D) Localization of polyP using the recombinant PPBD of E. coli exopolyphosphatase linked with an Xpress epitope tag. Samples were analyzed by confocal fluorescence microscopy as described under “Experimental Procedures”. Fluorescent (left) and its corresponding merge (center), and bright field (BF, right) images, are shown. (A-D) show representative experiments (n = 4). Bars in (A), (B), and (D) = 10 µm.

FIGURE 2. Urea-PAGE analysis of polyP extracted from mast cells and mast cell granules. (A) PolyP extracted from RBL-2H3 cells was electrophoresed by 6% urea-PAGE and stained with DAPI (negative staining). Chain lengths of commercial standards (PolyP25, PolyP45, and PolyP65) are on the left. An arrow on the right shows the position of migration of RBL-2H3 sample (PolyP_RBL-2H3). In addition, extracted mast cell polyP was treated with an excess of ScPPX1 (PPX) and/or heparinase III (Hepase). Left lane is a DNA ladder. Note that DAPI bound to DNA did not photobleach. DNA remained positively stained while polyP was negatively stained. (B) Densitometric analysis of the labeled gel shown in (A), at the zone where mast cell polyP was found (arrow). Analysis was performed using ImageJ 1.43u software. (C) PolyP extracted from RBL-2H3 isolated granules was electrophoresed and treated as described in (A). An arrow on the right shows the position of migration of polyP granule sample (PolyP_granules). (D) Densitometric analysis of the labeled gel shown in A, at the zone where mast cell polyP was found (arrow). Analysis was performed as above. (A-D) show representative experiments (n = 3).

FIGURE 3. Localization of polyP, serotonin and histamine in mast cells. PolyP co-localizes with serotonin (A) but not with histamine (B) in RBL-2H3 cells while serotonin only partially co-localizes with histamine (C), as shown by confocal immunofluorescence analysis. Fixed RBL-2H3 cells were labeled with the recombinant PPBD of E. coli exopolyphosphatase linked with an Xpress epitope tag as shown in Figure 1 (D), and with specific antibodies for serotonin and histamine as described under “Experimental Procedures”. For each combination, independent fluorescent signals and their merged images are shown. Bars: 10 µm.

FIGURE 4. Decrease in intracellular polyP after mast cell activation. RBL-2H3 cells were stimulated to degranulate by sequential exposure to anti-DNP-IgE and DNP-BSA, as described under “Experimental Procedures”. (A) Confocal fluorescence microscopy of fixed cells before (Basal) and after activation (Activated), and labeled with DAPI to show polyP localization. Corresponding bright field images are shown in the right panels. A representative experiment is shown (n = 3). (B) Densitometric analysis of polyP labeling of 50 cells before (Basal) and after (Activ.) mast cell activation. Analysis was performed using ImageJ 1.43u software. Results show the mean ± S.D. for three experiments. (C) Percentage of β-hexosaminidase activity in the supernatant as compared to the total enzyme activity in the cell pellets before (Basal) and after (Activ.) mast cell activation. Results show the mean ± S.D. for 3 experiments.

FIGURE 5. Mast cells have electron dense, acidic and calcium-rich organelles. (A) Electron microscopy of whole intact RBL-2H3 cells allowed to adhere to a Formvar- and carbon-coated grid and observed by transmission electron microscopy. The pictures show a detail of the intracellular granules.
with intragranular regions of high density. Bars: 0.2 µm. (B) Intracellular calcium measurements. RBL-2H3 cells were loaded with fura 2/AM and suspended in a medium with 100 µM EGTA as described under “Experimental Procedures”. At the times indicated, 40 µM glycyl-L-phenylalanine-beta-naphthylamide (GPN), 5 µM nigericin (NIG), 20 mM NH₄Cl, and 1 µM thapsigargin (Thaps) were added. (C) The histograms summarize the changes in [Ca²⁺]ᵢ (n = 3). (D) Changes in Acridine Orange fluorescence. RBL-2H3 cells were incubated with Acridine Orange and their plasma membrane was permeabilized with digitonin, as described under “Experimental Procedures”. At the times indicated, 5 µM nigericin and 20 mM NH₄Cl were added. (E) The histograms summarize the changes in Acridine Orange fluorescence (n = 3).

FIGURE 6. PolyP content, acidity, and depletion of polyP after alkalinization, of isolated mast cell granules. (A) Short- (SC) and long-chain (LC) polyP in whole RBL-2H3 cells (cells) and isolated mast cell granules (granules). Results are the mean ± S.D. (n = 4). (B) Isolated mast cell granules were incubated with Acridine Orange (A.O.) and analyzed by fluorescence microscopy as described under “Experimental Procedures”. Bars = 5 µm. (C) SC and LC polyP levels were measured in isolated mast cell granules at different times after the addition of 10 µM nigericin (NIG) or 20 mM NH₄Cl. Results are the mean ± S.D. of n = 4.

FIGURE 7. Human basophils possess polyP and mast cell polyP induces bradikinin release in plasma. (A) Confocal fluorescence microscopy of human peripheral blood mononuclear cells (PBMC) labeled with specific anti-IgE FITC antibodies (IgE) and the recombinant PPBD of E. coli exopolyphosphatase (PPBD-PolyP) as described under “Experimental Procedures”. Independent fluorescent signals and their merged images are shown. Bars = 10 µm. (B) Flow cytometry analysis of whole fixed human blood cells after labeling with anti-IgE PE antibodies. Dot plot represents a typical pattern of expression of IgE in human blood cells, in which basophilic granulocytes subset (“Bas”) and other blood cells (“IgEneg”) are indicated. The measurement of a representative sample is shown (n = 4). (C) Fixed samples of labeled blood cells, as described in panel A, were incubated in the absence (Control) or presence of DAPI (+DAPI). Cell fluorescence was determined on gated basophils (“Bas”, upper panel), and other blood cells (“IgEneg”, lower panel), in the FL3 channel that is specific for DAPI-polyP complexes (n = 4). (D) Differences in the geometric Mean Fluorescence Intensity (ΔMFI) in the FL3 channel, after addition of DAPI in basophils (“Bas”), and other blood cells (“IgEneg”). Results show the mean ± S.D. of the measurements made from four individuals. Asterisk indicates significant differences, as determined by the Student’s t-test (P < 0.05). (E) Bradykinin release was quantified by ELISA in human plasma after incubation with synthetic polyP (polyP₆₅, 10 µg/ml) or with polyP extracted from RBL-2H3 cells (PolyP₉BL-2H3). Results show the mean ± S.D. of measurements made in samples from three individuals.
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J. Biol. Chem. published online July 3, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.385823

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