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Pyrophosphate-induced acidification of trans cisternal elements of rat liver Golgi apparatus

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Trans cisternal elements of the Golgi apparatus from rat liver, identified by thiamin pyrophosphatase cytochemistry, were isolated by preparative free-flow electrophoresis and were found to undergo acidification as measured by a spectral shift in the absorbance of acridine orange. Acidification was supported not only by adenosine triphosphate (ATP) but nearly to the same degree by inorganic pyrophosphate (PP_i). The proton gradients generated by either ATP or PP_i were collapsed by addition of a neutral H^+/K^+ exchanger, nigericin, or the protonophore, carbonyl cyanide *m*-chlorophenylhydrazone, both at 1.5 μM. Both ATP hydrolysis and ATP-driven proton translocation as well as pyrophosphate hydrolysis and pyrophosphate-driven acidification were stimulated by chloride ions. However, ATP-dependent activities were optimum at pH 6.6, whereas pyrophosphate-dependent activities were optimum at pH 7.6. The Mg^{2+} optima also were different, being 0.5 mM with ATP and 5 mM with pyrophosphate. With both ATPase and especially pyrophosphatase activity, both by cytochemistry and analysis of free-flow electrophoresis fractions, hydrolysis was more evenly distributed across the Golgi apparatus stack than was either ATP-or PP_i-induced inward transport of protons. Proton transport colocalized more closely with thiamin pyrophosphatase activity than did either pyrophosphatase or ATPase activity. ATP- and pyrophosphatate-dependent acidification were maximal in different electrophoretic fractions consistent with the operation of two distinct proton translocation activities, one driven by ATP and one driven by pyrophosphate.

Introduction

Proton translocating enzymes driven by ATP hydrolysis (H^+-ATPases) have been associated with several components of the endomembrane system (e.g. endosomes, coated vesicles, trans Golgi apparatus elements and lysosomes) which develop acidic interior compartments [1]. The proton translocating ATPase demonstrated with isolated Golgi apparatus was electrogenic and required chloride or some other permeant anion for maximal activity [2–4]. Two lines of evidence indicated that the H^+-ATPase was located in the trans cisternae. The in situ effects of the ionophore, monensin, proposed to be driven by a pH gradient [5], were located at the trans-face. DAMP, a basic congener of dinitrophenol which concentrates in acidic compartments, accumulated only in the trans cisternae and secretory vesicles [6].

In this report, we demonstrate proton translocation by isolated Golgi apparatus driven as well by hydrolysis of inorganic pyrophosphate. Pyrophosphate-driven acidification is known from bacterial chromatophores [7,8] and tonoplast vesicles from plant cells [9–11].

The H^+ pyrophosphatase activities were present in trans cisternal fractions of Golgi apparatus separated by free-flow electrophoresis. These activities may represent an alternative energy source for membrane transport through the Golgi apparatus.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DAMP, 3-(2,4-dinitroanilino)-3'-amino-*N*-methyl-dipropylamine; DTT, dithiothreitol; INT, 2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium; PP_i, inorganic pyrophosphate; TPP, thiamin pyrophosphate; TPPase, thiamin pyrophosphatase.

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Materials and methods

Golgi apparatus isolation and free-flow electrophoresis

Golgi apparatus were prepared from the livers of male Holtzman rats by the rapid procedure of Morré et. al. [12]. Unstacking and subsequent free-flow electrophoresis were as described [13,14] using a VAP-5 continuous free-flow electrophoresis unit (Bender and Hobein, Munich, FRG).

Cytochemistry

Cytochemistry was according to Novikoff and Goldfischer [15]. Samples were prefixed in 0.2% glutaraldehyde prepared in 0.1 M sodium cacodylate (pH 7.2) for 2 h at 4°C and then incubated for 90 min at 37°C in a medium containing 3.3 mM thiamin pyrophosphate-Cl (cocarboxylase), 4 mM lead nitrate, 15 mM $MgCl_2$ in 80 mM Tris-maleate/5% sucrose (pH 8.0). The filtered medium was changed every 30 min. Postfixation was with 1% osmium tetroxide for 2 h and samples were embedded in Epon [16]. Thin sections were viewed and photographed with a Phillips electron microscope.

Marker enzyme determinations

Thiamin pyrophosphatase. The assays contained 50 mM Tris-HCl (pH 8.0), 15 mM $CaCl_2$, 3 mM thiamin pyrophosphate and 50 to 100 μg protein, in a total volume of 1 ml [17]. Incubations were at 37°C for 30 min. and the reaction was terminated with cold 10% trichloroacetic acid. Inorganic phosphate was determined by the method of Harris [18].

NADP phosphatase. The standard assay conditions were 50 mM sodium acetate (pH 5.0), 4 mM NADP and 250 μg protein in a final volume of 1 ml [19]. Incubations were at 37°C for 60 min. The reaction was terminated and inorganic phosphate determined as for thiamin pyrophosphatase.

NADH-cytochrome-c reductase (EC 1.6.99.3). The procedure of Crane and Löw [20] was used. Assays were in the presence of 50 mM sodium phosphate (pH 7.0), 0.5 mM KCN and 30 to 50 μg of protein in a final volume of 2.8 ml. The NADH concentration was 2 mM and and the cytochrome *c* was 0.033%. Absorbance was followed at 550 nm with reference at 541 nm.

Succinate–INT reductase (EC 1.3.99.1) and cytochrome-c oxidase (EC 1.9.3.1). Activities were determined as described by Pennington [21] and Sun and Crane [22], respectively. All specific activities were corrected for zero-time determinations and for an incubated blank containing all components except for sample. Proteins were assayed according to Lowry et al. [23] with bovine serum albumin as the standard.

Phosphatase assays

The standard assay conditions were 2.5 ml of 20 mM Tris-Mes buffer containing 0.2 M sucrose and 100 μg protein incubated 30 min at 37°C. Sodium azide (2.5 mM) and ammonium molybdate (0.25 mM) were included also as inhibitors of non-specific phosphatase activity. $MgCl_2$, pH and substrate concentration were varied over a range of concentrations to determine optimum conditions for both ATPase and pyrophosphatase activities. Linearity with respect to time and protein concentration was verified. Phosphate liberated was determined as described [18].

Proton transport assays

Proton transport in the Golgi apparatus was measured in 2.5 mM Tris-Mes (pH 7.0) containing 10 mM potassium thiocyanate or 50 mM chloride (KCl or NaCl), 3 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 6 μM acridine orange and 75 to 100 μg protein in a total volume of 1.5 ml [3]. Proton transport in cisternae of the subfractionated Golgi apparatus was measured directly with fractions isolated by free-flow electrophoresis, which contained 10 mM triethanolamine-acetic acid (pH 6.5), 0.25 M sucrose and 0.5 mM $MgCl_2$. KCl, $MgCl_2$, DTT and acridine orange were added to match the transport conditions for whole Golgi apparatus.

Transport was initiated by the addition of sodium ATP or sodium pyrophosphate from stock solutions of 30 mM for a final concentration of 3 mM. The difference between the absorbance at 492 nm and that at 540 nm was measured using a dual wavelength SLM DW2000 spectrophotometer.

Results

Phosphatase activity in the Golgi apparatus

The pyrophosphatase and Mg^{2+}-ATPase activity of the Golgi apparatus of rat liver were assayed under conditions where activity was proportional to time of incubation (5 to 30 min) and protein concentration (10 to 100 μg). ATPase activity was stimulated maximally by 0.5 mM Mg^{2+} with an optimum pH of 6.6 (Fig. 1 and Table I). The optima for pyrophosphatase activity were less defined, however. Activity was maximal with 5 mM Mg^{2+} at pH 7.6. Neither succinate–INT reductase nor cytochrome-*c* oxidase (two mitochondrial marker enzymes) were present in detectable quantities in the isolated Golgi apparatus preparations.

Phosphatase activity of the Golgi apparatus was stimulated by chloride ions and protonophore (Table II). Chloride ions stimulated Mg^{2+}-ATPase (20 to 45%) depending on the counter ion (NH_4 > Na > K). Mg^{2+}-pyrophosphatase was stimulated by choride ions (20%) except for NaCl which inhibited the activity (12%). The protonophore, CCCP, stimulated both Mg^{2+}-ATPase activity (16%) and Mg^{2+}-pyrophosphatase activity (95%).

ATP- and pyrophosphate-dependent phosphatase activities of freshly prepared Golgi apparatus were not

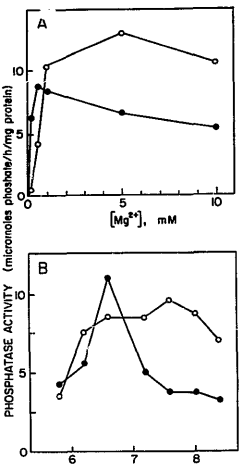

Fig. 1. Dependency of hydrolysis of ATP (solid symbols) and of pyrophosphate (open symbols) of liver Golgi apparatus as a function of Mg^{2+} (A), as $MgCl_2$, and pH (B). Optimum $MgCl_2$ concentration was determined at pH 6.6 for ATPase and 7.6 for pyrophosphatase activities. Likewise, optimum pH was determined at 0.5 mM $MgCl_2$ for ATPase and 5 mM $MgCl_2$ for pyrophosphatase activities.

stimulated by membrane permeabilizing concentrations of detergent (Triton X-100) or by repeated freeze-thaw treatment (Table III). However, thiamin pyrophosphatase activity was stimulated 5-fold by detergent and 50% by freezing and thawing.

Acidification of isolated Golgi apparatus

Acidification of isolated Golgi apparatus, indicated by the decreasing absorbance of acridine orange, was driven by both Mg^{2+}ATP and Mg^{2+}-pyrophosphate (Fig. 2). ATP-driven proton transport into the Golgi apparatus lumen was observed under conditions optimized for ATPase activity. However, the proton transport activity was low, especially with cisternae separated by free-flow electrophoresis. Proton transport activity was enhanced 2–4-fold by a higher substrate concentration and therefore 3 mM Mg^{2+}ATP was used rather than 0.5 mM. Upon addition of ATP, isolated Golgi apparatus acidified rapidly during the initial 1 to 2 min and then more slowly for up to 20 min (Fig. 2A). Pyrophosphate-driven acidification was not as rapid as ATP-driven acidification (Fig. 2B) but usually continued for a longer period of time before reaching equilibrium (data not shown).

TABLE I

Optimum conditions for phosphatase activity in Golgi apparatus

All samples contained 20 mM Tris-Mes buffer, 0.2 M sucrose, 100 μg protein and 1 mM substrate. Values are for Mg^{2+}-dependent activity in the presence of non-specific phosphatase inhibitors ($(NH_4)_6Mo_7O_{24}$ (0.25 mM) and NaN_3 (2.5 mM).

Condition	ATPase	Pyrophosphatase
Mg^{2+}	0.5 mM	5 mM
pH	6.6	7.6
Cl^-	50 mM (NaCl)	50 mM (KCl)

TABLE II

Stimulation of phosphatase activity by chloride and protonophore

Phosphatase activity was determined as described in Table I except using 0.5 mM $MgSO_4$ and with chloride salts as indicated. Carboxyl cyanide *m*-chlorophenylhydrazone, CCCP, a H^+ specific ionophore was added from a concentrated stock in ethanol. Values are based on results representative of three separate experiments and represent averages of Mg^{2+}-dependent activity ± standard deviations. Values in parentheses are percentages relative to determinations without additions.

Addition	Concn.	μmol/h per mg protein (% control)			
		ATPase		pyrophosphatase	
None		1.65	(100)	0.94	(100)
NaCl	50 mM	2.32 ± 0.30	(141)	0.83 ± 0.10	(88)
KCl	50 mM	1.96 ± 0.18	(119)	1.13 ± 0.16	(120)
NH_4Cl	10 mM	2.38 ± 0.33	(144)	1.10 ± 0.01	(117)
CCCP	1.5 μM	1.91 ± 0.10	(116)	1.83 ± 0.46	(195)
Ethanol	0.5%	1.68 ± 0.03	(102)	1.39 ± 0.07	(148)

TABLE III

Effect of detergent and freezing on phosphatase activity of Golgi apparatus

Phosphatase activity was determined in samples containing 100 μg Golgi apparatus, Tris-Mes (pH 7.5), 0.2 M sucrose, 50 mM KCl, 1 mM DTT, 0.5 mM $MgCl_2$ and 1 mM substrate, with or without detergent, after a 20 min incubation at 37°C. For freeze/thaw, samples were frozen (−20°C) and warmed to 37°C twice before addition of substrate. Values based on results of two experiments with different preparations and represent Mg^{2+}-dependent activity using controls minus $MgCl_2$. Values in parenthesis are percentages relative to determinations without additions.

Treatment	μmol/h per mg protein (% control)		
	ATPase	pyrophosphatase	TPPase
None	6.3 (100)	5.2 (100)	0.36 (100)
Triton X-100 0.05%	6.3 (100)	5.4 (104)	1.90 (528)
Triton X-100 0.10%	6.1 (97)	5.1 (98)	2.00 (555)
Freeze/Thaw	7.8 (123)	5.4 (104)	0.87 (150)

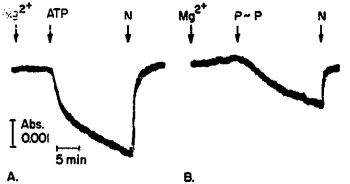

Fig. 2. Spectrophotometric tracings of proton pumping induced by ATP (A) and inorganic pyrophosphate (B). After 10 min 1.5 μM nigericin (N) was added to collapse the proton gradients.

Phosphatase activity of free-flow electrophoretic subfractions

Unstacked Golgi apparatus separated into 12 to 16 subfractions by free-flow electrophoresis. Cytochemistry with thiamin pyrophosphate marked the trans cisternae and possibly portions of the trans Golgi apparatus network in whole tissue (Fig. 3) and the most electrophoretically mobile subfractions of Golgi apparatus separated by free-flow electrophoresis (Fig. 4A–C). Golgi apparatus subfractions were further characterized with thiamin pyrophosphatase, NADP phosphatase and NADH-cytochrome-c reductase, marker enzymes for trans, medial and cis cisternae, respectively (Fig. 5). Mg^{2+}-ATPase specific activity was maximal in the most mobile fractions corresponding to the highest specific activity of thiamin pyrophosphatase (Fig. 6). Pyrophosphatase specific activity also was maximal in the trans region of the electrophoretic separation of Golgi apparatus (Fig. 7), but was absent from the most mobile fractions (which may represent the trans Golgi apparatus network), with the highest specific activities of ATP-dependent activities. Moreover, as seen also with cytochemistry (Fig. 3B), a significant amount of pyrophosphatase activity was present throughout the Golgi apparatus and on free-flow electrophoretic separation migrated with fractions corresponding to the cis and medial regions as well.

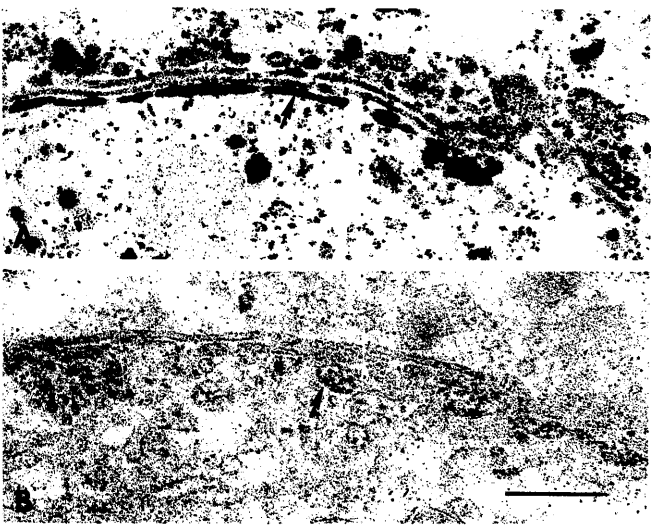

Fig. 3. Thiamin pyrophosphatase cytochemistry in Golgi apparatus of rat liver parenchyma. Reaction products with thiamin pyrophosphate (A) and inorganic pyrophosphate (B) as substrate were localized most heavily in the trans-most cisternae (arrows). Bar = 0.5 μm.

Acidification of Golgi apparatus subfractions

ATP-driven proton transport was highest in the trans region of the electrophoretic separation, significantly less in the medial fractions and was absent from cis fractions (Fig. 6). Pyrophosphate-driven proton transport was found exclusively in electrophoretic fractions 33 to 35 of Fig. 7, a subset of the trans Golgi apparatus membrane from which elements of the trans Golgi apparatus reticulum may be excluded. This represented a difference between the activity profiles for Mg^{2+}ATP-driven acidification and pyrophosphate-driven acidification in that the two pooled most electrophoretically mobile fractions, with the highest Mg^{2+}ATP-driven specific activities (Fig. 6), had little

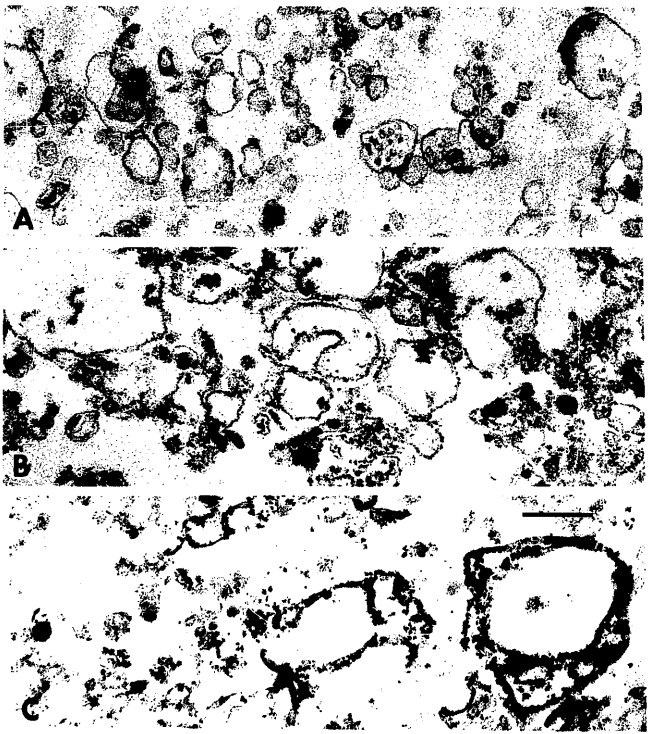

Fig. 4. Thiamin pyrophosphatase cytochemistry in fractions of rat liver Golgi apparatus separated by free-flow electrophoresis. Fraction 41 (A) representative of cisternae of the cis face, the least electrophoretically mobile portion of the separation, showed no evidence of staining. Cisternal fraction 36 (B) from the center of the separation showed some cytochemical reactivity. Cisternae from the most electrophoretically mobile fraction 31 (C) showed strong reaction product indicative of thiamin pyrophosphatase activity. Bar = 0.5 μm.

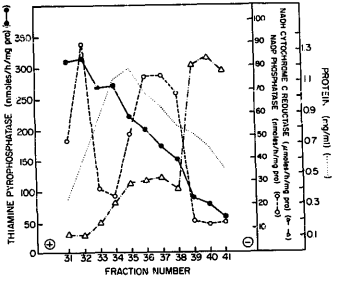

Fig. 5. Electrophoretic separation of unstacked rat liver Golgi apparatus comparing specific activities of thiamin pyrophosphatase with those of NADP phosphatase and NADH–cytochrome-c reductase as markers of specific Golgi apparatus domains.

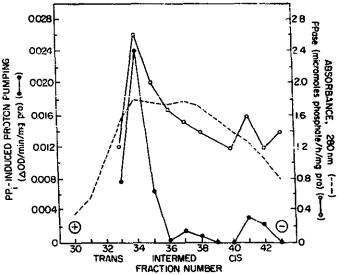

Fig. 7. Specific activities of pyrophosphatase and pyrophosphate-dependent proton pumping in rat liver Golgi apparatus subfractionated by free-flow electrophoresis. These activities are distributed differently from those of ATPase and ATP-dependent proton pumping of Fig. 6. The pyrophosphatase activity is widely distributed among Golgi apparatus subfractions in parallel with the cytochemical observations (Fig. 3B). The PP_i-induced proton pumping is concentrated in the Golgi apparatus subfractions of greatest electrophoretic mobility corresponding to trans-derived elements.

or no capacity for pyrophosphate-driven acidification (Fig. 7). Pyrophosphatase activity also was low or absent in these fractions but thiamin pyrophosphatase remained high (Fig. 5).

Discussion

A correlation between the localization of thiamin pyrophosphatase and ATPase activities in free-flow electrophoresis fractions was observed in that both activities had cis to trans profiles of increasing activity. As well, pyrophosphatase activity was the highest in the trans cisternae of subfractionated Golgi apparatus.

The Mg^{2+}-dependent activities of ATPase and pyrophosphatase differed greatly in optimal conditions.

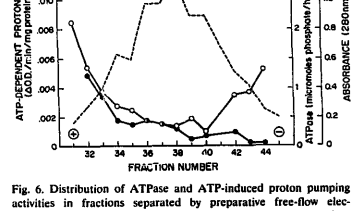

Fig. 6. Distribution of ATPase and ATP-induced proton pumping activities in fractions separated by preparative free-flow electrophoresis. ATPase activity showed two peaks but ATP-dependent proton pumping paralleled the hydrolysis of thiamin pyrophosphate of Fig. 5.

ATPase functioned best at pH 6.6 with 1 mM ATP and 0.5 mM $MgCl_2$ and with sodium as the counter ion for chloride. Pyrophosphatase activity was optimal at pH 7.6, with 1 mM inorganic pyrophosphate and 5 mM $MgCl_2$. Potassium was the preferred counter ion for pyrophosphatase. The differences in conditions may not represent sufficient evidence to conclude that ATPase and pyrophosphatase are distinct enzymes but both by cytochemistry and by free-flow electrophoresis, ATP- and PP_i-hydrolyzing activities distributed independently one from another.

The lack of stimulation by detergent of either ATPase or pyrophosphatase, in contrast to the stimulation of TPPase, would suggest that the substrate binding site for ATPase and pyrophosphatase are cytoplasmic as would be expected for an inward pumping mechanism of energy driven acidification. In contrast the substrate binding site for thiamin pyrophsophate is luminal (Table III). The stimulation by chloride and ionophore indicates that the hydrolysis activity for cisternal fractions from the trans Golgi apparatus is coupled electrogenically to ion transport for both ATP and PP_i [3]. The reasons for the differences in optimal substrate concentrations for phosphatase and proton transport activities are not clear. However, the higher substrate concentrations at which proton transport is more easily measured, represent those used widely by others [2–4].

ATP-driven proton pumping by Golgi apparatus is now well established [2–4]. A spectrophotometric assay

using acridine orange has been employed [3,24] to demonstrate the development of an acidic pH within isolated Golgi apparatus elements. Using this assay we find that, in addition to ATP, inorganic pyrophosphate supports proton pumping. This possibility was indicated from studies with bacterial chromatophores [7,8] and was supported later with results from plant membranes [9–11] where hydrolysis of inorganic pyrophosphate was shown to generate the energy required for proton transport in these systems.

A trans localization of ATP- and pyrophosphate-driven proton pumping activities was established by direct measurements in subfractions of Golgi apparatus prepared by free-flow electrophoresis. Comparison with the trans distribution of thiamin pyrophosphatase activity, determined both cytochemically and biochemically, placed the peak of proton transport activity in the trans cisternae with only slight overlap with the medial cisternae. The difference between the localization of maximal ATP-driven activities and maximal pyrophosphate-driven activities may reflect two separate sites of proton translocation activity, with the pyrophosphate-driven activities being reduced in the most electronegative fractions of greatest electrophoretic mobility.

Monensin, nigericin and CCCP dissipated the ATP- and pyrophosphate-induced proton gradients formed within the isolated and subfractionated Golgi apparatus. These ionophores have been shown previously to arrest or block the intracellular transport of proteins by disruption of Golgi apparatus secretory functions [25–32]. The proposed requirement for a proton gradient to sustain monensin-induced swelling of trans Golgi apparatus cisternae [5] would be consistent with the cis to trans distribution of phosphatase activity and trans localization of ATP- and pyrophosphate-driven proton pumping.

Identification of energy sources for secretion and transport by Golgi apparatus membranes has not received much clarification beyond initial studies that demonstrated that these processes were susceptible to blockage by respiratory inhibitors and compounds that led to the depletion of cellular ATP stores [33,34]. A role for ATP in vesicle acidification of Golgi apparatus [2–4] has provided one specific example to help explain these early observations. In the present study, evidence is provided for the utilization by Golgi apparatus of yet another abundant energy source, pyrophosphate, to support acidification.

Acknowledgments

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