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Membrane-Associated ATPases in Isolated Secretory Vesicles[1]

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ABSTRACT

Polysaccharide-containing vesicles were collected from secretory cells maintained in liquid culture. Characterization of membrane-associated nucleosidephosphatases revealed that the vesicles specifically hydrolyze ATP, have a pH optimum between 6.0 and 6.5, and are stimulated by inorganic cations, especially K^+. The ATPase activity in these vesicles was inhibited by orthovanadate and *N,N'*-dicyclohexylcarbodiimide; other inhibitors, such as oligomycin, sodium azide, and diethylstilbestrol were generally ineffective. Results from these studies are consistent with the notion that vesicles derived from the Golgi apparatus have partially differentiated into plasmalemma before they fuse with the plasma membrane.

Cellular secretion, which provides for the export of metabolic products, represents one aspect of a dynamic process known as membrane flow (12, 13). According to this hypothesis, membrane assembly and differentiation take place during movement of membrane material from ER, through the Golgi apparatus, to the plasma membrane. Although there are clear physical (8, 10, 14) and biochemical (4, 10, 14) differences in membrane composition, there is no satisfactory explanation for the mechanism by which these changes occur during exocytosis.

Inasmuch as ER, Golgi, and plasma membrane can be considered as nonmigrating elements in the endo-membrane system, it follows that examination of nonstationary, Golgi-derived vesicles may provide clues concerning the transitions between cytoplasmic membranes. The population of secretory vesicles represents a small percentage of the total membrane in a cell, thus efforts to isolate this fraction have been attempted on cell types that have prolific rates of secretion. VanDerWoude *et al.* (18) showed that polysaccharide-containing secretory vesicles could be isolated from pollen tubes of lily sp. Similar results have been obtained with pollen tubes of other species (6). However, in both these preparations the amount of vesicles obtained was too small to contemplate biochemical analysis of vesicle membranes.

We recently described the culture of hypersecretory cells from which large quantities of intact secretory vesicles can be isolated (5). Morphometric analysis of electron micrographs of vesicle pellets indicated that the preparation consisted of at least 80% intact, polysaccharide-containing vesicles. We have now extended these earlier observations to include biochemical characterization of vesicle membranes. This paper presents evidence that an important plasma membrane enzyme is present and active in isolated secretory vesicles.

MATERIALS AND METHODS

Cultured Cells. Liquid suspension cultures of *Avena sativa* L. cv Garry were initiated and maintained using standard sterile culture techniques that have been described previously (5). The cells in these cultures grow slowly in small clumps and initiate massive polysaccharide release, rather abruptly, after 4 to 6 weeks in liquid culture. The secreting cells are undifferentiated and densely cytoplasmic, with large populations of Golgi, mitochondria, and cytoplasmic vesicles.

Preparation of Secretory Vesicles. Suspension cells were collected by filtration through Miracloth, weighed, and resuspended at a ratio of 5 ml:1 g of cells in a homogenization medium containing: 25 mM Tris-Hepes at pH 7.2, 0.5 M sucrose, 3 mM EDTA, 1 mM DTT and 1% PVP (insoluble form). Resuspended cells were broken in a cold French press at 5,000 p.s.i. Apparently this method tends to spew cytoplasm through ruptures in the cell wall, since light microscopic views of pressure-broken cells showed masses of cytoplasm protruding from empty cell wall enclosures. Evidence at the light or electron microscopic level indicated that this procedure minimized breakage of organelles.

The following steps in the isolation procedure were carried out at 0 to 5°C. Material collected from the press was centrifuged at 500*g* for 5 min to remove cell walls and intact cells. The supernatant was centrifuged at 10,000*g* for 20 min to remove larger organelles, especially mitochondria. The 10,000*g* supernatant was filtered first through a 1.2-μm filter (Millipore RAWP 025, Bedford, MA) and then through a 0.45-μm filter (Millipore HAWP 025), using a 10-ml glass syringe and appropriate filter holders. The 0.45-μm filtrate was centrifuged at 55,000*g* for 30 min. A pellet was collected and washed once in a solution of 25 mM Tris-Hepes (pH 7.2), 0.5 M sucrose, and 1.0 mM DTT, and repelleted at 55,000*g* for 30 min.

Nucleoside Phosphate Assays. ATPase activity was assayed by the methods of Hodges and Leonard (9), using membrane material equivalent to 5 to 20 μg protein. Protein content was ascertained by the method of Bradford (3), using BSA as a standard.

The reaction components for the Mg^{2+}-ATPase assay consisted of 30 mM Tris-Hepes (pH 6.3 or otherwise indicated), 0.5 M sucrose, 3 mM $MgSO_4$, and 3 mM ATP (Tris form); 50 mM KCl was added to this mixture for the KCl/Mg^{2+}-ATPase assay. Total reaction volume was always 1 ml. Reactions were initiated by the addition of membranes and allowed to proceed at 37°C for 30 min. Pi produced during ATP hydrolysis was determined by the Fiske and SubbaRow method (7). KCl-stimulated ATPase (KCl-ATPase activity) was obtained by subtracting the Mg^{2+}-ATPase from the KCl/Mg^{2+}-ATPase activity. In a similar manner, other phosphate substrates, including CTP, GTP, ITP, UTP, ADP, and AMP, were administered to the enzyme preparation. All phosphate substrates were obtained from Sigma.

Inhibitor Studies. The inhibitors sodium orthovanadate (Fisher Scientific), DES[2] (Sigma), DCCD (Aldrich Chemical Co.), oligomycin (Sigma), and NaN_3 (Eastman Chemical Co.) were added as 10-μl additions to the reaction mixture. Inhibitors were dissolved in 50% ethanol prior to final dilution in the reaction

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[2] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol.

mixture. Control treatments for these studies consisted of similar additions of 50% ethanol.

RESULTS

Studies of ATPase Activity. The vesicle fraction demonstrated Mg^{2+}-ATPase activity that was dramatically stimulated by 50 mM KCl. The pH optimum of KCl/Mg^{2+}-ATPase activity had a broad peak from pH 6.0 to 6.5 (Fig. 1). There was also a small peak of KCl stimulation at pH 8.5, which is the pH optimum for mitochondrial ATPases (16). The vesicle ATPase specifically required nucleoside phosphates as substrates; there was no Pi release without such substrates.

The enzyme preferentially hydrolyzed ATP over other nucleoside triphosphates, ADP and AMP (Table I). In particular, the KCl-stimulated component of the enzyme activity was noticeably substrate selective. With the exception of CTP, all other substrates evidenced less than 17% stimulation with KCl and ATP.

Table II shows that, compared to other monovalent cation-chloride salts, KCl most stimulated the vesicle ATPase. Not surprisingly, RbCl produced 70% of the activity obtainable with KCl. However, other cations, such as NaCl, LiCl, and NH_4Cl stimulated the ATPase by less than 30%. One salt, CsCl, actually inhibited Mg^{2+}-ATPase by an average of 50% (data not shown). Anions other than chloride were not tested; however, the range of

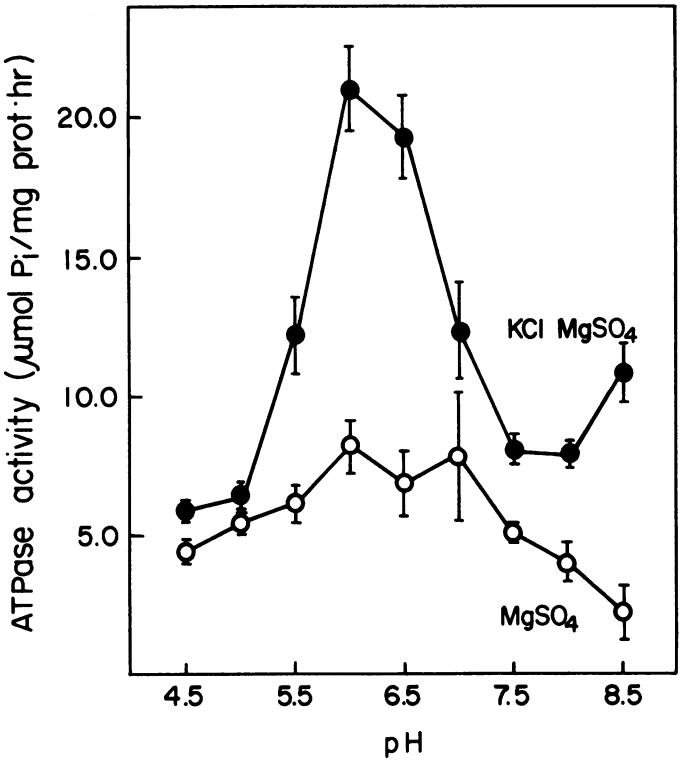

FIG. 1. ATPase activity of the vesicle fraction as a function of pH. The assay medium contained 30 mM Tris-Hepes (proportions varied to achieve desired pH), 3 mM Tris-ATP, 0.5 M sucrose, 3 mM $MgSO_4$, and, when added, 50 mM KCl. Errors are SE.

Table I. *Activity of the Secretory Vesicle Fraction with Various Substrates*

The assay medium contained 3 mM substrate (Tris salts), 30 mM Tris-Hepes (pH 6.3), 0.5 M sucrose, 3 mM $MgSO_4$, and 50 mM KCl (when added). Errors are SE.

Substrate	Mg^{2+}-Dependent Activity		KCl-Dependent Activity	
	μmol Pi/mg·h	%	*μmol Pi/mg·h*	%
ATP	19.3 ± 2.8	100	11.0 ± 1.9	100
CTP	2.5 ± 0.7	13	4.4 ± 0.6	40
GTP	6.9 ± 0.7	36	1.8 ± 1.2	16
ITP	8.0 ± 1.3	41	1.5 ± 0.6	14
UTP	6.1 ± 1.4	32	1.2 ± 0.5	11
ADP	8.5 ± 1.4	44	0.7 ± 0.3	6
AMP	3.0 ± 0.3	15	1.1 ± 0.7	10

Table II. *Effect of Monovalent Ions on ATPase Activity of the Vesicle Fraction*

Assay contained 30 mM Tris-Hepes (pH 6.3), 3 mM Tris-ATP, 0.5 M sucrose, 3 mM $MgSO_4$, and 50 mM chloride salts as indicated. Errors are SE.

Additions	Ion Stimulation	
	μmol Pi/mg·h	%
KCl	14.5 ± 2.0	100
RbCl	10.3 ± 2.2	71
NaCl	4.1 ± 2.1	28
LiCl	2.5 ± 1.2	17
NH_4Cl	3.9 ± 1.2	27
CsCl	—[a]	

[a] Nonstimulatory; CsCl inhibited ATPase activity (see "Results").

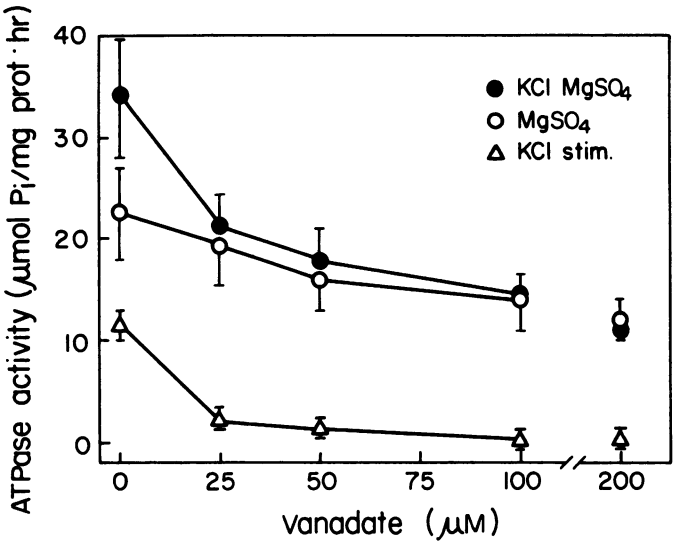

FIG. 2. Vesicle fraction ATPase activity as a function of increasing sodium orthovanadate concentration. Assay medium contained 30 mM Tris-Hepes (pH 6.3), 3 mM Tris-ATP, 0.5 M sucrose, 3 mM $MgSO_4$, and, when added, 50 mM KCl. Ten μl additions of sodium orthovanadate were combined with the assay medium to achieve the concentrations indicated. Errors are SE.

Table III. *Effect of Inhibitors on KCl-ATPase Activity of Vesicle Fraction*

Assay contained 30 mM Tris-Hepes (pH 6.3 or pH 8.5), 3 mM Tris-ATP, 0.5 M sucrose, 3 mM $MgSO_4$, and 50 mM KCl (when added). Final concentrations of sodium orthovanadate, oligomycin, NaN_3, and DCCD were 50 μM, 5 μg/ml, 0.5 mM, and 10 μm, respectively, in 0.5% ethanol. Errors are SE.

Additions	KCl-ATPase Activity			
	pH 6.3		pH 8.5	
	μmol Pi/mg·h	%	*μmol Pi/mg·h*	%
Control	12.4 ± 0.6	100	4.2 ± 0.4	100
Vanadate	3.4 ± 0.3	27	3.4 ± 0.3	81
Oligomycin	9.6 ± 0.7	77	1.5 ± 0.5	36
Azide	8.9 ± 0.9	72	1.6 ± 0.3	38
DCCD	4.0 ± 0.4	32	3.8 ± 0.8	90

stimulations obtained with different cation-chloride salts suggests that cations were most effective in stimulating this ATPase.

Inhibitor Studies. Both orthovanadate and DCCD were potent inhibitors of ATPase activity at pH 6.3. Orthovanadate particularly inhibited the KCl-stimulated component of the enzyme. Figure 2 shows that 25 μM orthovanadate removed approximately 80% of the KCl-ATPase activity. DES at 100 μM had no effect on the vesicle ATPase activity (data not shown).

Table III presents a comparison of four plasma membrane and mitochondrial ATPase inhibitors at pH 6.3 and 8.5. These data indicate that our vesicle pellet contained membranes which were sensitive to mitochondrial ATPase inhibitors (oligomycin and sodium azide) and those which were sensitive to inhibitors of plasma membrane ATPase (orthovanadate) (2, 17). Moreover, the effects of the mitochondrial and plasma membrane inhibitors were more pronounced at the optimal pH for each ATPase. In addition, DCCD, which is thought to block proton passage through membranes (11), had a greater inhibitory effect on secretory vesicles at the more acid pH.

DISCUSSION

The major finding of this study is that cation-sensitive ATPases are functional in membranes of secretory vesicles. If this vesicle ATPase is the one which ultimately resides in the plasma membrane, this study may provide support for the contention that the process of membrane differentiation, at least as it applies to ion transport proteins, occurs before or during the migration of vesicles to the cell periphery. The ATPase which has been characterized in this study has enzymic properties similar to cation-sensitive ATPases that are found in vesicles of reformed plasma membrane (9). The enzyme is specific for ATP, its activity is stimulated by monovalent cations, especially K^+, and it has a pH optimum of 6 to 6.5. Its behavior is different from the widely characterized plasma membrane ATPases in that it is inhibited by Cs^+, it has a relatively large affinity for CTP, and the pH optimum is slightly acidic. We are not certain if these details are indicative of actual performance dissimilarities between ATPases in secretory membranes and plasma membranes, or whether they can be accounted for by the obvious differences in vesicle preparation.

The vesicles were most sensitive to inhibitors which are known to have specific effects on the plasma membrane (orthovanadate) (17) or on proton passage through membranes (DCCD) (2, 11). In contrast DES, which has been shown to depress ATPase activity in a number of membrane systems, including fungal plasmalemma (2), higher plant plasmalemma (1), and higher plant tonoplast (19), had no effect on the secretory vesicle ATPase. The lack of a large inhibition of activity by NaN_3 and oligomycin at pH 6.3 indicates that the major portion of ATPase activity in our membrane fraction is not mitochondrial. Larger inhibitions by these chemicals at pH 8.5 confirm the presence of a certain amount of mitochondrial contamination in these preparations. Previous morphometric analysis showed that mitochondria comprised no more than 20% of all membrane-bound bodies in electronmicrographs (5). It should be possible to improve the purity of these vesicles by density-gradient centrifugation, or by increasing the speed of prefiltration spins. These avenues were not pursued in this study since we wished to keep vesicle yields as high as possible for enzyme analysis.

We are, of course, aware that identification of the membranes of secretory vesicles depends heavily on the inclusion of polysaccharides within the vesicle membrane, as has been demonstrated by electron microscopy and by colorimetric determinations of sugars in the final vesicle pellet (5, 6, 18). Our reliance on these types of information arises mostly from a lack of more definitive methods for identifying membranes from fractionated cells. The so-called marker enzymes for various membrane fractions are not as specific as one might wish; this is particularly true for components of the secretory pathway. There is considerable evidence that purported marker enzymes of the ER, Golgi, and plasma membrane have significant overlap of activities into adjoining fractions (14). Furthermore, the use of marker enzymes seems especially unsuitable for a transitory membrane element such as secretory vesicles, as they might well contain active enzymes of both Golgi and plasma membrane. The application of labeled polysaccharide precursors to secreting cells would probably label the interior of the vesicles; however, the finding that sugar labels are rapidly incorporated and dispersed throughout the cell (15) would make the timing of measurements and interpretation of results treacherous. More promising identification methods might be determinations of sterol:phospholipid ratios and density of vesicle membranes, since both are known to increase between ER and plasma membrane (4, 10).

With future modifications, vesicles isolated from suspension-cultured oat cells should be useful both for examining the process of membrane differentiation in cells and, conceivably, the function of the membrane-associated ATPases themselves. Because incorporation of ion-sensitive ATPases is crucial to the function of the plasma membrane, they are suitable markers with which to track the flow of membrane material through the endomembrane system. Moreover, it is clear that for the ATPases to reside on the inside of the plasmalemma following vesicle fusion, they must be located on the outside of the secretory vesicles. This, coupled with the fact that these vesicles are intact, unlike the broken and resealed plasma membrane preparations (16, 17), may make them superior for certain studies of ion transport physiology.

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