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Topical Review

Proton Pumping in the Secretory Pathway

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Introduction

Protons are pumped into intracellular compartments (e.g., lysosomes, endosomes, coated vesicles, synaptic vesicles and secretory granules) to create a proton motive force across the membrane or to acidify the lumen, which is necessary to perform cellular processes such as hydrolysis of macromolecules, receptor-mediated endocytosis and processing of preproproteins. The proton gradient is established and maintained by the H^+-pumping vacuolar-type ATPase (V-ATPase). Here we discuss this multisubunit enzyme, the importance of proton pumping in the various organelles of the secretory pathway, and the possible types of regulatory mechanisms that may control V-ATPase activity.

Proton Pumping Across Membranes

Cells are compartmentalized by lipid bilayers that separate the inside of the cell from the external environment and in eukaryotic cells also define internal compartments. The bilayer forms the basic structure of a biological membrane and acts as a highly selective filter and a device for active transport; it controls the transport of nutrients and waste products, and generates differences in ion concentrations between the outside and inside of the membrane-enclosed environment. Protons are pumped across a membrane by proton-ATPases that hydrolyze ATP, thereby generating a proton-motive force (pmf). Alternatively, the ATPases may utilize the energy of the pmf for ATP formation [84]. Proton-ATPases have been divided into three main classes, namely P-, F- and V-ATPases [101]. The P-type H^+-ATPase, found in plant and animal plasma membranes, is structurally and mechanistically distinct from the F- and V-ATPases in that it consists of only a single 100-kDa polypeptide that, like all other P-ATPases, operates via a phospho-enzyme intermediate [89]. In contrast, F- and V-ATPases are structurally related multisubunit enzymes that function without any apparent involvement of a phosphate intermediate. F-ATPases have been found in the plasma membrane of bacteria, the inner membrane of mitochondria and the thylakoid membrane of chloroplasts [2, 67], where they primarily synthesize ATP at the expense of the free energy of the pmf. V-ATPases function exclusively as ATP-dependent proton pumps, and are present in membranes of archaebacteria and some eubacteria, in endomembranes of all eukaryotic cells and at the plasma membrane of a subset of cells [86, 132]. In this review, we will focus on V-ATPase activity in the secretory pathway, and summarize the mechanisms that may be responsible for establishing and maintaining the proton gradient across organellar membranes.

The V-ATPases

Since a number of reviews that deal with the general structure, the pumping mechanism and the function of

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Abbreviations: ARF, ADP-ribosylation factor; Baf, Bafilomycin A1; CCV, clathrin-coated vesicle; COP, coatomer protein; ER, endoplasmic reticulum; F_0, membrane sector of the F-ATPase; F_1, peripheral sector of the F-ATPase; GFP, green fluorescent protein; ISGs, immature secretory granules; pmf, proton motive force; TGN, *trans*-Golgi network; V-ATPase, vacuolar H^+-ATPase; V_0, membrane sector of the V-ATPase; V_1, peripheral sector of the V-ATPase; VSV, vesicular stomatitis virus; VTCs, vesicular tubular clusters

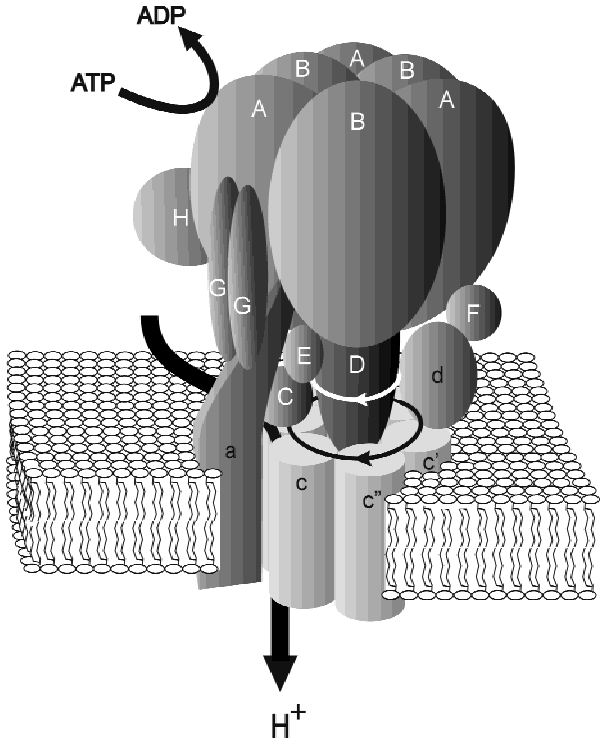

Fig. 1. Arrangement of the various subunits in the V-ATPase. Subunits of the peripheral catalytic V_1 sector and of the integral proton-conducting V_0 sector from bovine brain CCV V-ATPase are indicated by white and black letters, respectively.

the V-ATPases have been published recently [27–29, 86], we will only briefly discuss these issues.

Structure of the V-ATPase

Electron microscopy on isolated enzyme complexes has revealed that the V-ATPase is organized in two main parts (as is the F-ATPase): a cytoplasmic ball-like structure which is attached via a cytoplasmic stalk (together designated V_1; F-ATPase analog is F_1) to a membrane sector (V_0; F_0 in F-ATPase) [27]. The peripheral catalytic V_1 sector (molecular mass of ~840 kDa) is responsible for the hydrolysis of ATP, whereas the 260-kDa integral V_0 sector is responsible for proton translocation across the membrane (Fig. 1). Biochemical characterization of the enzyme isolated from bovine chromaffin granules, kidney microsomes and clathrin-coated vesicles (CCV) in combination with yeast genetics allowed the identification of the V-ATPase subunit composition [87]. Subunits could be assigned to the V_1 or V_0 sector by dissociation of the isolated holoenzyme, using urea [137], KI plus MgATP [5] or cold inactivation in the presence of NaCl and MgATP [79]. The V_1 sector consists of eight different subunits (A–H), with a stoichiometry of $A_3B_3C_1D_1E_1F_xG_2H_z$ (copy numbers of F (x) and H (z) have not been determined yet) (Table) [6, 133]. For the V-ATPases isolated from bovine coated vesicles, the molecular weights of the subunits A to H of the V_1 sector are ~73, 58, 40, 34, 33, 14, 15 and 50 kDa, respectively [29]. Five different subunits form the V_0 sector, namely subunits a (100 kDa) and d (38 kDa), and proteolipids c (17 kDa), c′ (17 kDa) and c″ (19 kDa), with a stoichiometry of a_1d_1 (c, c′ and c″)$_6$ (Table) [6, 29, 103]. The exact subunit composition may vary since isoforms of various subunits have been found in a number of species (*see below*).

Mechanism of V-ATPase Pumping

Based on the structural similarity with the F-ATPase [84] and the knowledge of F-ATPase mechanics as a rotary motor [1, 92], and analogous mechanistic model for V-ATPase pumping has been suggested (reviewed by Finbow and Harrison [27]). As for the F-ATPase subunits α and β, three dimers of the corresponding V-ATPase subunits (B and A, respectively) would form a hexameric ball (Fig. 1). Hydrolysis of ATP by the catalytic site of subunit A forces the rotation of a central stalk inside the A_3B_3 hexamer. Since, like the F-ATPase γ subunit, the D-subunit is predicted to have a very high α-helical content, it is thought to act similar to γ as a central stalk in the V-ATPase [11, 88]. The idea is then that the central stalk, which is linked to the ring of proteolipid subunits [93], causes rotation of this ring relative to the membrane-bound a-subunit (a in F-ATPase), thereby pumping protons across the membrane. Simultaneous rotation of subunits a and A_3B_3 with the central stalk and the proteolipid ring is prevented by a connecting second stalk, possibly formed by subunits a, E and G (a, δ and b in the F-ATPase), respectively [56, 86].

Function of the V-ATPase

In early studies concerning the importance of acidification for cellular processes, pH-altering drugs have been used that accumulate in acidic compartments and increase the lumenal pH by consuming protons, thereby causing osmotic swelling of the compartment, which may lead to undesirable side effects [71]. The discovery of the bafilomycins (Bafs) and later the concanamycins, two groups of macrolide antibiotics that inhibit the V-ATPase with high affinity, allowed more specific experiments [10, 23]. With these inhibitors, the V-ATPase was found to play an important role in intracellular processes such as trafficking, endocytosis, recycling, hydrolysis, processing and secretion of proteins, and in energizing the plasma membrane of some specialized cells [28, 86]. Another important tool for studying the V-ATPase has been the use of yeast mutants with a phenotype that can only be complemented by an acidic medium. The me-

Table Subunits of the V-ATPase.

Sector	Subunit	Yeast homolog	F-ATPase homolog	Molecular mass (kDa)	Proposed function	Mammalian isoforms	Ref. no.
V_1							
	A	VMA1	β	73	ATP hydrolysis	Va-68, HO-68 (human)	127
	B	VMA2	α	58	ATP binding	B1, B2 (bovine, human)	8,85,105,119
	C	VMA5		40	V_1-V_0 assembly		
	D	VMA8	γ?	34	Central stalk		
	E	VMA4	δ?	33	Connecting stalk		
	F	VMA7		14	V_1-V_0 assembly		
	G	VMA10	b	15	Connecting stalk	G1, G2 (bovine)	16
	H	VMA13		50	ATPase regulation	SFDα, SFDβ (bovine)	142
V_0							
	a	VPH1/STV1	a?	100	Proton transport, assembly, targeting	a1, a2, a3 (mouse)	91,102,125
	c	VMA6	c	17	Proton translocation?		
	c′	VMA3	c	17	Proton translocation		
	c″	VMA11	c	19	Proton translocation?		
	d	VMA16		38	Assembly?		

The molecular weights refer to the subunits of the bovine brain CCV V-ATPase [29].

dium is taken up by the yeast cell to compensate for the lack of V-ATPase-mediated acidification [81] The genes that were found to be mutated in these affected strains encode subunits of the V-ATPase or polypeptides implicated in the assembly, targeting or regulation of the pump [39, 86]. Inactivation of V-ATPase genes in several multicellular organisms indicated the importance of the pump for cell functioning and embryogenesis. By insertional mutagenesis via the transposable P-element, *Drosophila* genes encoding V_1 subunits A, B, C and E, and V_0 subunit c have been inactivated, causing lethality of the flies in the embryonic or larval stages [22]. Moreover, when gene expression of V-ATPase subunits was silenced in *C. elegans* by RNA interference, ovulation failed and embryogenesis was arrested [94]. Early embryonic death was also observed in mice lacking a functional proteolipid subunit c gene [45] A less severe phenotype was observed in patients with mutations in the human ATP6B1 gene encoding the B-subunit of the apical proton pump that mediates distal nephron acid secretion. These patients suffer from distal renal tubular acidosis and sensorineural hearing loss [49]. These studies illustrate the important role of V-ATPase activity for cell functioning and survival.

V-ATPases in the Secretory Pathway

Eukaryotic cells can secrete proteins continuously via the so-called constitutive pathway, whereas some cells that are specialized in secretion are also equipped with a regulated pathway of secretion. In the regulated pathway, selected proteins or small molecules are stored in specialized secretory vesicles, which secrete their contents only when the cell is triggered by the appropriate extracellular signal [50]. Secretory proteins are synthesized in the endoplasmic reticulum (ER) and pass during their transport to the plasma membrane different compartments of the secretory pathway, in which they can undergo modifications. To provide the proper microenvironment for these modifications, the pH within the secretory compartments decreases gradually from the ER to the plasma membrane (Fig. 2).

ER

Of the subcompartments of the secretory pathway, the ER is thought to have the highest pH, i.e., similar to the cytosol, which is close to neutral [71]. In an elegant manner, using targeted avidin in combination with pH-sensitive fluorescein-biotin, Wu and coworkers [135] found a pH value of 7.2 ± 0.2 in the ER of living HeLa cells. The fact that the pH of the ER is thought to be in equilibrium with the cytosolic pH does not necessarily imply that V-ATPases in the ER are inactive; they may well be active to generate a membrane potential [71, 117]. However, to our knowledge, there are no conclusive studies reporting the V-ATPase to be active in the ER and the importance of a proton gradient in this compartment. Apparently, ER processes such as the correct folding of polypeptides, assembly of protein complexes and *N*-linked glycosylation occur efficiently at neutral pH. From studies in yeast, we know that the first step in V-ATPase complex formation is the assembly of the membrane sector V_0, which is assisted by assembly factors (for review *see* [39, 86]). Molecular chaperones such as the binding protein BiP and calnexin may provide additional help in the folding and assembly of the newly synthesized V-ATPase [60].

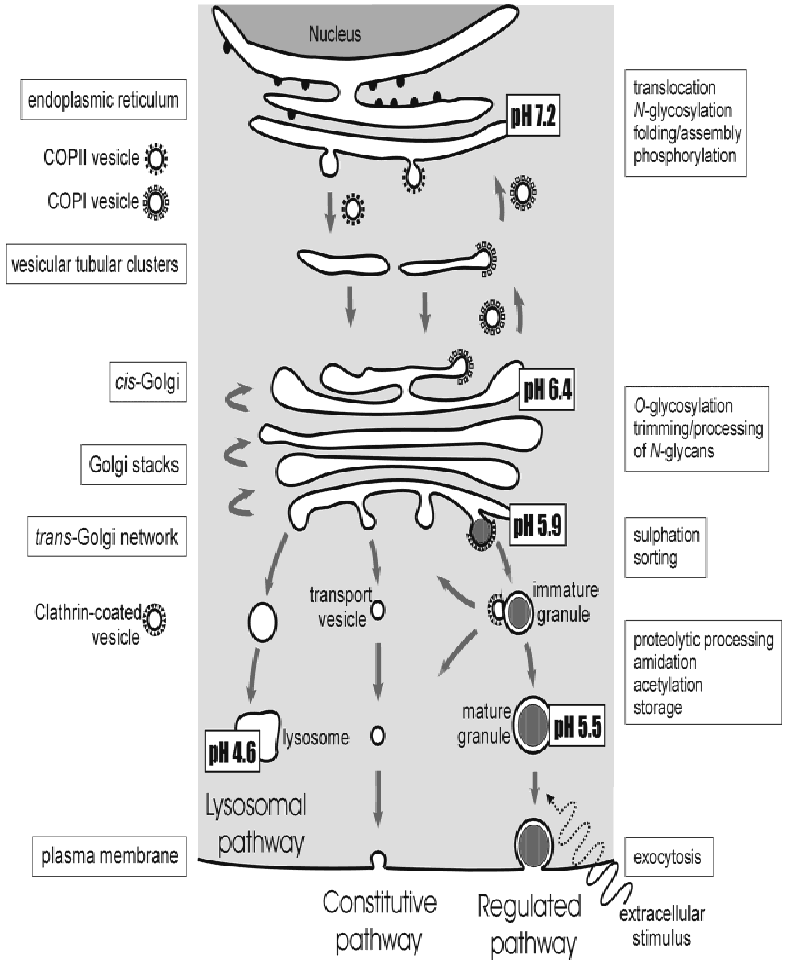

Fig. 2. pH-gradient in the secretory pathway. pH values of the lumen of the secretory compartments and of the lysosome (pH 4.6; [53]) are indicated.

Although the V_1 sector can be built onto the assembled V_0 subcomplex, it may not directly do so. Free V_1 sectors have been detected in the cytoplasm [82], and disassembly and reassembly of both sectors has been described as a dynamic mechanism to control V-ATPase activity (*see below*) [48, 120]. Furthermore, Morel et al. [77] found that the V_1 and V_0 are transported separately through the axon and only associate when they have both arrived at the nerve ending. Thus, the V_0 sector is assembled in the ER membrane, but the interaction with the V_1 sector and the actual proton pumping of the holoenzyme may well occur at a later stage in the secretory pathway.

Vesicular Tubular Clusters (VTCs)

Proteins that enter the secretory pathway are transported from the ER to the Golgi via vesicular tubular clusters (VTCs) also known as the ER-Golgi intermediate compartment (ERGIC) [114]. The transport from the ER to VTCs is mediated by cytoplasmic coatomer protein (COP) II-coated transport vesicles, whereas the vesicle coat protein complex COPI acts in the retrograde transport from VTCs and Golgi to the ER [59] and may participate in anterograde transport through the Golgi complex [90, 98]. Different effects of V-ATPase inhibitors on ER to Golgi transport have been reported. Concanamycin B caused no effect on ER to Golgi transport in human hepatoma HepG2 cells [140], whereas Baf only slightly affected the transport of the vesicular stomatitis virus (VSV) G glycoprotein from the ER to the *cis-/medial*-Golgi in BHK-21 cells [100]. Given the amount of the weak base amine 3-(2,4-dinitroanillino)-3′-amino-*N*-methyldipropylamine (DAMP) that accumulated in subpopulations of pre-Golgi structures in NRK cells, these structures appear to become progressively more acidic during their transfer to the Golgi region [100]. An active V-ATPase regulating retrograde transport from the VTCs and the Golgi complex to the ER has been demonstrated with Baf. The V-ATPase appeared to control the distribution of coatomer and the formation of COPI-coated vesicles [100] The assembly of coats is regulated by small GTPases like ADP-ribosylation factor (ARF), whose redistribution to the membrane depends

on intravesicular acidification [141]. These studies indicate that V-ATPase activity plays a role in coat-mediated transport processes [100]. Other processes that require a low pH in pre-Golgi compartments are the dissociation of the T cell receptor-associated protein (TRAP) from receptor subunits [9] and the interaction between KDEL-containing proteins and the KDEL-receptor, which is a prerequisite for their retrieval [110, 134]. Together, these results suggest that proton pumping in the early secretory pathway is needed for regulating protein-protein interactions, and anterograde and retrograde transport, possibly via pH-dependent coat assembly/disassembly.

Golgi

Almost two decades ago, Glickman et al. [34] reported that Golgi membranes contain an active proton pump that is capable of creating a transmembrane pH gradient. Since then, different techniques have been used to measure intra-Golgi pH. Using a marker for acidic vacuolar structures, Anderson and Pathak [4] and Orci et al. [97] showed that the distal subcompartments of the Golgi become progressively more acidic. In HeLa cells transfected with mutant green fluorescent proteins (GFPs) fused to the Golgi-enzyme galactosyltransferase, the *medial-/trans*-Golgi was calculated to have a pH of 6.58 at steady state [62] The avidin/fluorescein-biotin system allowed Wu et al. [135] to estimate the pH of the Golgi in HeLa cells to be 6.4 ± 0.3. A similar pH was measured in the Golgi of intact Vero cells with FITC-conjugated verotoxin that was delivered to the Golgi via endocytosis [51]. In many cell types, the passage of secretory proteins through the Golgi is affected when cells are treated with drugs that efficiently neutralize Golgi compartments (such as monensin, ammonium chloride and concanamycin B) [71, 75, 128, 140]. In BHK-21 cells, an active V-ATPase in the Golgi apparatus is essential for protein transport and in the *trans*-Golgi for viral glycoprotein maturation [99]. However, Baf did not inhibit transport from the *trans*-Golgi to the cell surface, suggesting that the block was before the *trans*-Golgi network (TGN). A low pH in the Golgi is a prerequisite for the specific interaction between a carrier protein and the molecule to be delivered to a storage or lysosomal vesicle [50]. The pH in the *trans*-Golgi is important for the binding of lysosomal enzymes to the mannose-6-phosphate receptor, while after lysosomal acidification the enzymes dissociate from the receptor and the receptor then recycles back to the Golgi [108]. Furthermore, disruption of the *trans*-Golgi pH by ammonium chloride or monensin indicated that the pH is important for the polymerization of von Willebrand Factor (vWF) and its subsequent targeting into Weibel-Palade bodies, which may be receptor-mediated [129]. The requirement of a low pH for Golgi enzyme activity appears to be enzyme dependent, since processing of N-linked glycans by sialyltransferases relies upon a low pH [140], whereas the activity of *cis/medial*-Golgi processing enzymes, such as endoglycosidase H, is not affected by Baf [100].

TGN

The TGN appears to be more acidic than the Golgi cisternae [51, 115]. Using the TGN resident proteins TGN38 and furin to ferry fluorescent pH-sensitive indicators into the TGN via endocytosis, an average pH of 5.91 in HeLa cells and 5.95 in CHO cells was calculated [20]. In HeLa cells transfected with pH-sensitive GFP, a somewhat higher pH was found (6.21 ± 0.39) [74]. Work from several laboratories has indicated that the low pH in the TGN is important for the processing of proproteins, sorting of secretory cargo and retrograde transport of components of the biosynthetic pathway from the TGN to earlier compartments. In yeast cells treated with V-ATPase inhibitors or in mutant yeast cells with a disrupted V-ATPase subunit gene, vacuolar precursor proteins are not processed and accumulate in the *trans*-Golgi complex or in post-Golgi vesicles [139]. Expression of the influenza M2 proton channel in polarized Madin-Darby canine kidney (MDCK) cells perturbs the pH and interferes selectively with the release of newly synthesized apical proteins from the TGN [41]. Furthermore, in Baf-treated elastogenic cells, tropoelastin secretion was inhibited and an intracellular accumulation of tropoelastin was detected in the TGN and small secretory vesicles [18]. Although the precise mechanism by which proteins are conveyed to the regulated secretory pathway is elusive, self-condensation of regulated secretory proteins in the acidic microenvironment of the TGN/immature granules is considered to be an important factor in the sorting process [12, 13]. Self-condensation is thought to be dependent on local protein concentrations, pH and Ca^{2+} levels. Fifteen exocrine pancreatic secretory proteins have been found to aggregate under conditions of acidic pH (5.5) and calcium concentrations that mimic those in the TGN [30]. Moreover, in vitro the zymogen granular contents aggregate in the presence of cations and in an acidic environment, which is believed to exist in the pancreatic TGN [58]. Likewise, a decrease in pH together with an increase in calcium are sufficient to trigger selective aggregation of carboxypeptidase E [116], prolactin [14], chromogranin B and secretogranin II in the TGN [13]. Interestingly, a peptide corresponding to the proregion of the prohormone convertase enzyme PC2 was found to undergo aggregation and membrane association in a pH- and calcium-dependent manner [46]. The acidic lumen of the TGN has also been found to trigger the processing of secretory proteins. For instance, Moore, Gumbiner and Kelly [76]

and Stoller and Shields [118] demonstrated that the treatment of cultured neuroendocrine cells with chloroquine or ammoniumchloride not only disrupts the sorting of peptide hormone precursors to the regulated secretory pathway, but also inhibits their endoproteolytic processing to mature bioactive peptides. In anterior pituitary-derived AtT-20 cells, an acidic pH in the TGN favours the sorting and proteolytic processing of the prohormone pro-opiomelanocortin (POMC) [112, 122]. The processing of POMC to adrenocorticotropic hormone (ACTH) can occur in earlier compartments of the secretory pathway, once the lumenal pH drops below 6.0 and millimolar concentrations of calcium are present [111]. A pH between 6 and 6.2 in the TGN was found to be optimal for the cleavage of prosomatostatin in retrovirally transfected rat anterior pituitary GH3 cells [138]. In contrast, in some cases acidotropic weak bases dissipate the pH gradient, but had no discernable effect on prohormone processing [66, 83]. However, as mentioned above, these weak bases may cause undesirable side effects because of their acidotropic behavior. In conclusion, the acidic lumen of the TGN creates the environment for aggregation-induced sorting and processing of regulated secretory proteins, which is thought to continue during the biogenesis and maturation of secretory granules.

Secretory Granules and Synaptic Vesicles

In eukaryotic cells, chromaffin granules were the first vacuolar structures that have been found to contain a H^+-ATPase [7, 52]. The proton pump in these granules generates a transmembrane proton gradient, which drives the uptake of monamines, ATP and various ions from the cytosol [70]. V-ATPase activity has also been found to drive the uptake of histamine by isolated mast cell granules [64], serotonin (5-hydroxytryptamine, 5-HT) by posterior pituitary neurosecretory granules [78] and the neurotransmitter glutamic acid by synaptic vesicles and CCVs [104]. Furthermore, V-ATPases are involved in the acidification of the lumen of endocrine and neuroendocrine secretory granules, thereby providing the proper conditions for packaging and/or processing of secretory proteins [71]. For instance, the lumenal pH of 6.3 ± 0.1 in isolated immature secretory granules (ISGs) from PC12 cells, which is comparable to the pH in the TGN and higher than the pH of mature secretory granules (pH 5.0–5.5), allows the processing of secretogranin II [126]. Comparable pH values were found for insulin-containing granules (pH 5.0), mast cell granules (pH 5.2 ± 0.55), and synaptic vesicles from hippocampal neurons (5.67 ± 0.71) and the bovine intermediate lobe (pH lower than 5.6) [44, 63, 74]. Orci et al. [96, 97] and Anderson and Orci [3] have demonstrated that the endoproteolytic conversion of proinsulin to insulin occurs in coordination with a progressive acidification of ISGs. Furthermore, Baf affected the proteolytic processing of POMC during the maturation of the secretory granules in AtT-20 cells [122], and perturbed the intracellular transport of newly synthesized prolactin and maturation of secretory granules in rat pituitary-derived GH3 cells [42]. As a consequence, both POMC and prolactin were secreted from these cells in a constitutive-like fashion, i.e., constitutive secretion originating from ISGs [54]. Recently, we found in a post-TGN compartment, most likely the ISG, a Baf-sensitive action of V-ATPases that is pivotal for the transport, sorting, processing and secretion of regulated secretory proteins by *Xenopus laevis* intermediate pituitary cells [113]. The secretion of unprocessed POMC from these Baf-treated cells seems to occur also in a constitutive-like fashion. Like in the TGN, the microenvironment in ISGs favors the condensation of secretory proteins (*see above*) and subsequently the capture of the aggregate into a secretory granule by pH-dependent interaction of the aggregate with a receptor or membrane lipids [15, 57]. Thus, V-ATPase may drive the uptake of various compounds by synaptic vesicles and secretory granules, and progressively acidify secretory granules for the sorting and processing of secretory proteins. Concerning the granular pH in exocrine cells some controversy exists [19, 70, 97] and additional studies are necessary to clarify this issue.

Exocytosis

Following fusion of a vesicle with the plasma membrane, the interior of the vesicle contacts the extracellular environment. Consequently, the contents of the vesicle are released and the low pH inside the vesicle is immediately neutralized. Secretory granules, but probably also synaptic vesicles, re-acidify upon their retrieval from the plasma membrane [74]. Inhibition of V-ATPase activity with Baf has been shown to affect regulated exocytosis. Baf inhibits the release of insulin from insulin-containing granules, probably by blocking the V-ATPase-coupled glutamate uptake by the granules (a process that is thought to make the granule fusion-competent; [65]) and the exocytosis of eosinophil granules [55]. However, secretion can also be accelerated by pH-altering drugs. For example, ammonium chloride and Baf have been shown to stimulate secretion from rat parotid cells [128] and the pituitary tumor cell line GH3, respectively [42]. On the other hand, in exocrine secretory granules, a low pH is not required for the exocytotic event [19] These discrepancies in results may be explained by the different types of inhibitors and cells that were used, but again imply that further studies have to be performed to elucidate the role of lumenal pH in exocytosis.

Regulation of V-ATPase Activity

As discussed above, V-ATPases are involved in a wide variety of physiological processes. Control of these pro-

cesses requires the regulation of V-ATPase activity and a number of such regulatory mechanisms have been proposed.

Disulfide-bond Formation in the Catalytic Site

Disulfide bond formation between cysteines Cys^{254} and Cys^{532} (numbers refer to the bovine A subunit) in the catalytic portion of the V-ATPase subunit A leads to the inactivation of the enzyme [25, 26], suggesting that the formation of this disulfide bond is part of the mechanism regulating the V-ATPase. This is in agreement with the findings that a significant fraction of the V-ATPase isolated from native CCVs is in the inactivated oxidized state [25] and that in synaptic vesicles the active V-ATPase is in the fully reduced state [108]. In the yeast Golgi, some other mechanism should regulate V-ATPase activity, since disulfide bond formation does not seem to be essential for the acidification of this compartment [61].

Assembly/Disassembly

Besides fully assembled V-ATPase complexes, pools of free V_1 and V_0 sectors have been found in eukaryotic cells [21, 82, 120, 123, 140]. In yeast, the regulation of the assembly and disassembly of the two sectors is glucose dependent [47]. Moreover, the ratio between assembled and disassembled sectors changes during moulting in the tobacco hornworm (*Manduca sexta*) [120] and during starvation in insects [38]. These studies suggest that association and dissociation of the two sectors is a general mechanism for controlling V-ATPase activity. At present, it is not clear whether such a regulatory mechanism also occurs in mammalian cells and which factors determine the assembly state of the V-ATPase [48].

Conformational Change of c Subunits

Grabe, Wang & Oster [37] proposed a model in which the pump 'changes gears,' i.e., adjust its activity depending on the lumenal pH of the organelle. This model is based on the assumption that the F-ATPase rotary mechanism of proton translocation also applies to the V-ATPase and on the finding that the proton-binding c-subunit of the F-ATPase has a pH-dependent conformation [106]. The lower the pH, the more c-subunits change to a conformation that does not allow proton binding. In an acidic lumen, the pumping capacity of the V-ATPase would then be reduced. In this way, pH-sensing c-subunits would directly influence V-ATPase activity.

Variable Proton-ATP Coupling

The V-ATPase can change its efficiency of the coupling between proton transport and ATP hydrolysis [30], thereby varying the number of protons that are transported per ATP hydrolyzed. This change in capacity may depend on the cytoplasmic and lumenal pH [19], implying a kind of feedback mechanism that regulates the pH gradient across organellar membranes.

Isoforms of V-ATPase Subunits

Besides conformational changes of the V-ATPase subunits, a diversity in the molecular composition of the enzyme may change pumping activity. Isoforms of subunits have been identified in several species. In yeast, two isoforms of subunit a (Vphp1 and Stvp1) have been found [68, 69]. Since Vphp1 is localized to the vacuolar membrane and Stv1p to the Golgi and/or endosomal membrane, the a-subunit may target V-ATPases to different compartments [69]. In vertebrates, three isoforms of subunit a have been found, each with a specific tissue expression pattern [91, 102, 125]. Isoforms of other V-ATPase subunits have also been described [73] (for review *see* [32, 72]). They originate from different genes or are the result of differential RNA processing (alternative splicing/polyadenylation). Alternative splicing has been described for the subunit a gene from bovine brain [102], the subunit B and d genes from *Manduca sexta* midgut [73], and the catalytic subunit A gene from chicken osteoclasts [43]. The choice of subunits during V-ATPase biosynthesis may determine the intrinsic activity of the pump, its ability to respond to regulatory signals and the targeting of the pump to its proper intracellular location.

Regulation of Organelle-specific V-ATPase Activity

The above-mentioned mechanisms regulate the intrinsic activity of the V-ATPase enzyme. To achieve the diversity in lumenal pH values among the different organelles, additional regulatory processes must be available.

Targeting of V-ATPases to Specific Organelles

The V-ATPase has to be transported to an intracellular location where its activity is required. Organelle-specific routing signals may target the V-ATPase in different quantities or with different intrinsic properties (*see above*) to the appropriate cellular compartment. In this way, the cell may regulate the capacity of proton pumping in an organelle-specific manner. The rate of proton transport through the plasma membrane of some specialized cells, such as renal intercalated cells and osteoclasts,

depends on the density of V-ATPases in the membrane, which is regulated by the insertion or retrieval of H^+-ATPase-containing vesicles [35, 40]. Likewise, membrane remodelling events may affect the density of V-ATPases in the organellar membrane [124, 126]. The appealing idea that V-ATPases with different pumping properties (*see* under "Regulation of V-ATPase activity") are selectively targeted to organelles is supported by the finding that in carrot, antisense RNA directed against a subunit A isoform prevents the expression of the V-ATPase in tonoplasts, but not in the membranes of Golgi-enriched microsomes [36]. Furthermore, V-ATPases have been isolated from different organelles of the kidney and were found to differ significantly in their enzymatic properties, possibly due to a variation in the B-subunit isoforms [131]. Yeast V-ATPases containing different isoforms of the a-subunit have a different intracellular localization, in spite of the fact that these isoforms appear to be functional homologues [69].

Targeting signals have not yet been identified in the structures of the V-ATPase subunits. However, enzyme targeting may also be provided by V-ATPase-associated proteins with specific routing signals. In yeast, a peripheral ER membrane protein (Lst1p) is involved in the selective export of the plasma membrane ATPase out of the ER [107]. Supek et al. [121] isolated from bovine chromaffin granules a novel 45-kDa protein, named Ac45, which copurified with the membrane sector of the pump. When compared to the amount of Ac45 found in the chromaffin granular membrane, V-ATPases purified from kidney microsomes contained reduced amounts of Ac45 and kidney membrane V-ATPase preparations were devoid of the protein [121]. Based on these results, it was hypothesized that Ac45 targets the V-ATPase to a subset of intracellular compartments. Interactions between V-ATPases and transport proteins have indeed been reported. The 50-kDa subunit of the AP-2 adaptin complex associates specifically and stoichiometrically with the coated-vesicle V-ATPase [82]. Galli and coworkers [33] could coprecipitate the V_0 sector with proteins of the vesicle docking fusion complex (synaptobrevin and synaptophysin), and hypothesized that this interaction may play a role in recruiting the pump to synaptic vesicles.

Activator and Inhibitory Proteins

V-ATPase activity may also be regulated in an organelle-specific manner by activator or inhibitory proteins. In yeast, one candidate may be the KEX2 gene, which encodes a serine protease localized to the late Golgi compartment. Mutations in this gene lead to an inhibition of the V-ATPase, despite the fact that the enzyme is present in a fully assembled state and in principle enzymatically active [95]. How kex2 affects V-ATPase activity is not known, but presumably a kex2-dependent endoproteolytic cleavage activates some protein in the intracellular cascade of V-ATPase activation. Bovine CCVs and kidney cytosol were also found to contain proteins that either activate or inhibit the activity of the purified V-ATPase [136, 140]. It is likely that additional proteins will be found that exhibit a role in V-ATPase regulation. In this connection, the V-ATPase accessory subunit Ac45 should be tested, not only as a chaperone-like protein (*see above*), but also as an inhibitor or activator of the enzyme.

Signaling Molecules

A number of studies point to a role of signaling molecules in the regulation of V-ATPase activity (for review *see* [72]). Kane [47] demonstrated that, in yeast, glucose deprivation results in a rapid dissociation of the V_1 and V_0 domains, and that this effect is reversed upon readdition of glucose, suggesting the involvement of a signaling pathway that transmits the signal from the extracellular environment to the V-ATPase enzyme. Furthermore, hydrogen peroxide (H_2O_2) produced by several regions in the brain can inhibit V-ATPase activity in bovine brain synaptic vesicles, thereby affecting glutamate uptake [130]. At present, it is unclear how these signals are transmitted to the V-ATPase and how the enzyme is responding to these molecules. Chloride channels may play a role in this process. Because V-ATPases establish a membrane potential, proton transport must be accompanied by the movement of another charged ion [5]. The major ion conductance involved in dissipation of the membrane potential is mediated by chloride channels [5] that are regulated by protein kinase A-dependent phosphorylation [80]. Thus, a signaling pathway via the chloride channel may control V-ATPase activity.

Regulating Organellar pH via Membrane Permeability

Recent studies indicate that the V-ATPase is not quiescent when the pH of the Golgi is at steady state or even below resting pH [24, 109]. These recent findings support an earlier idea that the proton gradient across the membrane of an organelle is not solely controlled by the V-ATPase, but rather by keeping a balance between H^+-pumping of the V-ATPase and an endogenous proton leak [31, 51]. Regulation of the extent of leakage could represent a mechanism of controlling the pH of individual subcellular compartments. For instance, the Golgi is more acidic than the ER, because it has an active pump and an apparent H^+-permeability three times smaller than that of the ER [133]. Although the nature of the leak

pathways remains to be clarified, leakage may occur through the vacuolar membrane (partially via a conductive component [109]) or via the membrane sector of the V-ATPase. Thus, the variety in proton gradients that exist among the different membranes may be explained by changing the activity of the V-ATPase or altering the magnitude of proton leakage through the membrane via variations in the lipid or protein composition.

Concluding Remarks and Future Prospects

During the last decade, considerable progress has been made concerning the elucidation of the structure, mechanism and function of the proton-pumping V-ATPase. Both at the plasma membrane and intracellularly this enzyme plays an important role in a variety of biological processes. In the pre-Golgi area of the secretory pathway, V-ATPase activity may be involved in retrograde vesicle transport, while in the TGN and post-TGN compartments, the activity of the V-ATPase is important for the sorting, transport, processing and release of regulated secretory proteins and the uptake of monoamines, ATP and certain ions from the cytosol. Different mechanisms have been proposed to explain how the activity of the V-ATPase is controlled and how the proton gradient across the membrane of the different organelles is regulated. However, the exact regulatory mechanisms are still not fully understood. Finding new V-ATPase-interacting proteins that could inhibit, activate or target the pump in an organelle-specific way will be a major challenge for solving this issue. The application of modern approaches, such as knockout technology, antisense RNA and RNA interference, in combination with microarray gene expression profiling, may lead to the identification of proteins the expression of which is affected upon silencing of a V-ATPase subunit gene. Such a link in gene expression suggests a functional interaction between these gene products and the V-ATPase, making them good candidates for a role in the mechanism that regulates the organelle-specific activity of the pump. One major drawback of these experiments could be that the activity of the V-ATPase appears to be essential for embryonic development. Thus, the reduction in the expression of a V-ATPase subunit may well lead to an early death of the transgenic organism. These problems may be circumvented by disturbing the expression of the gene in a tissue- or time-specific mode, e.g., via the use of conditional knockouts, or by using transfected cell lines. The application of these and other approaches will certainly teach us more about proton pumping across biological membranes.

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