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The Insect Plasma Membrane H^+ V-ATPase: Intra-, Inter-, and Supramolecular Aspects

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The plasma membrane H^+ V-ATPase from the midgut of larval *Manduca sexta*, commonly called the tobacco hornworm, is the sole energizer of epithelial ion transport in this tissue, being responsible for the alkalinization of the gut lumen up to a pH of more than 11 and for any active ion movement across the epithelium. This minireview deals with those topics of our recent research on this enzyme that may contribute novel aspects to the biochemistry and physiology of V-ATPases. Our research approaches include intramolecular aspects such as subunit topology and the inhibition by macrolide antibiotics, intermolecular aspects such as the hormonal regulation of V-ATPase biosynthesis and the interaction of the V-ATPase with the actin cytoskeleton, and supramolecular aspects such as the interactions of V-ATPase, K^+/H^+ antiporter, and ion channels, which all function as an ensemble in the transepithelial movement of potassium ions.

KEY WORDS: Vacuolar ATPase; insects; *Manduca sexta*; midgut; subunit topology; bafilomycin; concanamycin; regulation of biosynthesis; transepithelial K^+ transport; actin.

INTRODUCTION

H^+ transporting V-ATPases are found in two principal locations, in endomembranes and in plasma membranes (Stevens and Forgac, 1997; Wieczorek *et al*., 1999). The enzyme from the midgut of larval *Manduca sexta*, commonly called the tobacco hornworm, is a plasma membrane H^+ V-ATPase that occurs in the apical membrane of goblet cells (see Fig. 4), one of the two major types of differentiated cells in the midgut (Wieczorek *et al*., 1999). Eight different subunits constitute its cytosolically oriented V_1 complex and four different subunits its membrane bound V_0 complex. The primary structures of all 12 subunits are known (Merzendorfer *et al*., 2000), and a low resolution quaternary structure of the V_1 complex has been explored by small angle X-ray scattering and electron microscopy (Grüber *et al*., 2000b; Radermacher *et al.*, 2001).

The lepidopteran midgut turned out to be a model tissue for studies of general aspects of V-ATPases, not only because it is a rich source for purification of the enzyme in milligram amounts, but also because the V-ATPase plays a central role in midgut ion tranport physiology (Wieczorek *et al*., 2000). In this minireview we report on recent work from our group on the biochemistry and physiology of the insect plasma membrane V-ATPase. In the first part we discuss intramolecular aspects such as facets of subunit topology and the binding site for macrolide antibiotics. The second part sheds light on intermolecular aspects such as the regulation of V-ATPase biosynthesis by ecdysteroid hormones and the specific interaction of the V-ATPase and its subunits with the actin cytoskeleton. In the last part we switch to midgut physiology and deal with supramolecular aspects such as the interactions of V-ATPase, K^+/H^+ antiporter and ion channels which all function as an ensemble in the transepithelial movement of potassium ions.

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INTRAMOLECULAR ASPECTS

Topology of Subunits

Early models for subunit topology relied completely on the evident similarity of V- and F-ATPases which both consist of a V_1/F_1 complex being attached to the membrane bound V_0/F_0 complex (Nelson, 1989). More recent approaches revealed low resolution structural data, especially on the V_1 part (Grüber *et al*., 2000a,b). While progress was made particularly on its head subunits *A* and *B*, many questions remain to be solved about the arrangement of its stalk subunits in the isolated V_1 complex and in the V_1V_0 holoenzyme. One of the central questions regards the identity of the central V_1 stalk subunit, the homologue of the F-ATPase γ-subunit which undergoes rotation during ATP hydrolysis and serves an essential function in rotary catalysis. V_1 subunits *D* and *E*, respectively, have been discussed as alternative candidates for such a role (for a review see Margolles-Clark *et al.*, 1999).

For several years our group had favored the *E* subunit as the central stalk subunit, mainly because of its relative insensitivity to proteolysis and to chaotropic effects indicating that it is shielded, in the center of the V_1 stalk, by other surrounding subunits (Grüber *et al*., 2000a). Recently, mutational analysis of the yeast subunit *D* indicated its influence on the coupling efficiency between ATP hydrolysis and proton transport (Xu and Forgac, 2000), and a report on the combination of cysteine mutagenesis and covalent cross-linking (Arata *et al*., 2002) also compellingly led to the conclusion that subunit *D* is the most likely homologue to the γ-subunit of F_1. In addition, results from the V_1-ATPase of Thermus thermophilus clearly demonstrated that subunit *D* that exhibits considerable similarity to that of eukaryotic V-ATPases constitutes part of the rotor shaft (Imamura *et al*., 2003). Thus, our present topological model for the V-ATPase from *Manduca sexta* also favors subunit *D* as the central stalk subunit (Fig. 1). The neighborhood of subunits was chosen according to the results of cross-linking experiments for the yeast and the *Manduca* enzyme. The results obtained from these studies indicate direct contacts of subunit *D* to the head subunit *B* and to subunit *F* that appears to be located near the V_0 complex; on the other hand, subunit *E* joins subunits *A*, *B*, *C*, *F*, and *G* as direct neighbors (Grüber *et al*., 2000a; Tomashek *et al*., 1997; Xu *et al*., 1999; S. Reineke, D. Friedrichs, H. Merzendorfer and H. Wieczorek, unpublished).

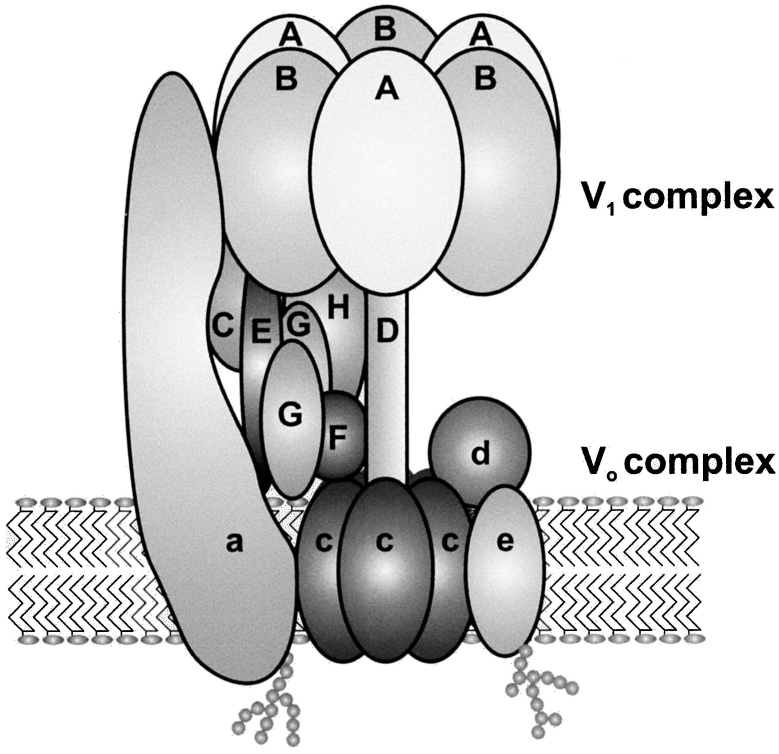

Fig. 1. Hypothetical model of the V-ATPase from larval *Manduca sexta* midgut.

Binding of Plecomacrolide Antibiotics

Although the inhibitory effect of plecomacrolides like bafilomycin and concanamycin on V-ATPases was already discovered more than 10 years ago (Bowman *et al*., 1988; Dröse *et al*., 1993), little is known about their mode of action or their binding-site within the V-ATPases. Both antibiotics reversibly inhibit V-ATPases at nanomolar concentrations (Bowman *et al*., 1988; Dröse *et al*., 1993, Dröse and Altendorf, 1997; Huss *et al*., 2002). First insights that bafilomycin may interact with the membrane bound V_0 complex emerged from two sets of experiments involving reconstituted V_0 complexes. Crider *et al*. (1994) detected a latent proton conductance at low pH which was inhibited by bafilomycin, and Zhang *et al*. (1994) showed that the addition of reconstituted V_0 complexes to the holoenzyme preparation led to the disappearance of the inhibitory effect of the plecomacrolide. The interaction of bafilomycin with the V_0 complex was finally confirmed by copurification of the V_0 complex from chicken osteoclasts with a ^{3}H-labeled bafilomycin A_1 (Mattsson and Keeling, 1996).

The fact that already the addition of reconstituted subunit *a* to the reconstituted V_1V_0 holoenzyme of bovine clathrin coated vesicles resulted in a protection against bafilomycin led to the hypothesis that the binding site resides in this subunit (Zhang *et al*., 1994). Contrasting results from chicken osteoclasts pointed to subunit *c* as a candidate for plecomacrolide binding. Affinity chromatography using a bafilomycin C_1 column resulted in the purification of the V_1V_0 holoenzyme with an excess of subunit *c*, and preincubation with DCCD, a covalent inhibitor of the V-ATPase binding to the essential glutamic acid in subunit *c* (E139, see also Fig. 2), prevented the binding to the bafilomycin column, suggesting a colocalization of the binding sites of these two inhibitors in subunit *c* (Rautiala *et al*., 1993). The idea of bafilomycin interacting with subunit *c* was supported by sequence analysis of mutated subunits *c* from *Neurospora crassa* where single amino acid exchanges at three different positions led to a rather high tolerance for bafilomycin as compared to the wild type (Bowman and Bowman, 2002). To finally prove that plecomacrolides bind to subunit *c*, a semisynthetic derivative of concanamycin A (9-*O*-[*p*-(trifluoroethyldiazirinyl)-benzoyl]-21,23-dideoxy-23-[^{125}**I**]iodo-concanolide A, J-concanolide A) was used which generates, upon UV-irradiation, a covalent bond in its binding pocket (Huss *et al*., 2002). This probe labeled in a concentration dependent manner only subunit *c* in the purified V_1V_0 holoenzyme as well as in the purified V_0 complex and even in native ATPase containing vesicles. Since labeling could

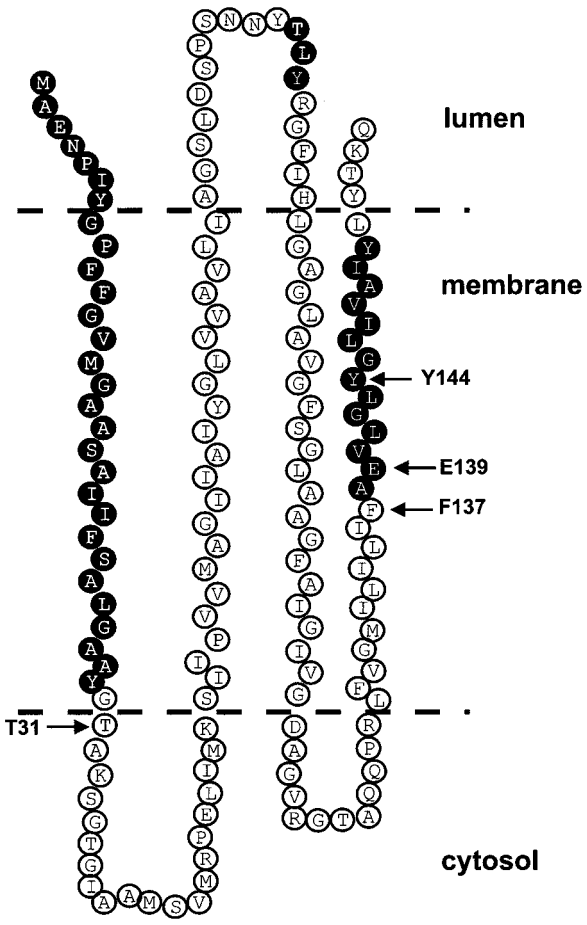

Fig. 2. Putative secondary structure of subunit *c* from *Manduca sexta* (Accession No. P31403) as calculated by NiceProt view (SWISS-PROT). Amino acids of the fragments labeled with 9-*O*-[*p*-(trifluoroethyldiazirinyl)-benzoyl]-21,23-dideoxy-23-[^{125}**I**]iodo-concanolide A found by MALDI-MS analysis are shaded. Conserved amino acids whose exchange causes a partial resistance to bafilomycin in *Neurospora crassa* (T31, Y144, F137) as well as the essential glutamate (E139) are indicated by arrows.

be prevented in the presence of concanamycin A as well as of bafilomycins A_1 and B_1, respectively, it was obvious that the binding site for plecomacrolide antibiotics is formed by subunit *c*.

To analyze the binding site in more detail we labeled subunit *c* with J-concanolide A. After digestion with chymotrypsin, MALDI-TOF analysis led to the identification of five fragments which contained covalently bound J-concanolide A (amino acids 1–7, 8–22, 12–29, 85–87, and 138–151; M. Huss, S. König and H. Wieczorek, unpublished; see Fig. 2). The most interesting fragment 138–151 included one of those mutation sites (Y144) responsible for bafilomycin resistance of the *Neurospora* enzyme (Bowman and Bowman, 2002) and was directly flanked

by another mutation site, F137, implying a putative hot spot for the antibiotic efficiency.

INTERMOLECULAR ASPECTS

Hormonal Control of V-ATPase Gene Expression

The reversible dissociation of the V_1 complex from the membrane bound V_0 complex is the only regulatory property of V-ATPases which has been shown so far to occur in a physiological context (Kane, 1995; Sumner *et al*., 1995). In *Manduca* larvae, dissociation occurs during molt and as a consequence of starvation. Interestingly, control of V-ATPase activity seems to be interrelated with the control of V-ATPase biosynthesis. Examination of transcript levels by Northern blotting during periods of molt and starvation revealed a decrease of the mRNA amounts for almost every V-ATPase subunit (Reineke *et al*., 2002). The coordinated reduction of V-ATPase transcripts suggested the existence of either a transcriptional or posttranscriptional control mechanism that minimizes V-ATPase biosynthesis during periods of enzyme inactivation and therefore may help to save cellular energy. Since ecdysteroids are known as key regulators of the insect molt by controlling the expression of numerous regulatory genes (for a review see Thummel, 1996), it was tempting to speculate that the observed decline of transcript amounts for V-ATPase subunits may be mediated by ecdysteroids. Sequence analysis of three 5′ regions of the genes *mvB*, *mvG*, and *mvd* encoding respective V-ATPase subunits from *Manduca* provided first indications for the involvement of ecdysteroids (Reineke *et al*., 2002), because all upstream regions contained putative ecdysterone-responsive elements corresponding to the consensus sequence KNTCANTNNMM (Luo *et al*., 1991).

First experimental evidences for an ecdysteroid-mediated control of V-ATPase biosynthesis were deduced from injection experiments (Reineke *et al*., 2002). When ecdysterone was injected into the dorsal vessel of feeding larvae, a short-term increase and a long-term decrease of the transcripts encoding subunits *B*, *G*, and *d* was observed. Immunohistochemical studies of the midgut epithelium revealed that ecdysterone injection leads to changes in goblet cell morphology and in subcellular distribution of the V_1-complex comparable to the situation during molt and during starvation. The regulatory function of ecdysterone was finally established by reporter gene assays, which were performed with plasmids containing different 5′ upstream regions of *mvB*, *mvG*, and *mvd* for the control of luciferase gene expression in transfected *Sf*21 cells. When ecdysterone was added to the culture medium a short-term increase and a long-term decrease of promoter activities was found. Thus the results of the reporter gene assays were in line with those of the injection experiments (Reineke *et al*., 2002). In conclusion, ecdysteroids appear to have a dual effect on the transcription of V-ATPase subunits, whereby the long-term effect is to down-regulate the amounts of V-ATPase transcripts during moult and starvation. The discovered ecdysterone responsive element within the upstream region of the investigated V-ATPase genes may be only one of several regulatory elements. In addition, ecdysteroids may also indirectly affect transcription rates via the control of other transcription factors as it was suggested for the *Manduca* dopa decarboxylase gene (Hiruma *et al*., 1997) or may influence transcript stabilities by a posttranscriptional mechanism similar to the gene product of *esr20* which is expressed in the trachea of *M. sexta* (Meszaros and Morton, 1997).

Interaction of the Insect Plasma Membrane V-ATPase With the Actin Cytoskeleton

Many transport proteins such as diverse ion channels, glucose or serotonin transporters and the Na^+/H^+ antiporter interact, in their physiological environment, with the actin cytoskeleton (e.g. Cantiello, 1995; Kurashima *et al*., 1999; Sakai *et al*., 2000; Wang *et al*., 1994, 1998). This may also be true for V-ATPases since it was shown in a recent report that the osteoclast ruffled membrane V-ATPase binds to actin filaments during osteoclast activation (Lee *et al*., 1999). The novelty of this interaction was that binding to F-actin did not depend on the presence of a linker protein but was a direct interaction between both proteins. Binding was shown to be mediated by the amino-terminal part of the V_1 subunit *B* (Holliday *et al*., 2000). In yeast, however, binding of the endomembrane V-ATPase and F-actin could not be verified (Xu and Forgac, 2001) and therefore we asked ourselves whether this type of interaction might be a general phenomenon of plasma membrane V-ATPases. To investigate this question, we performed studies using the plasma membrane V-ATPase from *Manduca* (Vitavska *et al*., in press). We confirmed the colocalization of F-actin and the plasma membrane V-ATPase not only in goblet cell apical membranes of the midgut, but also in luminal brush border membranes of Malpighian tubules and salivary glands, respectively, which are known to contain V-ATPase as well (Wieczorek *et al*., 1999). We demonstrated that in vitro both, the V_1 complex and the V_1V_0 holoenzyme, bind directly to F-actin. As in the osteoclast V-ATPase the interaction is mediated by subunit *B*, but by contrast, the insect V-ATPase binds to actin filaments also via its *C* subunit. The latter subunit is part of the V_1 complex in the holoenzyme, but gets lost, during molt or starvation, upon dissociation of

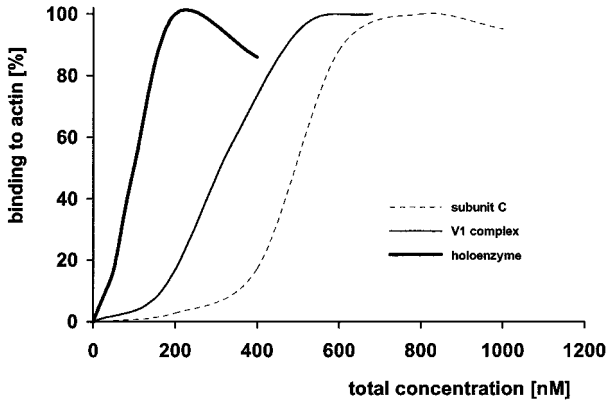

Fig. 3. Concentration dependent binding of the V_1V_0 holoenzyme, the V_1 complex and the recombinant V_1 subunit *C* to F-actin as revealed by pelleting assays (incubation with 0.2 μM F-actin, followed by centrifugation at 220,000g for 1 h; see Vitavska *et al*., in press).

the V_1 complex from the membrane bound V_0 complex (Gräf *et al*., 1996). From the finding that cytosolic V_1 complexes which lack subunit *C* do no colocalize with F-actin, we concluded that the V_1 complex does not bind to F-actin in vivo. When we reassociated the V_1 complex with recombinant subunit *C*, we observed increased binding of the resulting V_{1C} complex as compared to the *C*-free V_1 complex. From this result we concluded that subunit *C* stabilizes actin binding in the V_1V_0 holoenzyme. Indeed the interaction of the V-ATPase with actin appears to be strongly dependent on the presence of both subunits, *B* and *C*: actin binding of the V_1V_0 holoenzyme which contains both, subunits *B* and *C*, occurs at clearly lower concentrations than binding of the V_1 complex which contains only subunit *B* or binding of the recombinant subunit *C* alone (Fig. 3). Thus subunit *C* may act as a linker protein between the goblet cell apical membrane and the actin based cytoskeleton.

SUPRAMOLECULAR ASPECTS

The V-ATPase as Membrane Energizer in the Insect Midgut

Most animal cell membranes facing a Na^+ rich extracellular medium are equipped with a Na^+/K^+-ATPase that serves, simultaneously, to keep intracellular Na^+ low while securing a high intracellular K^+ content. The presence of the Na^+/K^+-ATPase is of utmost importance and obligatory for many essential processes, such as the control of cell ion content and cell volume, membrane excitability or membrane transport of various solutes, as well as the fine-adjustment of intracellular regulators like pH and Ca^{2+} via Na^+ coupled mechanisms in the plasma membrane. In epithelia, the basolateral Na^+/K^+-ATPase plays, in conjunction with apical Na^+ channels, a pivotal role in transepithelial Na^+ uptake as well as in powering K^+ secretion in concert with apical K^+ channels. Other Na^+ coupled solute movements, e.g. nutrient uptake or Cl^- secretion, also need a functional Na^+/K^+-ATPase.

These systems fail, however, in a Na^+ poor environment which may be unilateral, as is the case for animal tissues which are contiguous to e.g. an apical medium with "freshwater" quality. Such a medium may be extracorporal or, for instance, in the gut. Caterpillar midguts are, indeed, faced with a luminal low Na^+, but high K^+ content and a pH of 12 in the anterior and 9 in the posterior part (Dow, 1986); moreover, their hemolymph is also Na^+ poor and has a K^+ concentration up to 30 mM (Chamberlin, 1989; Dow, 1986). The bilateral Na^+ deficiency is very likely a consequence of the K^+ rich plant diet; as a further consequence, there is no Na^+/K^+-ATPase present in the midgut epithelium (Jungreis and Vaughn, 1977). Instead, the plasma membrane V-ATPase developed as the major player in this tissue, steering ion transport, ionic balance, and generating features that are quite uncommon in non-insect tissues (Wieczorek, 1992).

The larval midgut monolayered epithelium of *Manduca* consists ot two main types of differentiated cells, K^+ secreting goblet cells (Harvey *et al*., 1983) and amino acid absorbing columnar cells (Hanozet *et al*., 1989). The apical membrane of goblet cells contains at least three ion transporters (Wieczorek *et al*., 1989): the V-ATPase which creates a membrane voltage far above 100 mV (Dow and Peacock, 1989; Moffett and Koch, 1988b), cytosol negative; a $K^+/2H^+$ antiporter (Azuma *et al*., 1995) secreting K^+ in exchange for H^+; a Cl^- channel which is active only at high Cl^- concentration (Wieczorek *et al*., 1989). Basolaterally, K^+ channels of different types are prominent (Moffett and Koch, 1991). For transepithelial K^+ secretion the following scenario turned out to be true (see Zeiske, 1992, and Fig. 4): K^+ enters via basolateral channels, driven in passively, partly facilitated by a comparably not too unfavorable chemical K^+ gradient (Moffett and Koch, 1992), and partly by the cytosolic negativity generated by the apical V-ATPase (Moffett and Koch, 1988a,b). Once cytosolic, K^+ exits apically using the electrophoretic antiporter, driven by the apical voltage (Azuma *et al*., 1995; Wieczorek *et al*., 1991).

Midgut V-ATPase: An Energizer of a Facet of Disparate Events

Depending on neighboring, apical or basolateral ion transporters that rely either on the chemical or the

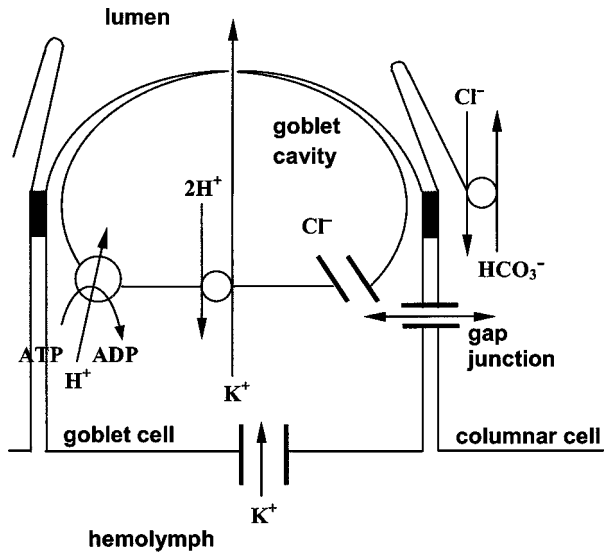

Fig. 4. Ion transport elements in the midgut epithelium of larval *Manduca sexta*. The V-ATPase in the goblet cell apical membrane powers electrophoretic secretion of K^+ via a $K^+/2H^+$ antiport (see Wieczorek *et al*., 1999). Further elements are a Cl^- channel in the goblet cell apical membrane (Wieczorek *et al*., 1989), a Cl^-/HCO_3^- antiport in the columnar cell apical membrane (Chao *et al*., 1989) and K^+ channels in the basal membrane of goblet and/or columnar cells (Zeiske *et al*., 1986).

electrical gradient generated by the V-ATPase, a multitude of different processes powered by the H^+ pump as primary input element can be expected such as intracellular pH regulation versus HCl secretion (Dow, 1992), luminal alkalinization (Dow, 1992) and electrogenic K^+ secretion (Harvey *et al*., 1983). This paragraph will concentrate on the V-ATPase as a key regulator of cell pH.

Effective down-regulation of cytosolic acid threat (caused e.g. by proton influx or excess metabolism) is mandatory for any cell. So far, the caterpillar exhibits, with a Cl^-/HCO_3^- antiporter in the apical membrane of columnar cells (Chao *et al*., 1989), an instrument for syncytial acidification. Clearly, the V-ATPase, depending only on the availability of the chemical (ATP) and transportable (proton) substrate, is prone to eliminate H^+ from the cytosol. To be effective, this must be done by either simultaneously importing a strong cation or by joint exclusion of HCl from the cytosol, as otherwise H^+ transport is stopped early when the pump electromotive force is reached. Chemical or electrical relaxation of the electromotive force is thus required to allow the V-ATPase to function as cell alkalizer by enabling mass fluxes of protons. The apical $K^+/2H^+$ antiporter will tend to collapse the pump electromotive force, at least partly, and so enable also K^+ secretion, whereas the clearance of the cytosol from H^+ will be minimized as a consequence of H^+ reimport after export by the H^+ pump.

The number of transporters and their turnover rates will determine the final outcome, here with the pump, the antiporter and the Cl^- channel in rivalry, as pump cooperation with the antiporter will not, but co-operation with the channel will, eliminate cytosolic protons. As estimated from negatively stained vesicles, the V-ATPase density in the goblet cell apical membrane is extremely high, about $5000/\mu m^2$ (M. Huss and H. Wieczorek, unpublished). While the antiporter density is unknown, their overall transport capacity must not be too small to allow for the very large K^+ fluxes; high turnover rates are most likely (Rosskopf *et al*., 1991) since SDS-PAGE of goblet cell apical membrane does not show major bands except those constituting the V-ATPase. Since Cl^- channels are supposed to have turnover rates of more than 10^6/s (Begenisich and Melvin, 1998), their number may be very small, escaping detectability by SDS-PAGE, but still allow efficient fluxes of Cl^-, and therefore of H^+ with a pump in parallel. In this view, most of the pumped H^+ may recycle via the antiporter and drive K^+ secretion, and a comparably small if not minute fraction will draw Cl^- via the channel and so contribute to deprotonization of the cytosol. The result may be satisfying enough for the needs of the cell as, using artificial K^+ rich saline as the ambient medium, no significant K^+ or pH-gradient across the goblet cell membrane could be observed in electrophysiological experiments (Moffett and Koch, 1992); thus, cell pH is regulated and massive K^+ secretion occurs at the same time. When basolateral K^+ is omitted from the saline, K^+ secretion stops but the goblet cavity pH turns from neutral to acidic (Dow, 1992). We now see the remaining concerted activity of the H^+ pump and the Cl^- channel in the goblet cell membrane while the antiporter falls still. Moreover, the resulting macrosopic process (HCl secretion) is electroneutral and no more electrogenic (active K^+ secretion).

What happens if the cytosolic pH becomes acidic? Intuitively, one expects that a drop in cell pH stimulates the pump, as shown for the yeast vacuolar V-ATPase (Parra and Kane, 1998) but also for that in the kidney intercalated cells (Brown and Breton, 1996), but that at the same time impairs the antiporter activity. We could observe that extracellular acidification below pH 6 reversibly inhibits K^+ secretion (Zeiske *et al*., 2002). Using nystatin-permeabilized epithelia we found, moreover, that basolateral K^+ channels as well as the apical machinery involved in K^+ secretion were inhibited, in a concerted manner, by intracellular acidic pH with a half-maximal effect at pH 5.5 (Zeiske *et al*., 2002). Inhibition of K^+ channels by intracellular H^+ is well-described (Doupnik *et al*., 1995; Harvey, 1995) and thus accounts for the effect of internal H^+ on the basolateral side. Assuming pump stimulation

by H^+ in this epithelium as well, the blocking effect of the internal pH drop should be due to a halt of the antiporter as previously expected. Using cell pH changes induced by salts of weak acids and bases, we could confirm the above mentioned data while keeping the extracellular medium neutral and the membranes intact (Zeiske *et al*., 2002).

CONCLUSION

The results described here illustrate the utility of a comparative physiological approach to the biochemistry and physiology of V-ATPases. The tobacco hornworm model has contributed to our knowledge on V-ATPases by adding novel subunits such as the V_1 subunit F (Gräf *et al*., 1994), by establishing the location of subunits such as that of subunit *G* in the V_1 complex (Lepier *et al*., 1996), by the detection of the only so far known mechanism of physiological regulation, the reversible dissociation of V_1 subunits from the V_0 complex (Gräf *et al*., 1996; Sumner *et al*., 1995), by elucidation of low resolution structures of the V_1 complex (Grüber *et al*., 2000a,b; Radermacher *et al*., 2001), by the examination of macrolide binding to subunit *c* (Huss *et al*., 2002) or by the analysis of the interaction with the actin cytoskeleton (Vitavska *et al*., in press). Part of the research was enabled by the fact that the caterpillar midgut is a rich source for the purification of the V_1V_0 holoenzyme, of the V_1 complex and of the V_0 complex in milligram amounts. We also would like to stress that the caterpillar midgut offers the possibility to investigate a plasma membrane V-ATPase in its physiological context (Zeiske *et al*., 2002). Understanding the mechanism of K^+ secretion in full detail and its role in the context of cell physiology is a challenge for the coming years.

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