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THE INSECT V-ATPase, A PLASMA MEMBRANE PROTON PUMP ENERGIZING SECONDARY ACTIVE TRANSPORT: IMMUNOLOGICAL EVIDENCE FOR THE OCCURRENCE OF A V-ATPase IN INSECT ION-TRANSPORTING EPITHELIA

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Summary

Active electrogenic K^+ transport in insects serves as the energy source for secretion or absorption in gastrointestinal epithelia or for the receptor current in sensory epithelia. In the larval midgut of the tobacco hornworm *Manduca sexta*, a vacuolar-type proton pump (V-ATPase) and a K^+/nH^+ antiport represent the functional elements of the potassium pump. Several immunological findings support the hypothesis that active K^+ transport in other insect epithelia may also be energized by a V-ATPase. In immunoblots, crude homogenates of sensilla-rich antennae and Malpighian tubules of *M. sexta* cross reacted with an immune serum directed to the purified plasma membrane V-ATPase from the midgut; the *M. sexta* midgut V-ATPase cross reacted with polyclonal antibodies to endomembrane V-ATPases from xenic origin. In immunocytochemical investigations of larvae of *M. sexta* and adults of *Antheraea pernyi*, monoclonal antibodies to defined subunits of the purified midgut V-ATPase or polyclonal antibodies to xenic endomembrane V-ATPase labelled the sites of active K^+ transport: the goblet cell apical membrane in the midgut, the brush border of Malpighian tubules and the apical projections of the auxiliary cells in antennal sensilla. The functional mechanism of a primary H^+-pumping V-ATPase and a secondary H^+-dependent K^+ transport postulated for K^+-transporting insect epithelia may be further applicable to active Na^+ or Cl^- transport and would provide a unifying concept for all ouabain-insensitive electrogenic ion transport in insects. The findings from the midgut investigations, however, are the first instance in which a V-ATPase provides an alternative to the Na^+/K^+-ATPase in energizing secondary active transport in animal plasma membranes.

How many primary ion pumps are there in insect ion-transporting epithelia?

In multicellular organisms, regulation and control of intracellular and extracellular fluid volumes and their osmotic and ionic concentrations are accomplished by polarized epithelia interfacing the organism with its environment. Water and solute transport are provided by the integrated function of primary ion pumps, classically the ouabain-sensitive Na^+/K^+-ATPase, carriers and ion channels. In insect gastrointestinal epithelia, reports of a ouabain-insensitive electrogenic apical cation pump have accumulated (Harvey *et al.* 1983). Insects, however, are thought to be unusual not only because they

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are able to secrete K^+ actively, but they have also been shown to be able to reabsorb Cl^- actively (Phillips *et al.* 1986), suggesting the still controversial existence of a primary active apical anion pump in animal cells (Gerencser *et al.* 1988).

In the insect model epithelium, the larval midgut of *Manduca sexta,* transport studies with isolated apical membrane vesicles, biochemical analysis of the apical membrane and purification of the ion-transporting ATPase led recently to the identification of the molecular correlates of the electrogenic cation pump: a H^+-pumping V-ATPase and an electrogenic K^+/nH^+ antiport (Schweikl *et al.* 1989; Wieczorek *et al.* 1991; see Wieczorek, 1992). Since no Na^+/K^+-ATPase was found in the midgut (Jungreis and Vaughan, 1977), the V-ATPase is solely responsible for the energization of epithelial transport. Unusually, the midgut V-ATPase is constitutively localized in the apical plasma membrane and functionally substitutes for the common Na^+/K^+-ATPase, a hitherto extraordinary situation in animal epithelia.

All phytophagous insects, like the *M. sexta* larva, ingest excess potassium and have to control potassium levels by excretion, as most plant tissues contain more potassium than sodium. This circumstance might be one reason why gastrointestinal organs such as midgut and Malpighian tubules and salivary glands and even sensory epithelia, have made use of a controlled transepithelial electrochemical K^+ potential for different purposes. After the discovery of a V-ATPase as the primary ion pump in the model system of the lepidopteran midgut, it seemed reasonable to postulate that a similar transport mechanism is applicable to insect K^+-transporting epithelia in general. Moreover, by replacing the secondary K^+/nH^+ antiport, realized in the midgut, by suitable transport mechanisms, the same molecular scheme is also applicable to secondary active transport of other ion species.

Immunological evidence for a V-ATPase in insect K^+-transporting epithelia

One possible strategy to test this hypothesis is provided by immunocytochemical investigations of the tissues in question, for which a direct biochemical analysis of the ion-transporting membrane would be cumbersome or impossible. The purification of the insect V-ATPase from the plasma membrane of *M. sexta* midgut offered the possibility to produce polyclonal and monoclonal antibodies against the enzyme (U. Klein, A. Lepier, B. Förg-Brey and H. Wieczorek, in preparation).

In a first attempt to probe other insect ion-transporting epithelia for the occurrence of a V-ATPase, crude homogenates of larval Malpighian tubules and adult antennal sensory epithelium of *M. sexta* were tested in immunoblots after sodium dodecyl sulphate polyacrylamide gel electrophoresis with the immune serum directed to the holoenzyme. The polyclonal antibodies were found to cross react with protein bands corresponding in size to the main subunits of the midgut V-ATPase (Klein *et al.* 1991). Furthermore, immune sera directed against the whole enzyme or single subunits of xenic V-ATPases from plant tonoplasts and bovine chromaffin granules cross reacted in immunoblots with corresponding subunits of the midgut V-ATPase (Russell *et al.* 1992) and supported the close immunological relationship of the insect plasma membrane V-ATPase to classical endosomal representatives of the V-ATPases.

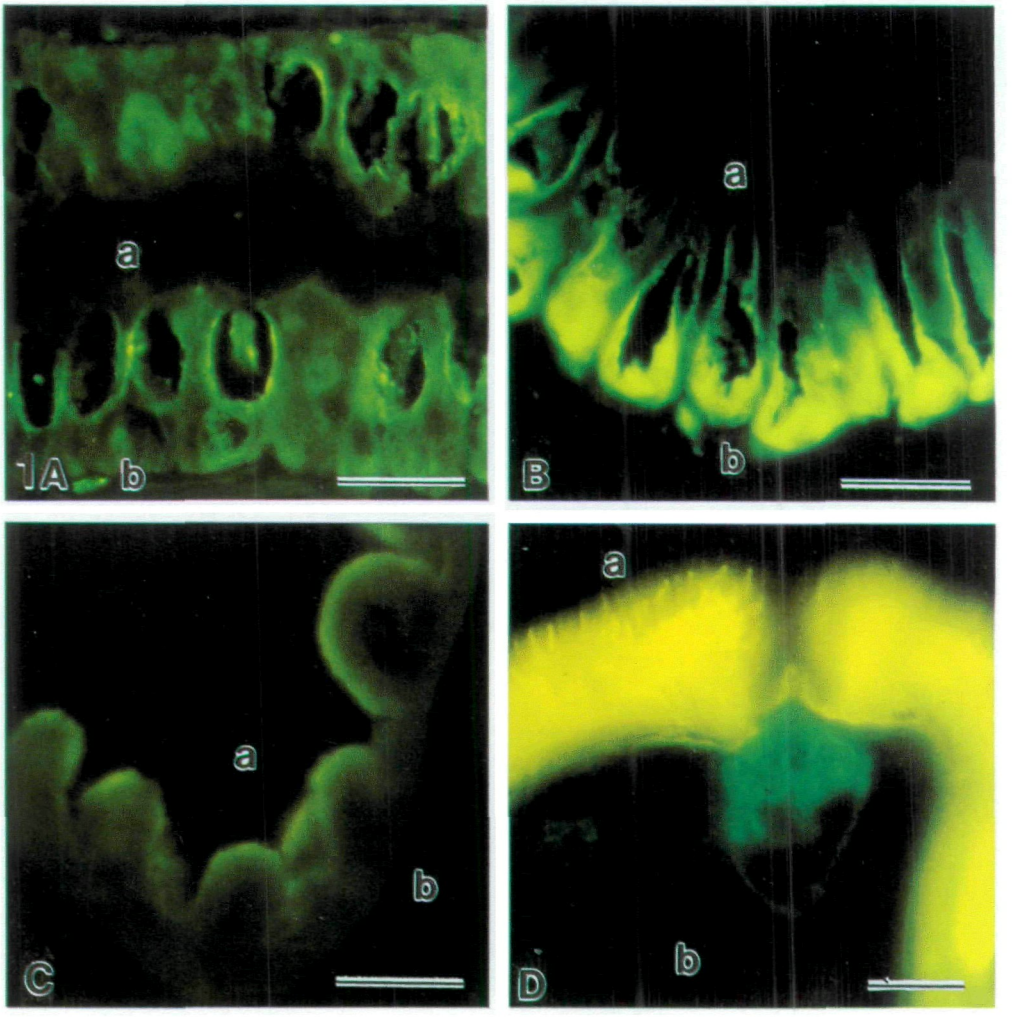


Fig. 1. Apical localization of plasma membrane V-ATPase in insect ion-transporting epithelia: midgut, Malpighian tubules and sensilla. Light microscopy, about 10 μm cryosections, immunofluorescence by monoclonal antibodies to the midgut V-ATPase and FITC-conjugated secondary antibodies (for details of methods, see Klein *et al.* 1991; Klein and Zimmermann, 1991). (A) Posterior; (B) middle midgut of larval *Manduca sexta*, labelling of the goblet cell apical membrane; (C) Malpighian tubule of larval *M. sexta*; labelling of the apical brush border; (D) antennal sensillum coeloconicum of adult *Antheraea pernyi;* labelling of the goblet cell apical membrane. Note the strong autofluorescence of the cuticle. a, apical; b, basal. Scale bars, 10 μm.

When monoclonal antibodies directed against defined subunits of the midgut V-ATPase were used to probe microscopical sections of the midgut epithelium, they demonstrated the localization of the V-ATPase in agreement with the biochemical findings (Wieczorek *et al.* 1986; Schweikl *et al.* 1989). As analysed by light and electron microscopy, clear labelling of the antibodies was found in the foldings of the goblet cell apical membrane (Figs 1A,B, 2A; Klein *et al.* 1991; U. Klein, A. Speiser and H. Wieczorek, in preparation). Similar localization of cross reacting epitopes was obtained by labelling cryosections with a monospecific immune serum to the 56×10^3 M_r subunit of plant tonoplast V-ATPase when analysed by light microscopy (Russell *et al.* 1992). In both cases, slight background staining in the cells' interior was found, which may be attributed either to V-ATPases of endomembrane origin or to the plasma membrane V-ATPase on its biosynthetic route. However, cross reaction with mitochondria was never observed (Fig. 2A,B) despite sequence homologies of F- and V-ATPase in several subunits derived from postulated common ancestor genes (see Nelson, 1992). The apical membrane was labelled in all goblet cells throughout the whole length of the midgut when monoclonal antibodies to the midgut V-ATPase (Fig. 1A,B; U. Klein, A. Speiser and H. Wieczorek, in preparation) or polyclonal antibodies to the plant tonoplast V-ATPase (Russell *et al.* 1992) were applied. Therefore, it is very probable that the V-ATPase serves as the primary energy source all along the midgut, regardless of possible differences in the physiological functions of the three morphologically distinct regions (Dow, 1986).

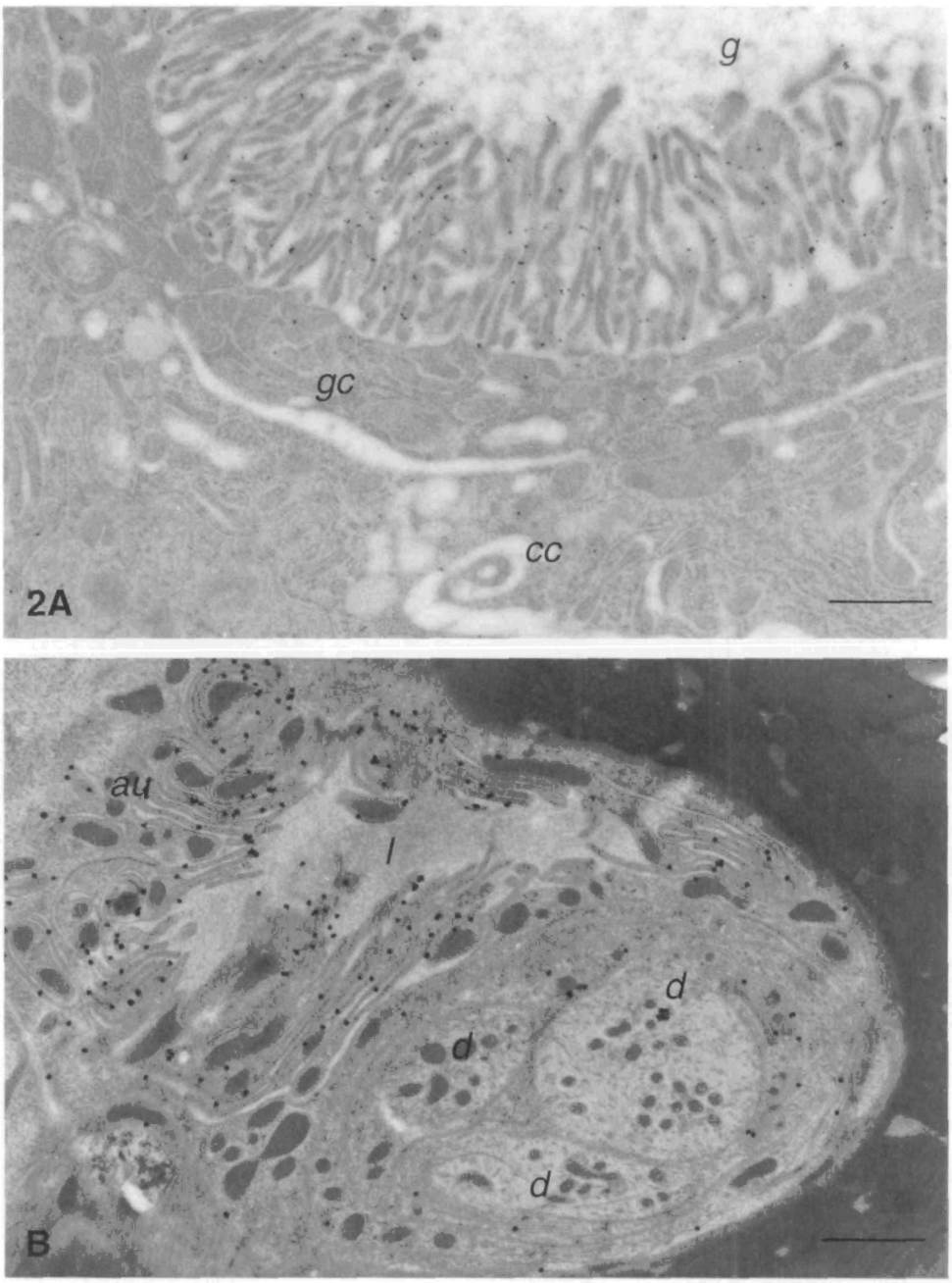
Other insect ion-transporting epithelia were found to contain a V-ATPase on their apical membrane when they were probed immunocytochemically. Malpighian tubules of *M. sexta* were labelled at their brush border by the monoclonal antibodies to the midgut V-ATPase as well as by the polyclonal antibodies to the plant tonoplast V-ATPase (Fig. 1C; Klein *et al.* 1991; Russell *et al.* 1992). As an example of a sensory epithelium, the antennal sensilla of the saturniid moth *Antheraea pernyi* were examined with monoclonal antibodies to the midgut V-ATPase by light and electron microscopy (Figs 1D, 2B; Klein and Zimmermann, 1991). Immunogold labelling was found at the apical membrane of the auxiliary cells in all types of sensilla, regardless of their respective receptor modalities. The localization of the gold particles correlated with typical features of the suspected ion-transporting membranes: intense membrane folding and dense particle studding.

In summary, these immunological investigations of midgut, Malpighian tubules and sensilla provide clear evidence for the occurrence of a V-ATPase in those apical epithelial membranes that are the designated sites of the electrogenic potassium pump. This evidence supports the hypothesis that a V-ATPase is the energizing element for insect epithelial ion transport in general.

Insect ion-transporting epithelia: are they variations on a common theme?

Many insect ion-transporting epithelia are well described morphologically (reviewed by Cioffi, 1984; Chapman, 1985). Functional analysis of the organs as a whole was performed by measuring fluid and solute transport and transepithelial voltages (TEVs) or

short-circuit currents. Variation of saline composition and application of drugs, along with radioisotopic flux and microelectrode analysis, helped to localize ion channels, carriers and pumps at the apical or basolateral face of the epithelial layer. X-ray microanalysis of electrolyte element concentrations in single cellular compartments (e.g. Gupta *et al.* 1977) provided a more substantial basis for functional cellular models (see



Harvey, 1980, 1982). In the lepidopteran midgut, however, intracellular measurements with ion-sensitive microelectrodes complemented by the biochemical analysis led to a well-founded functional model for the apical potassium pump (Wieczorek *et al.* 1991; see also Harvey, 1992; Moffett and Koch, 1992; Wieczorek, 1992; Wolfersberger, 1992; Zeiske, 1992).

In various insect ion-transporting epithelia with many different functions, active electrogenic transport has been demonstrated or proposed, entraining one ion uphill against its electrochemical gradient. The primary active ion pump has invariably been localized in the apical membrane of the respective cells. The area of this membrane is usually highly enlarged by microvilli or membrane foldings, in most cases closely associated with mitochondria (Bradley, 1984). The membrane is studded with 10 nm particles ('portasomes'; Harvey, 1980; Harvey *et al.* 1983) at its inner face, which resemble V-ATPase particles from other sources (see Klein *et al.* 1991; Bowman *et al.* 1992). All epithelia exhibit the common subset of epithelial intercellular junctions; the only features specific to insects are intercellular septate junctions and intracellular scalariform junctions between mitochondria and lateral cell membranes (Noirot-Timothée and Noirot, 1980; Hakim and Baldwin, 1984). However, the coupling or isolating function of the respective junctions is variable and cannot be deduced unequivocally from their morphological appearance (Hakim and Baldwin, 1984). An apical cuticular lining may be present in tissues of ectodermal origin (rectum, sensilla) and a viscous polyanionic matrix may be localized outside the apical membrane (midgut, salivary gland, sensillum, rectum). The meaning of this charged matrix is not yet fully understood (see Gupta, 1989; Harvey, 1992), and even the applicability of the Donnan membrane equilibrium for the calculation of ion concentrations can be questioned (Wiggins *et al.* 1991; Moffett and Koch, 1992).

In the *midgut* of larval *M. sexta*, the electrochemical K^+ gradient, produced by the goblet cells, is used for absorption of amino acids by symporters in the apical microvilli of the neighbouring columnar cells (Giordana *et al.* 1989), for the production and regulation of the highly alkaline pH in the midgut lumen (Dow and O'Donnell, 1990) and for potassium homeostasis in haemolymph and gut lumen (Dow and Harvey, 1988). The activity of the primary proton pump, localized in the goblet cell apical membrane (see above) leads to the uphill transport of K^+ by a K^+/nH^+ antiport, building up a TEV of about 120 mV (Harvey *et al.* 1983; Wieczorek *et al.* 1991).

In the *Malpighian tubules* of plant feeders, such as the fruit fly *Drosophila hydei*, a K^+ gradient, accompanied by a 50 mV TEV, drives the formation of primary urine in the

Fig. 2. Apical localization of plasma membrane V-ATPase in insect ion-transporting epithelia: midgut and sensilla. Electron microscopy, ultrathin sections, immunogold labelling by monoclonal antibodies to the midgut V-ATPase and gold-conjugated secondary antibodies (for details of methods, see Klein *et al.* 1991; Klein and Zimmermann, 1991). (A) Posterior midgut of larval *Manduca sexta*; labelling of the goblet cell apical membrane; *cc*, columnar cell; *g*, apical goblet cavity; *gc*, goblet cell; (B) antennal sensillum styloconicum of adult *Antheraea pernyi*; labelling of the auxiliary cell apical membrane; *l*, apical outer receptor lymph cavity; *au* , auxiliary cell; *d*, sensory dendrites. Note that mitochondria were not labelled. Scale bars, 1 μm.

proximal segment (Wessing *et al.* 1987). Inhibition of urine production by bafilomycin A_1 and amiloride strongly suggested the existence of a H^+-pumping V-ATPase and a K^+/H^+ antiport as molecular elements for the potassium pump (Bertram, 1989; Bertram *et al.* 1991). In the Malpighian tubules of the ant *Formica polyctena* urine production was also inhibited by bafilomycin A_1 and *N*-ethyl maleimide (NEM) (Weltens *et al.* 1992). The immunological evidence described above supports the occurrence of a V-ATPase in the Malpighian tubules of the plant-feeding *M. sexta* larvae. In the Malpighian tubules of the blood-feeding yellow fever mosquito *Aedes aegypti,* a Na^+- and K^+-rich fluid is actively secreted (Aneshansley *et al.* 1988). Preliminary observations showed that in these tubules bafilomycin A_1 also caused the TEV to drop from about 50 mV to 20 mV (T. L. Pannabecker and K. W. Beyenbach, personal communication). In other blood-sucking insects, the bug *Rhodnius prolixus* (Maddrell and Overton, 1989) and the tsetse fly *Glossina morsitans* (Gee, 1976), Na^+ and K^+ are transported uphill to drive the secretion of the isosmotic primary urine with the aid of a presumed apical cation pump. Thus, there is accumulating evidence that a H^+-pumping V-ATPase powers the electrochemical cation gradients for Malpighian tubule secretion.

Most insect *sensilla* contain a high K^+ concentration in the receptor lymph and exhibit TEVs, ranging from 20 to 80 mV, that are thought to energize receptor currents, and an electrogenic potassium pump was proposed in the apical membrane of the auxiliary cells surrounding the sensory cells (Thurm and Wessel, 1979; Küppers und Thurm, 1979). Biochemical investigations of the blowfly proboscis demonstrated the existence of a ouabain- and azide-insensitive K^+-stimulated ATPase in the sensilla-rich labellum but not in the sensilla-poor haustellum (Wieczorek, 1982; Wieczorek and Gnatzy, 1985). Finally, immunological cross reactions of midgut V-ATPase antibodies with the apical membrane of the auxiliary cells in antennal sensilla of *A. pernyi* could be shown (see above). These findings make it very likely that a V-ATPase also serves as the primary ion pump in insect sensilla.

The *rectum* of locusts as well as the *ileum* actively absorb a hypo-osmotic fluid for the production of dry faeces (Phillips *et al.* 1986; Peach and Phillips, 1991). In these epithelia, Cl^- is transported uphill and not a cation. In the rectum, principal cells build up a TEV of about 100 mV. There is good recent evidence for the existence of a vanadate-insensitive apical electrogenic H^+ pump (Thomson and Phillips, 1992). These physiological studies together with preliminary immunocytochemical investigations of the locust rectum (U. Klein, unpublished data) and of crustacean gills (Putzenlechner *et al.* 1992) point to the possibility that a V-ATPase might also energize epithelial transport of anions.

Further insect epithelia thought to contain an apical cation pump are the *salivary glands* of blowflies (Berridge *et al.* 1976), the salivary glands of cockroaches (Gupta and Hall, 1983) and the labial glands of saturniid moths (Kafatos, 1968), which all secrete K^+-rich fluids. Several types of rectal epithelia contain apical ion pumps for reabsorption or secretion of chloride and water: the *rectal complex* of mealworms (O'Donnell and Machin, 1991), the *anal sac* of silverfishes (Küppers *et al.* 1986), the *rectal salt glands* of larval mosquitos (Strange and Phillips, 1985) and the *rectal gills* of larval dragonflies (Komnick, 1977), the latter two being insect larvae living in fresh water.

Although the ionic species transported uphill may vary in the different insect epithelia, I propose that an apical proton pump is the primary energizing element in all cases. Following this hypothesis, the proton-motive force would then entrain secondary active transport of strong ions, which do not interfere with the pH of the milieu, such as K^+ (in midgut, sensilla or salivary glands and Malpighian tubules of plant feeders), Na^+ (in salivary glands and Malpighian tubules of blood-feeders) or Cl^- (in the locust rectum, guiding a passive distribution of Na^+ or K^+). The proton-motive force provides energy in two different ways, which are used to varying extents for the different epithelial functions: e.g. in salivary glands or Malpighian tubules, mainly concentration work is performed in a mass flux of salt entraining water movement, whereas, in sensilla, electrical work drives the receptor current, running through ion channels of the sensory cell after stimulation. Remarkably, it is possible to switch an epithelium's characteristics temporarily by modulating the activity of apical anion channels accompanying uphill cation transport: when salivary glands of the blowfly were stimulated with cyclic AMP, which activates the cation pump but not the apical Cl^- channels, the TEV increased, but no secretion occurred (Berridge, 1980; Gupta, 1989).

Implications: insect ion transport as a general model for the energization of animal plasma membranes

Harvey (1980) and Harvey *et al.* (1983) postulated several different ion pumps, though closely related, to describe the different ion transport mechanisms in insect gastrointestinal epithelia. With the application of the proton pump hypothesis to all ouabain-insensitive insect electrogenic ion transport, the problem of different ion pumps is reduced to a common principle, satisfying the researchers' expectation of a 'simplicity of nature'. The primary energizing element, the proton pump, is identical in all cases. However, the different transepithelial electrochemical ion gradients have to be accomplished by different types of secondary active H^+-dependent ion transport. The early invention of the primary proton pump seems to be more fundamental and conservative than the evolution of a variety of energetically coupled transporters, by which the regulation of osmotic and ionic conditions could specifically be adapted to the various requirements imposed by the different biotopes and diets of the numerous insect species.

In addition, increasing knowledge about other plasma membrane V-ATPases (see Gluck, 1992; Brown *et al.* 1992; Harvey, 1992) may even permit one to speculate that many of the controversial epithelial transport mechanisms in vertebrate tissues, such as the K^+ transport in the stria vascularis of the inner ear (Runhaar *et al.* 1991), are energized by a V-ATPase and not, or not exclusively, by the common Na^+/K^+-ATPase. Thus, V-ATPases in animal plasma membranes may serve not only for acid secretion, as in urinary epithelia (see Gluck, 1992; Brown *et al.* 1992) or osteoclasts (see Chatterjee *et al.* 1992), but also for energizing water and solute transport by secondary active transport processes. A more widespread membrane energization by a V-ATPase would break the dogma of the Na^+/K^+-ATPase as the single energizing ion pump in animal plasma membranes, irrespective of the discovery of further new primary ion pumps (such as the chloride

pump in *Acetabularia acetabulum;* Ikeda *et al.* 1990). It looks as if Harvey's (1980) foreseeing statement turned out to be true: 'It is not that the insect gut is an unfavorable object of study but rather that the analysis of the gut has been uneven'; Wigglesworth's faith in the value of insects as suitable objects for physiological analysis is justified once again (see Wigglesworth, 1948).

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