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Distribution and serotonin-induced activation of vacuolar-type H^+-ATPase in the salivary glands of the blowfly *Calliphora vicina*

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Summary

Secretory activity in blowfly salivary glands is activated by the hormone serotonin. We have investigated the distribution and activity of two cation pumps that are possibly involved with transepithelial ion transport, i.e. Na^+/K^+-ATPase and vacuolar-type H^+-ATPase (V-ATPase). By immunofluorescence labelling of secretory cells, Na^+/K^+-ATPase was localized on the basolateral plasma membrane and V-ATPase on the highly folded apical membrane. Activities of both ATPases were probed in salivary gland homogenates by applying specific inhibitors for these ion pumps, namely ouabain and bafilomycin A_1. In control glands, bafilomycin-A_1-sensitive V-ATPase activity and ouabain-sensitive Na^+/K^+-ATPase activity accounted for 36% and 19%, respectively, of the total ATPase activity. V-ATPase activity increased approximately twofold after stimulation with serotonin, whereas Na^+/K^+-ATPase activity was not significantly affected. Biochemical assays provided evidence that the serotonin-induced activation of V-ATPase activity was accompanied by a recruitment of peripheral V_1 subunits from the cytosol to the plasma membrane, indicative of the assembly of V_0V_1 holoenzymes.

These data show that a V-ATPase located in the apical plasma membranes of the secretory cells is a component of the apical 'potassium pump' that has been identified previously by physiological approaches. The V-ATPase energizes the apical membrane and provides the primary driving force for fuelling a putative K^+/nH^+ antiporter and, thus, for fluid secretion. Serotonin-induced assembly of V_0V_1 holoenzymes might constitute a regulatory mechanism for the control of pump activity.

Key words: vacuolar ATPase, V-ATPase, portasome, bafilomycin, Na^+/K^+-ATPase, serotonin, 5-hydroxytryptamine, regulation, assembly, immunocytochemistry, insect, blowfly, *Calliphora vicina*.

Introduction

The salivary glands of the blowfly are thin tubules that extend as a pair into the abdomen of the animal. The abdominal portions of the gland are secretory and produce a K^+- and Cl^--rich primary saliva. The thoracic portions of the tubule are reabsorptive and modify the ionic composition of the saliva (Oschman and Berridge, 1970). Because of their structural simplicity and the ease in which they can be isolated and manipulated, these organs have been used as a model system for studying the mechanisms involved in transepithelial fluid transport and for their modes of regulation (e.g. Berridge, 1979).

Secretion in the abdominal portion of the salivary glands is stimulated by serotonin [5-hydroxytryptamine (5-HT)], which acts as a hormone in this system (Berridge and Patel, 1968). Exposure to 5-HT results in the activation of two intracellular messenger pathways, namely the cyclic AMP (cAMP) system and the inositol(1,4,5)-trisphosphate/Ca^{2+} system (Heslop and Berridge, 1980; Berridge and Heslop, 1981; Berridge et al., 1983; Litosch et al., 1985; Zimmermann and Walz, 1997, 1999). The 5-HT-induced increase in cAMP activates an electrogenic active K^+ transport mechanism (Berridge et al., 1976; Berridge, 1977). Cl^- movement across the epithelial layer occurs passively through Cl^- conductances that open in response to the 5-HT-dependent rise in cytosolic Ca^{2+} (Berridge et al., 1976; Berridge, 1977). The resulting transepithelial KCl transport then provides the osmotic driving force for fluid secretion.

The molecular correlate of the active K^+ transport mechanism in blowfly salivary glands has not been determined so far. For other insect epithelia that secrete a K^+-rich fluid into a luminal space, such as the midgut, Malpighian tubules and antennal sensilla, evidence has been presented that a vacuolar-type H^+-ATPase (V-ATPase) is localized in the apical membrane of the epithelial cells and is involved with the extrusion of K^+ (Schweikl et al., 1989; Wieczorek et al., 1989; Klein and Zimmermann, 1991; Klein, 1992; Maddrell and O'Donnell, 1992; Harvey and Wieczorek, 1997). V-ATPase is a multi-subunit transporter composed of a catalytic ATP-binding V_1 component that resides on the cytoplasmic side of

the membrane and a membrane-bound V_0 component that forms an H^+ channel (Stevens and Forgac, 1997; Wieczorek et al., 1999; Nishi and Forgac, 2002). The electrochemical H^+ gradient generated by the V-ATPase may then drive a secondary K^+ transport *via* a K^+/nH^+ antiporter, resulting in a net extrusion of K^+ (Wieczorek et al., 1991; Lepier et al., 1994; Harvey and Wieczorek, 1997).

The present study examines the hypothesis that, as in other insect epithelia, K^+ secretion in blowfly salivary glands is powered by a V-ATPase. Ultrastructural methods, ATPase assays and immunotechniques in conjunction with a panel of antibodies against various subunits of V-ATPase have been used to demonstrate that V-ATPase is present within the secretory cells and that it is highly enriched on their apical membranes. Stimulation of the salivary glands with 5-HT results in an activation of V-ATPase activity, whereas Na^+/K^+-ATPase, residing on the basolateral membranes of the secretory cells, is unaffected by hormone treatment. Furthermore, we present the results of experiments that indicate the way in which the V-ATPase is activated upon 5-HT stimulation.

Materials and methods

Animals and preparation

Blowflies (*Calliphora vicina* Robineau-Desroidy) were reared at the Institute. One to four weeks after the emergence of the flies, the abdominal portions of their salivary glands were dissected in Ringer solution containing 155 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $MgCl_2$, 2.7 mmol l^{-1} sodium glutamate, 2.7 mmol l^{-1} malic acid, 10 mmol l^{-1} D-glucose and 10 mmol l^{-1} Tris (pH 7.2).

Electron microscopy

For high-pressure freezing, glands were placed in the 50-μm-deep cavity of aluminium platelets, surrounded with 1-hexadecene and covered with a second platelet. The samples were transferred to a high-pressure freezing apparatus (HPM 010, Balzers Union, Liechtenstein), frozen in liquid nitrogen at a hydrostatic pressure of 2×10^8 Pa (Moor et al., 1980) and freeze-substituted in dry acetone containing 2% OsO_4 as described in detail previously (Klein and Zimmermann, 1991; Zimmermann, 2000). After being washed in dry acetone at room temperature, specimens were embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a CM100 electron microscope (Philips, Eindhofen, The Netherlands) operated at 80 kV or 100 kV.

ATPase activity assays

After dissection, glands were incubated for 3 min in Ringer solution or stimulated for 3 min with 1 μmol l^{-1} 5-HT. Subsequently, glands were homogenized on ice in 0.3 mol l^{-1} sucrose, 0.1 mmol l^{-1} EGTA, 0.1 mol l^{-1} imidazol (pH 7.2), 0.1% β-mercaptoethanol and proteinase inhibitors (leupeptin, 10 μg ml^{-1}; pepstatin A, 10 μg ml^{-1}; Pefabloc SC, 1 mg ml^{-1}). ATPase activity was probed in aliquots of 10 μl, representing the equivalent of four glands. The aliquots were adjusted to 112 mmol l^{-1} NaCl, 30 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} EGTA, 0.1 mol l^{-1} imidazol (pH 7.2), 30 mmol l^{-1} sucrose, 5 mmol l^{-1} Na_2ATP, 0.01% β-mercaptoethanol and 4% dimethyl sulfoxide (DMSO; carrier solution for bafilomycin A_1), resulting in a final volume of 100 μl. In the case of 5-HT-stimulated tissue, 1 μmol l^{-1} 5-HT was added to the homogenizing medium and the assay mixtures. To resolve bafilomycin A_1-, ouabain-sensitive and the remaining ATPase activity, 1 μmol l^{-1} bafilomycin A_1 (Sigma, Deisenhofen, Germany) or 1 mmol l^{-1} ouabain (Sigma) was added to the respective assay mixtures. After incubation for 60 min at 30°C, reactions were stopped by adding 100 μl of 1 mol l^{-1} H_2SO_4. The preparations were then centrifuged for 10 min at 18 000 ***g*** at 4°C; inorganic phosphate (P_i) liberated during the experiment was determined in an aliquot of the supernatants as a phosphomolybdate complex (Bonting et al., 1961). The concentration of phosphomolybdate was measured photometrically; 10 nmoles P_i formed in the 100 μl assay led to a change in light extinction (ΔE) of 0.36 at 707 nm. Enzyme activity values were corrected for background P_i levels by subtracting the extinction values of homogenate-free controls.

Antibodies

Na^+/K^+-ATPase was detected with monoclonal mouse antibody α5–IgG (Takeyasu et al., 1988), which is known to cross-react with the Na^+/K^+-ATPase α-subunit of various insects, including dipteran flies (Lebovitz et al., 1989; Baumann, 1997). Antibody α5 was applied at a concentration of 20 μg ml^{-1} for immunofluorescence labelling and at 1.5 μg ml^{-1} on western blots. Subunits A and E of V-ATPase were identified by polyclonal antibodies made in rabbits against the C-terminus of bovine V-ATPase subunits A and E (Roussa et al., 1998). The antigens exhibit 73% amino acid identity with the corresponding regions in *Drosophila* homologues (*Drosophila melanogaster* vacuolar ATPase subunit A gene, GenBank Acc. No. U19742; Guo et al., 1996). Both antibodies were used at a dilution of 1:200 for immunofluorescence labelling and at 1:2500 for western blotting. Subunit B of V-ATPase was identified on western blots (dilution 1:10 000) by a polyclonal antibody raised in rabbits against a 279-amino-acid region (residues 79–357) of the V-ATPase subunit B from the insect *Culex quinquefasciatus* (Filippova et al., 1998). This region of the *Culex* V-ATPase subunit B shares 98% amino acid identity with that published for *Drosophila* V-ATPase subunit B (Davies et al., 1996). Subunits a and d of V-ATPase were detected with polyclonal antibodies that were raised in guinea pigs by injection of the N-terminal 390-amino-acid-long region of subunit a or complete subunit d of *Manduca sexta* V-ATPase (H. Wieczorek, unpublished). Sequence identity between the *Manduca* antigens and the corresponding *Drosophila* polypeptides amounts to approximately 90% for each of the two subunits (Merzendorfer et al., 1997, 2000; Adams et al., 2000). These antibodies were applied at 1:5000

to 1:10 000 on western blots and at 1:1000 for immunofluorescence labelling. Monoclonal antibody DM 1A against α-tubulin was purchased from Sigma and diluted 1:4000 for western blot analysis. Secondary antibodies conjugated to Cy3 or horseradish peroxidase (HRP) were obtained from Rockland (Gilbertsville, PA, USA) and American Qualex (La Mirada, CA, USA).

Fluorescence microscopy

Salivary glands were fixed for 2 h either in 3% paraformaldehyde and 0.2% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer (PB), pH 7.4, or in 3% paraformaldehyde in PB supplemented with 1 mmol l^{-1} of the cross-linking reagent dithiobis(succinimidyl proprionate) (Pierce, Rockland, IL, USA). After a 10-min wash in PB, specimens were labelled with F-actin probe OregonGreen–phalloidin and the DNA stain DAPI, both used according to the manufacturers instructions (Molecular Probes, Eugene, OR, USA). For indirect immunofluorescence staining, fixed specimens were washed in PB, transferred to 10% (w/v) sucrose in PB for 1 h, incubated overnight in 25% sucrose in PB and frozen in melting isopentane (–165°C). Sections (10 μm thick) were cut on a cryostat and placed on cover slips coated with poly-L-lysine. The sections were successively incubated in (1) 0.01% Tween 20 in phosphate-buffered saline (PBS), (2) 50 mmol l^{-1} NH_4Cl in PBS, (3) PBS and (4) blocking solution containing 1% normal goat serum, 0.8% bovine serum albumin (BSA), 0.1% fish gelatin and 0.5% Triton X-100 in PBS, each step lasting 5 min at room temperature. The samples were then incubated overnight at 4°C with primary antibodies diluted in blocking solution, washed for 3×10 min in PBS and reacted with fluorochrome-tagged secondary antibodies and OregonGreen–phalloidin for 1 h at room temperature. Sections were washed again for 3×10 min in PBS and mounted in Mowiol 4.88 (Farbwerke Hoechst, Frankfurt, Germany) containing 2% n-propyl gallate. Fluorescence images were recorded with a Zeiss LSM 510 or a Zeiss LSM 5 PASCAL confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

Immunoblot analysis

Salivary glands were homogenized on ice in reducing sample buffer (Carl Roth, Karlsruhe, Germany). The preparations were heated for 5 min to 60°C and centrifuged for 10 min at 18 000 ***g*** to remove non-solubilized material. Proteins were separated on sodium dodecylsulphate (SDS) 12% polyacrylamide gels in the Laemmli buffer system (Laemmli, 1970) and electrotransferred onto nitrocellulose sheets. After being incubated for 30 min with 5% low-fat dry milk in Tween buffer (0.1% Tween 20, 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris, pH 7.5), blots were reacted for 2–16 h with primary antibodies diluted in Tween buffer, washed several times with Tween buffer and an intermittent treatment in a solution containing 2 mol l^{-1} urea, 1% Triton X-100 and 0.1 mol l^{-1} glycine (pH 7.5), reacted for 1 h with the appropriate HRP-coupled secondary antibody and finally rinsed extensively with Tween buffer. Bound antibody was visualized by enhanced chemiluminescence (Pierce, Rockland, IL, USA).

Biochemical analysis of V-ATPase distribution

Non-stimulated or 5-HT-stimulated glands were homogenized in extraction buffer containing 70 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 50 mmol l^{-1} Tris (pH 7.5), 20 mmol l^{-1} dithiothreitol and a cocktail of protease inhibitors (see above). Membranes and cytosolic proteins were then separated by centrifugation for 30 min at 150 000 ***g*** at 4°C. Proteins within the supernatant fraction were precipitated by the method of Wessel and Flügge (1984), solubilized in sample buffer and heated to 60°C for 3 min. The pellet fraction was washed twice with extraction buffer, resuspended in sample buffer and also heated to 60°C. Western blots were carried out as described above. To determine the relative amount of each protein within the pellet and the supernatant, images of the western blots were recorded with a cooled CCD camera and analysed densiometrically by use of the software program Metamorph (Universal Imaging Corp., West Chester, PA, USA).

Statistical analysis

Data are presented as means ± S.D. Statistical comparisons were made by an independent Student's *t*-test. *P* values of <0.001 were considered as highly significant; *P* values of >0.2 were considered as nonsignificant.

Results

Morphology of the secretory cells

The secretory portion of the salivary glands is composed of a single layer of uniformly differentiated epithelial cells (Oschman and Berridge, 1970; Fig. 1A). The cells form a simple tubule with a central lumen into which saliva is secreted upon 5-HT stimulation. Laterally, the cells are tightly coupled by septate junctions (Skaer et al., 1975; Zimmermann, 2000), providing a border between the apical and the basolateral domains of the plasma membrane. On the basal side of the cell, interdigitating infoldings of the plasma membrane form a labyrinth that extends approximately one-tenth of the height of the cell towards the apical pole. On the luminal side of the cell, the surface is involuted deeply into the cell to form a branching system of canaliculi that almost reach to the basal labyrinth. The apical surface is further enlarged by elaborate microvilli that cover both the luminal portion and the canaliculi. These microvilli are conspicuously flattened, resulting in a moiré pattern in some areas of the sections (Fig. 1A).

High magnification views reveal that the cytoplasmic aspect of the apical membrane is almost entirely covered by an electron-dense coat approximately 15 nm in thickness (Fig. 1B). This coat appears to be composed of densely packed 10-nm-wide particles attached to the membrane by a short stalk (arrowheads in Fig. 1B). The particles thus resemble portasomes, the structural correlate of V-ATPase (Gupta and Berridge, 1966; Harvey, 1980; Klein and Zimmermann, 1991;

Klein, 1992). No particles have been detected on the basolateral domain of the plasma membrane (Fig. 1C), suggesting that portasomes are localized exclusively on the apical domain.

Identification of V-ATPase and Na^+/K^+-ATPase within the salivary gland

Transepithelial electrolyte and water transport in animals is energized by active ion transport mechanisms, such as the Na^+/K^+-ATPase or the V-ATPase. Western blot analysis of blowfly salivary gland with an antibody against the catalytic α-subunit of Na^+/K^+-ATPase and antibodies against various subunits of the V_0 portion and of the V_1 portion of V-ATPase suggests that both ion pumps are present within the secretory cells (Fig. 2). The V-ATPase antibodies used for these

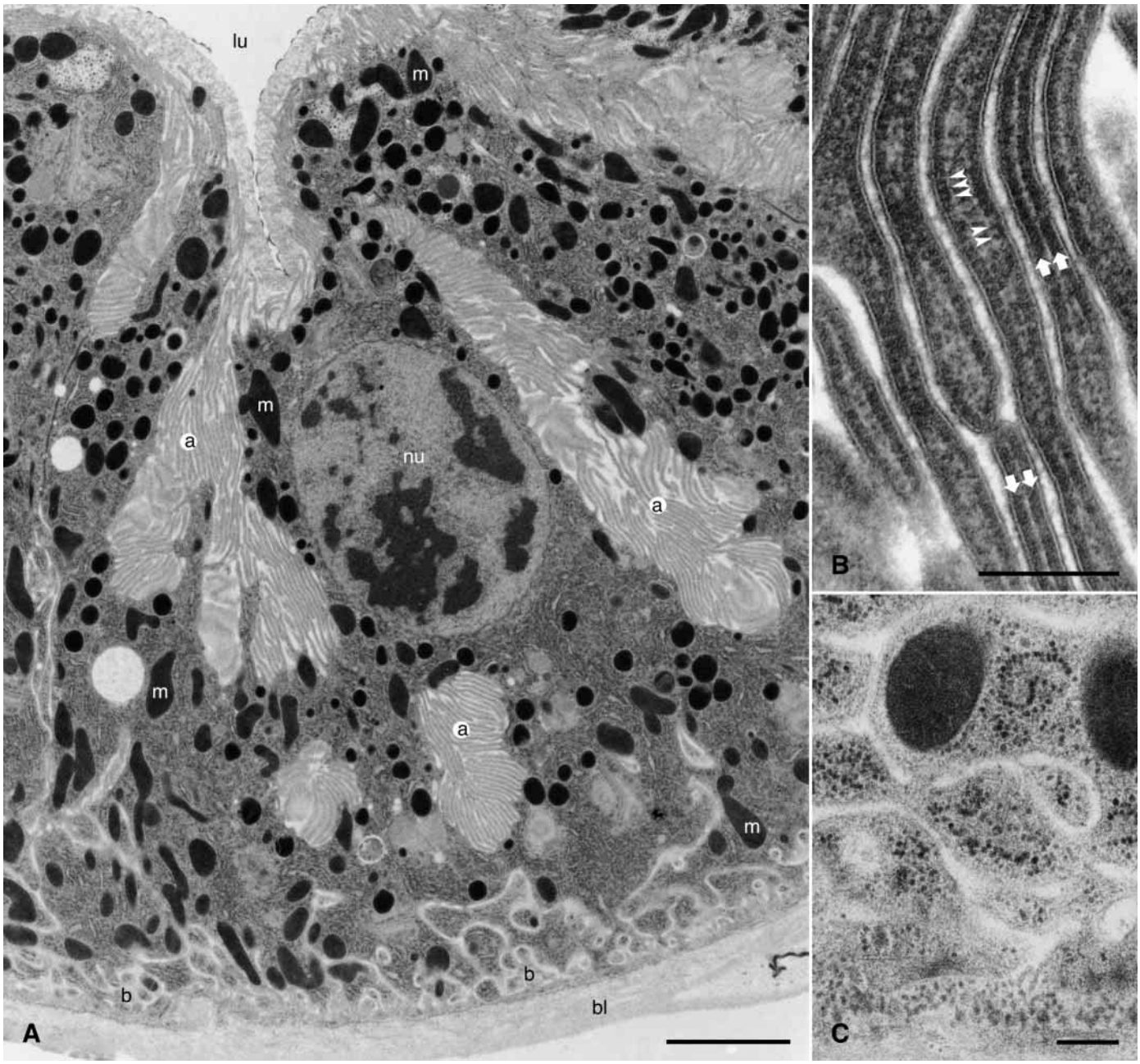

Fig. 1. Morphology of secretory cells in a high-pressure-frozen freeze-substituted salivary gland. (A) Part of a cross-section through a non-stimulated salivary gland. The gland is composed of a single layer of secretory cells and enclosed by a basal lamina (bl). The apical surface (a) of the secretory cells is covered by spatulate microvilli and invaginates deeply into the cell. Numerous mitochondria (m) reside close to the apical (a) and the basolateral (b) domain of the plasma membrane. lu, lumen of the salivary gland; nu, nucleus. (B) High-magnification view of spatulate microvilli on the secretory cell apical domain. The cytoplasmic aspect of the plasma membrane is studded with particles (arrowheads). In most areas of the plasma membrane, these particles are too densely packed to be revealed individually (arrows). (C) The basolateral domain of the plasma membrane lacks such particles. Scale bars, 2.5 μm (A), 200 nm (B,C).

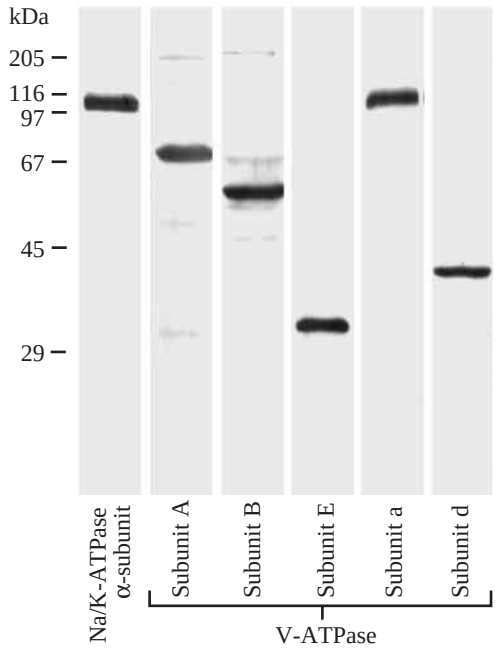

Fig. 2. Detection of Na^+/K^+-ATPase and V-ATPase within salivary glands. Western blots of salivary gland proteins were probed with an antibody against Na^+/K^+-ATPase α-subunit and antibodies against various subunits of V-ATPase. All antibodies intensely label a band of the appropriate molecular mass.

experiments were raised against V-ATPase subunits of other insects or of vertebrates; the antigens, however, display a high homology with the corresponding regions in the respective V-ATPase subunits of *Drosophila*, a dipteran fly closely related to *Calliphora* (see Materials and methods). Each of the antibodies intensely labelled a band of the appropriate electrophoretic mobility on western blots of blowfly salivary glands (anti-Na^+/K^+-ATPase α-subunit, ~110 kDa; anti-V-ATPase subunit A, ~74 kDa; anti-subunit B, ~58 kDa; anti-subunit E, ~32 kDa; anti-subunit a, ~115 kDa; anti-subunit d, ~39 kDa).

To examine the expression of Na^+/K^+-ATPase and V-ATPase within salivary glands by an alternative method, we measured the ATPase activity of homogenized glands in the absence and presence of a selective inhibitor for each transporter. When bafilomycin A_1, a highly specific inhibitor of V-ATPase (Bowman et al., 1988; Dröse and Altendorf, 1997), was applied to homogenized salivary glands, it inhibited ATPase activity with an IC_{50} of approximately 3 nmol l^{-1} (Fig. 3A). At a saturating bafilomycin concentration of 1 μmol l^{-1}, ATPase activity was reduced by 43.1±5.7%, suggesting that a relatively large amount of the total ATPase activity was provided by the bafilomycin-sensitive V-ATPase under our assay conditions. When ouabain, a specific inhibitor of Na^+/K^+-ATPase, was applied at 1 mmol l^{-1}, a concentration easily within the saturating range in other systems (e.g. Lebovitz et al., 1989), ATPase activity was reduced by 18.6±4.5% (Fig. 3B). These findings suggest that approximately 60% of the P_i detected within the assay was due to the activity of V-ATPase and Na^+/K^+-ATPase, supporting the conclusion that secretory cells are equipped with both ion transporters. The remaining P_i may have been liberated by other ATPases present within the homogenate and/or may have resulted from endogenous P_i trapped within vesicles.

Distribution of V-ATPase and Na^+/K^+-ATPase within the secretory cells

The localization of V-ATPase and Na^+/K^+-ATPase within non-stimulated secretory cells was determined by immunofluorescence labelling of cryostat sections (Fig. 4). In order to provide a spatial reference for the position of the immunoreactive structures, specimens were co-labelled with OregonGreen–phalloidin, a probe for filamentous actin. By this procedure, the apical membrane, including the canaliculi, was visualized as a brightly fluorescent structure that embraced the

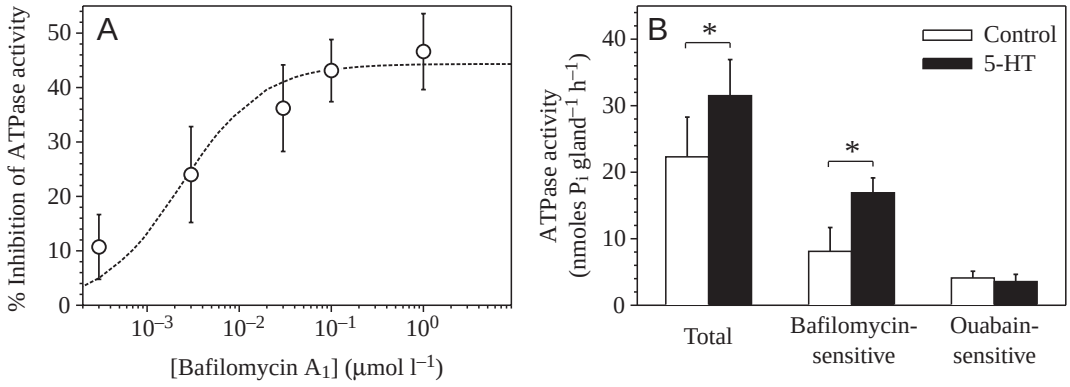

Fig. 3. Bafilomycin A_1-sensitive and ouabain-sensitive ATPase activity in homogenized salivary glands. (A) Dose–response curve for the effect of bafilomycin A_1 on the total ATPase activity within the homogenate. Values represent the means ± S.D. of 4–11 experiments for each concentration. Half-maximal inhibition (IC_{50}) is reached at approximately 3 nmol l^{-1} bafilomycin A_1. (B) Effect of 5-hydroxytryptamine (5-HT) on total ATPase activity, bafilomycin-sensitive ATPase activity and ouabain-sensitive ATPase activity. Values represent the mean ± S.D. of 5–16 experiments; asterisks indicate a significance level of $P<0.001$. In 5-HT-stimulated salivary glands, bafilomycin-sensitive ATPase activity is enhanced by a factor of approximately 2, whereas ouabain-sensitive ATPase activity does not change significantly.

nuclei, whereas the basal surface of the secretory cells was identified by weak reactivity with phalloidin (Fig. 4A,E,I; Zimmermann, 2000).

Anti-Na^+/K^+-ATPase stained the basal and lateral sides of the secretory cells, whereas the apical domain exhibited no immunoreactivity for Na^+/K^+-ATPase (Fig. 4B,F,J). By contrast, antibodies against V-ATPase subunit A (data not shown), subunit E (Fig. 4C,G,K) and subunit d (Fig. 4D,H,L) bound to the apical membrane including the invaginations. Staining of the invaginations with anti-V-ATPase antibodies was not homogeneous over the entire membrane domain but varied in intensity (Fig. 4C,D). This may have been due to the compact structure of the microvilli and cross-linkage of proteins within the microvilli, resulting in poor accessibility of the antibodies to the antigens. Anti-V-ATPase antibodies further bound to vesicular structures within the secretory cells (Fig. 4C,D,K,L), indicating the presence of V-ATPase on cell organelles.

These results suggest that Na^+/K^+-ATPase is restricted to the basolateral membrane. V-ATPase is concentrated at the apical

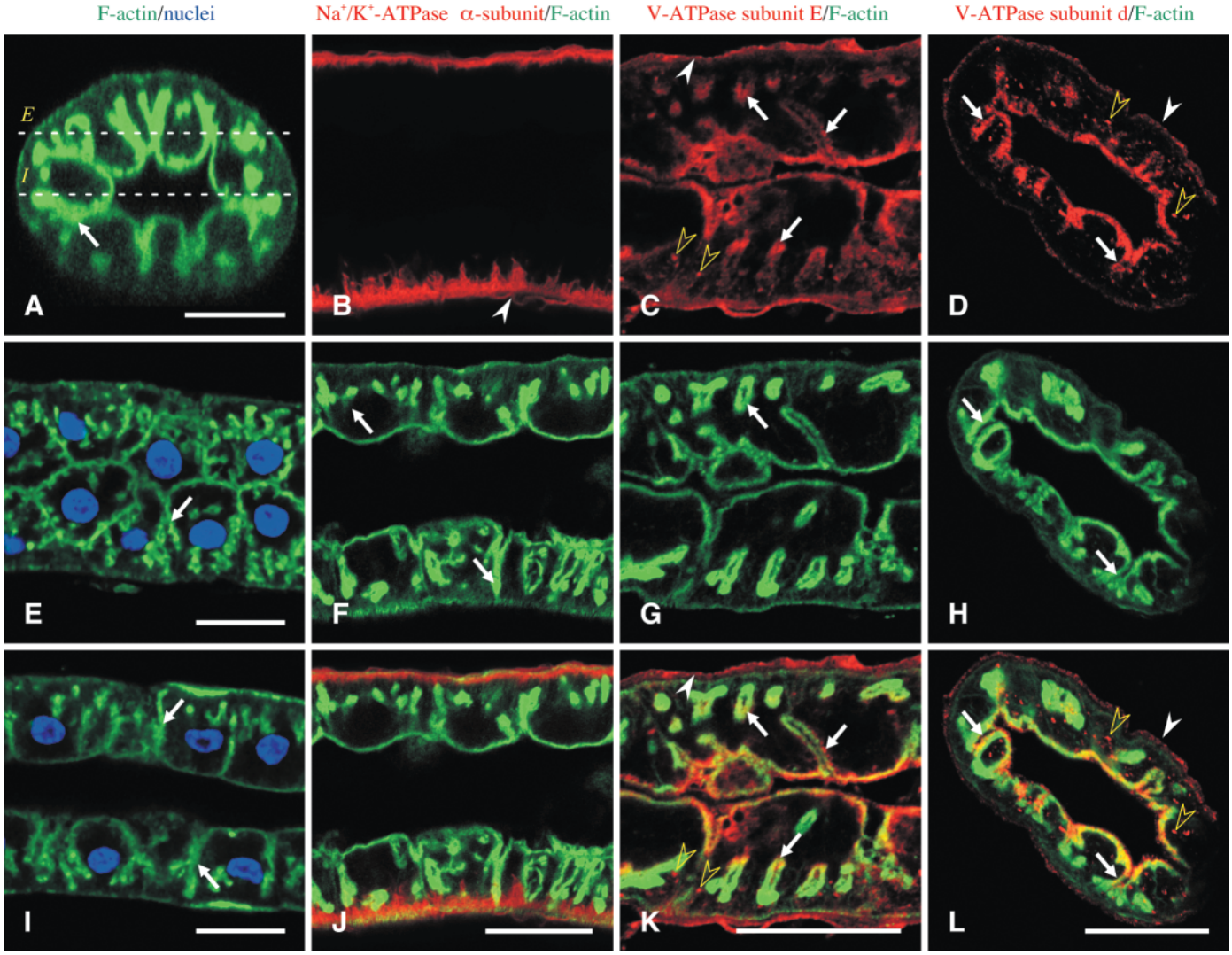

Fig. 4. Immunofluorescence localization of Na^+/K^+-ATPase and V-ATPase. (A,E,I) Labelling with OregonGreen–phalloidin (green) reveals the organization of the apical domain of the secretory cells. (A) An optical cross-section through an entire salivary gland and (E,I) two horizontal planes through a gland as indicated by broken lines in (A). The apical surface of the secretory cells is involuted deeply into the epithelium to form a branching system of canaliculi (arrows) that embrace the DAPI-stained nuclei (blue in E,I). (B,F,J) Double-labelling of a longitudinal section through a salivary gland with antibody α5 against Na^+/K^+-ATPase α-subunit (red) and phalloidin (green); (C,G,K) a longitudinal section co-stained with an antibody against V-ATPase subunit E (red) and phalloidin (green); (D,H,L) a cross-section co-labelled with an antibody against V-ATPase subunit d (red) and phalloidin (green); (J,K,L) composite images of phalloidin and antibody labelling. The basolateral domain but not the apical domain of the plasma membrane is intensely labelled for Na^+/K^+-ATPase. Antibodies against the various V-ATPase subunits stain the luminal side of the cell and the canaliculi (arrows). Moreover, some vesicular structures within the secretory cells are labelled by V-ATPase antibodies (yellow arrowheads in C,D,K,L). Immunofluorescence associated with the basal lamina (white arrowheads) surrounding the epithelial tubule is due to nonspecific binding of secondary antibodies. Scale bars, 20 μm.

domain of the plasma membrane and is present in smaller amounts on cell organelles. Staining patterns with Na^+/K^+-ATPase and V-ATPase antibodies and with phalloidin were similar in non-stimulated (Fig. 4) and 5-HT-stimulated glands (data not shown), indicating that exposure to 5-HT does not lead to dramatic reorganization of the membrane domains or redistribution of pump molecules within the cells.

Effect of 5-HT on ATPase activity

If any of the transporters identified above is involved in driving 5-HT-induced fluid secretion, the activity of the transporter(s) should increase upon stimulation with 5-HT. To examine this possibility, the ATPase activity of both transporters was determined in salivary glands treated with 5-HT at a concentration known to be saturating in physiological assays (Berridge and Prince, 1972). Under these conditions, total ATPase activity within the homogenate increased from 22.3±6.0 nmol P_i $gland^{-1}$ h^{-1} to 31.5±5.4 nmol P_i $gland^{-1}$ h^{-1} (Fig. 3B). Ouabain-sensitive ATPase activity did not differ significantly between control and 5-HT-stimulated tissue, whereas bafilomycin-sensitive ATPase activity was increased by a factor of approximately 2 after exposure to 5-HT. The increase in bafilomycin-sensitive ATPase activity (8.8 nmol P_i $gland^{-1}$ h^{-1}) compares well with the increase in total ATPase activity (9.2 nmol P_i $gland^{-1}$ h^{-1}), suggesting that V-ATPase may be the main ATPase that is activated in response to 5-HT in our experimental assay.

Effect of 5-HT on the assembly state of V-ATPase

It has been proposed that assembly/disassembly of the V_0V_1 holoenzyme is involved in the regulation of V-ATPase activity in various systems (Sumner et al., 1995; Kane, 1995; Kane and Parra, 2000; Wieczorek et al., 2000). To examine whether this mode of regulation also occurs in blowfly salivary glands, non-stimulated and 5-HT-stimulated glands were homogenized, a crude membrane preparation was isolated by high-speed centrifugation, and the relative distribution of the respective proteins between the pellet and the cytosolic supernatant was probed on western blots.

In control tissue, only a minor fraction of the V_1 subunits A (24.4±13.4%) and E (41.7±7.6%) was detected within the pellet (Fig. 5). More than 90% of the V_0 subunits a and d and essentially all Na^+/K^+-ATPase α-subunits were retained in the pellet, demonstrating that membranes were almost entirely recovered in this fraction. Upon 5-HT stimulation, the relative amount of V_1 proteins was significantly increased within the pellet fraction (subunit A, 56.6±8.3%; subunit E, 63.6±7.3%), whereas no change could be detected in the relative distribution of the V_0 subunits or of the Na^+/K^+-ATPase α-subunit. To exclude the possibility that cytosolic proteins were generally enriched in the pellet after 5-HT stimulation, we also determined the relative distribution of α-tubulin between both fractions. Moreover, we analysed the total amount of protein in the pellet and the supernatant on SDS gels stained with Coomassie Blue. Neither method showed any significant difference between the pellet and supernatant in response to 5-HT stimulation. We thus conclude that 5-HT stimulation leads to a specific recruitment of V_1 subunits to the membranes.

Discussion

V-ATPase energizes K^+ transport across the secretory cells

Electrophysiological studies performed in the 1970s have demonstrated that fluid secretion in blowfly salivary glands is powered by an active electrogenic K^+ transport mechanism (Berridge et al., 1976; Berridge, 1977). The present results provide evidence that an apically localized V-ATPase is involved in this K^+ transport system. First, electron microscopy

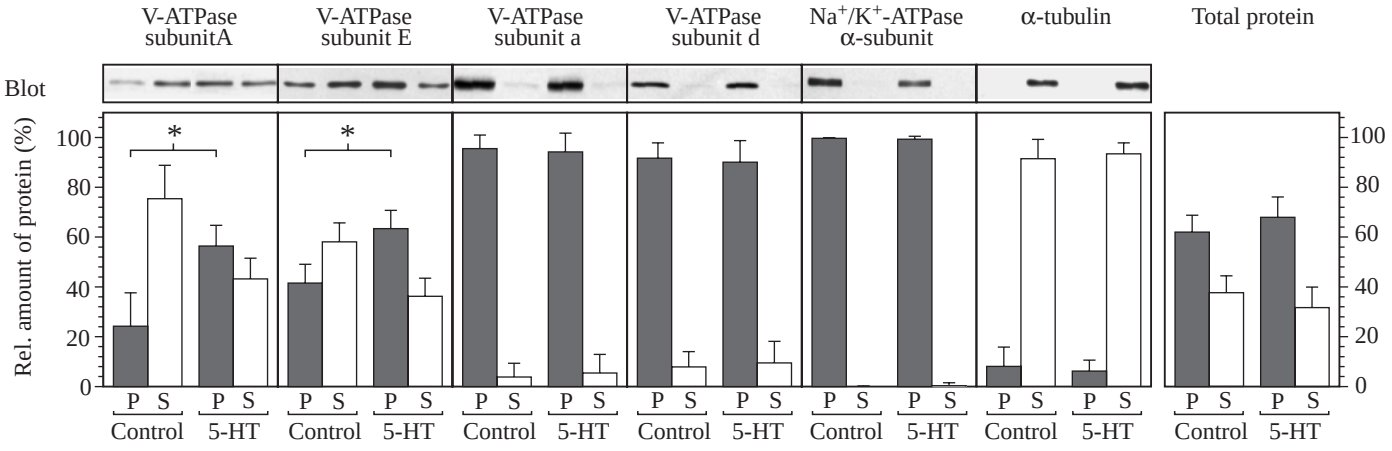

Fig. 5. Assembly state of V-ATPase in control and 5-hydroxytryptamine (5-HT)-stimulated salivary glands. The glands were homogenized and separated by high-speed centrifugation into a pellet fraction (P) and a supernatant (S). The relative distribution of various proteins between the two fractions was then probed by western blot analysis. Upon stimulation with 5-HT, the amount of the V_1 component proteins (subunits A and E) increases within the pellet and diminishes in the supernatant. No significant change in response to 5-HT stimulation is observed in the distribution of integral membrane proteins (Na^+/K^+-ATPase α-subunit; V_0 component proteins, subunits a and d), of α-tubulin or of the total amount of protein between both fractions. Values represent the mean ± S.D. of six independent experiments. Asterisks indicate a significant difference (P<0.001).

has visualized portasomes, the structural correlate of V-ATPase, on the apical membrane of the secretory cells. Although portasomes have been described previously in chemically fixed blowfly salivary glands (Oschman and Berridge, 1970), application of cryofixation in combination with freeze-substitution provides a new aspect. In chemically fixed tissue, individual portasomes can be easily identified as they are well separated from each other (figs 7 and 9 in Oschman and Berridge, 1970; B. Zimmermann, unpublished results). In cryofixed tissue, however, the entire cytoplasmic face of the apical membrane is covered by an electron-dense coat, thus making it almost impossible to identify individual portasomes. This finding suggests that portasomes are very tightly packed on the apical membrane *in vivo*. Second, on Western blots of salivary glands, antibodies against several subunits of the V_0 and V_1 components of V-ATPase cross-react with proteins of the appropriate molecular mass. On cryostat sections, these antibodies label the apical membrane of the secretory cells, the membrane area that is covered by portasomes, which has been implicated previously in housing the K^+ transport mechanism. V-ATPase is also detected on intracellular organelles that may represent acidic organelles, such as endosomes, lysosomes and Golgi-derived vesicles (Stevens and Forgac, 1997; Nishi and Forgac, 2002). However, considering the large area of the apical membrane and the small number of V-ATPase-positive organelles, it is reasonable to assume that the relative amount of V-ATPase on intracellular organelles is low compared with the amount of V-ATPase on the apical domain and that changes in V-ATPase activity and assembly during our experiments can be largely attributed to pump molecules within the apical domain of the secretory cells. Third, ATPase activity in homogenized salivary glands is partially blocked by the V-ATPase-specific inhibitor bafilomycin A_1. Sensitivity to bafilomycin A_1 is within the nanomolar range and, thus, similar to that reported for V-ATPase in other systems (Dröse and Altendorf, 1997). The finding that total ATPase activity is reduced by almost 45% at saturating bafilomycin A_1 concentrations supports the concept that V-ATPase is present at numbers high enough to provide the driving force for fluid secretion. Finally, 5-HT stimulates total ATPase activity in our *in vitro* assay, and V-ATPase accounts for the majority if not all of the additional ATPase activity. We thus conclude that the apical membrane of the secretory cells is highly enriched with V-ATPase and that 5-HT treatment leads to an activation of this ion pump.

Further support for the concept that V-ATPase-driven H^+ transport energizes the apical membrane for K^+ extrusion into the lumen of the salivary glands could be provided by *in vivo* experiments with bafilomycin A_1. Application of bafilomycin A_1 at concentrations as high as 10 μmol l^{-1}, however, has no obvious effect on the 5-HT-induced transepithelial potential changes of isolated salivary glands, the electrophysiological response accompanying secretion (B. Zimmermann, unpublished results). We suggest that this results from the limited accessibility of bafilomycin A_1 to V-ATPase *in vivo*. Since the drug was applied from the bath side and since the paracellular pathway is occluded by septate junctions (Skaer et al., 1975; Zimmermann, 2000), bafilomycin A_1 must cross the basolateral membrane and the cytoplasm in order to reach its target site. Because of its high lipophilicity (Dröse and Altendorf, 1997), however, bafilomycin A_1 may accumulate in the basolateral membrane and become entrapped in this membrane domain. Similar problems have been reported for other systems with externally applied bafilomycin A_1 (Dröse and Altendorf, 1997; Beyenbach et al., 2000; Boudko et al., 2001).

In conclusion, active K^+ transport across the apical membrane of blowfly secretory cells may occur by a mechanism similar to that in other insect epithelia. In the goblet cells of the midgut of *Manduca sexta*, the system that has been studied most thoroughly in this respect, V-ATPase establishes an electrochemical gradient across the apical membrane that is then used for K^+ transport *via* a K^+/nH^+ exchanger (Wieczorek et al., 2000). The combined action of both transporters, V-ATPase and K^+/nH^+ exchanger, results in a net flux of K^+ from the cytosol into the midgut lumen. Future studies of the blowfly salivary gland should determine whether this system also has a K^+/nH^+ exchanger.

Regulation of V-ATPase

Several mechanisms have been proposed to play a role in the control of V-ATPase activity (Stevens and Forgac, 1997; Wieczorek et al., 2000; Nishi and Forgac, 2002): (1) the density of V-ATPase molecules within the plasma membrane may be changed by shuttling pump molecules between an internal vesicular pool and the cell surface; (2) disulphide bond formation between cysteine residues in subunit A may lead to reversible inhibition of V-ATPase activity; (3) the coupling efficiency of proton transport and ATPase activity may change; (4) V-ATPase may be activated or inhibited by regulatory proteins; and (5) the active V-ATPase holoenzyme may reversibly dissociate into its inactive V_0 and V_1 components.

Our observations suggest that the first of the above mechanisms, namely changes in pump density within the plasma membrane *via* exo/endocytotic pathways, does not play a prominent role in the regulation of V-ATPase activity in blowfly salivary glands. Electron microscopy has not provided evidence of any 5-HT-induced changes in cell morphology indicative of the shuttling of pump molecules between a vesicular pool and the cell surface. Moreover, immunofluorescence microscopy has not visualized a redistribution of V-ATPase-immunoreactive vesicles within the secretory cells upon exposure to 5-HT. Concerning mechanisms 2–4, there is no evidence so far that supports or undermines their occurrence in salivary glands. The finding that 5-HT treatment stimulates a recruitment of V_1 sector proteins to the membranes suggests, however, that mechanism 5, an assembly/disassembly of the V_0V_1 holoenzyme, might be used to control V-ATPase activity within the secretory cells. Since both the amount of membrane-associated V_1 sector subunits and V-ATPase activity roughly double upon 5-HT stimulation, it may be further proclaimed that the assembly of

the V-ATPase holoenzyme is directly linked to the activation of V-ATPase.

Attempts to observe 5-HT-induced assembly of V-ATPase by electron microscopy or immunofluorescence microscopy have failed. The reason for this may be that the 5-HT-dependent change in the state of V-ATPase assembly is not an all-or-nothing effect. As deduced from the biochemical assay, the amount of membrane-bound or cytosolic V_1 sectors changes by a factor of about two, and neither of the above methods may be sensitive enough to detect this shift. Alternatively, unassembled V_1 sectors may not be freely diffusible and able to spread throughout the cytoplasm but may remain in the vicinity of the apical membrane. V_1 sectors could be spatially restrained in their mobility by binding to actin filaments within the microvilli or by interaction with other membrane-associated proteins. Studies on osteoclasts have demonstrated that V-ATPase subunit B interacts with actin filaments *in vitro* and *in vivo* (Lee et al., 1999; Holliday et al., 2000). Moreover, an isoform of V-ATPase subunit B has been shown to contain a C-terminal PDZ-binding motif and to associate with the PDZ protein Na^+/H^+ exchanger regulatory factor (Breton et al., 2000).

A reversible assembly/disassembly of the V-ATPase holoenzyme has been demonstrated previously in two other systems. In goblet cells of *Manduca* midgut, V-ATPase dissociates into its components during moulting or starvation (Sumner et al., 1995; Gräf et al., 1996). Similarly, a rapid dissociation of the V_1 sector from the V_0 sector occurs on the yeast vacuole upon glucose deprivation (Kane, 1995; Parra and Kane, 1998). In both cases, the intracellular messenger system that regulates the status of V-ATPase assembly has remained elusive. For yeast, conventional second messenger systems, such as the cAMP and the protein kinase C pathways, have been excluded (Parra and Kane, 1998). Recent studies on yeast have identified a protein complex involved with the regulation of V-ATPase assembly (Seol et al., 2001); the final link between the glucose level in the medium and the assembly of the V-ATPase holoenzyme, however, is still missing.

For the blowfly salivary gland, it is well documented that the active K^+ transport mechanism is regulated *via* the cAMP pathway (Berridge et al., 1976; Berridge, 1977). It may thus be presumed that the assembly state and activity of V-ATPase are controlled by cAMP and protein kinase A. We have started to examine this hypothesis but preliminary results indicate that the modes of V-ATPase regulation are far more complex than expected.

Function of Na^+/K^+-ATPase in blowfly salivary gland

In addition to the apical V-ATPase, the plasma membrane of the secretory cells contains another ion pump, Na^+/K^+-ATPase, that resides on the basolateral membrane. Stimulation with 5-HT does not affect Na^+/K^+-ATPase activity, suggesting that Na^+/K^+-ATPase does not have a fundamental role in secretion. This conclusion is further supported by pharmacological experiments on isolated salivary glands, demonstrating that the rate of fluid secretion and the ionic composition of the saliva during 5-HT stimulation do not change after addition of ouabain (Berridge and Schlue, 1978). Na^+/K^+-ATPase may thus be required only for maintaining the electrochemical Na^+ and K^+ gradient across the basolateral membrane in resting glands, as suggested by Berridge and Schlue (1978).

Conclusions

Our results implicate a V-ATPase as the ion pump that provides the driving force for K^+ extrusion into the lumen of blowfly salivary glands. The apical membrane of blowfly salivary gland cells houses a massive number of V-ATPase molecules, and V-ATPase activity is stimulated by 5-HT. The results of biochemical assays suggest further that the 5-HT-dependent regulation of pump activity involves the assembly of V_0 and V_1 sectors to the V_0V_1 holoenzyme. Since there is extensive knowledge on second messenger pathways activated upon 5-HT stimulation, blowfly salivary glands might provide an attractive model system for analysing the detailed sequence of events that occur between the arrival of an external stimulus and the activation of V-ATPase within animal cells.

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