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CHANGES IN H^+-TRANSLOCATING VACUOLAR-TYPE ATPase IN THE ANTERIOR SILK GLAND CELL OF *BOMBYX MORI* DURING METAMORPHOSIS

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Summary

A proton-translocating vacuolar-type ATPase (V-ATPase) was identified and characterized in the anterior silk gland of *Bombyx mori*. By incubating the intact tissue with the fluorescent dye Acridine Orange, the acidified compartment was detected at the apical pole of the epithelial cells. This was observed throughout the feeding period of the fifth-instar larva until the onset of spinning. Acidification was prevented completely and reversibly by 0.8 μmol l^{-1} bafilomycin A_1, a specific inhibitor of V-ATPase. The presence of V-ATPase in a microsomal fraction was verified by immunoblots using an antiserum to the V-ATPase holoenzyme from *Manduca sexta* midgut. The antiserum localized the V-ATPase to the apical plasma membrane of the anterior silk gland cells, suggesting that the enzyme is functionally active in pumping protons out of the cell towards the glandular lumen of feeding silkworm larvae. In spinning larvae, the acidification produced by the V-ATPase appears to cease, because acidic compartments were seen rarely and only in the periphery of basal cytoplasm, and because immunocytochemical staining for the V-ATPase was greatly reduced at the apical surface. The metamorphic changes in relation to the occurrence of V-ATPase corresponded well with the ultrastructural changes in the anterior silk gland cell of *Bombyx mori* larvae.

Key words: H^+-translocating vacuolar-type ATPase, V-ATPase, acidification, plasma membrane, silk gland, silkworm, *Bombyx mori*, metamorphosis.

Introduction

Evidence that proton-translocating vacuolar-type ATPases (V-ATPases) occur in animal plasma membranes is accumulating steadily (Harvey and Wieczorek, 1997). In insect systems that cannot utilize Na^+-motive forces, proton-motive forces produced by V-ATPases are indispensable for the energization of membrane transport. Studies in the midgut of the tobacco hornworm *Manduca sexta* have provided the basis for our understanding of the role of V-ATPases in plasma membrane energization by the proton-motive force (Wieczorek, 1992); in this system, the V-ATPase plays a vital role in the generation of a transmembrane voltage and in energizing secondary active transport processes such as $K^+/2H^+$ antiport and amino acid:K^+ symport across the apical plasma membrane (Lepier *et al.* 1994; Azuma *et al.* 1995). Plasma membrane V-ATPases are now well-documented in a variety of other insect tissues, i.e. sensory sensilla (Klein and Zimmermann, 1991), salivary glands (Just and Walz, 1994), Malpighian tubules (Klein *et al.* 1991; Pietrantonio and Gill, 1995) and developing ovaries (Janssen *et al.* 1995).

The silk gland of *Bombyx mori* is a protein factory specialized in silk production. It develops during larval growth, synthesizes vast amounts of several silk proteins and secretes them into the glandular lumen until the onset of spinning (Prudhomme *et al.* 1985; Suzuki, 1994). The silk gland consists of a pair of tissue masses, each consisting of three parts, the anterior, middle and posterior regions, opening at the mouthparts as a spinneret. The middle and posterior parts of the gland are responsible for producing the silk proteins, sericin(s) and fibroin, respectively. The ultrastructure of the middle and posterior silk gland cells shows enormously active exocytosis, in particular at the apical cytoplasm, numerous Golgi bodies, secretory vesicles and regularly arranged microtubules (Akai, 1984). The silk proteins are secreted and temporarily deposited in the glandular lumen as ‘liquid silk’, a solution containing over 30 % protein. This gel-like solution is maintained within the glandular lumen without irreversible coagulation and/or denaturation before spinning. The gelation of fibroin solution *in vitro* is pH-dependent (Ayub *et al.* 1993). Therefore, it would be of interest to explore the system regulating pH in the silk gland *in vivo*.

In this investigation, we focused on the anterior silk gland, which has no role in the production of the silk protein. This region guides the excytosed silk proteins from the middle and posterior glands into the spinneret. It has long been believed that this part of the silk gland is a simple mechanical duct connected to the spinneret (Akai, 1984). The silk gland of

Bombyx mori serves as a model system in which gene regulation and expression have been studied extensively (Suzuki, 1994). There is only one report describing physiological aspects of the *Bombyx mori* silk gland, although that investigation was limited to the posterior part (Nakagaki and Sasaki, 1988). In the present study, we identify a plasma membrane V-ATPase in the anterior silk gland of *Bombyx mori*, demonstrate its localization and its metamorphic changes, and discuss its potential physiological function.

Materials and methods

Insects

Hybrid races (Shunrei × Shogetsu and Kinshu × Showa) of the silkworm *Bombyx mori* were reared on fresh mulberry leaves or an artificial diet at 24–26 °C. The final (fifth) larval instar stadium lasts 10 days: 7 days for feeding, followed by 3 days for spinning to build a cocoon. Larvae from two physiologically different stages were studied: 6-day-old fifth-instar larvae in which the deposition of liquid silk in the glandular lumen reaches its maximum rate ('feeding larvae'); and 8-day-old fifth-instar larvae in which the physiological changes for larval–pupal metamorphosis have just begun ('spinning larvae').

Fluorescence microscopy

The anterior silk gland was dissected from feeding larvae and spinning larvae and rinsed carefully with Grace's insect cell culture medium (pH 7.0, GIBCO BRL, USA). Using a Probe-Clip (500 µl chamber, Grace Bio-Labs Inc., USA), the anterior silk gland as a whole was immediately incubated at 25 °C in Grace's medium containing 2.5 µg ml^{-1} Acridine Orange (Sigma, USA) and 0.5 % dimethyl sulphoxide. The gland was observed using conventional fluorescence light microscopy (Olympus BH-RFL, Japan) during the incubation (maximum up to 3 h). For the negative control experiments, bafilomycin A_1 (Wako Pure Chemical Industries, Ltd, Japan) dissolved in dimethyl sulphoxide was included in the Grace's medium at 0.8 µmol l^{-1} (final concentration). Dimethyl sulphoxide (final concentration 0.5 %) does not itself affect the occurrence of Acridine Orange fluorescence.

Conventional electron microscopy

The anterior silk glands from feeding larvae and spinning larvae were prefixed in 3.75 % glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.4) for 60 min on ice, washed with 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.4) and postfixed in 1 % OsO_4 in 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.4) for 30 min on ice. All specimens were dehydrated in a graded series of ethanols and embedded in Epon–Araldite resin (TAAB laboratories, USA). The blocks were sectioned on an RMC MT7000M ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Hitachi H-600A electron microscope operated at 75 kV.

Antibody specificity

Rabbit antiserum against the V-ATPase holoenzyme from *Manduca sexta* midgut (Wieczorek *et al.* 1991) was used for all immunological experiments. The antiserum was diluted 1:10 000 or 1:20 000 with 10 % normal goat serum in phosphate-buffered saline (PBS, 0.15 mol l^{-1} NaCl, 10 mmol l^{-1} sodium phosphate buffer, pH 7.4). To check antibody specificity, an immunological protein blotting test was performed using a microsomal fraction extracted from the anterior silk gland of 50 feeding larvae. Tissue was homogenized in an ice-cold buffer consisting of 0.3 mol l^{-1} sucrose, 5 mmol l^{-1} EDTA, 10 mmol l^{-1} Mops–Tris (pH 7.0) including 5 µg ml^{-1} leupeptin and 5 µg ml^{-1} pepstatin A. The homogenate was centrifuged at 5000 ***g*** for 10 min and the supernatant was centrifuged at 102 000 ***g*** for 60 min. The resulting pellet (microsomal fraction) was suspended in the same buffer but without proteinase inhibitors. The gel electrophoresis and protein blotting procedures were performed as described previously (Azuma *et al.* 1991).

Immunocytochemistry

Anterior silk glands from feeding larvae and spinning larvae were collected and fixed in Bouin's solution (water-saturated picric acid:formalin:acetic acid, 15:5:1 by volume) for 5–6 h at room temperature (20–25 °C). After fixation, tissue pieces were dehydrated through a graded series of ethanols and embedded in a Paraplast embedding medium (Paraplast-Regular, Sigma, USA). Serial sections of approximately 5 µm thick were mounted on slides coated with 3-aminopropyltriethoxysilane and dried overnight at 40 °C. Sections were dewaxed, rehydrated, equilibrated with PBS and then treated with 10 % normal goat serum in PBS for 1–2 h at room temperature in order to block the non-specific binding of the V-ATPase antibody. After removing the goat serum, sections were incubated overnight (at 4 °C) with the V-ATPase antiserum (1:10 000 dilution). Normal rabbit serum (1:1000 dilution) was used for control staining. After rinsing with PBS, sections were treated successively with biotinylated anti-rabbit secondary antibody and avidin-biotinylated peroxidase complex (ABC) reagent (Vectastain ABC kit, Vector Laboratories, Inc., USA). The reaction products were finally observed as deposits of 3,3′-diaminobenzidine (DAB).

Immunoelectron microscopy

Anterior silk glands from feeding larvae were collected and fixed in a periodate–lysine–paraformaldehyde fixative (PLP; McLean and Nakane, 1974) for 4–5 h at 4 °C. The tissues were subsequently washed with 10 %, 20 % and 30 % sucrose dissolved in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.4), each step for 2 h, embedded in Tissue-Tek (O.C.T. compound, Miles Inc., USA) and frozen quickly. Tissue blocks were kept at –80 °C until use. Frozen sections (10 µm) were cut on a Bright cryostat (Instrument Company Ltd, UK), placed on 3-aminopropyltriethoxysilane-coated slides, and dried overnight at 40 °C. Sections were rehydrated with PBS and then soaked in 0.3 % H_2O_2 in 100 % methanol for 30 min to destroy endogenous tissue peroxidase. After re-equilibration with PBS, sections were processed using the immunoperoxidase staining

procedure described above. Sections were postfixed with 1 % OsO_4 in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.4) for 1 h at room temperature to enhance the DAB-stained deposits. Finally, sections were dehydrated through a graded series of ethanols, Epon-embedded, ultrathin-sectioned, as described above, and stained with lead citrate.

Results

Acidification in the anterior silk gland cell from feeding larvae and spinning larvae

Acridine Orange is a useful probe for identifying acidic intracellular compartments (Yoshimori *et al.* 1991; Dow, 1992; Moriyama, 1996). The anterior silk gland consists of uniform hexagonal cells joined together as a monolayer, building up a very thin tubular duct (approximately 0.5 mm in diameter). The apical cell surface is lined with a thick cuticular layer, and the glandular lumen is filled with the liquid silk (Akai, 1984).

Intact anterior silk glands were incubated in Grace's insect cell culture medium containing Acridine Orange. Since the silk gland is very thin and transparent, it was possible to observe whole mounts (Fig. 1). In the gland from the feeding larva, orange fluorescence appeared in the cytoplasm after 10 min of incubation as a weak granular pattern and, within 1 h of incubation, its intensity increased strongly and the stain accumulated in the apical cytoplasm (Fig. 1A). In the gland from the spinning larva, orange fluorescence appeared in the periphery of the basal cytoplasm after 10 min of incubation, but its intensity did not increase and we could detect no change in orange fluorescence at the apical region (Fig. 1B). In both feeding and spinning larvae, no significant differences in the distribution pattern of orange fluorescence could be detected along the whole 3–4 cm length of the anterior silk gland. No orange fluorescence was observed when the gland was incubated in a medium containing bafilomycin A_1, a specific inhibitor of V-ATPases (feeding larva, Fig. 1C; spinning larva, Fig. 1D). Since the intensity of the orange fluorescence was much stronger and sharper in glands from feeding larvae than in those from spinning larvae, we checked the reversibility of acidification in feeding larvae. Glands were first incubated with a bafilomycin-A_1-containing medium for 1 h and then transferred to a bafilomycin-A_1-free medium (30 min later, Fig. 1E; 120 min later, Fig. 1F). The acidic compartments reappeared at the apical surface as a sharp zone of orange fluorescence. When the gland was once again transferred to the bafilomycin-A_1-containing medium, this orange zone disappeared within 30 min (data not shown). These data indicate that the acidic compartments are produced by the

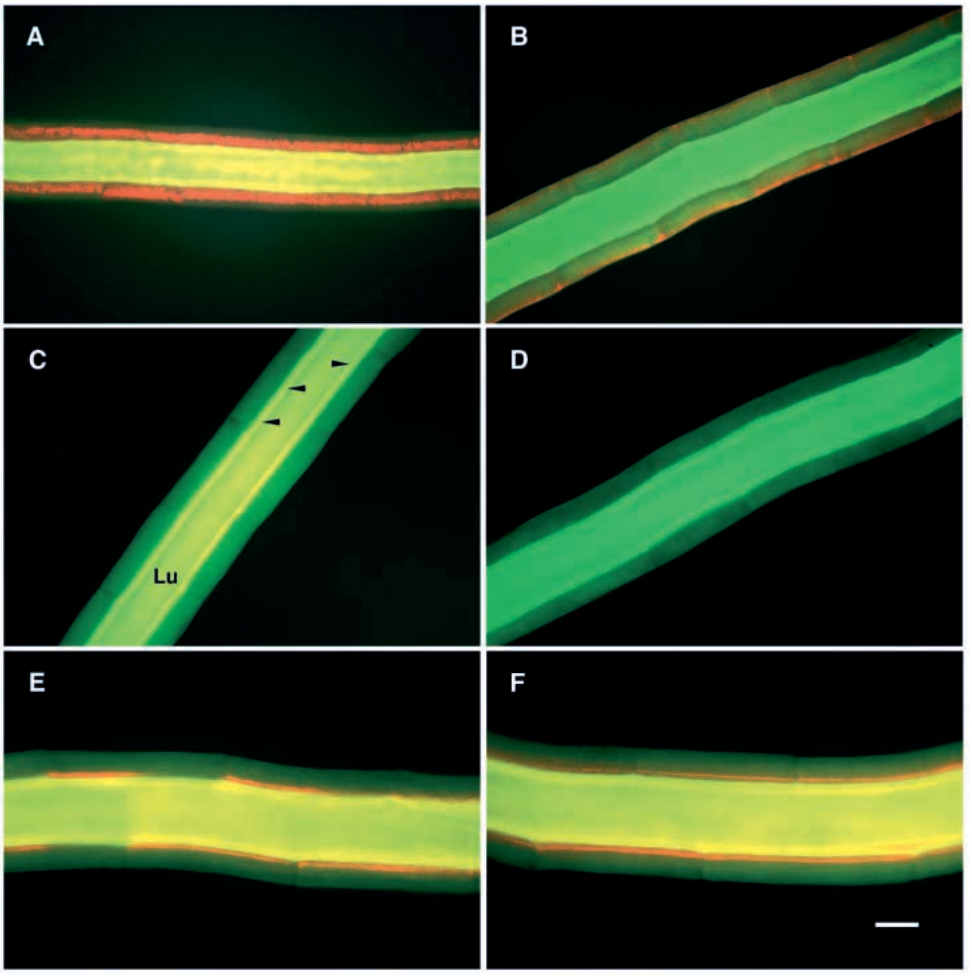

Fig. 1. Acridine Orange staining of the anterior silk gland of *Bombyx mori*. The intact gland was incubated with Grace's insect cell culture medium including 2.5 µg ml^{-1} Acridine Orange and 0.5 % dimethyl sulphoxide. (A,B) Orange fluorescence after 60 min of incubation in the apical cytoplasm of the silk gland cell from a feeding larva (A) or at the periphery of the basal cytoplasm of the gland from a spinning larva (B). Lack of orange fluorescence after incubation of the gland in a medium containing 0.8 µmol l^{-1} bafilomycin A_1 (C, feeding larva; D, spinning larva). The cuticular layers are marked by arrowheads. Lu, lumen of the gland. (E,F) Reversible appearance of orange fluorescence at the periphery of the apical cytoplasm of the gland from a feeding larva. The gland was first incubated with a medium containing 0.8 µmol l^{-1} bafilomycin A_1 for 1 h, and then transferred to the bafilomycin-A_1-free medium for 30 min (E) and 120 min (F). Scale bar, 100 µm.

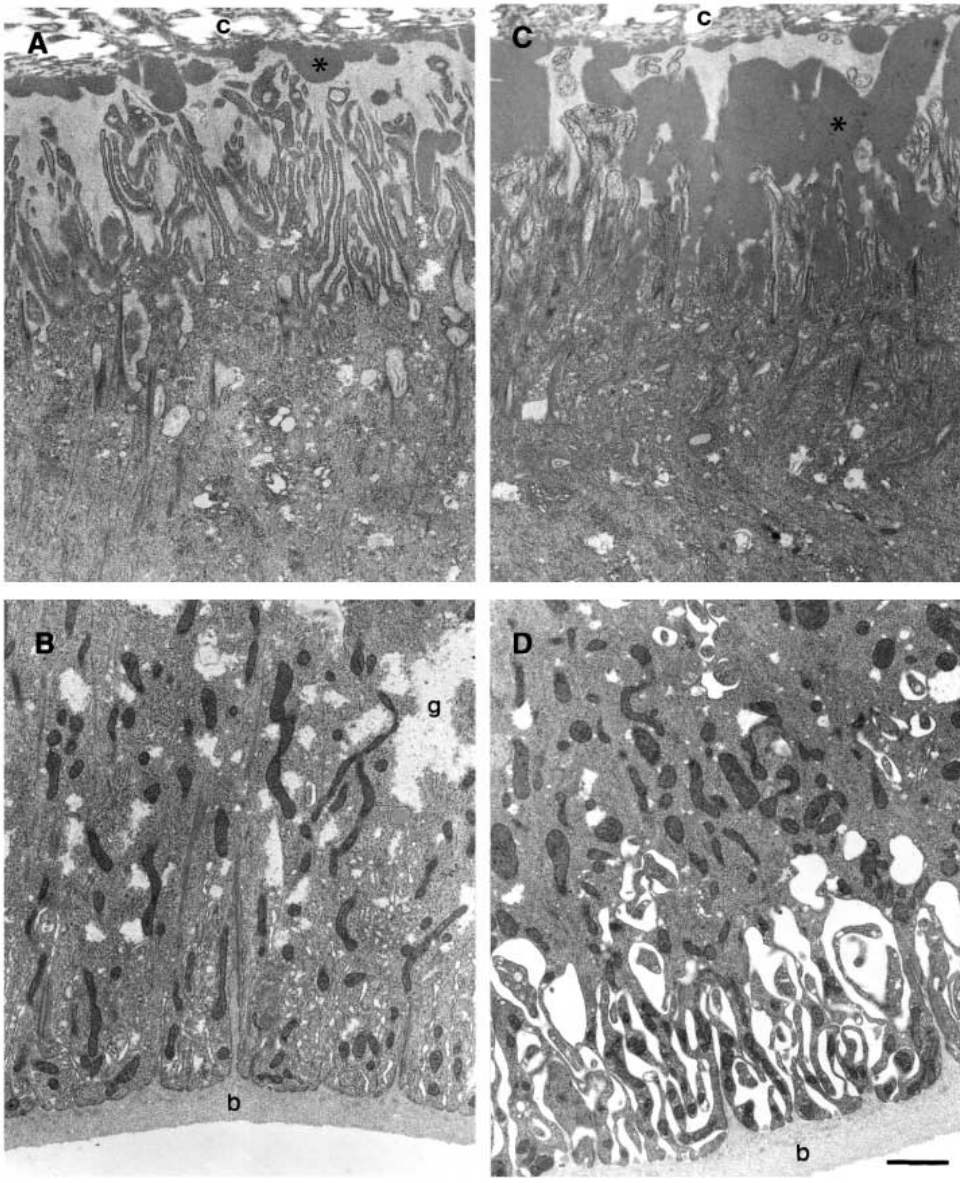

Fig. 2. Electron micrographs of the anterior silk gland cell from a feeding larva and a spinning larva of *Bombyx mori*. (A) Apical region from a feeding larva. (B) Basal region from a feeding larva. (C) Apical region from a spinning larva. (D) Basal region from a spinning larva. b, basal lamina; c, cuticular layer; g, glycogen granules; *, dense bodies. Scale bar, 1 μm.

proton-motive force established by a V-ATPase and that there is greater expression of V-ATPase in the apical plasma membrane and/or in intracellular vesicles beneath the plasma membrane in the gland of the feeding larva.

Ultrastructure of the anterior silk gland cell from feeding larvae and spinning larvae

Electron micrographs showed that the ultrastructure of the anterior silk gland cell underwent drastic changes, not only apically but also basally, at the final larval stage (Fig. 2). In the feeding larva, the apical plasma membrane showed well-developed villous projections, and electron-dense bodies were observed along the outside of the apical plasma membrane close to the cuticular layer (Fig. 2A). The basal cytoplasm resembled that of 'mitochondria-rich cells' (Brown and Breton, 1996). This region was characterized by many basal infoldings of the plasma membrane and by clusters of glycogen granules (Fig. 2B). In both regions of the cytoplasm, microtubules were distributed regularly along the axis of the cell, and Golgi bodies as well as some vesicles were observed frequently (Fig. 2A,B). In the spinning larva, the apical projections appeared to be shortened or were absent, and the electron-dense bodies increased in area at the zone between the apical plasma membrane and the cuticular layer (Fig. 2C). The basal infoldings were more widely spaced and invaded deeply into the cytoplasm; in addition, numerous intracellular vacuoles were present (Fig. 2D). Some mitochondria were phagocytosed within these vacuoles; this may correspond to the orange fluorescence detected basally in the spinning larvae (Fig. 1B). Clusters of glycogen granules were greatly reduced in number. The array of microtubules appeared to be less organized in both apical and basal domains.

In the apical cytoplasm of feeding larvae, we could find no vesicles that might correspond to the strong orange

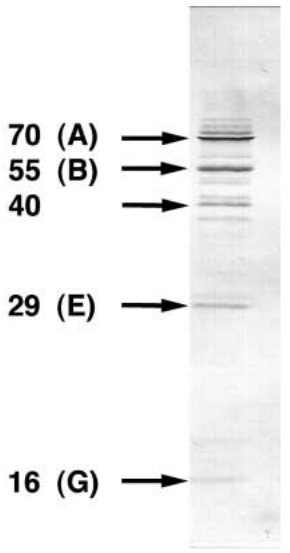

Fig. 3. Immunoblot of microsomal proteins from the anterior silk gland of a feeding larva of *Bombyx mori*. The antigen was subjected to SDS–polyacrylamide gel electrophoresis on 12.5 % gels and was treated with the antiserum to the V-ATPase holoenzyme from *Manduca sexta* midgut. The relative molecular masses of putative V-ATPase subunits in *Bombyx mori* are indicated. Names of known subunits are given by letters (A, B, E, G).

fluorescence zone at the apical surface (Fig. 1E,F). Therefore, we assume that the fluorescence was derived from the zone between the apical plasma membrane and the electron-dense bodies (Fig. 2A), which appeared as a rather bright matrix. This suggests that V-ATPase in the anterior silk gland of the feeding larva is located in the apical plasma membrane and pumps protons out of the cell towards the glandular lumen.

Immunocytochemistry of V-ATPase in the anterior silk gland cell from feeding larvae and spinning larvae

To determine whether V-ATPase occurs in the apical plasma membrane, we first checked the specificity of the antiserum raised against the *Manduca sexta* midgut V-ATPase (Wieczorek *et al.* 1991) by immunoblotting microsomal proteins from the anterior silk gland of feeding larvae (Fig. 3). As a result of the coagulation of the liquid silk, it was practically impossible to perform the immunoblot experiment using crude cell extracts of the gland. Strong staining was detected at bands of approximately 70 kDa and 55 kDa, most probably corresponding to the peripheral (V_1) subunits A and B, respectively (Wieczorek *et al.* 1991). Other minor bands were also detected at approximately 40 kDa, 29 kDa (subunit E, Gräf *et al.* 1994) and 16 kDa (subunit G, Lepier *et al.* 1996). Thus, this antiserum could be used as a specific probe for V-ATPase in the anterior silk gland.

V-ATPase was localized immunocytochemically in anterior silk gland cells at the light microscopic level (Fig. 4). In the feeding larva, strong immunolabelling could be detected at the apical surface facing the cuticular layer (Fig. 4A). Notably the

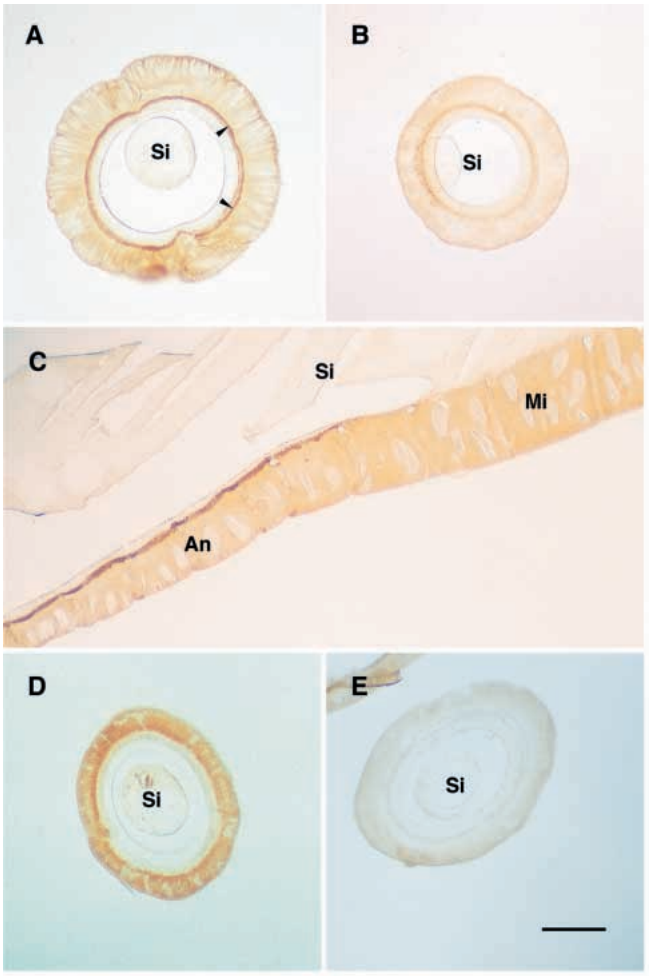

Fig. 4. Immunocytochemistry of V-ATPase in the anterior silk gland cell from a feeding larva and a spinning larva of *Bombyx mori*. (A) Cross section of the silk gland from a feeding larva treated with the *Manduca sexta* V-ATPase antiserum. The specific immunoreaction is marked by arrowheads. (B) Cross section from a feeding larva treated with normal rabbit serum as a control. (C) Longitudinal section at the boundary between the anterior silk gland (An) and the middle silk gland (Mi). (D) Cross section from a spinning larva treated with the *Manduca sexta* V-ATPase antiserum. (E) Cross section from a spinning larva treated with normal rabbit serum as a control. Si, liquid silk fixed in the glandular lumen. Scale bar, 100 μm.

outermost periphery facing the cuticular layer was not specifically immunolabelled where we observed a non-specific immunoreaction in the control experiment (Fig. 4B). It should be noted that this outermost thin layer may originate from expression of the sericin 1 gene in the anterior silk gland cell (Couble *et al.* 1987) and this may correspond ultrastructurally to the electron-dense bodies (Fig. 2A). A significant immunoreaction was also detected at the periphery of the basal cytoplasm (Fig. 4A). This basal staining corresponds to some vesicles at the basal cytoplasm (Fig. 2B), but these vesicles do not seem to produce the acidified compartment because the vital staining with Acridine Orange never showed as orange fluorescence in the basal cytoplasm of the gland from feeding

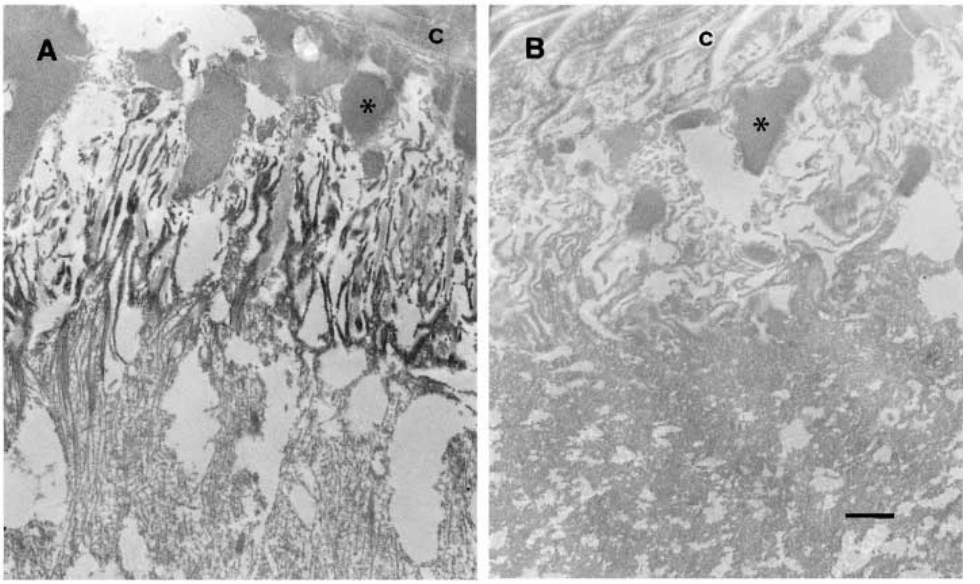

Fig. 5. Immunoelectron micrographs of V-ATPase in the anterior silk gland cell of a feeding larva of *Bombyx mori*. (A) A PLP-fixed frozen section treated with the *Manduca sexta* V-ATPase antiserum. The presence of large invaginations beneath the apical plasma membrane seems to be an artefact of the fixation process. (B) A section treated with normal rabbit serum. c, cuticular layer; *, dense bodies. Scale bar, 1 μm.

larvae (Fig. 1). Fig. 4C shows a longitudinal section at the boundary with the middle silk gland. Immunolabelling at the apical surface was not detected in the middle silk gland, but was limited to the anterior silk gland. In the spinning larva, this apical immunolabelling was greatly reduced and the cytoplasm was immunolabelled as a whole (Fig. 4D). The control showed no significant staining (Fig. 4E).

Immunoelectron microscopy of V-ATPase in the anterior silk gland cell from feeding larvae

The strongest immunoperoxidase reaction product observed using light microscopy was limited to the apical surface of the anterior silk gland cell (feeding larvae, Fig. 4A,C). We examined this reaction product at the electron microscopic level and confirmed that V-ATPase was localized at the apical plasma membrane (Fig. 5). The electron-dense reaction product was detected only at the villous projections of the plasma membranes (Fig. 5A), whereas the apical cytoplasm beneath the plasma membrane was stained less densely. No specific DAB deposits could be detected in the control (Fig. 5B). The membrane ultrastructure was unclear in the cytoplasm owing to incomplete fixation. Glutaraldehyde was not used because we were interested in preserving the antigenicity prior to tissue fixation and because glutaraldehyde-based fixation increased the non-specific immunoperoxidase staining (data not shown). In the sections treated with *Manduca sexta* antiserum (Fig. 5A), it is clear that the matrix substances of microtubules at the apical cytoplasm are clearer than in the control (Fig. 5B). Immunoperoxidase reaction products (DAB with OsO_4) may have diffused into the microtubules, enhancing the contrast in Fig. 5A.

Discussion

The present report has demonstrated that a V-ATPase is located in the apical plasma membrane of the anterior silk gland cell and that its distribution is changed during the metamorphic phase of silkworm larvae. The silk gland of *Bombyx mori* is a highly specialized and differentiated labial gland, producing vast amounts of only a few specific secretory proteins. The gland develops at the final larval stage in parallel with the production of silk proteins, when larval growth reaches its maximum rate (Prudhomme *et al.* 1985). To perform osmoregulatory work in such a highly energy-requiring tissue, the V-ATPase in the silk gland may be as indispensable in generating a proton-motive force as the V-ATPase in the midgut (Dow, 1992; Wieczorek, 1992). The Na^+ concentration in *Bombyx mori* larvae is not high enough for the generation of a Na^+-motive force in the silk gland (Nakagaki and Sasaki, 1988) or in the haemolymph (Shimizu, 1982).

We could detect V-ATPases in the bafilomycin-sensitive acidic compartments of the living intact gland. The acidic compartments were most prominent at the periphery of the apical cytoplasm. The ultrastructure of the anterior silk gland was typical of a polarized epithelial cell, with a regular array of microtubules not only at the apical cytoplasm but also at the basal cytoplasm (Fig. 2). Microtubules may, as in kidney epithelial cells (Brown *et al.* 1992), provide a 'V-ATPase shuttle', since we observed a gradual increase in orange fluorescence intensity at the most apical region of the cytoplasm within the first 1 h of incubation in the feeding larva (Fig. 1). This observation implies the targeting of vesicles which shuttle V-ATPase to the apical pole of the anterior silk gland cell. As the strongly acidic zone was limited to the apical border, the V-ATPase is functionally inactive in vesicles in the central cytoplasm.

The V-ATPase may be located in vesicles beneath the plasma membrane, as observed in gill epithelia (Lin *et al.* 1994; Sullivan *et al.* 1995). However, electron microscopy did not reveal regularly distributed vesicles close to the plasma membrane. In contrast, immunoelectron microscopy clearly showed a V-ATPase located in the apical plasma membrane. Therefore, it would be reasonable to conclude that the acidified compartment corresponds to the zone between the cuticular

layer and the apical plasma membrane. We have observed these apical acidic compartments throughout the feeding period of fifth-instar larvae (M. Azuma and Y. Ohta, unpublished observations). This indicates that the physiologically active V-ATPase functions in the anterior silk gland cell until the onset of spinning.

The plasma membrane V-ATPase loses its pump activity during the spinning phase. The disappearance of the acidic compartment and the ultrastructural changes at the apical region suggest that the rather drastic cellular transformation occurs after the onset of spinning (Figs 1, 2). The basal plasma membrane infoldings also showed drastic changes, with large, deep invaginations and many intracellular vesicles (Fig. 2D). This corresponds well with the newly emerging acidic compartments in the basal cytoplasm (Fig. 1B), which are acidified by an endomembrane V-ATPase, as in lysosomes (Yoshimori *et al.* 1991). When the cocoon is finally built up and larval–pupal metamorphosis proceeds, the cell becomes full of acidic endomembranes (M. Azuma and Y. Ohta, unpublished observations), suggesting that the histolysis of the gland is approaching. The temporal and spatial expression of V-ATPase during metamorphosis await further analysis.

The glandular lumen of the anterior silk gland is filled with secreted silk proteins, 'liquid silk'. The plasma membrane V-ATPase energizes the transport of acid out of the cells until the onset of spinning, acidifying the glandular lumen in feeding larvae. An *in vitro* study of the gelation mechanism of fibroin solution demonstrated that the critical point for gelation lies at approximately pH 5–6 (Ayub *et al.* 1993). This pH is potentially achievable by a V-ATPase (Harvey, 1992; Harvey and Wieczorek, 1997). The acidic lumen might affect the physicochemical nature of the unusually high concentration of silk proteins, perhaps causing the 'sol–gel transformation' of the liquid silk within the lumen. The liquid silk will increase in viscosity and be plugged in the canal of the anterior silk gland of feeding larvae. At the spinning phase, when the V-ATPase is switched off, acidification stops and the glandular lumen will become neutral. This pH change may cause the liquid silk to become mobile and to flow easily out of the spinneret. Although this speculation needs to be confirmed experimentally *in vivo*, it may explain why silk is prevented from spilling out of the spinneret during the feeding period.

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