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INTRACELLULAR ELECTROLYTE LEVELS AND TRANSPORT OF SECRETORY GRANULES IN EXOCRINE GLAND CELLS

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Summary

We demonstrate the intracellular transport of secretory granules of a silk protein, fibroin, from the Golgi region to the apical cytoplasm with special reference to microtubule organization, electrolyte concentrations and the acidic intragranular pH of normal and mutant posterior silk gland cells, using the techniques of electrophysiological microelectrode and microprobe analysis and of light and electron microscopic autoradiography. The silk gland cells of a recessive mutant making only flimsy cocoons were defective in the microtubule systems, did not stain with an anti-tubulin antibody in immunofluorescent microscopy, and accumulated intracellular granules in the apical and basal cytoplasm. The increase in intracellular calcium concentration and levels of chloride secretion were also reduced in the mutant cells. A carboxylic ionophore, monensin, which collapsed the granular H^+ gradient, induced the transport of chloride and an increase in the intracellular calcium concentration, while it blocked the intracellular transport of granules from the Golgi region to the apical cytoplasm in normal cells. Thus, we conclude that the H^+ gradient across the membrane of secretory granules is responsible for the intracellular transport of the secretory granules along the microtubule systems in silk gland cells, while Ca^{2+} is thought to be required for the exocytosis of the granules.

Key words: granule transport, microtubule system, granule H^+ gradient, silk gland, *Bombyx mori*.

Introduction

Various intracellular organelles in eukaryotic cells are transported along microtubules to their particular destinations. In endocrine or exocrine gland cells, secretory granules are transported along microtubule systems from the basal to the apical cytoplasm (Malaisse *et al.* 1975; Sasaki and Tashiro, 1976), although the mechanism of movement of the granules along the microtubule is still unclear. Microtubules, as well as various fusion proteins, have been implicated in intracellular vesicle transport and secretion by epithelial cells (Drubin and Nelson, 1996; Rothman and Wieland, 1996; Kondo *et al.* 1997).

In the posterior silk glands of the silkworm *Bombyx mori*, which are composed of very large hexagonal cells, there are two organized cytoskeletal systems. One system is a radial microtubule system in which the microtubules run radially from the basal to the apical cytoplasm, and the other is a circular microtubule–microfilament system that runs in a circular arrangement around the glandular lumen. The two systems are concerned with the intracellular transport of fibroin granules and the secretion of fibroin into the lumen, respectively (Sasaki and Tashiro, 1976).

A recessive mutant of the silkworm *Bombyx mori*, named 'flimsy cocoon', makes very small, thin cocoons of a lighter weight, which may be defective in the intracellular transport and/or secretion of fibroin (Adachi-Yamashita *et al.* 1980; Maekawa *et al.* 1980).

Secretory stimulants evoke an intracellular Ca^{2+} signal, which is mediated by Ca^{2+} release through inositol (1,4,5)-trisphosphate ($Ins P_3$) receptors on the membrane of intracellular Ca^{2+} stores, and secretory stimulants leading to enzyme and fluid secretion induce marked changes in the bioelectrical properties of the cell membrane of exocrine glands (Berridge, 1993; Petersen *et al.* 1994). Our previous work revealed that, when the luminal membrane potential of the silk gland cells was depolarized, a simultaneous decrease in luminal resistance was detected; cytoplasmic [Ca^{2+}] increased and Cl^- was secreted into the lumen after secretory stimulation with an anti-microfilament reagent, cytochalasin D (Nakagaki and Sasaki, 1988).

Golgi-derived vesicles, such as secretory granules, lysosomes and endosomes, are more acidic than the cytoplasm (Rudnick, 1986; Orci *et al.* 1987; De Lisle and Williams, 1987). It was found that recycling of the low-density lipoprotein receptor is interrupted by a carboxylic ionophore,

monensin, which collapses the Golgi vesicle H^+ gradient (Basu *et al.* 1981). The H^+ gradient of Golgi-derived vesicles may be concerned with solute storage, the processing of enzymes and/or the accumulation of proteins and recycling of surface receptors (Johnson *et al.* 1982; Rudnick, 1986; Mellman, 1992). However, the acidic interior of these organelles may also be used for another physiological function.

In the present study, we investigated the intracellular transport of secretory granules from the Golgi region to the apical cytoplasm in relation to cytoskeletal systems and levels of various electrolytes in the cells of posterior silk glands from normal and flimsy cocoon mutant *Bombyx mori* silkworms. The effects of monensin on the intracellular transport of granules, the membrane potential and electrolyte levels in the normal silk glands were also investigated.

Materials and methods

The fifth-instar larva of normal *Bombyx mori* silkworms and the recessive mutants that make only flimsy cocoons were used. The posterior silk gland cells of these silkworms are very large hexagonal cells, each of which extends for as much as half the circumference of the gland, the cell dimensions being (approximately) 1.3 mm×1.6 mm×0.17 mm at the maturation stage of the fifth instar. The strain of silkworm used was a hybrid of Gunpo and Shyogyoku for wild type. Larvae that are homozygous and heterozygous for the flimsy cocoon mutant (*flc*) were produced by crossing *Ze*+/+*flc* females with *flc*/+*flc* males. The posterior silk gland cells of the silkworm synthesize and secrete large amounts of fibroin.

Immunofluorescent microscopy

The normal and mutant posterior silk glands were fixed for 30 min at 4 °C with 1 % glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer, pH 7.4, containing 0.25 mol l^{-1} sucrose. When the glands had been washed in the same buffer and frozen, thin sectioning (10 μm thick) was carried out with a cryostat (Frigocut 2800, Reichert-Jung, Heidelberg, Germany). The sections were incubated in phosphate-buffered saline (PBS) (pH 7.3) in the presence of a guinea pig polyclonal antibody raised against anti-chicken brain tubulin (a gift from Dr Y. Fukui, Faculty of Sciences, Osaka Universtiy, Osaka, Japan) at a dilution of 1:25 for 30 min at 22 °C, followed by staining with rhodamine isothiocyanate-labelled goat immunoglobulins (IgG) raised against anti-guinea pig IgG. The sections were then washed with PBS and examined by fluorescence microscopy (Vanox-s, Olympus, Tokyo, Japan).

Immunoelectron microscopy

Silk glands were incubated in the presence of 50 μmol l^{-1} 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine (DAMP) in Eagle's minimum essential medium (MEM) for 20–30 min to allow the uptake of DAMP, and then 25 μmol l^{-1} monensin was added and the glands were incubated for 30 min. The tissues were fixed with 2 % paraformaldehyde in 0.1 mol l^{-1} sodium cacodylate buffer. The tissues were processed for indirect immunoperoxidase staining of intracellular sites, using the method of Anderson *et al.* (1984), with 50 μg ml^{-1} of monoclonal mouse anti-dinitrophenol (DNP) IgE and 0.5 mg ml^{-1} of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgE. To localize HRP, cells were incubated at room temperature for 10 min with 0.2 % diaminobenzidine and 0.01 % H_2O_2. The cells were fixed in 1 % osmium tetroxide and 1 % potassium ferrocyanide in 0.1 mol l^{-1} sodium cacodylate, dehydrated and embedded in Epon.

Conventional and high-voltage electron microscopy

Silk glands were fixed with glutaraldehyde and osmium, and processed for electron microscopy. For high-voltage electron microscopy, sections were cut at 2–3 μm thickness and examined under a high-voltage electron microscope (H1250M, Hitachi, Tokyo, Japan) at an accelerating voltage of 1000 kV, taken at tilting angles of ±8° (National Institute for Physiological Sciences, Okazaki, Japan).

Microelectrode measurement

Measurements of the basolateral and luminal membrane potentials, the transcellular potential difference and input resistance were carried out *in vitro*. The microelectrode was connected to a differential-type amplifier (MEZ-7101, Nihon Kohden, Tokyo, Japan) allowing current injection and the simultaneous measurement of membrane potential and input resistance (Nakagaki and Sasaki, 1988). The microelectrode was advanced into the cell and then further into the lumen. Monensin was added to the bath solution at a final concentration of 6 μmol l^{-1}.

X-ray microprobe analysis

Segments of the glands were quickly frozen in liquid nitrogen by pressing them against the wall of a metal block cooled to the temperature of liquid nitrogen. Thick cryosections (0.1–0.2 μm) were cut at −140 °C (MT-7000/CR21, RMC Co., AZ, USA) and mounted on gold or titanium grids, then promptly freeze-dried at −85 °C overnight before carbon coating (FTS Systems, Inc., Stone Ridge, NY, USA). The X-ray microanalysis was performed using a Hitachi H-500 or H-7100 electron microscope interfaced with a Kevex Si (Li) detector and 5100 multichannel analyzer, or an EMAX-3770 X-ray microanalyzer system (Horiba Co., Kyoto, Japan). The microanalyzer was operated at 75 kV. A probe current of 10^{-9} to 10^{-10} A was used, and the analysis was usually carried out for 100–200 s. For the estimation of local dry mass fractions, an analysis of the frozen hydrated sections was performed. The grids with frozen sections were placed in the cooled specimen holder of the electron microscope (GATAN 626-DH, Institute, Warrendale, PA, USA), and the X-ray microanalysis was carried out immediately (Sasaki *et al.* 1996). The X-ray energy spectra and further data processing (to obtain the final concentrations) were performed by an on-line computer system. The special utility programs include some statistical analyses. Details of these procedures have been reported elsewhere (Sasaki *et al.* 1983, 1996; Nakagaki *et al.* 1984). The dry mass fraction was estimated to be 25 % in the

cytoplasm of the normal and mutant glands, using frozen hydrated and dehydrated sections of the glands.

Light and electron microscopic autoradiography

Silk glands were pulse-labelled *in vitro* for 3 min with [^{3}H]glycine and then chased for 20, 40 and 120 min in MEM medium (pH 6.8, 22 °C) containing excess cold glycine. Monensin (6 μmol l^{-1}) was added after 20 min, when grains were already localized in the Golgi region (Sasaki *et al.* 1981). The glands were then fixed, dehydrated and embedded in the usual manner, followed by light and electron microscopic autoradiography (Sasaki *et al.* 1981).

Results

Intracellular transport of secretory granules in normal and mutant cells

Electron micrographs from the basal and apical cytoplasm of the normal posterior silk gland cell showed that the secretory granules (the fibroin globules) and elongated mitochondria aligned with the microtubules in the basal–apical direction, the long axis of the mitochondria always remaining parallel to this direction. Many such striae composed of microtubules, fibroin globules and mitochondria are found in the radial direction in the basal–apical cytoplasm. The Golgi apparatus was located in the basal cytoplasm, and we observed mature fibroin globules in the apical cytoplasm, where they had accumulated for exocytosis into the lumen (Fig. 1A,B). Microfilaments in a circular arrangement surrounding the glandular lumen have been reported and may be concerned with fibroin secretion (Sasaki and Tashiro, 1976).

When a frozen section of the lightly fixed normal gland cells was labelled with a fluorescent antibody against tubulin obtained from chick brain, cytoplasmic staining of the cells was observed, whereas the mutant cells showed no staining (Fig. 2A,B). The fluorescence in normal cells was ten times more intense than that from mutant gland cells (fluorescent

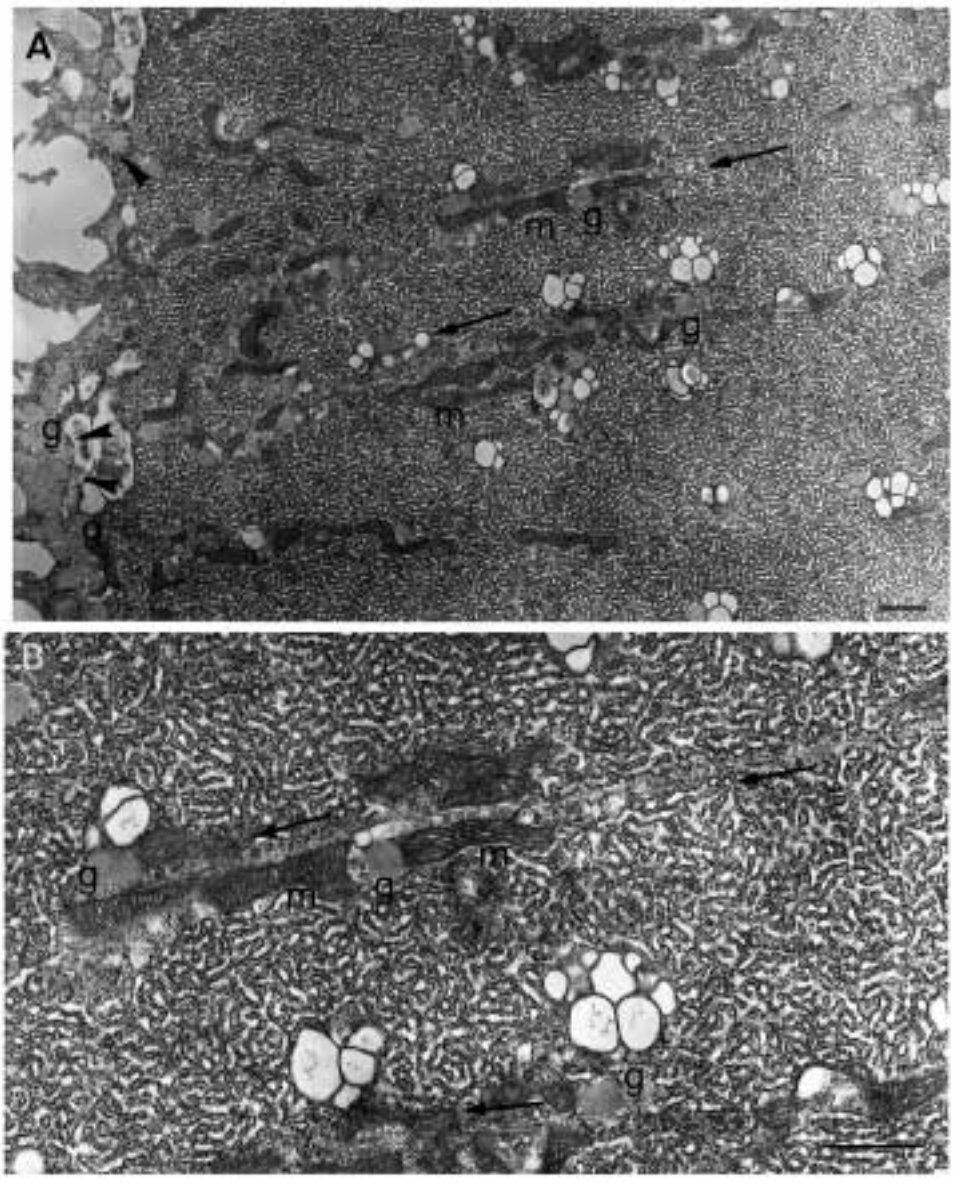

Fig. 1. Transmission electron micrographs of normal silk gland cells reveal the radial microtubule system. (A) Microtubules run from basal to apical cytoplasm (arrows), and mitochondria (m) and fibroin granules (g) are aligned with the microtubules, but are disordered for the circular microtubule–microfilament system at the apical region. At the peripheral part of the apical cytoplasm, the microtubules, granules and mitochondria are arranged irregularly, and fibroin granules are observed (arrowheads). (B) High-magnification view of A. Microtubules are observed clearly, and granules and mitochondria are aligned closely with microtubules. Scale bars, 1 μm.

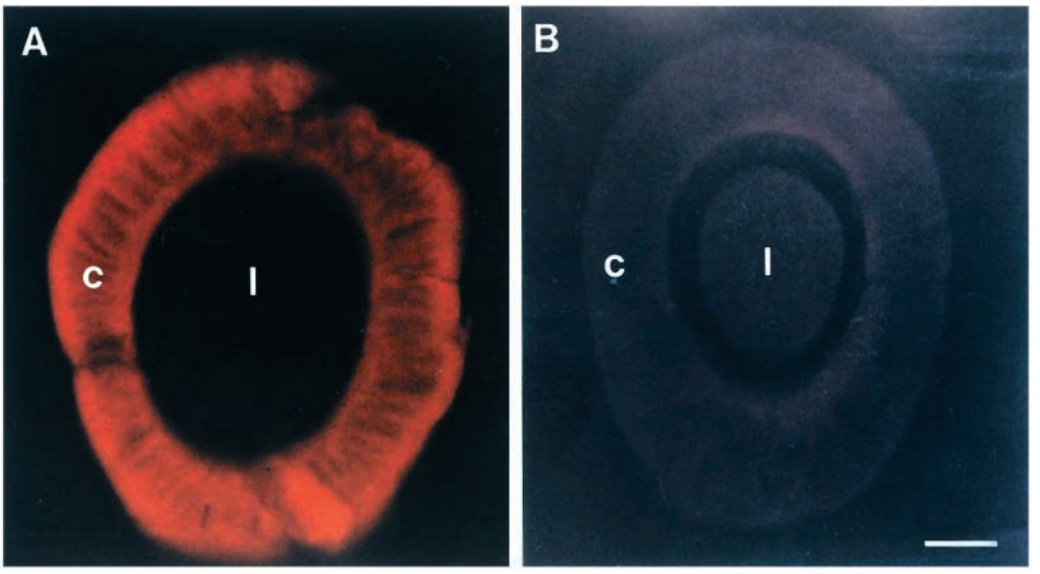

Fig. 2. Immunofluorescent light micrographs of silk glands stained with antibody to tubulin. Cross section of a normal gland showing strong staining in the basal and apical cytoplasm (A), whereas the basal and apical cytoplasm of the mutant gland is not immunostained (B). c, cytoplasm; l, lumen. Scale bar, 50 μm.

intensity; normal 848±11.4, mutant 80.3±2.0; means ± S.E.M., *N*=10), when the glands were measured in a fluorescent microspectroscopic photometer.

We studied the exocytosis of fibroin secretory granules into the lumen of normal cells using high-voltage electron microscopy of thick sections (3 μm) (Fig. 3A). The high-voltage electron micrograph of a thick section (3 μm) from mutant cells revealed the marked accumulation of fibroin secretory granules, sometimes arranged in the direction of the radial microtubule system, in the basal and apical cytoplasm (Fig. 3B). A marked accumulation of granules and the parallel alignment to the radial microtubule systems in the silk gland cells have been reported in the presence of vinblastine, an anti-microfilament reagent (Sasaki *et al.* 1981).

Microelectrode and X-ray microprobe measurement

A typical electrophysiological recording of membrane and transepithelial potentials from normal and mutant cells is shown diagrammatically in Fig. 4A. Transcellular potentials were lumen-negative in both glands, with the potential of the lumen of the mutant gland (–14±0.7 mV, mean ± S.E.M., *N*=40) being less negative than that of the normal gland (–25±0.9 mV). The basal and luminal membrane potentials of the mutant gland cells (–55±2.4 mV, –42±1.7 mV, respectively) were more hyperpolarized than those of the normal cells (–48±2.0 mV, –22±1.2 mV, respectively) (Fig. 4A), which secreted the silk protein fibroin vigorously.

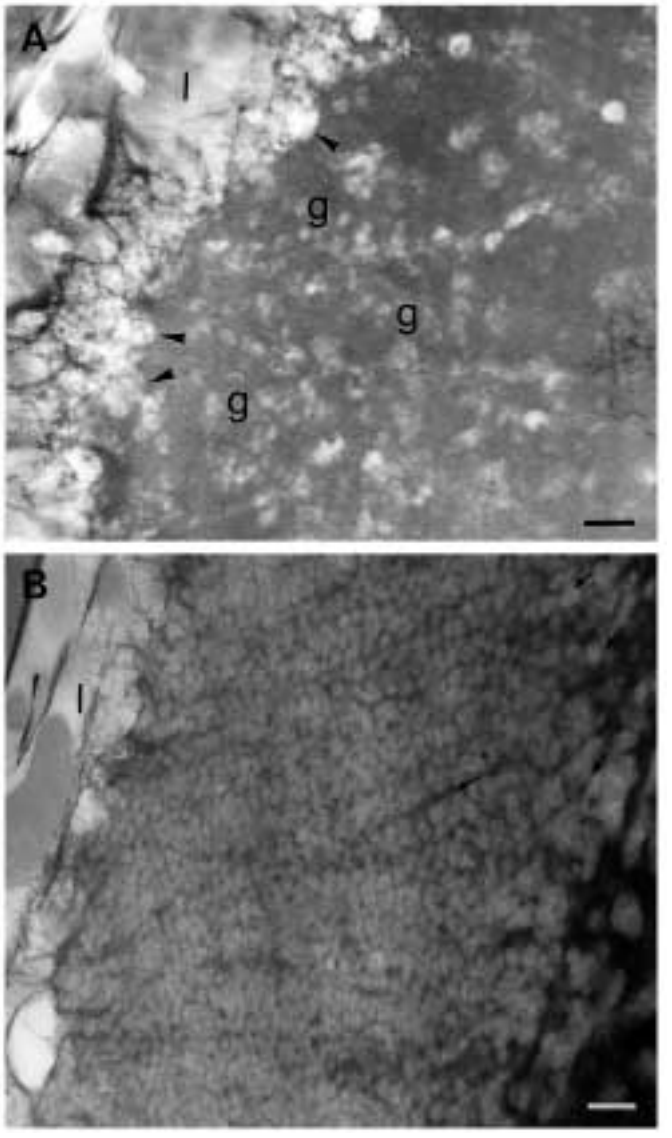

Fig. 3. Electron micrographs of sections 3 μm thick. (A) Secretion of fibroin granules (g) into the lumen is observed in normal gland cells (arrowheads). More microvilli are seen at the luminal portion compared with mutant cells. (B) The greater accumulation of secretory granules arranged in the direction of the radial microtubule system (arrows) found throughout the apical cytoplasm of mutant silk gland cells compared with normal cells. l, lumen; g, fibroin granule. Scale bars, 1 μm.

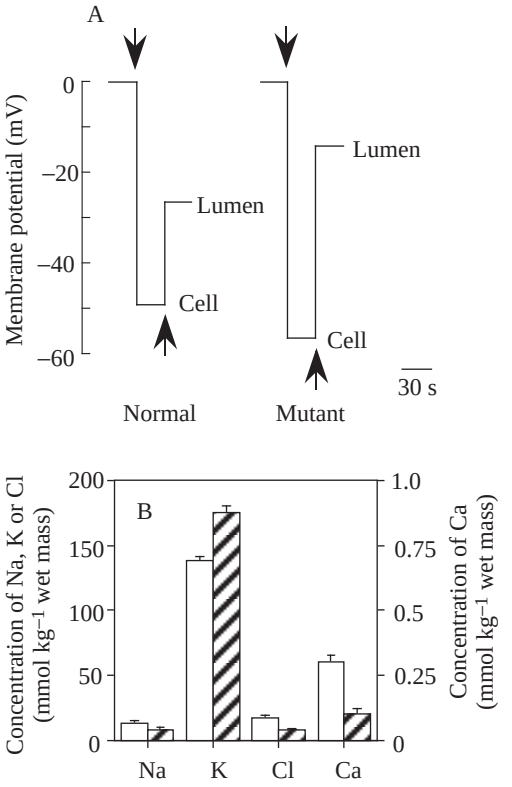

Fig. 4. Membrane potentials and elemental concentrations of normal and mutant cells. The microelectrode was advanced into the cell (downward-pointing arrows) and further into the lumen (upward-pointing arrows). (A) Diagram to illustrate a typical intracellular recording of membrane potentials and transepithelial potentials of normal and mutant glands. The voltage in the lumen of the normal gland is more negative than that of mutant cells. (B) Elemental concentrations in the cells measured by X-ray microanalysis. Cytoplasmic Ca, Na and Cl levels in normal cells (open columns) are significantly higher (P<0.05) than in mutant cells (hatched columns), while cytoplasmic K concentration in normal cells is lower (P<0.05) than in mutant cells (mmol kg^{-1} wet mass; values are means + S.E.M., N=20).

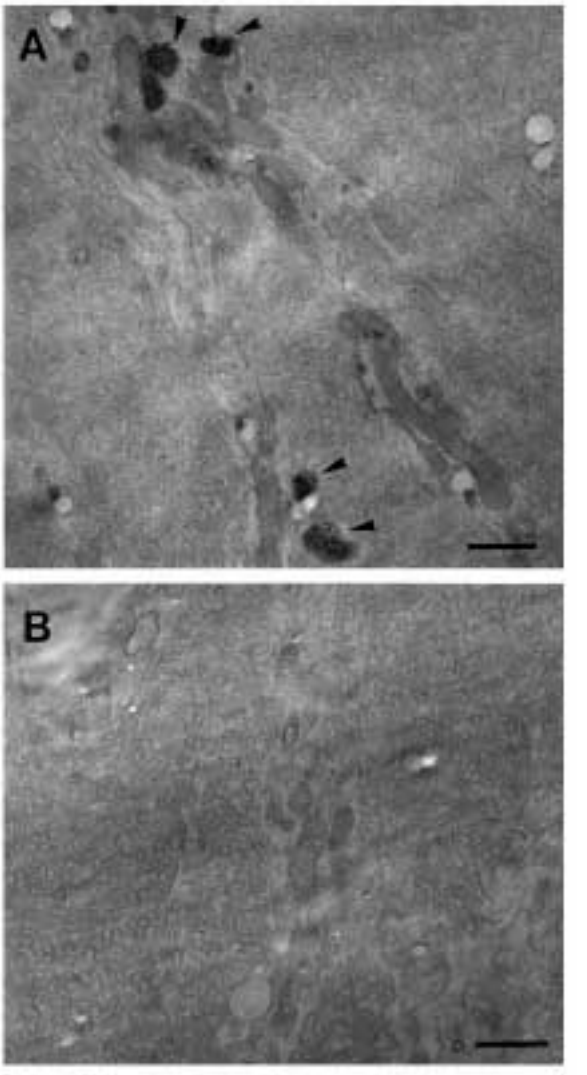

Fig. 5. Transmission electron micrographs of silk glands incubated with DAMP. Note the positive immunoactivity of DAMP in the fibroin secretory granules (arrowheads) of the basal cytoplasm (A), but that DAMP is not visualized within the cells when the silk glands had been exposed to monensin for 5 min (B). No post-staining. Scale bars, 1 μm.

The cytoplasmic concentrations of elements in the mutant and normal gland cells are shown in Fig. 4B. The cytoplasmic Ca, Na and Cl concentrations (Ca, 0.3 mmol kg^{-1} wet mass; Na, 13 mmol kg^{-1} wet mass; Cl, 17 mmol kg^{-1} wet mass, N=20) were significantly higher (P<0.05) and the K concentration (K, 138 mmol kg^{-1} wet mass) was significantly lower (P<0.05) in the normal gland cells than in the mutant cells (Ca, 0.1 mmol kg^{-1} wet mass; Na, 8 mmol kg^{-1} wet mass; Cl, 8 mmol kg^{-1} wet mass; K, 175 mmol kg^{-1} wet mass, N=20). The fibroin secretory granules contain relatively high Ca concentrations (4–8 mmol kg^{-1} wet mass) and act as the intracellular Ca^{2+} store, as do pancreatic exocrine glands (Sasaki *et al.* 1996).

The effect of monensin on normal posterior silk gland cells

DAMP is concentrated in cellular compartments that have an acidic pH (Anderson *et al.* 1984; Orci *et al.* 1987). Fig. 5A shows the immunoelectron microscopic localization of DAMP in the fibroin secretory granules in the cytoplasm of normal silk gland cells. When normal cells were allowed to take up DAMP, washed and then exposed to monensin for 5 min, DAMP was not detected within the cells (Fig. 5B).

We studied the effects of monensin, which eliminates the electrochemical potential gradient of H^+ across the membrane of secretory granules (De Lisle and Williams, 1987), on the membrane potentials and cytoplasmic electrolyte concentrations of normal silk gland cells. The basal membrane potential (−47 mV) was depolarized (by 5 mV) and the input resistance decreased simultaneously with the application of monensin (N=15) (Fig. 6A). The Ca, Na and Cl concentrations (Ca, 0.45 mmol kg^{-1} wet mass; Na, 19 mmol kg^{-1} wet mass; Cl, 51 mmol kg^{-1} wet mass; N=20) in

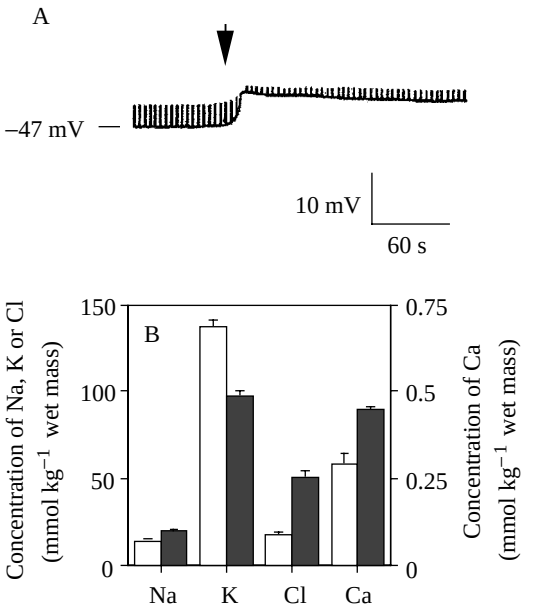

Fig. 6. (A) The basal membrane potential (–47 mV) is depolarized (by 5 mV) and input resistance decreases in response to the application of monensin (6 μmol l^{-1}) (at the arrow) in normal cells. (B) In the presence of monensin (filled columns) (6 μmol l^{-1}), cytoplasmic Ca, Na and Cl concentrations increase significantly (P<0.05) in normal cells (open columns) and cytoplasmic K concentration decreases significantly (P<0.05) (mmol kg^{-1} wet mass; means ± S.E.M., N=20).

cytoplasm treated with monensin were significantly increased compared with those of normal glands (Ca, 0.29 mmol kg^{-1} wet mass; Na, 13 mmol kg^{-1} wet mass; Cl, 17 mmol kg^{-1} wet mass; N=20) (P<0.05), while the cytoplasmic K was significantly decreased from 138 mmol to 97 mmol kg^{-1} wet mass (N=20) (Fig. 6B). The Ca concentrations measured by X-ray microanalysis are total (bound + free) concentrations. Almost all of the Ca^{2+} in the intracellular component is in the bound rather than the free form, and the increase in Ca^{2+} activity would be accompanied by a large increase in the amount of bound Ca^{2+}, so that the changes in Ca concentration measured by X-ray microanalysis may reflect changes in levels of intracellular free Ca^{2+} (Somlyo *et al.* 1985; Tsunoda *et al.* 1988).

Light and electron microscopic autoradiographs

The movement of secretory protein pulse-labelled with [^{3}H]glycine was observed by autoradiography in normal and mutant cells. The effect of monensin was also observed, to determine the effect of H^+ gradients across the membrane of secretory granules and/or plasma membrane on the intracellular transport of secretory granules along the microtubule system from the Golgi region to the apical cytoplasm of normal gland cells.

Electron microscopic autoradiographs confirmed the localization of grains in the secretory granules near the Golgi regions in normal, mutant and monensin-treated (data not shown) gland cells chased for 20 min after a 3 min pulse label (Fig. 7A). After incubation for 120 min in the chase medium, most grains were localized over the apical cytoplasm and partly over the newly discharged fibroin in the normal gland cells only (Fig. 7B).

In light microscopic autoradiographs, we observed grains throughout the cytoplasm after a 20 min chase, also in the apical cytoplasm after a 40 min chase, and mostly in the lumen after a 120 min chase in normal gland cells (Fig. 8A, arrowheads). In mutant gland cells, the grains were observed in the basal cytoplasm after both a 20 min and a 40 min chase. After a 120 min chase, while most grains were observed in the basal cytoplasm, some were found in the apical cytoplasm (Fig. 8B, arrowheads). In contrast, many grains were found in the basal cytoplasm and few grains were found in the apical cytoplasm of normal gland cells chased for 20, 40 and 120 min and treated with monensin (Fig. 8C, arrowheads).

Discussion

In the present study, the immunofluorescent light micrographs clearly indicated that mutant silk gland cells had a reduced quantity of polymerized tubulin in the basal and apical cytoplasm compared with wild-type cells. High-voltage electron microscopic observations showed a marked accumulation of secretory granules in the basal and apical cytoplasm of mutant cells. These light and electron microscopical findings suggested that there is a serious deficiency in the microtubule system, such that the secretory granules in the Golgi region cannot migrate along the radial

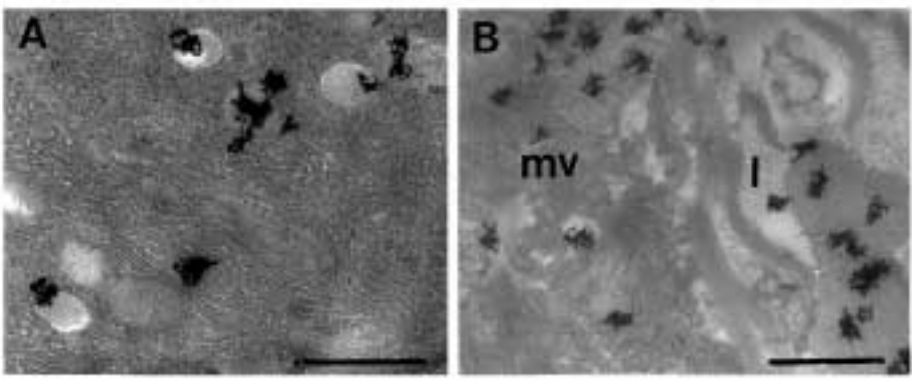

Fig. 7. Electron microscopic autoradiographs of a normal silk gland chased for 20 min (A) and 120 min (B) after a 3 min pulse of [^{3}H]glycine. (A) Note the localization of grains over the fibroin secretory granules. (B) Note the localization of grains over the apical cytoplasm and the discharged fibroin in the lumen. mv, microvilli; l, lumen. Scale bars, 1 μm.

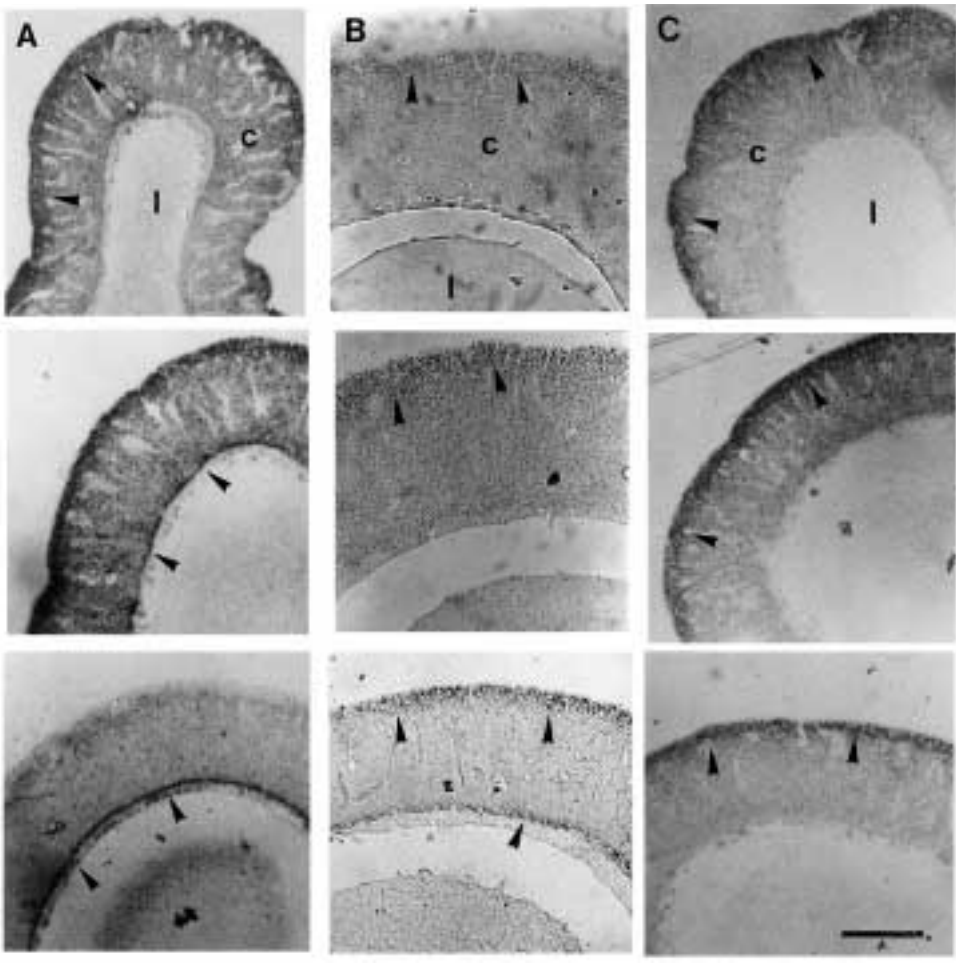

Fig. 8. Light microscopic autoradiographs of [^{3}H]glycine in normal, mutant and monensin-treated gland cells (A, normal cells, B, mutant cells, C, monensin-treated normal cells). (A) Note the accumulation of grains (arrowheads) over the cytoplasm after a 20 min chase (top), over the apical cytoplasm after a 40 min chase (middle) and over the lumen after a 120 min chase (bottom). (B) The ^{3}H-labelled grains are located over the basal cytoplasm after a 20 min chase (top) and throughout the cytoplasm after a 40 min chase (middle); after a 120 min chase, the grains are still localized at the basal and apical cytoplasm (bottom). (C) No ^{3}H-labelled grains are localized in the apical cytoplasm or lumen in the presence of monensin even after 40 min (middle) and 120 min (bottom) in the light microscopic autoradiographs. No post-staining. c, cytoplasm; l, lumen; arrowheads, accumulation of grains. Scale bars, 50 μm.

microtubule systems to the apical cytoplasm, resulting in the accumulation of fibroin globules in the basal and apical cytoplasm near the Golgi region of mutant cells. This supports the idea that the intracellular transport of secretory granules requires well-organized microtubule systems in silk gland cells.

Our microelectrode recordings showed that the basal and luminal membrane potentials of normal cells are depolarized compared with those of mutant cells. The electron microprobe data revealed that the cytoplasmic Ca, Na and Cl concentrations were higher in normal cells than in mutant cells. The results suggest that normal gland cells have a secretory potential in which Na^+ and Cl^- influx into the basal cytoplasm, Cl^- secretion into the lumen and Ca^{2+} mobilization are involved (Nakagaki and Sasaki, 1988). Fluid secretion in the *Calliphora erythrocephala* salivary gland is controlled by 5-hydroxytryptamine (5-HT), using cyclic AMP to drive K^+ pumps, whereas Cl^- follows passively through channels regulated by Ca^{2+} (Berridge *et al.* 1975). A transepithelial potential oscillation due to intracellular Ca^{2+} mobilization was reported in response to 5-HT (Berridge, 1994). The mutant gland cells have no such secretory potential. In addition, there is a possibility that the mutant cells have some abnormalities of Cl^- conductance in the membranes as a result of the inhibition of secretory granule transport, as in the case of epithelial cells in cystic fibrosis, which have been investigated using patch-clamp and whole-cell recordings (Barasch *et al.* 1991; Egan *et al.* 1992). However, it is likely that the secretory potential itself bears some relationship to the exocytotic mechanism of secretory granules that accumulated in the apical cytoplasm, but bears little relationship to the intracellular transport of secretory granules from the Golgi region to the apical cytoplasm along the radial microtubule system of normal gland cells.

When normal gland cells were treated with monensin, which eliminated the electrochemical potential gradient of H^+ across the membrane of the secretory granules (Fig. 5), the basal membrane potential depolarized, the secretory potential was reduced and the membrane conductance increased (Fig. 6A). Monensin is considered to be an electroneutral Na^+/H^+ antiporter; however, it has been shown that monensin increased the conductance of a phospholipid bilayer membrane in the presence of Na^+ (Inabayashi *et al.* 1995). In the present study, the cytoplasmic Ca, Na and Cl concentrations were increased and K concentration was decreased by monensin (Fig. 6B). Monensin eliminated the H^+ gradient across the granule membrane, resulting in an increase in the cytoplasmic H^+ electrochemical potential, which is thought to activated the Na^+/H^+ antiporter on the plasma membrane in silk gland cells.

Na^+ entry is also thought to enhance the Na^+/Ca^{2+} exchange mechanism on the membrane of secretory granules. In bovine medullary chromaffin cells, monensin-mediated Na^+ entry activates InsP_3, and Ca^{2+} is then released from intracellular Ca^{2+} stores, resulting in a marked increase in $[Ca^{2+}]$ in the cytoplasm (Nassar-Gentina *et al.* 1994). Furthermore, monensin and the H^+/K^+ ionophore nigericin induced a reduction in the acidity of secretory granules; this would appear to inhibit the release of Ca^{2+} from granules, as a result of a reduction in the free intragranular Ca^{2+} concentration, and to inhibit the cytosolic Ca^{2+} spikes in the secretory granule area evoked by either InsP_3 or acetylcholine application in pancreatic acinar cells (Titievsky *et al.* 1996).

In the present study, the increase in cytoplasmic H^+ concentration is thought to have activated the Na^+/H^+ antiporter in the cell membrane and induced the membrane depolarization and intracellular elemental concentration changes, as if the secretory potential had been induced by secretory stimulation. These Ca^{2+} and H^+ concentration increases in cytoplasm treated with monensin activate Ca^{2+}-activated K^+ channels and the H^+ pump, following stimulation of the K^+/H^+ antiporter on the apical membrane. It was reported that this type of K^+/H^+ antiporter is present in the apical plasma membrane in the midgut of the tobacco hornworm *Manduca sexta* (Lepier *et al.* 1994).

Nevertheless, the migration of autoradiographically labelled secretory granules from the Golgi region to the apical cytoplasm was inhibited by monensin treatment. Even after chase periods of 40 min and 120 min after labelling with [^{3}H]glycine, secretory granules labelled with [^{3}H]glycine accumulated at the Golgi region but not at the apical cytoplasm, where the exocytosis of granules occurs (Fig. 8C). In experiments using antimycin or a typical uncoupler, dinitrophenol, which is thought to affect mitochondria and to decrease the cytoplasmic ATP level, such inhibitory effects on the intracellular transport of secretory granules were not detected in light or electron microscopic autoradiographs (data not shown). In mutant gland cells, in which the microtubule systems are defective, many labelled granules were retained in the basal cytoplasm; however, some labelled granules migrated to the apical portion after a 40 min and a 120 min chase (Fig. 8B), as in the case of colchicine- and/or vinbastine-treated normal silk gland cells (Sasaki *et al.* 1981).

These results suggest that the migration of secretory granules from the Golgi region to the apical cytoplasm along the microtubule systems is implicated in the formation of the electrochemical potential gradient of H^+ across the membrane of secretory granules, and that the secretory potential and cytoplasmic Ca^{2+} may not be involved in the migration of secretory granules in the posterior silk gland cells. It has been speculated that there is a mechanism and/or molecular 'machine' in the contact point between the radial microtubules and the secretory granules that migrate from the Golgi region to the apical cytoplasm in the posterior silk gland cells. The details of this 'machine' and its relationship to the well-known 'machine' kinesin (Vale *et al.* 1985; Howard *et al.* 1989; Ashkin *et al.* 1990; Block *et al.* 1990) are yet to be elucidated. It is possible that the structure of this machine resembles the flagellar motor of some bacteria (Berg and Anderson, 1973) and drives the secretory granules to migrate along the microtubule using energy derived from the electrochemical flow of H^+ from the inside to the outside of the membrane of secretory granules, while this electrochemical potential of H^+ could also be used for the uptake of Ca^{2+} into the intracellular store, i.e. the secretory granule (Mogami *et al.* 1997).

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