

Fluorescence measurements of serotonin-induced V-ATPase-dependent pH changes at the luminal surface in salivary glands of the blowfly *Calliphora vicina*

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Summary

Secretion in blowfly salivary glands is induced by the neurohormone serotonin and powered by a vacuolar-type H⁺-ATPase (V-ATPase) located in the apical membrane of the secretory cells. We have established a microfluorometric method for analysing pH changes at the luminal surface of the secretory epithelial cells by using the fluorescent dye 5-*N*-hexadecanoyl-aminofluorescein (HAF). After injection of HAF into the lumen of the tubular salivary gland, the fatty acyl chain of the dye molecule partitions into the outer leaflet of the plasma membrane and its pH-sensitive fluorescent moiety is exposed at the cell surface. Confocal imaging has confirmed that HAF distributes over the entire apical membrane of the secretory cells and remains restricted to this membrane domain. Ratiometric analysis of HAF fluorescence demonstrates that serotonin leads to a reversible dose-dependent acidification at the luminal

surface. Inhibition by concanamycin A confirms that the serotonin-induced acidification at the luminal surface is due to H⁺ transport across the apical membrane *via* V-ATPase. Measurements with pH-sensitive microelectrodes corroborate a serotonin-induced luminal acidification and demonstrate that luminal pH decreases by about 0.4 pH units at saturating serotonin concentrations. We conclude that ratiometric measurements of HAF fluorescence provide an elegant method for monitoring V-ATPase-dependent H⁺ transport in the blowfly salivary gland *in vivo* and for analysing the spatiotemporal pattern of pH changes at the luminal surface.

Key words: vacuolar ATPase, V-ATPase, 5-*N*-hexadecanoyl-aminofluorescein, pH, concanamycin A, serotonin, 5-hydroxytryptamine, insect, blowfly, *Calliphora vicina*.

Introduction

Salivary glands in the blowfly are thin tubules that extend as a pair into the abdomen of the animal. The abdominal portion of the glands produces a KCl-rich primary saliva upon stimulation with the neurohormone serotonin (5-hydroxytryptamine, 5-HT). Cl⁻ movement across the epithelial layer occurs passively through Cl⁻ channels that are opened by a 5-HT-induced rise in the intracellular Ca²⁺ concentration. K⁺, in contrast, is transported actively *via* an electrogenic mechanism (Berridge et al., 1976; Berridge, 1977).

Active K⁺ transport in blowfly salivary glands, like that in various insect epithelia, is thought to involve a vacuolar-type H⁺-ATPase and a parallel, functionally linked *n*H⁺/K⁺ exchanger (Wieczorek et al., 1991; Wieczorek et al., 1999; Wieczorek et al., 2000; O'Donnell et al., 1996; Zimmermann et al., 2003). V-ATPase is a multi-subunit transporter composed of a catalytic V₁ component that resides on the cytoplasmic side of the membrane and a H⁺-conducting V₀

component that spans the membrane (Stevens and Forgac, 1997; Nelson and Harvey, 1999; Wieczorek et al., 1999; Nishi and Forgac, 2002). The activity of this proton pump generates, across the apical membrane, an electrochemical gradient that has been proposed to drive secondary K⁺ transport *via* a *n*H⁺/K⁺ exchanger, resulting in a net extrusion of K⁺ (Wieczorek et al., 1991; Wieczorek et al., 1999; Lepier et al., 1994; Harvey and Wieczorek, 1997).

Evidence in support of this model is provided by ultrastructural and immunocytochemical studies, demonstrating that the apical membrane of the secretory cells in blowfly salivary glands is highly enlarged by canalicular infoldings and densely covered by V-ATPase molecules (Oschman and Berridge, 1970; Zimmermann et al., 2003). Moreover, biochemical experiments on salivary glands have shown that 5-HT stimulates the hydrolytic activity of V-ATPase and induces a reversible assembly of V-ATPase holoenzyme molecules from their V₀ and V₁

subcomplexes (Zimmermann et al., 2003). Since V-ATPase holoenzymes, but not the V_0 and V_1 components have the ability to hydrolyse ATP and to pump protons (Zhang et al., 1992; Gräf et al., 1996; Kane and Smardon, 2003), the 5-HT-induced assembly is also indicative of an activation of V-ATPase.

Biochemical experiments for analysing stimulus-dependent regulation of V-ATPase are laborious and, because of their poor time resolution, do not provide information on the kinetics of hormone-induced V-ATPase activation. To examine the 5-HT-dependent regulation of V-ATPase by an alternative, more direct method and to monitor the time course of V-ATPase activation in individual salivary glands, we have established an *in vivo* method that makes use of the H^+ transport activity of the V-ATPase. If H^+ transport into the lumen of the gland is augmented and the luminal surface pH decreases when 5-HT stimulates H^+ transport *via* V-ATPases in the apical membrane, then the fluorescent dye 5-*N*-hexadecanoyl-aminofluorescein (HAF) can conveniently be used as a pH sensor. The fatty acyl chain of this dye partitions into the outer leaflet of the plasma membrane, and its pH-sensitive fluorescent moiety is then exposed to the extracellular space in the vicinity of the plasma membrane (Genz et al., 1999). Here, we demonstrate that HAF remains restricted to the apical membrane of the epithelial cells after pressure-injection into the lumen of the salivary gland and that ratiometric analysis of HAF fluorescence provides an elegant method for recording luminal surface pH changes. Since the luminal acidification induced by a 5-HT stimulus is abolished by concanamycin A, an inhibitor of V-ATPase (Dröse et al., 1993; Dröse and Altendorf, 1997), these pH changes reflect the activity of the proton pump.

Materials and methods

Animals and preparation

Blowflies (*Calliphora vicina* Robineau-Desvoidy) were reared at our Institute. Imagines aged between 1 and 4 weeks were used for experiments. The abdominal segments of their salivary glands were dissected in standard physiological saline solution (PS) containing 128 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-HCl (pH 7.2). In some experiments, the pH of the PS was adjusted to other values as indicated.

Reagents

5-*N*-hexadecanoyl-aminofluorescein (HAF; Invitrogen, Karlsruhe, Germany) was dissolved in dimethylsulphoxide (DMSO) as a 20 mmol l^{-1} stock solution, portioned and stored at $-20^{\circ}C$. 5-HT and concanamycin A were purchased from Sigma (Taufkirchen, Germany). Oregon Green-Phalloidin was from Invitrogen. Dimethyl-trimethylsilylamine and the ion sensor (H^+ -cocktail, Fluka 95291) for pH-sensitive microelectrodes were from Fluka (Buchs, Switzerland).

Microfluorometric measurements of luminal surface pH

Salivary gland tubules were attached to the Cell-Tak-coated (BD Biosciences, San Jose, CA, USA) surface of a glass-bottomed perfusion chamber. For pH measurements at the basolateral surface, the gland was incubated for 5 min in PS containing 30 μ mol l^{-1} HAF. For measurements of pH changes at the luminal surface, a microelectrode filled with 30 μ mol l^{-1} HAF in PS was inserted through the cut end of the tubule into the lumen of the salivary gland. The microelectrode was connected to a pneumatic picopump (PV820; World Precision Instruments, Sarasota, FL, USA) and dye was ejected by application of multiple pulses of variable pressure (<10 psi) and duration (0.5–1 s). For digital imaging of HAF fluorescence, the perfusion chamber was placed on a Zeiss Axiovert 135. HAF was alternately excited through a Zeiss Fluor 20/0.75 objective at wavelengths of 470 nm and 410 nm provided by a VisiChrome monochromator unit containing a 75 W Xenon arc lamp (Visitron Systems, Puchheim, Germany). Excitation occurred every 30 or 15 s for 1–10 ms at 470 nm and for 2–20 ms at 410 nm, depending upon the concentration of HAF integrated into the membrane. After passage through a 515–565 nm band-pass filter, fluorescence images were recorded with a cooled charge-coupled device camera (CoolSnap HQ; Photometrics, Tucson, AZ, USA), digitized, and transferred to a personal computer for offline calculation of the fluorescence ratio at 470 and 410 nm excitation (F_{470}/F_{410} ratio) with the imaging software Metafluor 6.1 (Universal Imaging Corp., Downingtown, PA, USA).

Confocal imaging

A glass-bottomed perfusion chamber with a HAF-labelled salivary gland was placed on a Zeiss LSM510 confocal microscope and examined through a Zeiss Achroplan 40 \times /0.8 W objective. HAF fluorescence was excited with a 488-nm Argon laser and imaged through a 505 nm long-pass filter. For comparative purposes, salivary glands were fixed and labelled with Oregon Green-Phalloidin as described previously (Zimmermann et al., 2003).

Measurements of luminal pH changes with double-barrelled pH-sensitive microelectrodes

Double-barrelled pH-sensitive microelectrodes were pulled from theta borosilicate glass tubing (Hilgenberg, Malsfeld, Germany) with an outer diameter of 2 mm. The active barrel was silanized with dimethyl-trimethylsilylamine by the hot vapour method (Munoz et al., 1983). The tip of the active barrel was filled from behind (Munoz et al., 1983) with the ion sensor and backfilled with 100 mmol l^{-1} sodium citrate (pH 6.6). The reference barrel was filled with 3 mol l^{-1} KCl.

For electrical recordings, the active and reference barrels were connected to the two inputs of a differential amplifier (V86, List Medical, Darmstadt, Germany). The potential recorded by the reference barrel was subtracted from that recorded by the active barrel. This 'differential signal', which indicates ion activity, and the voltage recorded from the reference barrel were monitored on a chart recorder and stored

on a PC by using the software chart 8.30 (HEKA, Lambrecht/Pfalz, Germany) or TestPoint (Keithley, Germering, Germany). The bath electrode was an Ag/AgCl pellet connected to the bath *via* a 3 mol l⁻¹ KCl–agar bridge.

Salivary glands (gland lumen) were impaled under optical control (Leica DM IBB inverted microscope). The position of the electrode was checked optically and electrically. With the electrode positioned in the gland lumen, the differential voltage signal is proportional to luminal pH, and the reference barrel records the transepithelial potential. The preparation was continuously superfused with PS at a flow rate of about 1 ml min⁻¹.

For calibration of the pH-selective microelectrodes, we used the standard PS (pH 7.2), PS titrated to pH 7.6, and a Pipes-buffered PS (pH 6.8). The pH-sensitive microelectrodes were calibrated immediately in the recording chamber after a successful experiment following withdrawal of the microelectrode into the bath. The 'differential ion signals' (in mV) recorded in the calibration solutions were plotted over pH. The mean slope of the last batch of electrodes was 56±9 mV (N=6).

Results

Labelling of distinct surface domains of the secretory cells with HAF

A precondition for measuring pH at a distinct surface of an epithelium with a pH-sensitive fluorochrome is the exclusive localization of the dye to the respective surface domain. To examine whether HAF exhibited this property in blowfly salivary glands, the isolated glands were incubated with HAF in PS and then washed with PS and the dye distribution was examined by confocal microscopy (Fig. 1). Upon application of the dye from the bath side, the basal surface of the secretory cells with their basal labyrinth exhibited intense fluorescence. On the lateral surface of the cells, fluorescence ended abruptly halfway towards the lumen of the gland, at the site where septate junctions link adjacent epithelial cells (Zimmermann,

2000). These findings suggested that HAF became inserted into the basolateral membrane domain of the epithelial cells and remained restricted to this plasma membrane domain.

To label the apical surface of the epithelium with HAF, as required for monitoring luminal surface pH changes, the dye was pressure-injected into the lumen of salivary glands. Membrane insertion and localization of HAF was again checked by confocal fluorescence microscopy (Fig. 2).

As a reference for the structural organization of the epithelial cells, longitudinal optical sections through salivary glands that had been fixed in formaldehyde and stained with Oregon Green–Phalloidin are shown in Fig. 2D,E for comparison. The apical membrane in the secretory segment of the salivary glands is highly enlarged by canaliculi that extend towards the basal side of the cells. These canaliculi are densely covered by sheet-like microvilli and stain intensely with the F-actin probe phalloidin (Oschman and Berridge, 1970; Zimmermann, 2000; Zimmermann et al., 2003).

After injection of HAF into the lumen of live salivary glands, the luminal surface of the cells became intensely labelled with HAF. Within about 15 min after injection, HAF staining progressed deeply into the canaliculi (Fig. 2A), resulting in a labelling pattern that matched well with the phalloidin image of chemically fixed glands (Fig. 2B–E). The basolateral surface of the epithelial cells, however, remained unlabelled upon injection of HAF into the gland lumen.

We concluded that HAF could be inserted into a specific surface domain of the secretory cells, either the basolateral or the apical plasma membrane domain, and that the dye remained restricted to that domain.

pH sensitivity of HAF

The pH sensitivity of membrane-associated HAF was examined on salivary glands with HAF in their basolateral membrane. Since the excitation spectrum of HAF is pH-dependent, with the isosbestic point at about 455 nm (Genz et al., 1999), fluorescence intensity was sequentially measured at the excitation wavelengths of 410 nm (F₄₁₀) and 470 nm (F₄₇₀).

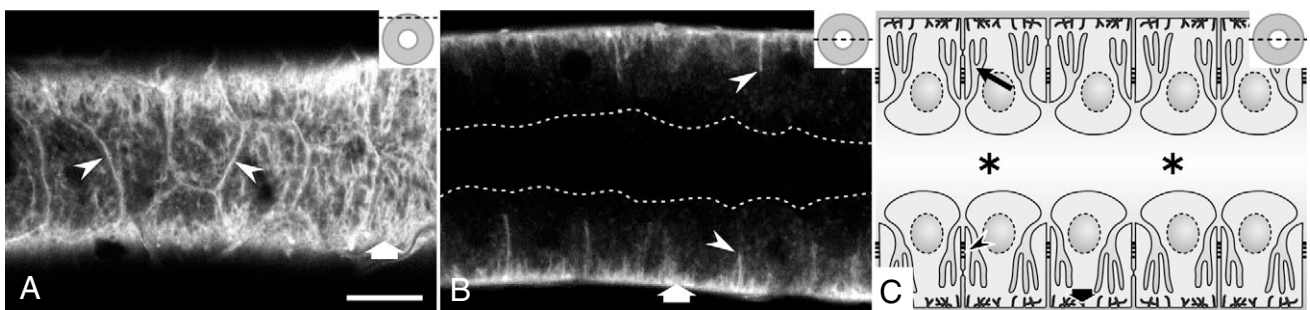


Fig. 1. (A,B) Confocal fluorescence images of a live salivary gland incubated with 30 $\mu\text{mol l}^{-1}$ HAF in PS. The optical section plane through the gland tubule is indicated (upper right of each image). The broken lines in B indicate the luminal surface of the salivary gland, as deduced from background fluorescence of the cells. HAF staining is present on the basal infoldings (broad arrows) and on the lateral domain of the plasma membrane (arrowheads). Note that staining ends abruptly halfway along the lateral domain, indicating that HAF does not diffuse across septate junctions. (C) The structural organization of the secretory portion of the salivary gland. Asterisks, lumen of the gland; arrowhead, septate junction; long arrow, apical infoldings (=canaliculi); broad arrow, basal labyrinth. Bar, 20 μm .

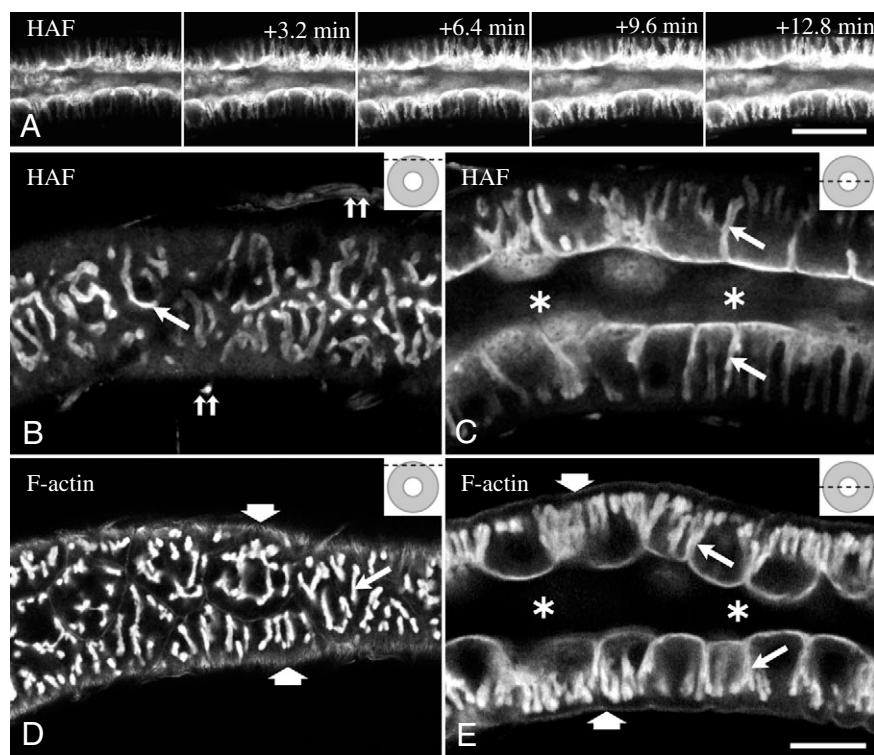


Fig. 2. HAF in the apical membrane domain. (A) Timed series of confocal images through a live salivary gland after pressure-injection of $30 \mu\text{mol l}^{-1}$ HAF in PS into its lumen. The first image was recorded ~ 3 min after dye injection. Subsequent images were taken at an interval of 3.2 min. Note that fluorescence intensity gradually rises within the first four images, indicating the insertion of more dye molecules into the membrane. (B,C) Confocal images of a live salivary gland ~ 20 min after injection of HAF into its lumen (asterisks). The optical section plane is indicated (upper right of each image). HAF staining is present on the luminal surface of the secretory cells and extends into the canaliculi, which are deep infoldings of the apical membrane (arrows). Double arrows indicate tracheoles that reside on the outer surface of the gland and that exhibit autofluorescence. (D,E) Confocal images through a chemically fixed, Oregon Green-Phalloidin-stained salivary gland (shown for comparative purposes). Phalloidin-labelled actin filaments line the canaliculi (long arrows). Asterisks indicate the lumen of the gland, and broad arrows indicate F-actin at the basal surface. Bars, $50 \mu\text{m}$ (A); $20 \mu\text{m}$ (B–E).

Changes in the bath pH produced anti-parallel changes in the F_{410} and F_{470} signals (Fig. 3A). In addition to these pH-dependent changes, a continuous pH-independent drift of both F_{470} and F_{410} signals towards higher intensities was noted (broken lines in Fig. 3A). Such a persistent increase in fluorescence was variable in extent and observed in many experiments. We considered that it was attributable to integration of more dye molecules into the membrane during the experiment. Calculation of the F_{470}/F_{410} fluorescence ratio largely eliminated the baseline drift and revealed that an acidification in the PS caused a decrease in the F_{470}/F_{410} ratio, whereas an alkalization led to an increase in this ratio (Fig. 3B).

5-HT-induced pH changes at the luminal surface

The effect of 5-HT stimulation on luminal surface pH was examined in salivary glands with HAF in their apical, canalicular membrane. In non-stimulated glands, the F_{470}/F_{410} ratio was variable between preparations. The ratio depended on the intensity of HAF staining or the concentration of HAF molecules in the plasma membrane. With our instrumental settings, an F_{470}/F_{410} ratio below 2.5 was indicative of insufficient staining, and such specimens were discarded. Fig. 4 shows further that the F_{470}/F_{410} ratio varied slightly along the salivary glands; this may be also attributed to differences in HAF concentration on the luminal surface along the tubule.

Within 1–2 min after bath application of 30 nmol l^{-1} 5-HT [a concentration that saturates the rate of fluid secretion (Berridge and Patel, 1968; Berridge and Prince, 1972)], the

F_{470}/F_{410} ratio consistently decreased (Fig. 4B, Fig. 5A–C), indicative of a pH drop at the luminal surface of the apical plasma membrane domain. The F_{470}/F_{410} ratio declined simultaneously and by an almost equal amount throughout the entire gland in the field of view (Fig. 4A,B), and it remained reduced as long as the glands were exposed to 5-HT. Within about 3 min after 5-HT washout, the fluorescence ratio recovered to its pre-stimulus value (Fig. 4B, Fig. 5A–C).

The 5-HT-induced luminal acidification was dose-dependent (Fig. 5A–D). At concentrations of $< 1 \text{ nmol l}^{-1}$, 5-HT had no measurable effect on the F_{470}/F_{410} ratio. A half-maximal effect was observed at 5.7 nmol l^{-1} 5-HT, whereas 5-HT concentrations of $\geq 10 \text{ nmol l}^{-1}$ led to a maximal decrease in the F_{470}/F_{410} ratio. Notably, even at sub-saturating 5-HT concentrations, the spatiotemporal pattern in the F_{470}/F_{410} ratio change was alike over the entire length of the salivary gland within the field of view (data not depicted).

Effect of concanamycin A on the 5-HT-induced HAF signal

To examine whether the 5-HT-induced acidification at the luminal surface resulted from an increase in H^+ transport via V-ATPase molecules in the apical membrane domain, we wanted to block V-ATPase activity by use of a specific inhibitor. We had noticed previously that bafilomycin A_1 , an inhibitor of V-ATPase (Bowman et al., 1988; Dröse and Altendorf, 1997), was ineffective if applied on the bath side of the salivary glands, probably because of limited access to V-ATPase in the apical membrane (Zimmermann et al., 2003). Therefore, we examined the effect of concanamycin A, another potent and highly specific inhibitor of V-ATPase (Dröse et al., 1993; Dröse and Altendorf,

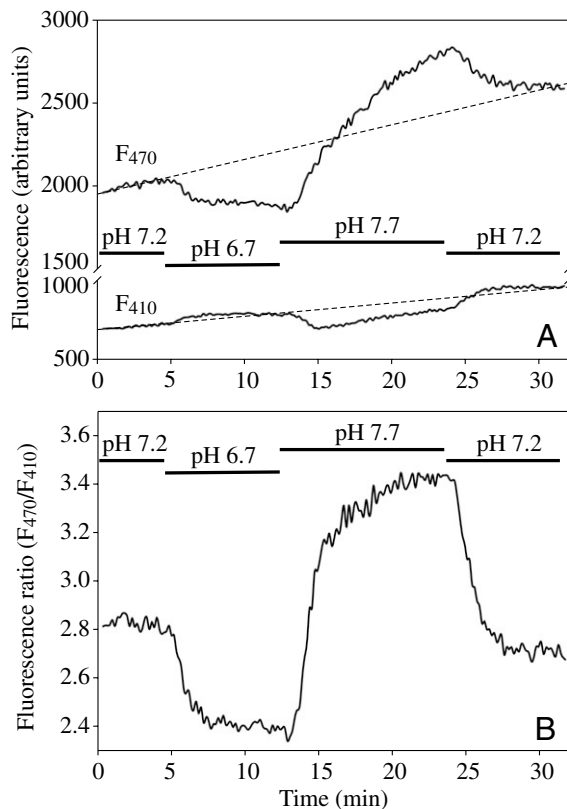


Fig. 3. pH-dependent changes in HAF fluorescence. A salivary gland was incubated for 5 min with $30 \mu\text{mol l}^{-1}$ HAF in PS in order to label the basolateral surface of the epithelial cells and was then superfused with PS of different pH as indicated. (A) HAF fluorescence upon excitation at 470 nm and 410 nm. (B) Ratio of the fluorescence signals obtained at the excitation wavelengths of 470 nm and 410 nm (F_{470}/F_{410}). Acidification is accompanied by a decrease in the ratio and alkalization by an increase in F_{470}/F_{410} .

1997), on 5-HT-induced changes in luminal surface pH. Upon superfusion with $1 \mu\text{mol l}^{-1}$ concanamycin A in the presence of 0.02% DMSO, we consistently observed a slow increase in the F_{470}/F_{410} ratio (Fig. 6A,C), indicative of an alkalization at the luminal surface. Moreover, the 5-HT-induced acidification was largely abolished by concanamycin A (Fig. 6A,D). Superfusion with 0.02% DMSO alone, in contrast, led to a slight decrease in the F_{470}/F_{410} ratio, probably because of photobleaching of the fluorochrome during these long recording periods (Fig. 6B,C). The luminal surface acidification upon a subsequent 5-HT stimulus in the presence of 0.02% DMSO was almost as large as that induced by a 5-HT stimulus applied before exposure to DMSO (Fig. 6B,D). We thus conclude that the 5-HT-induced luminal surface acidification results largely if not completely from H^+ transport *via* V-ATPase across the apical membrane into the lumen of the salivary gland.

Microelectrode measurements of 5-HT-induced luminal pH changes

Although the above microfluorometric measurements demonstrated that 5-HT caused a luminal acidification, they

did not provide information on the actual pH values within the gland lumen. HAF fluorescence can be calibrated *in vivo* if the pH can be reliably controlled and manipulated at the respective surface domain (Genz et al., 1999). Since this was rather difficult to accomplish or was even impossible in the luminal compartment of a salivary gland (see Discussion), we used double-barrelled pH-sensitive microelectrodes to substantiate the 5-HT-induced acidification in the gland lumen by obtaining quantitative data on the pH changes. It cannot be excluded, however, that pH changes at the luminal surface deviate slightly from pH changes in the bulk lumen, as reported for the colon (Genz et al., 1999).

In the lumen of unstimulated glands, a pH of 7.4 ± 0.2 (mean \pm s.d., $N=14$) was determined by pH-sensitive microelectrodes. 5-HT application induced a dose-dependent drop in luminal pH (Fig. 7A,B). The time courses of the pH changes recorded with pH-sensitive microelectrodes were almost identical to those recorded optically with HAF (compare Figs 5A–C and 7A). Despite the rather large standard deviations, the dose–response relationship obtained from pH measurements with pH-sensitive microelectrodes displayed the same characteristics as that obtained from HAF measurements (compare Figs 5D and 7B), with a steep dynamic range between 1 and 10 nmol l^{-1} 5-HT, and a maximum effect with 5-HT concentrations of $\geq 10 \text{ nmol l}^{-1}$. We thus conclude that the results of microfluorometric measurements reliably reflected the direction and the kinetics of the 5-HT-induced change in luminal pH and that the pH change at saturating 5-HT concentrations amounted to about -0.4 units.

Discussion

HAF as a probe for pH changes at the luminal surface

The pH-sensitive fluorescent dye 5-*N*-hexadecanoyl-aminofluorescein (HAF) has been used previously for pH measurements at the intestinal surface of the guinea-pig colon (Genz et al., 1999). This organ can be opened easily by longitudinal sectioning for subsequent superfusion of the luminal surface with HAF-containing solution. After insertion of the fatty acyl chain of HAF into the outer leaflet of the plasma membrane, the pH-sensitive fluorochrome is exposed on the intestinal surface and the pH microclimate can be recorded in the vicinity of the plasma membrane. The present study demonstrates that this microfluorometric method can be adapted for analysing pH changes at the luminal surface of the *Calliphora* salivary gland. Because of its minute size, however, it is technically impossible to open the gland tubule and to spread the epithelium into a flat sheet. Thus, labelling of the apical membrane of the secretory cells requires dye injection into the lumen of the gland by use of a micropipette.

Confocal imaging has confirmed that HAF inserts into the apical membrane of the secretory cells upon injection into the gland lumen. The dye molecules diffuse laterally in this membrane domain and stain the entire canalicular system within about 15 min, and this staining remains restricted to the apical domain. Correspondingly, application of dye from the

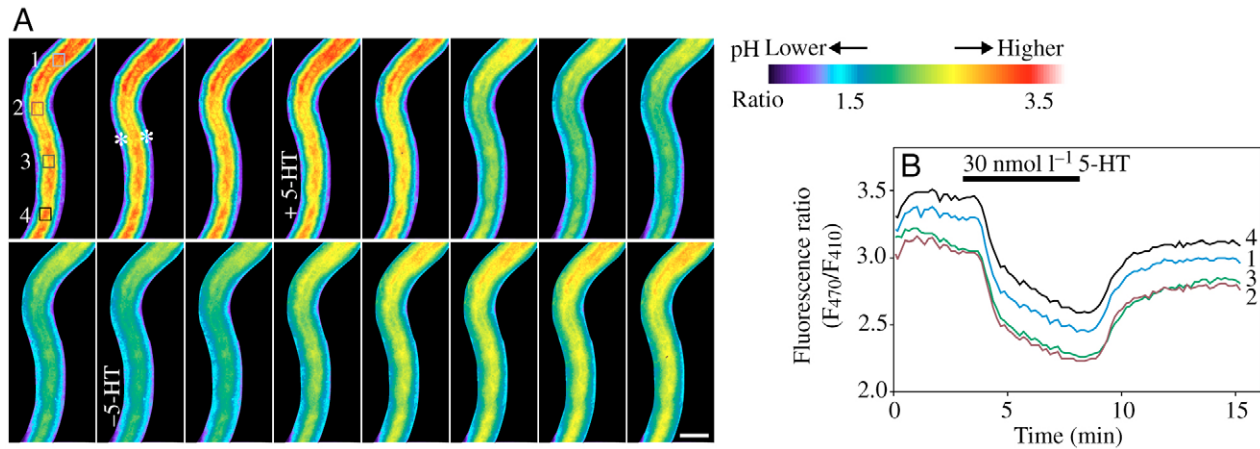


Fig. 4. Spatiotemporal analysis of pH changes at the luminal surface evoked by 30 nmol l^{-1} 5-HT. (A) Series of pseudocolour images of the F_{470}/F_{410} ratio in a salivary gland with HAF in the apical membrane. The squares in the first image indicate the areas selected to generate the graph. +5-HT, beginning of the 5-HT stimulus; -5-HT, washout of 5-HT. Note that the F_{470}/F_{410} ratio laterally along the salivary gland tube (asterisks) is not linked to pH because it results from cellular autofluorescence rather than HAF fluorescence. Only F_{470}/F_{410} in the mid-region of the salivary gland results from HAF and thus relates to luminal surface pH. Image intervals, 50 s; scale bar, $50 \mu\text{m}$. (B) Time course of 5-HT-induced changes in luminal surface pH in four different regions along a salivary gland (squares in A).

basolateral side results in exclusive labelling of the basolateral membrane domain. These results are in accordance with the finding that septate junctions positioned at the apicolateral side of the secretory cells (Oschman and Berridge, 1970; Zimmermann, 2000) provide a diffusional barrier for lipids in the outer leaflet of the plasma membrane (Wood, 1990; Aschenbrenner and Walz, 1998). Because of the spatially specific distribution of dye molecules, HAF can thus be used as a probe for measuring pH changes selectively at either the apical or the basolateral surface of the epithelium.

Unfortunately, the F_{470}/F_{410} ratio of HAF fluorescence cannot be translated into pH values in cases of luminal HAF

staining. Since differences have been observed between calibrations with HAF in solution and with HAF affixed to a membrane (Genz et al., 1999), reliable calibration must be performed *in vivo*. In cases of the basolateral localization of HAF, such a calibration can be readily performed by changing the pH in the bathing solution with free access to the basolateral surface (Fig. 3). In cases of the luminal localization of HAF, however, changing the pH of the luminal fluid in the intact gland tubule would require luminal perfusion of several different calibration solutions, which is a difficult task. Since the F_{470}/F_{410} ratio in unstimulated glands is apparently correlated with the amount of HAF integrated into the apical

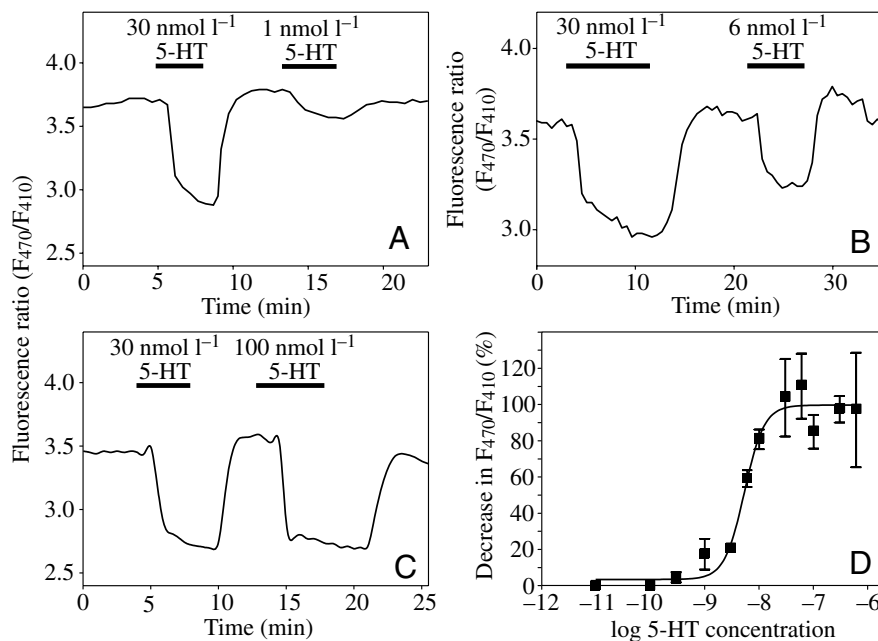


Fig. 5. Dose-response curve for 5-HT-induced changes in luminal surface pH. (A-C) Each salivary gland with HAF at its apical membrane domain was exposed first to 30 nmol l^{-1} 5-HT (reference stimulus), then to a test stimulus of variable 5-HT concentration. An area of about $20 \mu\text{m} \times 20 \mu\text{m}$, representing the size of one cell, was selected in the field of view to generate the graph. Note that the luminal compartment acidified reversibly in response to a 5-HT stimulus. (D) The decrease in F_{470}/F_{410} ratio evoked by the test stimulus was normalized to the F_{470}/F_{410} change induced by the reference stimulus. Values represent means \pm s.d. of 5 experiments for each concentration.

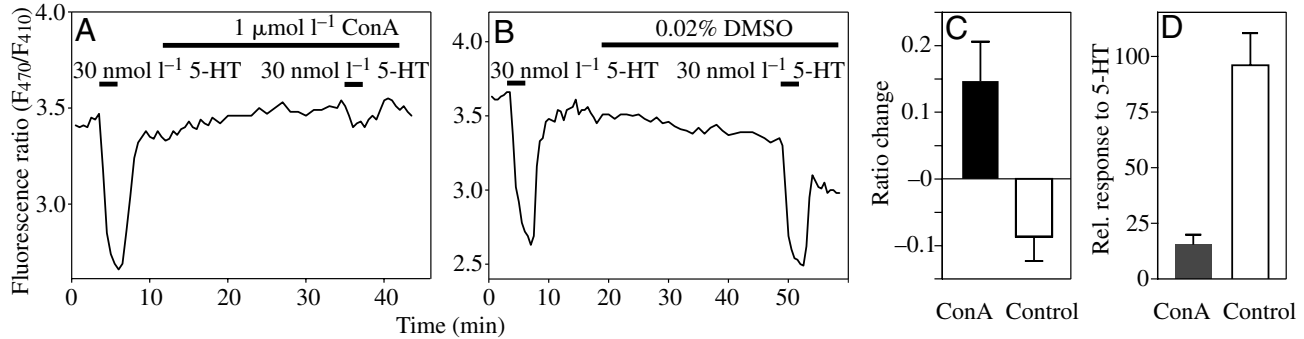


Fig. 6. Effect of concanamycin A on 5-HT-induced pH changes for salivary glands with HAF in their apical membrane domain. (A) Incubation in $1 \mu\text{mol l}^{-1}$ concanamycin A/0.02% DMSO (ConA) inhibited the 5-HT-induced changes in F_{470}/F_{410} ratio almost completely. (B) Control gland, exposed to the same experimental conditions but treated with DMSO only. (C) Quantitative analysis of the effect of concanamycin A (ConA) or 0.02% DMSO (Control) on the basal F_{470}/F_{410} ratio. Values (means \pm s.d., $N=6$) represent the difference in F_{470}/F_{410} ratio before and after exposure to concanamycin A or DMSO, but before the second 5-HT stimulus. (D) Quantitative analysis of the effect of concanamycin A on the 5-HT-induced acidification. Response to 5-HT in the presence of concanamycin A/0.02% DMSO (ConA) or 0.02% DMSO only (Control) was normalized to the response induced by the initial 5-HT stimulus. Values represent means \pm s.d. ($N=6$).

membrane domain, it is also not possible to deduce pH values on the basis of a calibration curve obtained from a different gland with HAF in the basolateral membrane domain. We consider that the F_{470}/F_{410} ratio depends upon dye concentration, because the relative contribution of autofluorescence to total fluorescence changes differently in the F_{470} and F_{410} channels with increasing dye concentration. This may also explain our observation that the basal F_{470}/F_{410} ratio decreases slightly during extensive recording times, as the fluorochromes become photobleached (see Fig. 7B,C).

Changes in ion concentrations can be recorded with ion-sensitive fluorochromes, provided that ion concentrations stay within the dynamic range of the respective fluorochrome. The linear range of changes in fluorescence ratio with membrane-inserted HAF lies between pH 6.5 and 8.0 (Genz et al., 1999). This fits well with our data concerning the pH sensitivity of HAF inserted into the basolateral membrane of the *Calliphora* salivary gland; a relatively linear dependence of the F_{470}/F_{410} ratio upon pH has been observed, at least between 6.7 and 7.7, the pH range tested (Fig. 3). Using double-barrelled pH-

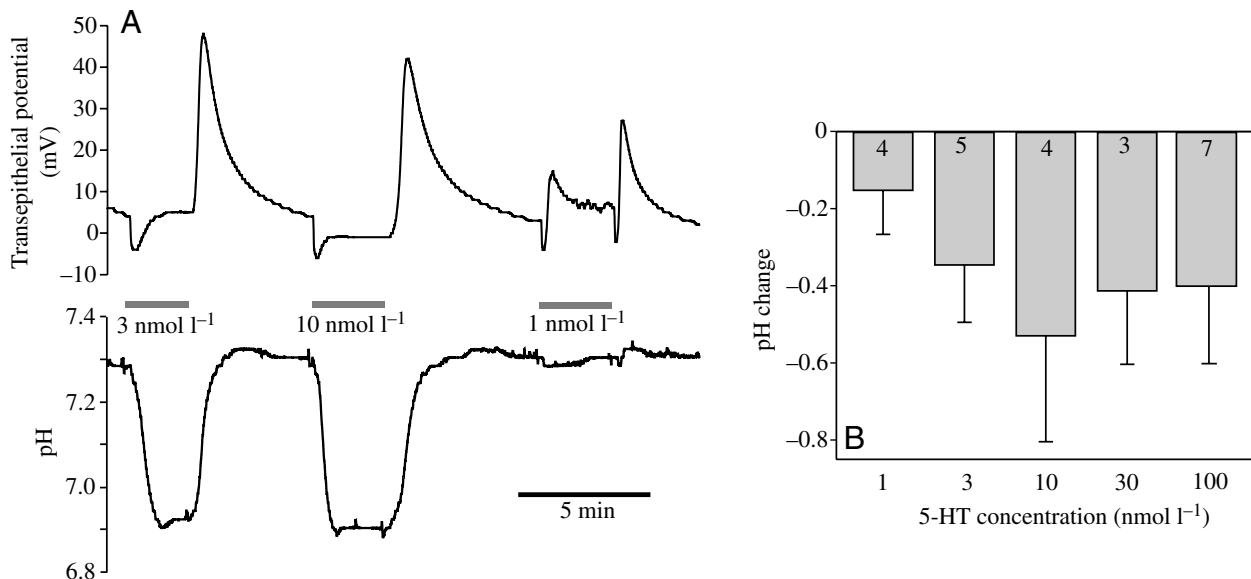


Fig. 7. 5-HT-induced pH changes in the lumen of the salivary gland as measured with double-barrelled pH-sensitive microelectrodes. (A) Effects of three different 5-HT concentrations (1, 3 and 10 nmol l^{-1}) on transepithelial potential (upper trace) and luminal pH (lower trace). The slightly positive transepithelial potential in the unstimulated gland became at least transiently negative upon 5-HT stimulation and displayed a large positive overshoot after 5-HT withdrawal as described previously (Berridge and Prince, 1971). In our hands the time courses of the transepithelial potential changes recorded by the reference barrel of the pH-sensitive microelectrodes differed slightly from those recorded by the classical paraffin oil-gap method (e.g. Berridge and Prince, 1971) for unknown reasons. However, similar differences were noted earlier in luminal recordings with double-barrelled K^+ -sensitive microelectrodes by the same group (see e.g. fig. 5 in Gupta et al., 1978). (B) Dose-response relationship of the 5-HT-induced pH changes obtained from recordings similar to those shown in A.

sensitive microelectrodes, we have determined a luminal pH of about 7.4 under resting conditions and a 5-HT-induced, dose-dependent pH drop of up to -0.4 pH units, fully within the linear range of pH-dependent HAF fluorescence. Although luminal pH may slightly deviate from luminal surface pH (Genz et al., 1999), HAF seems quite well fitted by its range of pH sensitivity for recording pH at the luminal surface in the salivary gland, at least qualitatively.

We conclude that microfluorometric measurements using HAF are an elegant and direct method for monitoring the kinetics of luminal surface pH changes in salivary glands of the blowfly *Calliphora vicina*. This microfluorometric technique is relatively easy to perform, convenient and fast, whereas the production of double-barrelled pH-sensitive microelectrodes is quite intricate and laborious. Moreover, HAF imaging allows the spatiotemporal dynamics of pH changes within a tissue or an entire mini-organ to be recorded non-invasively, whereas pH-sensitive microelectrodes provide information only on the pH at the site of impalement. Although our spatiotemporal analysis of HAF fluorescence suggests that the time course and the amplitude of 5-HT-induced luminal surface pH change are similar over the entire length of the salivary gland, this does not exclude pH gradients on a smaller scale. In particular, because of the limit of spatial resolution of our imaging system we cannot exclude that luminal surface pH on individual cells differs between the basal portion of the extensive canalicular system and the apical pole. Preliminary experiments indicate that spatial resolution of ratiometric HAF imaging can be improved further by confocal microscopy, by using 405-nm and 488-nm laser lines for sequential excitation of HAF fluorescence, providing the possibility of addressing this question.

5-HT-dependent regulation of V-ATPase activity at the apical membrane domain of the secretory cells

The present results demonstrate that the neurohormone 5-HT induces a luminal acidification in blowfly salivary gland, and that this acidification is (almost) entirely based on V-ATPase-dependent H^+ transport across the apical membrane of the epithelial cells. These findings are in agreement with the results of biochemical studies demonstrating that 5-HT and subsequent intracellular signalling pathways lead to an assembly of V-ATPase holoenzymes and an increase in V-ATPase activity (Zimmermann et al., 2003; Dames et al., 2006). In contrast to these biochemical studies, however, microfluorometric HAF measurements provide information not only on whether V-ATPase becomes activated, but also on the time course of its activation. We have demonstrated that luminal acidification reaches a plateau within about 1–2 min after onset of the 5-HT stimulus and, thus, within a similar timescale as other 5-HT-induced physiological responses, *viz.*, fluid secretion (1–1.5 min; Berridge and Prince, 1971; Berridge and Prince, 1972) and the change in transepithelial potential (~ 0.5 min; Heslop and Berridge, 1980). Similarly, the recovery phase after the removal of a saturating 5-HT stimulus lasts about 3 min, not only for the luminal pH, but also for the rate

of fluid secretion and the changes in transepithelial potential (Berridge and Prince, 1971; Berridge and Prince, 1972; Heslop and Berridge, 1980). Such temporal correlation is expected if this proton pump drives the 5-HT-induced changes in transepithelial potential and powers the 5-HT-induced fluid secretion.

Ratiometric measurements of HAF fluorescence also allow an examination of the concentration-dependence of 5-HT-induced V-ATPase activation in a practicable manner; this task would be far more laborious and troublesome by biochemical methods, *viz.* pelleting assays for analysing V-ATPase assembly status or ATPase assays for probing V-ATPase-dependent hydrolytic activity (Zimmermann et al., 2003). Also, pH recordings using double-barrelled pH-sensitive microelectrodes are laborious and may be susceptible to inconsistencies due to tissue damage during impalement.

We found that 5-HT elicits its half-maximal effect with respect to a luminal acidification at about 5.7 nmol l^{-1} , with a maximal effect at $\geq 10 \text{ nmol l}^{-1}$. The dose–response curve for 5-HT-induced luminal acidification thus compares well with dose–response curves for the 5-HT-induced transepithelial potential changes and fluid secretion (Berridge and Prince, 1972), further supporting our concept that V-ATPase activity is directly involved in eliciting the latter physiological responses.

The slight increase in luminal surface pH upon superfusion with concanamycin A in the absence of 5-HT may be due to inhibition of basal V-ATPase activity. This conclusion is in accordance with the results of our biochemical analyses, demonstrating assembled V-ATPase holoenzymes and V-ATPase-dependent ATP hydrolysis in homogenate of unstimulated glands, although at a lower amount or rate, respectively, than after 5-HT stimulation (Zimmermann et al., 2003). Furthermore, indirect support comes from the finding that the transepithelial potential in unstimulated glands is slightly positive (ca. $+5 \text{ mV}$), indicative of basal transepithelial cation transport (Berridge and Prince, 1971). Hence, 5-HT-dependent regulation of V-ATPase does not occur in an all-or-none mode, but rather represents a shift in the equilibrium state between inactive, probably disassembled transporters and active holoenzymes. A similar mode of V-ATPase regulation has been reported in midgut epithelial cells of *Manduca sexta* (Sumner et al., 1995; Gräf et al., 1996), in yeast (Kane, 1995; Parra and Kane, 1998; Kane and Smardon, 2003), in mammalian dendritic cells (Trombetta et al., 2003) and in renal epithelial cells (Sautin et al., 2005). Blowfly salivary glands examined by microfluorometric measurements of V-ATPase-dependent luminal surface pH changes thus provide an attractive experimental system for analysing this widespread mode of V-ATPase regulation in detail.

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