

Intracellular pH homeostasis and serotonin-induced pH changes in *Calliphora* salivary glands: the contribution of V-ATPase and carbonic anhydrase

Bettina Schewe^{1,2}, Elmar Schmälzlin³ and Bernd Walz^{1,2,*}

University of Potsdam, ¹Institute of Biochemistry and Biology, ²Department of Animal Physiology and ³Department of Chemistry, Physical Chemistry, University Campus Golm, Karl-Liebknecht-Strasse 24-25, 14476 Potsdam-Golm, Germany

*Author for correspondence (e-mail: walz@uni-potsdam.de)

Accepted 6 November 2007

SUMMARY

Blowfly salivary gland cells have a vacuolar-type H⁺-ATPase (V-ATPase) in their apical membrane that energizes secretion of a KCl-rich saliva upon stimulation with serotonin (5-hydroxytryptamine, 5-HT). We have used BCECF to study microfluometrically whether V-ATPase and carbonic anhydrase (CA) are involved in intracellular pH (pH_i) regulation, and we have localized CA activity by histochemistry. We show: (1) mean pH_i in salivary gland cells is 7.5±0.3 pH units (N=96), higher than that expected from passive H⁺ distribution; (2) low 5-HT concentrations (0.3–3 nmol l⁻¹) induce a dose-dependent acidification of up to 0.2 pH units, with 5-HT concentrations >10 nmol l⁻¹, causing monophasic or multiphasic pH changes; (3) the acidifying effect of 5-HT is mimicked by bath application of cAMP, forskolin or IBMX; (4) salivary gland cells exhibit CA activity; (5) CA inhibition with acetazolamide and V-ATPase inhibition with concanamycin A lead to a slow acidification of steady-state pH_i; (6) 5-HT stimuli in the presence of acetazolamide induce an alkalization that can be decreased by simultaneous application of the V-ATPase inhibitor concanamycin A; (7) concanamycin A removes alkali-going components from multiphasic 5-HT-induced pH changes; (8) NHE activity and a Cl⁻-dependent process are involved in generating 5-HT-induced pH changes; (9) the salivary glands probably contain a Na⁺-driven amino acid transporter. We conclude that V-ATPase and CA contribute to steady-state pH_i regulation and 5-HT-induced outward H⁺ pumping does not cause an alkalization of pH_i because of cytosolic H⁺ accumulation attributable to stimulated cellular respiration and AE activity, masking the alkalizing effect of V-ATPase-mediated acid extrusion.

Key words: intracellular pH, BCECF, salivary glands, blowfly, *Calliphora vicina*, serotonin, vacuolar H⁺-ATPase, V-ATPase, NHE, AE, carbonic anhydrase, oxygen consumption, Na⁺/amino acid cotransporter.

INTRODUCTION

The tubular salivary glands in the blowfly *Calliphora vicina* secrete a KCl-rich saliva when they are stimulated with the neurohormone, serotonin (5-hydroxytryptamine; 5-HT) (Berridge, 1970; Oschman and Berridge, 1970). A vacuolar-type proton ATPase (V-ATPase) in the apical membrane of the secretory epithelial cells is a key transporter that is indispensable for transepithelial K⁺ transport (Dames et al., 2006; Zimmermann et al., 2003). Active V-ATPase generates an electrochemical proton gradient that is used by a putative nH⁺/K⁺ antiporter for K⁺ transport into the glandular lumen (Zimmermann et al., 2003). Whether, and to what extent, V-ATPase-mediated proton pumping affects intracellular acid–base balance is unknown.

5-HT activates two parallel signalling cascades in *Calliphora* salivary glands: the cyclic AMP (cAMP) cascade and the inositol (1,4,5)-trisphosphate [Ins(1,4,5)P₃]/Ca²⁺ cascade (Berridge and Heslop, 1981; Berridge et al., 1983; Zimmermann and Walz, 2003). The 5-HT-induced activation of adenylyl cyclase leads to an increase in intracellular cAMP (Heslop and Berridge, 1980) and a cAMP-induced activation of the apical V-ATPase (Dames et al., 2006; Rein et al., 2008; Zimmermann et al., 2003). The 5-HT-induced and cAMP-mediated activation of V-ATPase causes a luminal acidification in the salivary glands (Dames et al., 2006; Rein et al., 2006; Rein et al., 2008). Therefore, one would expect an intracellular alkalization, as protons are pumped out of the cell.

Paradoxically, we have measured, in pilot experiments, an intracellular acidification, despite V-ATPase actively extruding protons into the gland lumen.

In this study, we have measured intracellular pH (pH_i) with the fluorescent dye BCECF to solve this paradox and to determine, in isolated *Calliphora* salivary glands, whether and to what extent V-ATPase activity contributes to steady-state pH_i regulation and to 5-HT-induced intracellular pH changes. In order to obtain information concerning the proton source that is responsible for the observed 5-HT-induced acidification, we have chosen two experimental strategies: (1) we have recorded 5-HT-induced changes in tissue O₂ content by using O₂-sensitive fluorescent microbeads; (2) we have identified the location of carbonic anhydrase (CA) activity in the salivary glands cytochemically and studied its effect on 5-HT-induced pH changes pharmacologically, because CAs catalyse the reversible hydration of CO₂, speeding up the formation of H⁺ and HCO₃⁻ in many tissues (Wagner and Geibel, 2002). In addition, we examined the possible involvement of Na⁺- and Cl⁻-dependent transporters in the generation of 5-HT-induced pH changes.

MATERIALS AND METHODS

Animals, preparation, solutions

Blowflies (*Calliphora vicina* Robineau-Desvoidy 1830) were reared at the institute. Between 5 days and 4 weeks after emergence of the

flies, the abdominal portions of their salivary glands were dissected in physiological saline (PS) containing (in mmol l⁻¹): NaCl 128, KCl 10, CaCl₂ 2, MgCl₂ 2.7, sodium glutamate 3, malic acid 2.8, D-glucose 10, and Tris 10 (pH 7.2, adjusted with NaOH). To produce Na⁺-free saline, we replaced NaCl by choline chloride (pH 7.2, adjusted with KOH). Cl⁻-free saline contained (in mmol l⁻¹): sodium isothionate 128, K₂SO₄ 5, CaSO₄ 2, MgSO₄ 2, malic acid 2.8, sodium glutamate 3, Tris 10 and glucose 10 (pH 7.2, adjusted with H₂SO₄). All experiments (unless otherwise stated) were performed at room temperature (~22°C).

Reagents

2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and nigericin were obtained from Invitrogen (Karlsruhe, Germany). Cell Tak was from BD Biosciences (San Jose, CA, USA). Serotonin (5-hydroxytryptamine; 5-HT), 6-ethoxyzolamide, acetazolamide, cAMP, isobutyl methylxanthine (IBMX), 5-(N-ethyl-N-isopropyl)amiloride (EIPA) and 4,4'-diisothiocyanatostilbene-2,2'-disulphonate (DIDS) were from Sigma (Taufkirchen, Germany). 8-(4-chlorophenylthio)adenosine-3',5'-cyclic AMP (8-CTP-cAMP) and forskolin were from Axxora Deutschland GmbH (Grünberg, Germany). The Sp-isomer of adenosine-3',5'-cyclic monophosphorothioate (Sp-cAMPS) was from Tocris Cookson Inc. (Ellisville, MO, USA). Concanamycin A was from Fluka (Buchs, Switzerland).

Confocal imaging

To characterize BCECF distribution within the cells of isolated salivary glands, confocal fluorescence images were recorded with a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) confocal scanning microscope (objective: Zeiss Achroplan 40/0.8 W water immersion objective). Unless otherwise stated, the salivary glands were loaded not only with BCECF, but also with tetramethylrhodamine ethyl ester perchlorate (TMRE) to label their mitochondria (Zimmermann, 2000). For dye loading, isolated gland tubules were incubated at room temperature for 20 min in PS containing 5 µmol l⁻¹ BCECF-AM and 0.2 µmol l⁻¹ TMRE. Loaded glands were then attached to the glass bottom of a superfusion chamber coated with Cell Tak. BCECF fluorescence was excited by an argon laser at 488 nm and imaged through a BP 505-530 nm bandpass filter. TMRE fluorescence was excited by a helium-neon laser at 543 nm and imaged through a LP 560 nm longpass filter.

Microfluorometric measurements of intracellular pH

For microfluorometric measurements of intracellular pH, the salivary glands were loaded for 20 min with 5 µmol l⁻¹ BCECF-AM at room temperature in darkness. BCECF-AM was diluted from a 1 mmol l⁻¹ stock solution containing dimethylsulphoxide (DMSO). The final concentration of DMSO in the loading medium was only 0.5%, a concentration that has no apparent effect on the physiology of the glands (Zimmermann and Walz, 1999). Dye-loaded salivary glands were attached to the Cell-Tak-coated surface of a glass-bottomed perfusion chamber and continuously superfused with PS at a rate of 2 ml min⁻¹.

The microfluorometer consisted of an upright Zeiss UEM/UMSP microscope stand with a photometer head (Zeiss MPM 03 with a type R 928 photomultiplier tube, PMT) and a 75 W xenon lamp monochromator unit (Polychrome II, T.I.L.L. Photonics, Planegg, Germany) coupled to the epifluorescence illumination port *via* a quartz-fibre light guide. A rectangular variable diaphragm in the photometer head was used to limit the area from which fluorescence was collected from the gland tubule to ca. 130 µm × 50 µm (includes

a group of approx. ten cells). Measurements were made with a Zeiss Neofluar 25/0.8 water immersion objective. BCECF fluorescence was alternately excited at 490 nm and 439 nm (isosbestic point) *via* a dichroic mirror (FT510) with a pair of brief 20 ms light pulses applied only every 5 s in order to reduce photobleaching. Fluorescence emission was passed through a long-pass filter (LP 515) to the PMT. The anode current of the PMT was converted to a voltage signal that was digitized at 1000 Hz with a DAS-1600 A/D board (Keithley, Germaring, Germany). Indeed, for data storage and display only, the 20-ms fluorescence signal (F, volts) excited every 5 s was digitized and the average was stored. Data acquisition, averaging, ratioing, display, storage and monochromator control were achieved by a program written with TestPoint programming software (Keithley, Germaring, Germany).

Intracellular pH was calculated from the F₄₉₀/F₄₃₉ ratios by using calibration data obtained with the nigericin-K⁺ method (Deitmer and Schild, 2000; Thomas et al., 1979). The high-K⁺ calibration solutions contained (in mmol l⁻¹): KCl 138, CaCl₂ 2, MgCl₂ 2, sodium glutamate 3, malic acid 2.8, D-glucose 10, Tris 10, and 10 µmol l⁻¹ nigericin. The pH of these calibration solutions was set to between 6.2 and 8.2 with KOH.

Microfluorometric measurement of tissue O₂ content

Microfluorometric O₂ measurements were performed optically as previously described (Schmäzlin et al., 2006). A general review of optical oxygen measurements is given in Papkovsky (Papkovsky, 2004). Briefly, 0.3-µm-diameter polystyrene beads doped with Pt(II)-tetra-pentafluorophenyl-porphyrin (PtPFPP) were used as oxygen probes. The phosphorescence of these beads is strongly quenched by molecular oxygen. The decay time, which depends on the ambient oxygen content, was determined by using a background-insensitive two-frequency phase modulation technique in which the respective phase shifts between sinusoidal excitation and emission signal at two different modulation frequencies are measured (Schmäzlin et al., 2005). Measurements based on decay time overcome some limitations of intensity measurements, such as dependence on the sensor concentration or absorption of the sample. The oxygen concentration was evaluated from the decay time by use of a calibration curve. The sensor beads were pressure-injected into the lumen of isolated salivary gland tubules. The injected glands were attached to a glass-bottomed recording chamber (as described above) and mounted onto the stage of a Zeiss UMSP 80 microscope spectrophotometer equipped with a 635 nm long-pass filter (LP 635, Semrock, Rochester, USA) in front of a PMT (Hamamatsu R 928). For phosphorescence excitation of the sensor beads, a high-power 405 nm LED (Roithner Lasertechnik, Vienna, Austria) with a light-focusing objective was mounted below the sample. Red components of the LED emission were blocked by a short-pass dichroic blue filter (FD1B, Thorlabs Europe GmbH, Karlsfeld, Germany). A rectangular variable diaphragm in front of the PMT allowed the area from which the luminescence was collected to be limited to the area that was injected with the oxygen sensor. The signal output of the PMT amplifier was tapped and connected to a dual-reference-type lock-in amplifier (EG&G, Signal Recovery 7260, Workingham, UK), which is able to measure the respective phase shifts at the two modulation frequencies simultaneously (Löhmansröben et al., 2006). The lock-in amplifier and the microscope were controlled by computers, which were also utilized for data acquisition and evaluation. The oxygen contents are specified in % air: 100% air denotes the oxygen content of air-saturated water, which is in equilibrium with water-vapour-saturated air. At 22°C and 101.3 kPa, 100% air corresponds to an oxygen concentration of 8.7 mg l⁻¹ (Benson and Krause, 1980).

Histochemical carbonic anhydrase localization

For the histochemical detection of CA activity, we used the cobalt/phosphate method of Hansson (Hansson, 1967) as modified by Brown (Brown, 1980). Dissected salivary glands were fixed for 2 h in 4% glutaraldehyde, 0.15 mol l⁻¹ sodium phosphate buffer (PB), pH 7.4, at 4°C, washed in PB, transferred to 10% (w/v) sucrose in PB for 30 min, infiltrated overnight with 25% sucrose in PB at 4°C, placed in cubes of boiled liver, surrounded by Tissue-Tek (Sakuma, Zoeterwoude, The Netherlands) and frozen in melting isopentane (-165°C). Sections (20 µm thickness) were cut on a Microm HM500 OM cryostat (Reichert-Jung) at -28°C and transferred to gelatinized slides, which were then incubated as described previously (Hansson, 1967; Just and Walz, 1994) with medium containing 1.75 mmol l⁻¹ CaSO₄, 11.7 mmol l⁻¹ KH₂PO₄, 157 mmol l⁻¹ NaHCO₃ and 53 mmol l⁻¹ H₂SO₄ (pH 5.8–6.0) by repeated dipping and periods in air. The sections were subsequently rinsed in water, immersed in a blackening agent (0.5% ammonium sulphide), rinsed again, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany). In control experiments, 10 µmol l⁻¹ acetazolamide, a specific CA inhibitor (Maren, 1967), was added

to the incubation medium. The sections were examined and photographed with a Zeiss Axiophot microscope equipped with differential interference contrast optics.

Statistical analysis

Data were presented as means ± s.e.m. in the text and figures. Statistical comparisons were made by an independent Student's *t*-test. *P* values of <0.05 were considered as significant.

RESULTS

Distribution of BCECF fluorescence

BCECF has been reported not to distribute homogeneously in the cytoplasm of some BCECF-AM-loaded cell types (e.g. Weinlich et al., 1998). Therefore, we inspected the intracellular distribution of BCECF fluorescence by confocal microscopy. Optical sections through BCECF-AM-loaded glands displayed a punctate BCECF fluorescence on a diffuse background (Fig. 1B). TMRE-stained mitochondria in the same cells gave rise to a staining pattern (Fig. 1C) that colocalized precisely with the punctate component of the BCECF staining (Fig. 1D). Thus, the distribution of

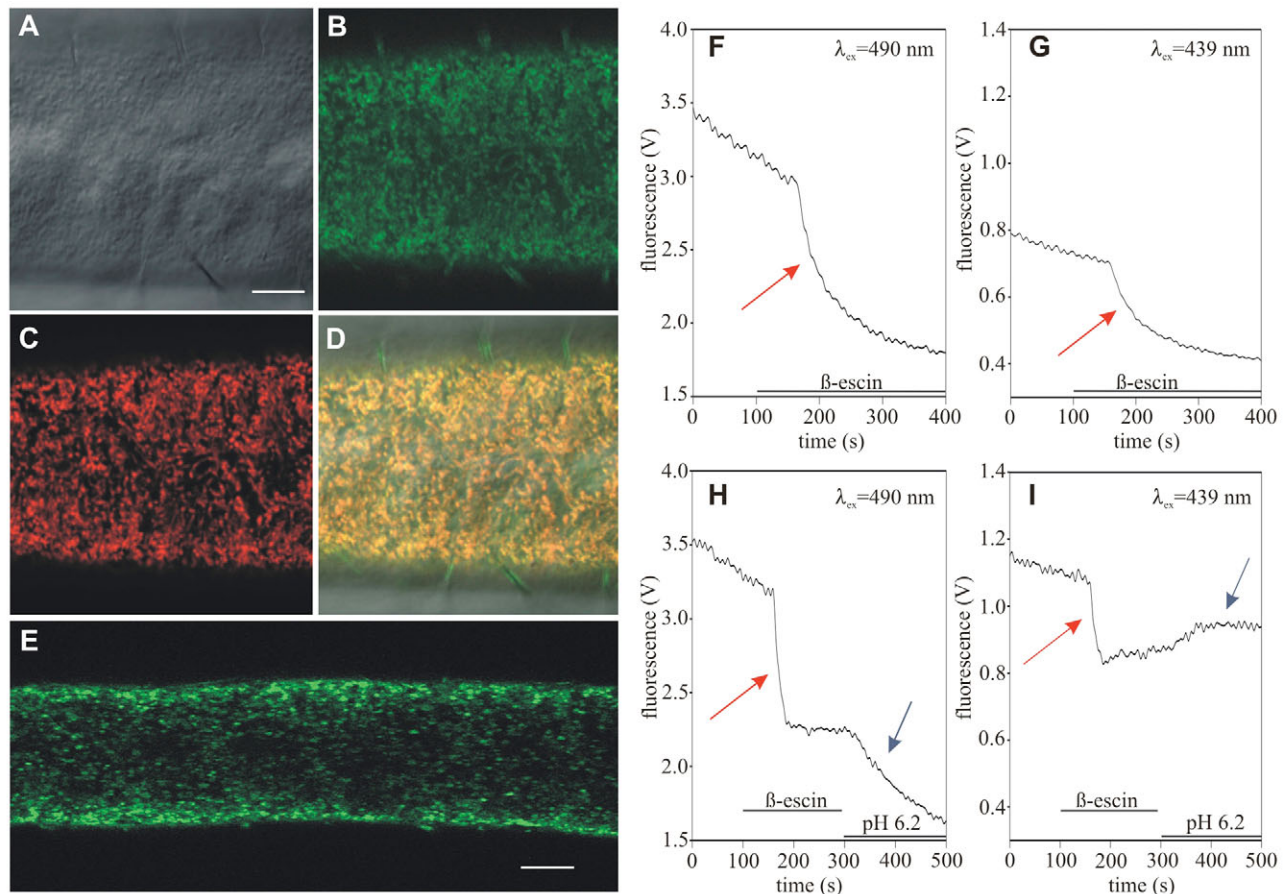


Fig. 1. (A–E) Distribution of BCECF and TMRE fluorescence in a double-labelled isolated salivary gland. (A) Tangential optical section of the gland tubule under differential interference contrast optics. (B–D) Confocal optical sections of the gland excited to display BCECF fluorescence (B) and TMRE fluorescence (C). BCECF-AM loading results in a punctate staining pattern on a diffuse background. (D) Overlaid of images of B and C in which the yellow colour indicates colocalization of BCECF-fluorescent spots and TMRE-stained mitochondria. (E) Punctate BCECF fluorescence in a permeabilized gland stained with BCECF-free acid; confocal optical section. Scale bars, 10 µm. (F,G) Drop in BCECF fluorescence excited at 490 nm and 439 nm after bath application of β -escin indicates loss of unbound dye from the cytoplasm resulting from permeabilization. (H,I) Traces showing BCECF fluorescence excited at 490 nm and 439 nm; β -escin permeabilization leads to loss of cytoplasmic dye because fluorescence emission drops at both excitation wavelengths (indicated by red arrows). A subsequent decrease in bath pH induces antiparallel changes in BCECF fluorescence (a drop in fluorescence excited at 490 nm; an increase in fluorescence excited at 439 nm, indicated by blue arrows) suggesting that the BCECF that remains after permeabilization records cytoplasmic pH changes.

BCECF fluorescence raised the possibility that some BCECF had accumulated within mitochondria, making attempts to measure cytoplasmic pH with this dye problematical. Another possible explanation for this staining pattern was the binding of BCECF to the cytoplasmic surface of mitochondria. To discriminate between these possibilities, we recorded the BCECF fluorescence microfluorometrically and permeabilized the cells by bath application of $200 \mu\text{g ml}^{-1}$ β -escin. Fig. 1F,G shows that the BCECF fluorescence excited at both 490 nm and at 439 nm decreased within 1–3 min after β -escin application because of dye loss from permeabilized cells. Nevertheless, even after permeabilization and dye loss from the cytoplasm, some punctate BCECF fluorescence persisted and colocalized with TMRE-stained mitochondria (data not shown). Two experimental tests were used to clarify whether this residual fluorescence arose from intramitochondrial BCECF or from dye bound to the mitochondrial surface. When the pH of the permeabilization medium was decreased to pH 6.2, the fluorescence excited at 490 nm decreased and that excited at 439 nm increased (Fig. 1H,I). These antiparallel fluorescence changes indicated that the residual punctate BCECF fluorescence was sensitive to cytoplasmic pH changes. In addition, we permeabilized glands and incubated them with BCECF-free acid, which is not able to enter mitochondria. Such BCECF-stained glands displayed the same punctate staining pattern as intact glands loaded with BCECF-AM (Fig. 1E). Together, these observations indicate that a fraction of the BCECF binds to the outer mitochondrial surface and, nevertheless, monitors cytoplasmic pH and its changes (not intramitochondrial pH).

Resting intracellular pH

BCECF fluorescence measurements were calibrated at the end of experiments by the nigericin- K^+ method (Deitmer and Schild, 2000; Thomas et al., 1979). The BCECF fluorescence ratio was recorded from cells that were superfused with high- K^+ -nigericin

calibration solutions with pH values ranging from 6.2 to 8.2. These fluorescence ratios were used to obtain calibration curves (data not shown). The BCECF fluorescence ratio was almost linearly related to pH_i between pH 7.0 and 8.0. The steady-state pH_i obtained in this way in Tris-buffered HCO_3^- -free PS was 7.5 ± 0.3 ($N=96$ flies).

5-HT- and cAMP-induced changes in pH_i

Stimulation of the isolated salivary glands with low concentrations of 5-HT (0.3 – 3 nmol l^{-1} 5-HT) induced a reversible and dose-dependent intracellular acidification of up to about 0.2 pH units (Fig. 2A–C,G). Higher 5-HT concentrations ($\geq 10 \text{ nmol l}^{-1}$; data are presented for 10 nmol l^{-1} 5-HT; data for 30 nmol l^{-1} and 100 nmol l^{-1} 5-HT are not shown) induced either a reversible monophasic acidification (11 out of 15 measurements) as shown in Fig. 2D or variable bi- or even multiphasic pH changes (Fig. 2E,F). Some pH changes were characterized by a transient alkalinization after a 5-HT washout (7 out of 15 measurements; Fig. 2E). Multiphasic 5-HT-induced pH changes began typically with a transient alkalinization followed by an acidification in the continuous presence of 5-HT. Then, after 5-HT washout, pH_i displayed a transient alkalinization once again (Fig. 2F). The magnitude of the multiphasic pH changes induced by 10 nmol l^{-1} 5-HT was large, spanning up to 1.4 pH units.

5-HT induces an increase in intracellular cAMP concentration ($[\text{cAMP}]_i$) in blowfly salivary glands (Heslop and Berridge, 1980). An artificial increase in $[\text{cAMP}]_i$ by bath application of cAMP, the inhibition of phosphodiesterase with IBMX or the stimulation of adenylyl cyclase with forskolin has been shown to induce fluid secretion from isolated glands (Berridge, 1970; Berridge and Patel, 1968; Berridge and Prince, 1971) and to stimulate V-ATPase-mediated H^+ -pumping into the gland lumen (Dames et al., 2006; Rein et al., 2006; Rein et al., 2008). Therefore, we tested whether and to what extent experimental conditions that elevate $[\text{cAMP}]_i$ also affect pH_i and mimic the effects of 5-HT on pH_i . Fig. 3A,E

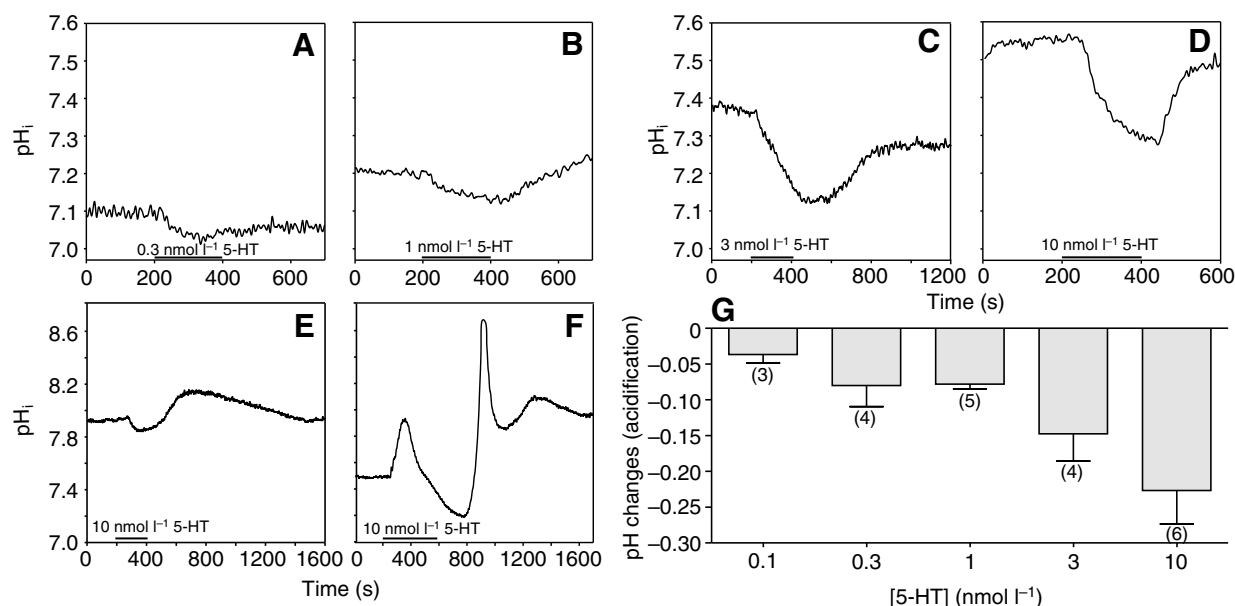


Fig. 2. (A–F) Changes in pH_i as a function of 5-HT concentration. 5-HT concentrations and the duration of 5-HT application are indicated in each panel. 5-HT concentrations $\leq 10 \text{ nmol l}^{-1}$ induce monophasic drops in pH_i . Only concentrations $\geq 10 \text{ nmol l}^{-1}$ produce bi- or multiphasic pH changes. (G) Summary data for acidifications induced by 0.1 – 10 nmol l^{-1} 5-HT (total pH_i changes). Summary data for 10 nmol l^{-1} 5-HT represent only monophasic acidification. Data in G are means \pm s.e.m.; the number of experiments is given in parentheses.

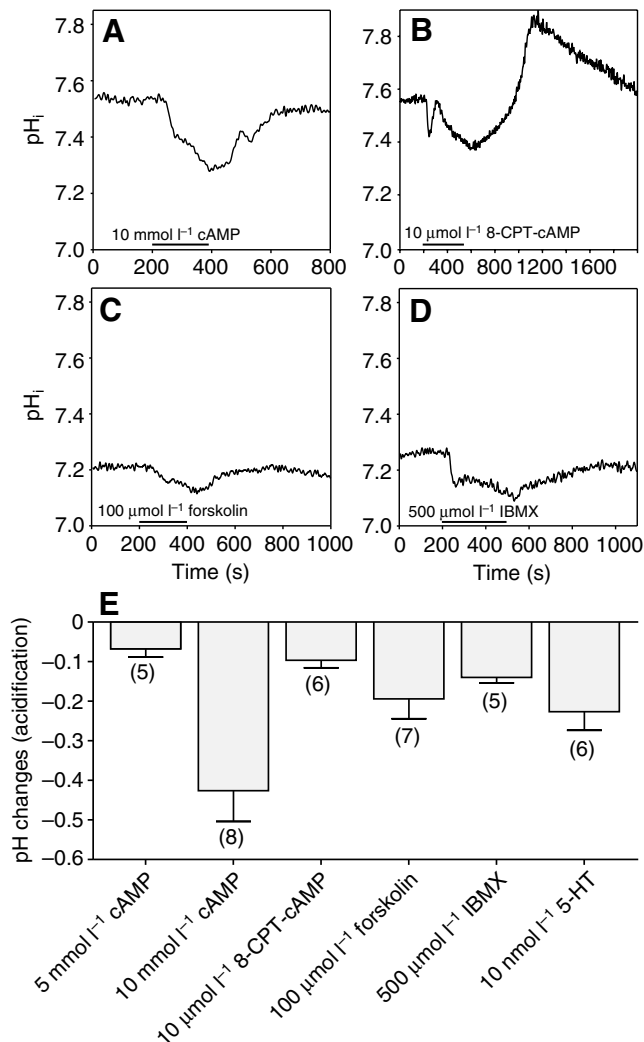


Fig. 3. (A–E) pH_i changes induced by bath application of 10 mmol l^{-1} cAMP (A), 10 μ mol l^{-1} 8-CPT-cAMP (B), 100 μ mol l^{-1} of the adenylyl cyclase inhibitor forskolin (C) and 500 μ mol l^{-1} of the phosphodiesterase inhibitor IBMX (D). These experiments show that increases in intracellular cAMP concentration mimic the acidifying effect of 5-HT stimuli. (E) Quantitative analysis of the experiments. Data are means \pm s.e.m.; the number of experiments is given in parentheses.

shows that bath application of 5 or 10 mmol l^{-1} cAMP induced a reversible intracellular acidification. The more membrane-permeable cAMP analogues cAMPS-Sp (200 μ mol l^{-1}) and 8-CPT-cAMP (10 μ mol l^{-1}) produced similar acidifications (Fig. 3B,E). pH_i changes at lower concentrations (Fig. 3B,E). pH_i was also decreased by bath application of 100 μ mol l^{-1} forskolin or 500 μ mol l^{-1} IBMX (Fig. 3C–E). Thus, experimental conditions that elevate $[cAMP]_i$ produce pH_i changes similar to those following 5-HT treatment. In this study we focus on the question of how 5-HT application or an increase in intracellular cAMP concentration cause the unexpected acidification, although cAMP is known to stimulate a protein kinase A-mediated outward H^+ pumping by the apical V-ATPase (Zimmermann et al., 2003; Dames et al., 2006; Rein et al., 2006; Rein et al., 2008; Voss et al., 2007). We make no attempts yet, to characterize the relative contribution of all mechanisms responsible for generating the complex waveforms of bi- or multiphasic pH changes.

5-HT and cAMP stimulate cellular respiration

What is the source of the acid equivalents that are responsible for the 5-HT-induced acidification? We supposed that the 5-HT- and cAMP-induced acidification may have been caused, at least partly, by increased respiration and CO_2 production, because we had recently measured a strong 5-HT-induced stimulation of cellular respiration in *Calliphora* salivary glands (Schmälzlin et al., 2005). We now tested whether elevated $[cAMP]_i$ and cAMP-induced V-ATPase-mediated H^+ pumping contributed measurably to the stimulation of O_2 consumption. We recorded tissue O_2 content microfluorometrically by evaluating the O_2 -sensitive luminescence lifetime of polystyrene beads containing PtPFP. The beads were pressure-injected into the lumen of isolated gland tubules (Schmälzlin et al., 2006). Fig. 4A–C illustrates three original recordings. In this figure, 100% O_2 concentration corresponds to the O_2 content in the bath PS, which is in equilibrium with ambient air. As the O_2 -sensitive beads lie within the lumen of a gland tubule, they sense O_2 that has diffused radially from the bath through the gland epithelium into the gland lumen. Because of cellular respiration, the resting O_2 content in the gland lumen varied between 60–80% air of that in the bath (Fig. 4A–C). We first tested whether 5-HT-induced stimulation of the apical V-ATPase contributed measurably to the 5-HT-induced activation of cellular respiration. Fig. 4A,D shows that luminal O_2 concentration dropped by about 10% upon stimulation of the glands with 10 nmol l^{-1} 5-HT, a concentration that saturates the rate of fluid transport. After 5-HT washout, we superfused the preparation for 400 s with the specific V-ATPase blocker concanamycin A and then applied 10 nmol l^{-1} 5-HT again in the presence of concanamycin A. The 5-HT-induced drop in luminal O_2 concentration was not significantly affected by concanamycin A (Fig. 4A,D). This result was not unexpected because cellular respiration is regulated by a highly complex set of variables (Brown, 1992; Boneh, 2006) and because 5-HT stimulates not only the apical V-ATPase, but also a number of ATP-consuming processes such as Na^+/K^+ -pump activity, SERCA-pump activity and the secretion of salivary enzymes. Therefore, in order to reduce complexity and to avoid an activation of the $Ins(1,4,5)P_3/Ca^{2+}$ -signalling pathway, we stimulated the V-ATPase by bath application of 10 μ mol l^{-1} 8-CPT-cAMP (Rein et al., 2006). Fig. 4B,D shows that 8-CPT-cAMP stimulated a drop in luminal O_2 concentration, as did a 5-HT stimulus. These figures also show that blocking V-ATPase activity with concanamycin A decreased the 8-CPT-cAMP-induced drop in luminal O_2 concentration significantly. Another way to minimize the possible contribution of a 5-HT-induced elevation in $[Ca^{2+}]_i$ or, as a consequence, ATP-consuming sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA)-pump activity to the activation of cellular respiration is to deplete the Ca^{2+} store by the inhibition of Ca^{2+} reuptake with the SERCA-pump inhibitor thapsigargin in the absence of extracellular Ca^{2+} . Under these conditions, 5-HT elicits only a transient Ca^{2+} elevation (Zimmermann and Walz, 1997). Fig. 4C shows that bath application of thapsigargin in Ca^{2+} -free PS caused luminal O_2 concentration to increase by almost 20%. We did not examine whether this decrease in cellular respiration was attributable to decreased ATP hydrolysis by SERCA pumps or to a changed cytoplasmic Ca^{2+} concentration. Fig. 4C,D shows, however, that the 5-HT-induced drop in luminal O_2 concentration was significantly reduced in Ca^{2+} -free thapsigargin-containing PS. Taken together, these experiments demonstrate that 5-HT or an elevation in $[cAMP]_i$ stimulate cellular respiration. They also show that (1) the cAMP and Ca^{2+} signalling pathways contribute in a complex manner to a (5-HT-induced) activation of cellular

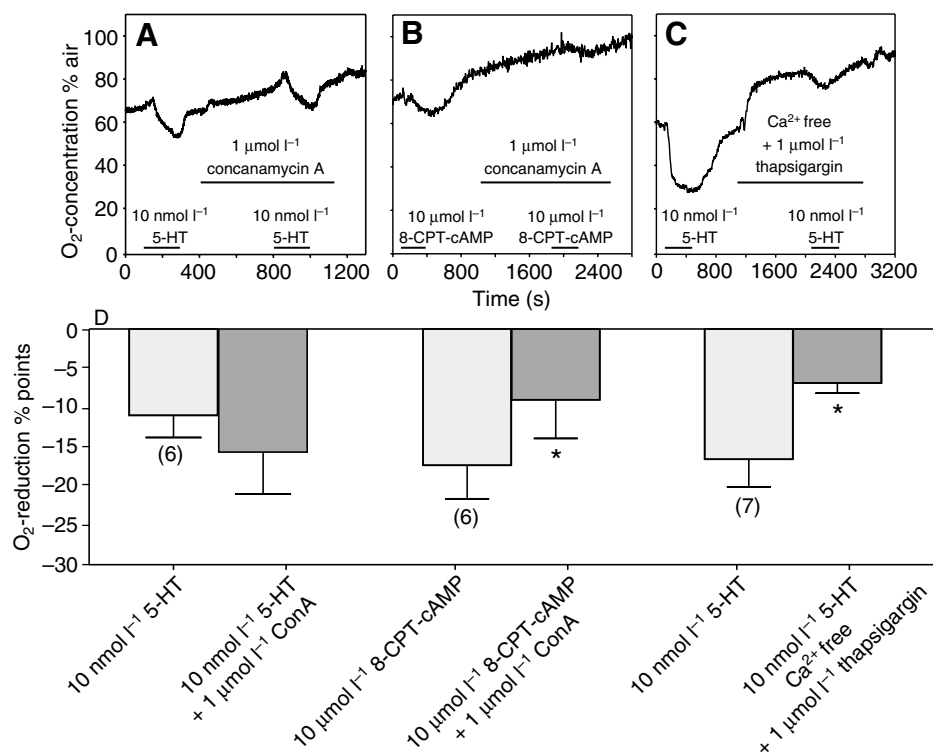


Fig. 4. Microfluorometric measurements of oxygen content in the lumen of isolated salivary gland tubules injected with polystyrene beads containing the O₂-sensitive luminescent dye PtPFPF. 100% O₂ concentration in A–C corresponds to the O₂ content in the bath PS, which is in equilibrium with ambient air. (A) 10 nmol l⁻¹ 5-HT stimulates cellular respiration and a drop in luminal O₂ concentration that is not significantly reduced in the presence of concanamycin A (A,D). The 8-CPT-cAMP-induced drop in luminal O₂ concentration is significantly reduced by concanamycin A (B,D). Application of 1 μmol l⁻¹ thapsigargin in Ca²⁺-free PS causes an increase in luminal O₂ concentration, and the 5-HT-induced drop in luminal O₂ concentration is significantly reduced under these conditions (C,D). Data in D are means ± s.e.m.; the number of experiments is given in parentheses; *P<0.05.

respiration and (2) that ATP consumption by V-ATPase activity contributes measurably to the stimulation of respiration in the absence of SERCA-pump activity.

Localization of CA activity

Sites of CA activity were identified in cryostat-sectioned salivary gland tubules by the classical cobalt/phosphate method of Hansson (Hansson, 1967) as modified by Brown (Brown, 1980). Fig. 5B,C shows that the epithelial cells contained the typical precipitates of the reaction product of CA activity, which was not detectable over the whole cell bodies but was restricted to the basal cell pole. Control sections, incubated in the presence of the CA inhibitor acetazolamide (10 μmol l⁻¹), were free of reaction product (Fig. 5A). Thus, the salivary gland cells contain CA, which is localized close to the basal plasma membrane domain.

Effect of CA and V-ATPase activity on 5-HT-induced pH changes

The above experiments indicate that CO₂ production by cellular respiration, which is at least partly attributable to V-ATPase activity,

may contribute to 5-HT-induced pH changes. Therefore, we subsequently examined in which way 5-HT-induced pH changes altered when CA and/or V-ATPase were blocked.

Fig. 6A shows that an inhibition of CA activity by bath application of acetazolamide led to a slow intracellular acidification. The CA-inhibitor ethoxzolamide (1 μmol l⁻¹) had a comparable effect (data not shown). Stimulation with 10 nmol l⁻¹ 5-HT in the continuous presence of acetazolamide induced an alkalinization (Fig. 6A,F) in all tested preparations (N=14) and never an acidification as under control conditions. Application of 10 μmol l⁻¹ 8-CPT-cAMP in the presence of acetazolamide also led to an intracellular alkalinization (Fig. 6B, N=7).

To examine whether V-ATPase-mediated proton pumping affected pH_i, we analysed the effect of the specific V-ATPase blocker concanamycin A on 5-HT-induced intracellular pH changes. Fig. 6C,D shows that bath application of 1 μmol l⁻¹ concanamycin A alone caused a slow intracellular acidification, probably because of inhibited basal V-ATPase activity and outward proton pumping in unstimulated cells (Zimmermann et al., 2003). In those preparations in which 10 nmol l⁻¹ 5-HT alone induced a

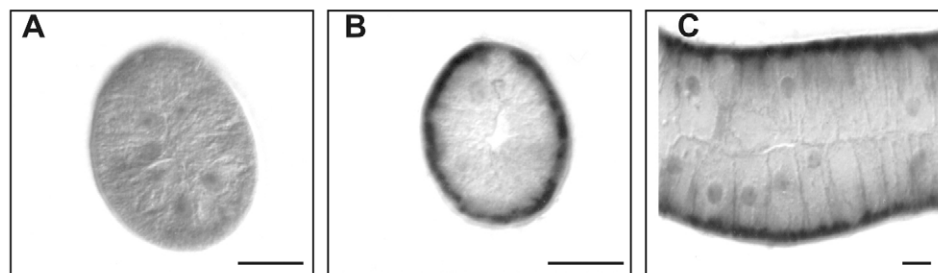


Fig. 5. (A–C) Location of carbonic anhydrase (CA) in cryostat sections of salivary gland tubules. The dark reaction product indicating CA activity is localized at the basal pole of the secretory cells as shown in a cross section (B) and a longitudinal section (C). Formation of the reaction product is completely inhibited by 10⁻⁵ mol l⁻¹ of the CA inhibitor acetazolamide (A). Scale bars, 20 μm.

monophasic acidification (Fig. 6C), concanamycin A had no apparent effect on the 5-HT-induced pH change (Fig. 6C). However, in preparations in which 10 nmol l⁻¹ 5-HT produced a multiphasic pH change, concanamycin A blocked the alkali-going response components; the 5-HT-induced pH responses became monophasic (Fig. 6D).

We next studied 5-HT-induced pH changes when both CA activity and V-ATPase were inhibited (Fig. 6E). When acetazolamide and concanamycin A were applied to unstimulated preparations together, these substances caused an additive acidification of resting pH_i as shown in Fig. 6E. 5-HT induced, in the presence of acetazolamide and concanamycin A, an alkalinization (Fig. 6E) but this alkalinization was about 0.2 pH units lower than that in the absence of concanamycin A (Fig. 6F).

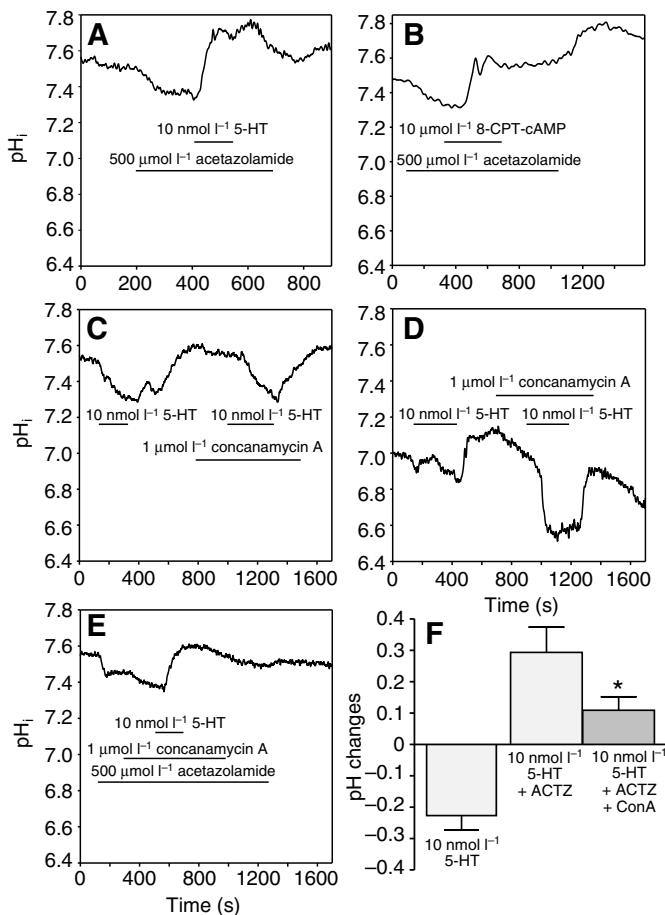


Fig. 6. Effects of acetazolamide and concanamycin A on 5-HT-induced pH_i changes. (A) Bath application of 500 μmol l⁻¹ acetazolamide causes a cytoplasmic acidification, whereas 10 nmol l⁻¹ 5-HT, applied in the presence of acetazolamide, produces an alkalinization. (B) In the presence of acetazolamide, 10 μmol l⁻¹ 8-CPT-cAMP induces an intracellular alkalinization. (C,D) Inhibition of the apical V-ATPase by 1 μmol l⁻¹ concanamycin A causes a small acidification. In preparations in which 10 nmol l⁻¹ 5-HT induces a monophasic acidification (C), this pH change is almost unaffected by concanamycin A. When 10 nmol l⁻¹ 5-HT induces a multiphasic pH change, concanamycin A blocks the alkalinizing response component (D). (E) Acetazolamide and concanamycin A applied together cause additive acidifications, and 10 nmol l⁻¹ 5-HT causes an alkalinization in the presence of these two inhibitors (E). The alkalinization produced by 10 nmol l⁻¹ 5-HT in the presence of acetazolamide is significantly ($P < 0.05$) smaller when the V-ATPase is simultaneously inhibited by concanamycin A (F). Data in F are means \pm s.e.m., $N = 9$.

Involvement of Na⁺/H⁺ antiporter (NHE) and anion exchanger (AE) activity on 5-HT-induced pH changes

The Na⁺/H⁺ antiporter (NHE) and anion exchanger (AE) are known to be involved in saliva secretion in mammals (Turner and Sugiya, 2002) and these transporters contribute to pH regulation in many cell types (Boron, 2004). Therefore, we tested whether these transporters are also involved in shaping 5-HT-induced intracellular pH changes. The simplest method for the inhibition of NHE activity is the removal of extracellular Na⁺. Unexpectedly, the removal of extracellular Na⁺ led to a strong alkalinization in *Calliphora* salivary gland cells (Fig. 7A). Stimulation of the salivary glands with 10 nmol l⁻¹ 5-HT in the absence of extracellular Na⁺ induced a small intracellular alkalinization, but no longer an acidification as seen under control conditions (Fig. 7A,E). To test whether this effect was related to NHE activity, we stimulated the cells with 10 nmol l⁻¹ 5-HT in the presence of 50 μmol l⁻¹ EIPA, a specific inhibitor of the NHE (Petzel, 2000). Bath application of EIPA alone had no effect

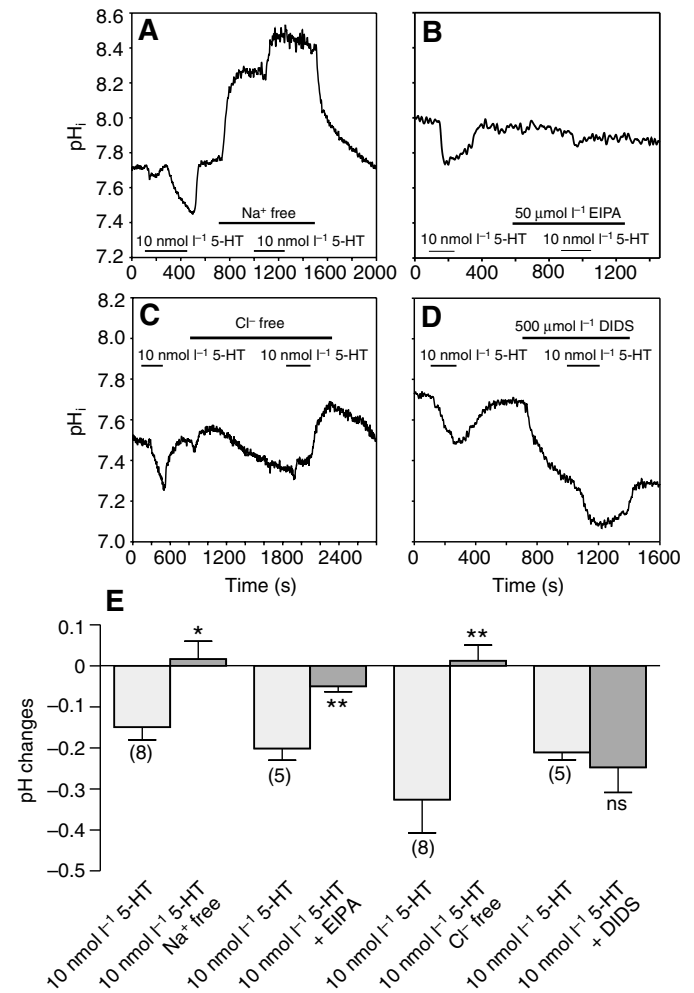


Fig. 7. Removal of Na⁺ from the bath causes an alkalinization (A). (A,E) Under Na⁺-free conditions, 10 nmol l⁻¹ 5-HT induces a small intracellular alkalinization ($*P < 0.05$). (B,E) In the continuous presence of 50 μmol l⁻¹ EIPA, 10 nmol l⁻¹ 5-HT induces a significantly smaller acidification than under control conditions ($**P < 0.01$). (C,E) In the absence of extracellular Cl⁻, 10 nmol l⁻¹ 5-HT induces a small alkalinization, which is significantly different from the control stimulation ($**P < 0.01$). (D,E) Application of 500 μmol l⁻¹ DIDS causes a strong acidification but does not significantly influence the 5-HT-induced acidification ($*P > 0.05$). Data in E are means \pm s.e.m. The number of replicates is given in parentheses.

on steady-state pH_i (Fig. 7B). However, under these conditions, the 5-HT-induced intracellular acidification was significantly reduced (Fig. 7B,E).

In order to obtain at least preliminary information on the involvement of an AE on 5-HT-induced pH changes, we examined the requirement of extracellular Cl^- for the 5-HT-induced acidification observed under control conditions. We observed that pH_i slowly became more acid upon the removal of extracellular Cl^- (Fig. 7C). Stimulation with 10 nmol l^{-1} 5-HT under Cl^- -free conditions led to a small intracellular alkalization, but no longer to an intracellular acidification as under control conditions (Fig. 7C,E). In addition, pH_i displayed a transient post-alkalinization after 5-HT washout (in five out of five preparations; Fig. 7C). This observation suggested that a $\text{Cl}^-/\text{HCO}_3^-$ antiporter was active and contributed to the 5-HT-induced acidification. As a further experimental test, we recorded 5-HT-induced pH changes in the presence of DIDS, an inhibitor of HCO_3^- transport (Boron, 2001). Fig. 7D shows that application of $500 \mu\text{mol l}^{-1}$ DIDS led to a strong acidification and that 10 nmol l^{-1} 5-HT induced an intracellular acidification in the continuous presence of DIDS, an acidification that was not significantly different from the acidification recorded under control conditions (Fig. 7D,E).

Taken together, these data indicate that an NHE and a Cl^- -dependent process contribute to the 5-HT-induced pH changes. The Cl^- dependence may be attributable to a DIDS-insensitive $\text{Cl}^-/\text{HCO}_3^-$ antiporter, which transports HCO_3^- out of the cell.

Alkalinization under Na^+ -free conditions

We described above that removal of extracellular Na^+ led to an intracellular alkalization of about 0.22 pH units (Fig. 7A, Fig. 8A). This observation was unexpected, because all known pH-regulating transporters that use the inwardly directed electrochemical Na^+ gradient either export H^+ or import HCO_3^- (Boron, 2004). Therefore, the withdrawal of extracellular Na^+ was expected to induce an intracellular acidification, and an explanation of the observed alkalization was not immediately obvious. At this point, we considered the possibility that salivary gland cells contained a Na^+ -driven organic acid import system as described for some insect Malpighian tubules (Ruiz-Sanchez and O'Donnell, 2006; Linton and O'Donnell, 2000; Maddrell et al., 1974) and mammalian cells (Kanai and Hediger, 2003). Our *Calliphora* physiological saline contains glutamate, and Na^+ -driven glutamate uptake acts as an acid loader. As a first experimental test of this hypothesis, we removed the amino acid glutamate from the bathing medium. This led to an intracellular alkalization resembling that seen after withdrawal of extracellular Na^+ . Under glutamate-free conditions, we observed an intracellular alkalization of about 0.13 pH units (Fig. 8B). The alkalization under glutamate-free conditions was not significantly different

from the alkalization under Na^+ -free conditions (Fig. 8C). This preliminary observation might be the first indication for the presence of a Na^+ -driven organic acid cotransporter in *Calliphora* salivary glands; its characterization will be the subject of a future study.

DISCUSSION

The major observations from the present study are that (1) V-ATPase, carbonic anhydrase (CA), and probably a Na^+ -driven organic acid cotransporter affect steady-state pH_i in unstimulated cells, (2) 5-HT-induced electrolyte and fluid secretion are accompanied by an intracellular acidification, (3) this acidification requires CA activity because 5-HT induces a concanamycin A-sensitive intracellular alkalization when CA is blocked, (4) the increased cellular respiration contributes to the 5-HT-induced acidification, (5) V-ATPase activity, NHE activity and a Cl^- -dependent process are involved in generating 5-HT-induced pH changes.

Methodological aspects

BCECF has been employed in numerous studies as a probe for cytoplasmic pH (e.g. Deitmer and Schild, 2000). However, in order for a fluorescent dye to be used as a specific probe for ion concentrations within a distinct subcellular compartment, the dye must be confined to that compartment. We have observed that BCECF-AM loading of *Calliphora* salivary glands leads to distinct punctate fluorescence on a diffuse background staining. The diffuse fluorescence represents freely mobile BCECF in the cytoplasm, as it is rapidly lost upon permeabilization of the plasma membrane. The punctate fluorescence component colocalizes with TMRE-stained mitochondria and remains after cell permeabilization. We thus have to consider that BCECF might accumulate within mitochondria, as has been described previously in a number of preparations (Slayman et al., 1994; Weinlich et al., 1998). Two observations indicate, however, that BCECF does not accumulate in the mitochondrial matrix in our preparations, but rather binds to the mitochondrial outer surface: first, incubation of β -escin-permeabilized preparations with membrane-impermeable BCECF stains mitochondria in a similar way as BCECF-AM loading of intact preparations and, second, a resting pH_i of ~ 7.5 (see below) argues against the possibility that we have recorded intramitochondrial pH. In the mitochondrial matrix, pH amounts to >8.0 (Abad et al., 2004). These observations and our conclusion that the punctate fluorescence in permeabilized salivary glands monitors pH changes in the bath reliably support a previous suggestion, derived from a slightly different experimental approach in other cell types (Weinlich et al., 1998), that BCECF reports cytoplasmic pH despite its non-homogeneous intracellular distribution.

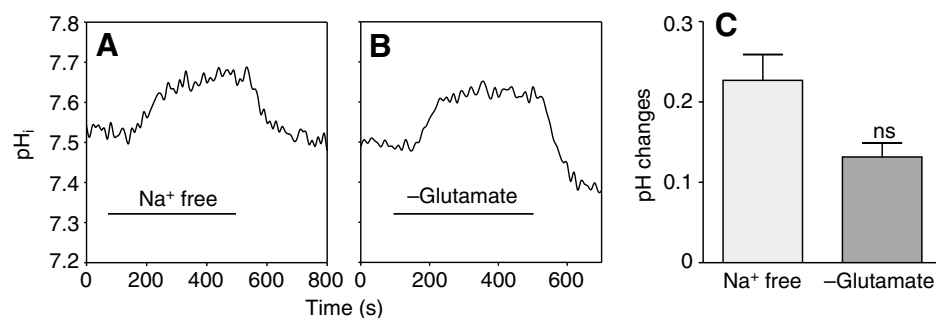


Fig. 8. (A) Removal of extracellular Na^+ leads to an intracellular alkalization (0.22 ± 0.03 pH units; $N=29$). (B) Removal of extracellular glutamate induces an intracellular alkalization (0.13 ± 0.02 , $N=6$) that is not significantly different from that observed under Na^+ -free conditions ($P>0.05$). (C). Data in C are means \pm s.e.m.

Steady-state pH_i

In HCO₃⁻-free PS, we have determined a steady-state pH_i of 7.5±0.3 in our salivary gland preparations. Insect Malpighian tubules can usefully serve as a structure for comparison. Bertram and Wessing (Bertram and Wessing, 1994) have measured, with double-barrelled pH-sensitive microelectrodes, almost the same pH in the proximal (pH 7.7) and distal (pH 7.4) segments of the Malpighian tubules in *Drosophila* larvae and, with the same method, a more acidic pH of 7.0 has been determined in Malpighian tubule cells of *Rhodnius prolixus* (Ianowski and O'Donnell, 2006).

Luminal pH in unstimulated *Calliphora* salivary glands is 7.4±0.2 (Rein et al., 2006). The bath pH is 7.2 in our experiments. With the known basolateral and apical membrane potentials of *Calliphora* salivary gland cells [−44 mV and −59.5 mV, respectively (Prince and Berridge, 1972; Berridge et al., 1975)], the Nernst equation reveals that the intracellular H⁺ concentration is approximately tenfold less than that expected from passive H⁺ distribution across either membrane domain. Thus, the cells maintain their pH_i at a value that requires active pH regulation, even in unstimulated glands. Our observation that the V-ATPase inhibitor concanamycin A causes a slow acidification of unstimulated gland cells indicates that this acid extruder is active, even in unstimulated glands, and contributes to steady-state pH_i regulation (Fig. 9A). Four further lines of evidence support this conclusion. In unstimulated glands, (1) the transepithelial potential (TEP) is about +15.5 mV [lumen with respect to the bath (Berridge et al., 1975)] because of net transepithelial cation transport in resting glands, (2) bafilomycin-A₁-sensitive V-ATPase activity accounts for 36% of the total

ATPase activity in homogenates of unstimulated glands (Zimmermann et al., 2003), (3) a fraction of about 25% of the available V₁ subcomplexes is assembled in the active V₀V₁ holoenzyme at the apical membrane (Zimmermann et al., 2003; Dames et al., 2006), (4) luminal pH changes in the presence of concanamycin A (Rein et al., 2006). Thus, the apical V-ATPase contributes in a significant way to pH regulation in unstimulated *Calliphora* salivary glands.

We observed also that carbonic anhydrase inhibition by acetazolamide application led to a small acidification. At present, we cannot explain this observation, because the concerted action of acid extruder (V-ATPase, Na⁺-dependent HCO₃⁻ transporter) and acid loader (Na⁺-glutamate cotransporter; see below and Fig. 9A) as well intracellular CO₂ caused by cellular respiration at rest and/or CO₂ diffusion affect acid–base balance and the carbonic anhydrase catalyzed equilibrium reaction in an extremely complex way.

However, our observation that steady-state pH_i does not change upon application of the NHE-inhibitor EIPA suggests that this transporter is not involved in the regulation of resting pH. However, we have observed a slow acidification upon the removal of extracellular Cl⁻ and a strong and fast acidification upon bath application of the AE-inhibitor DIDS. These observations indicate that a DIDS-sensitive AE contributes to steady-state pH_i. This interpretation is supported by the analysis of the kinetics of pH_i recovery after application of an extracellular NH₄Cl pulse: recovery from the resulting acidification is significantly slowed in the presence of DIDS (B.S. and B.W., unpublished). The DIDS-sensitive transporter is unlikely to be a Cl⁻/HCO₃⁻ antiporter, because this AE is an acid loader rather than an acid extruder. Possible candidates for the DIDS sensitivity of steady-state pH_i are a Na⁺/HCO₃⁻ cotransporter or a Na⁺-driven Cl⁻/HCO₃⁻ antiporter; both of which could contribute to keeping resting pH_i high (Fig. 9A). However, the characterization of the nature of the DIDS-sensitive anion exchanger has not been the immediate aim of this study and requires a more detailed investigation, because it is difficult to distinguish between these transporters only on the basis of pharmacological experiments.

Finally, we provide preliminary evidence that a Na⁺/organic acid cotransporter may be active in importing acid equivalents into the cells, as shown for insect Malpighian tubules (Ruiz-Sanchez and O'Donnell, 2006; Linton and O'Donnell, 2000; Maddrell et al., 1974) (see below and Fig. 9A).

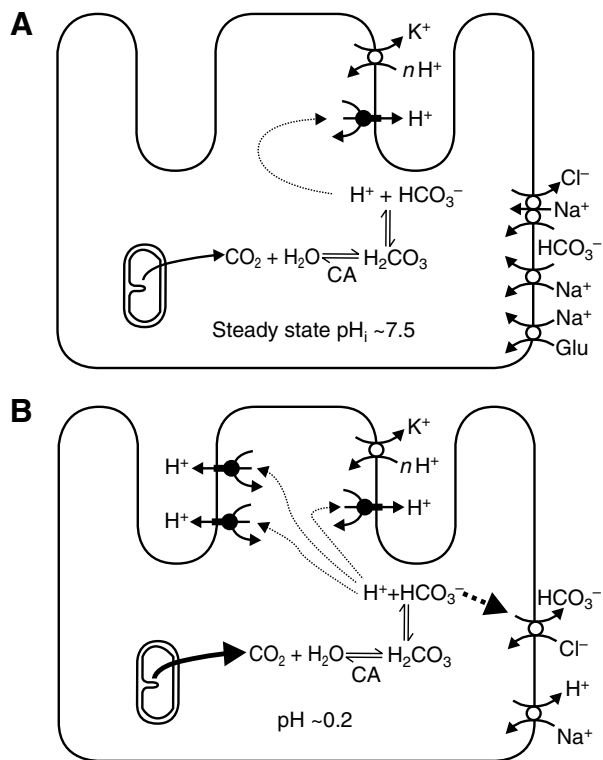


Fig. 9. Schematic representation of transporters that we discuss in conjunction with steady-state pH_i regulation (A) and the 5-HT- and cAMP-induced acidification (B). Note: 5-HT causes an acidification, a cAMP-mediated increase in the number of H⁺-pumping V-ATPase holoenzymes in the apical membrane of the gland cells and stimulates cellular respiration (B). For details, see Discussion.

5-HT-induced pH changes: the contribution of V-ATPase and CA activity

In *Calliphora* salivary glands, 5-HT stimulates bafilomycin-sensitive V-ATPase activity, the recruitment of V-ATPase complex V₁ to the apical membrane, the assembly of the V-ATPase V₀V₁ holoenzyme at the apical membrane and, as a result, enhanced H⁺ transport across the apical membrane into the gland lumen (Dames et al., 2006; Rein et al., 2006; Rein et al., 2008; Zimmermann et al., 2003). Despite this enhanced 5-HT-induced acid extrusion, we have observed that 0.3–10 nmol l⁻¹ 5-HT causes a dose-dependent intracellular acidification. 5-HT concentrations higher than 10 nmol l⁻¹ (and 10 nmol l⁻¹ 5-HT in some preparations) produce more complex bi- or even multiphasic pH changes. The result that 10 nmol l⁻¹ 5-HT elicits bi- or multiphasic pH changes in some preparations can be explained by our observation that different batches of flies differ somewhat in their sensitivity to 5-HT.

Why do the cells become more acidic despite 5-HT-induced and V-ATPase-mediated acid extrusion? We have recently found that 5-HT stimulates an increase in oxygen consumption by the salivary

gland tubules (Schmäzlin et al., 2006). Here, we have identified and localized CA activity in the gland cells. Moreover, we show that 5-HT no longer induces an acidification but rather an intracellular alkalization when CA is inhibited with acetazolamide. Because this 5-HT-induced alkalization can be reduced by concanamycin A, it must be attributable to V-ATPase-mediated outward proton pumping (Fig. 9B). These findings suggest also that the proton source for the 5-HT-induced acidification is CO₂ (from cellular respiration), which is hydrated by CA. Thus, cytosolic proton accumulation attributable to cellular respiration masks the alkalization that could be expected because of stimulated outward proton pumping. Only the multiphasic pH changes that are induced by high 5-HT concentrations (10 nmol l⁻¹ in some preparations and generally at 5-HT concentrations higher than 10 nmol l⁻¹) contain alkali-going response components that can be blocked by concanamycin A and are therefore directly attributable to V-ATPase activity.

All these effects seem to be mediated by cAMP, the messenger that stimulates the apical V-ATPase (Zimmermann et al., 2003; Dames et al., 2006), because an elevation of intracellular [cAMP] by bath application of cAMP, 8-CPT-cAMP, forskolin or IBMX produces a similar intracellular acidification as 5-HT and, as shown here for 8-CPT-cAMP, a concanamycin A-sensitive increase in oxygen consumption. We have observed that 5-HT induces the stimulation of oxygen consumption in the absence of SERCA-pump activity in Ca²⁺-free thapsigargin-containing PS [thapsigargin is used in order to reduce the complex contribution of the Ins(1,4,5)P₃/Ca²⁺ signalling pathway to the stimulation of cellular respiration]. This supports the view that the cAMP signalling pathway that stimulates ATP-consuming V-ATPase activity contributes to the intracellular acidification as a consequence of activated cellular respiration.

If the 5-HT-induced acidification discussed above is indeed caused by CO₂ from cellular respiration and by CA-mediated H⁺ and HCO₃⁻ production, the HCO₃⁻ produced must be removed efficiently. Therefore, we have tested whether AE activity contributes measurably to the 5-HT-induced acidification. We have found that the 5-HT-induced acidification is strongly Cl⁻ dependent, because 5-HT produces only a small alkalization under Cl⁻-free conditions, followed by a larger transient alkalization upon 5-HT washout. The Cl⁻/HCO₃⁻ antiporter is the only acid loader among the anion exchangers and is DIDS-sensitive in many systems. We have observed, however, that the 5-HT-induced acidification is Cl⁻ but not DIDS sensitive. Thus, *Calliphora* salivary glands could well contain a DIDS-insensitive Cl⁻/HCO₃⁻ antiporter (Fig. 9B), as has been described in β -intercalated cells in rabbit kidneys and other systems (Boron, 2001; Tsuganezawa et al., 2001). The transient post-alkalinization after 5-HT washout under Cl⁻-free conditions can be explained by ongoing V-ATPase-mediated outward H⁺ pumping, a process that is also responsible for the transient and large positive-going changes in transepithelial potential after 5-HT removal (Berridge and Prince, 1971). Physiologically, stimulated Cl⁻/HCO₃⁻ activity would be useful not only for HCO₃⁻ export, but also for basolateral Cl⁻ import in order to keep intracellular Cl⁻ high enough for apical Cl⁻ secretion.

We have also tested whether NHE activity contributes to the observed 5-HT-induced acidification. We have recorded that stimulation of the salivary glands with 10 nmol l⁻¹ 5-HT under Na⁺-free conditions leads to a small 5-HT-induced alkalization but no longer to an acidification. In addition, in the presence of the specific NHE-inhibitor EIPA (Petzel, 2000), the 5-HT-induced acidification is significantly reduced. At first glance, this result is counterintuitive, because if 5-HT activates an NHE as an acid extruder in some way, the 5-HT-induced acidification would be expected to become larger,

instead of smaller, upon NHE inhibition. However, if Na⁺/H⁺ exchange were coupled functionally to Cl⁻/HCO₃⁻ exchange (Fig. 9B), the lower parallel HCO₃⁻ export would lead to a lower acidification as observed.

Alkalinization under Na⁺-free conditions

The inwardly directed electrochemical Na⁺ gradient is of key importance for intracellular pH regulation. Na⁺-dependent transporters such as the Na⁺/H⁺ antiporter or Na⁺/HCO₃⁻ cotransporter use the electrochemical Na⁺ gradient for H⁺ export or HCO₃⁻ import (Fig. 9). In the absence of extracellular Na⁺, the described transporters are blocked and an intracellular acidification is to be expected (Boron, 2004). We have observed, in *Calliphora* salivary glands, that removal of extracellular Na⁺ leads to intracellular alkalization, suggesting the presence of a Na⁺-dependent acid loader. Known Na⁺-dependent acid loaders are Na⁺-driven amino acid transporters (Kanai and Hediger, 2003), which have been shown to be present in insect Malpighian tubules (Ruiz-Sanchez and O'Donnell, 2006; Linton and O'Donnell, 2000; Maddrell et al., 1974) and, for example leech giant glial cells (Deitmer and Schneider, 1997). In the latter preparation, glutamate application causes an intracellular acidification due to Na⁺-driven glutamate uptake (Deitmer and Schneider, 1997). Our observation that removal of glutamate from the bath leads to an alkalization similar to that following Na⁺ removal suggests that *Calliphora* salivary glands may indeed contain a Na⁺-dependent glutamate transporter. This suggestion is supported by the observation (Berridge, 1970) that only a small activation of fluid secretion occurs in the absence of glutamate. The major metabolic substrates in these cells are concluded to be certain amino acids that pass directly into the citric acid cycle rather than those entering the glycolytic pathway, such as glucose or trehalose (Berridge, 1970; Rapp and Berridge, 1980). Taken together, we favour the hypothesis that *Calliphora* salivary glands express a Na⁺-dependent glutamate transporter. We are currently searching for molecular evidence of its presence.

This work was supported by the Deutsche Forschungsgemeinschaft (Wa 463/9-5 and Graduiertenkolleg 837). We thank Drs O. Baumann and C. Hille for critically reading the manuscript and for helpful suggestions.

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