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RESEARCH ARTICLE

Intracellular pH regulation in unstimulated *Calliphora* salivary glands is Na^+ dependent and requires V-ATPase activity

Bettina Schewe[1,*], Wolfgang Blenau[2] and Bernd Walz[3]

[1]University of Potsdam, Institute of Nutrition Science, Department of Biochemistry of Nutrition, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany, [2]Oberursel Bee Research Institute, Goethe-University Frankfurt/Main, Department of Life Science, Karl-von-Frisch-Weg 2, 61440 Oberursel, Germany and [3]University of Potsdam, Institute of Biochemistry and Biology, Department of Animal Physiology, University Campus Golm, Karl-Liebknecht-Str. 24-25, 14476 Potsdam, Germany

*Author for correspondence (schewe@uni-potsdam.de)

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SUMMARY

Salivary gland cells of the blowfly *Calliphora vicina* have a vacuolar-type H^+-ATPase (V-ATPase) that lies in their apical membrane and energizes the secretion of a KCl-rich primary saliva upon stimulation with serotonin (5-hydroxytryptamine). Whether and to what extent V-ATPase contributes to intracellular pH (pH_i) regulation in unstimulated gland cells is unknown. We used the fluorescent dye BCECF to study intracellular pH_i regulation microfluorometrically and show that: (1) under resting conditions, the application of Na^+-free physiological saline induces an intracellular alkalinization attributable to the inhibition of the activity of a Na^+-dependent glutamate transporter; (2) the maintenance of resting pH_i is Na^+, Cl^-, concanamycin A and DIDS sensitive; (3) recovery from an intracellular acid load is Na^+ sensitive and requires V-ATPase activity; (4) the Na^+/H^+ antiporter is not involved in pH_i recovery after a NH_4Cl prepulse; and (5) at least one Na^+-dependent transporter and the V-ATPase maintain recovery from an intracellular acid load. Thus, under resting conditions, the V-ATPase and at least one Na^+-dependent transporter maintain normal pH_i values of pH 7.5. We have also detected the presence of a Na^+-dependent glutamate transporter, which seems to act as an acid loader. Despite this not being a common pH_i-regulating transporter, its activity affects steady-state pH_i in *C. vicina* salivary gland cells.

Key words: *Calliphora vicina*, salivary gland, intracellular pH regulation, Na^+/H^+ antiporter, NHE, vacuolar H^+-ATPase, V-ATPase, intracellular pH, insect, blowfly, BCECF, NH_4Cl prepulse.

INTRODUCTION

The tubular salivary glands in the blowfly *Calliphora vicina* consist of a single layer of epithelial cells that surround a central lumen. The glands secrete a KCl-rich primary saliva when they are stimulated with the neurohormone serotonin [5-hydroxytryptamine (5-HT)] (Berridge, 1970; Oschmann and Berridge, 1970). A vacuolar H^+-ATPase (V-ATPase) located in the apical membrane of the gland cells is of fundamental importance for fluid secretion. Active V-ATPase generates an inward-directed electrochemical H^+-gradient that is used by a putative nH^+/K^+ antiporter for K^+ transport into the glandular lumen (Dames et al., 2006; Rein et al., 2008; Wieczorek et al., 1991; Zimmermann et al., 2003).

Previously, we have shown that stimulation of the gland with 5-HT induces an intracellular acidification (Schewe et al., 2008). Furthermore, salivary gland cells have been demonstrated to possess carbonic anhydrase activity, and the observed 5-HT-induced acidification is attributable to stimulated cellular respiration that masks outward-directed H^+ pumping by V-ATPase (Schewe et al., 2008). However, whether and to what extent V-ATPase and other intracellular pH (pH_i)-regulating transporters contribute to pH_i regulation in unstimulated salivary gland cells remains largely unknown.

A tight regulation of the acid–base balance in the unstimulated gland is fundamental for the maintenance of cell function, because pH_i is an important parameter for the regulation of a wide range of cell processes. pH_i affects physiological processes as diverse as cellular metabolism, contractility, ion channel conductivity, ion transport and cell cycle control (Madshus, 1988). A physiological pH_i set between 6.9 and 7.6 (Ilundain, 1992) has to be maintained by intracellular buffer systems and pH_i-regulating transport mechanisms. pH_i-regulating transporters are divided into acid extruders and acid loaders. Acid extruders are alkalescent and transport protons (H^+) out of the cell or bicarbonate (HCO_3^-) into the cell. The Na^+/H^+ antiporter (NHE), V-ATPase, an inward-directed Na^+/HCO_3^- cotransporter (NBC), a Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE) and a Cl^-/H^+ antiporter (ClC) belong to this group of transporters. The Cl^-/HCO_3^- antiporter [anion exchanger (AE)] and outward-directed NBC transport HCO_3^- out of the cell and act as acid loaders (Boron, 2001; Boron, 2004). The HCO_3^- transporters mentioned here belong to the solute-linked carrier 4 (*SLC4*)-like transporters. To date, only two *SLC4*-like transporters from insects have been cloned and identified, the Na^+-driven anion exchanger (NDAE) of *Drosophila* (Romero et al., 2000; Sciortino et al., 2001) and an AE of *Aedes aegypti* (Piermarini et al., 2010). *Drosophila* NDAE seems to be analogous in function to the mammalian NDCBE, and to date no orthologs of the mammalian NBCs have been found in insects.

The aim of this study was to identify pH_i-regulating mechanisms that maintain the steady-state pH_i in the salivary glands of *C. vicina*, by focusing on the role of the V-ATPase. We measured pH_i using

the fluorescent dye BCECF and characterized mechanisms that maintain normal pH_i values in the unstimulated gland.

MATERIALS AND METHODS

Animals, preparation and solutions

Blowflies (*Calliphora vicina* Robineau-Desvoidy 1830) were reared at the Institute of Biochemistry and Biology, Department of Animal Physiology at the University of Potsdam, Germany. 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}) 128 NaCl, 10 KCl, 2 $CaCl_2$, 2.7 $MgCl_2$, 3 sodium glutamate, 2.8 malic acid, 10 D-glucose and 10 TRIS (pH 7.2, adjusted with NaOH). Na^+-free saline contained (in mmol l^{-1}) 128 choline chloride, 10 KCl, 2 $CaCl_2$, 2.7 $MgCl_2$, 3 sodium glutamate, 2.8 malic acid, 10 D-glucose and 10 TRIS (pH 7.2, adjusted with KOH). Cl^--free saline contained (in mmol l^{-1}) 128 sodium isothionate, 5 K_2SO_4, 2 $CaSO_4$, 2 $MgSO_4$, 2.8 malic acid, 3 sodium glutamate, 10 TRIS and 10 D-glucose (pH 7.2, adjusted with H_2SO_4). All experiments were carried out at room temperature (~22°C).

Reagents

2′-7′-Bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was obtained from Invitrogen (Karlsruhe, Germany), Cell Tak was from BD Biosciences (San Jose, CA, USA), and 5-(*N*-ethyl-N-isopropyl)amiloride (EIPA) and 4,4-diisothiocyanatostilbene-2,2-disulphonate (DIDS) were from Sigma (Taufkirchen, Germany). Dimethyl sulfoxide (DMSO) as a solvent and concanamycin A were from Fluka (Buchs, Switzerland).

Microfluorometric measurements of pH_i

For microfluorometric measurements of pH_i, the salivary glands were loaded for 20 min with 5 µmol l^{-1} BCECF-AM at room temperature in darkness. BCECF-AM was diluted in PS from a 1 mmol l^{-1} stock solution containing 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; Jena, Germany) and a 75 W xenon lamp monochromator unit (Polychrome II, T.I.L.L. Photonics, Planegg, Germany) coupled to the epifluoresence 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 approximately 130×50 µm (includes a group of approximately 10 cells). Measurements were made with a Zeiss Neofluar 25/0.8 water immersion objective. BCECF fluorescene was alternately excited at 490 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. In some experiments, DIDS and EIPA were used, which are themselves fluorescent. In these cases, the preparation was alternately excited at 490 and 450 nm. 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, Germering, Germany). Indeed, for data storage and display only, the 20 ms fluorescence signal (F, in V; 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). Intracellular pH was calculated from the F_{490}/F_{439} or F_{490}/F_{450} ratios by using calibration data obtained with the nigericin-K^+ method (Schewe et al., 2008; Thomas et al., 1979). The high-K^+ calibration solutions contained (in mmol l^{-1}) 138 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 3 sodium glutamate, 2.8 malic acid, 10 D-glucose, 10 TRIS and 10 µmol l^{-1} nigericin. The pH of these calibration solutions was set to be between 6.2 and 8.2 with KOH.

Cloning of a *C. vicina* EAAT fragment

Degenerate primers (sense: 5′-GGMACKGCIYTITAYGARGC-3′, antisense: 5′-CGRTCSARIADCCARTC-3′) corresponding to highly conserved amino acid sequences of *Aedes aegypti* (*Aa* EAAT, AAP76304), *Apis mellifera* (*Am* EAAT, AAD34586), *Diploptera punctata* (*Dp* EAAT, AAF71701), *Trichoplusia ni* (*Tn* EAAT, AAB84380) and *Drosophila melanogaster* (*Dm* EAAT, AAD09142) were designed in order to amplify EAAT fragments of *C. vicina*. PCR was performed using blowfly salivary gland cDNA as the template. Amplification was carried out for 90 s at 94°C (one cycle) followed by 35 cycles of: denaturation for 40 s at 94°C, primer annealing for 40 s at 40–60°C, elongation of 1 min per kilobase (kb) at 72°C and a final extension of 10 min at 72°C. A 194 bp fragment was cloned into pGEM-T vector (Promega, Mannheim, Germany). Sequencing was performed by GATC Biotech (Konstanz, Germany). The nucleotide sequence of the *C. vicina* EAAT fragment has been submitted to the European Bioinformatics Institute database (accession no. FN689418).

Statistical analysis

Statistical analysis was undertaken using GraphPad Prism 4 (Version 4.01, GraphPad Software, San Diego, CA, USA). Data are presented as means ± s.e.m. in the text and figures. All data sets were tested for deviation from the normal distribution. Statistical comparisons were made using a Student's paired t-test and (for Fig. 1A) by a one-sample t-test to test whether the changes shown are significantly different from zero. P-values <0.05 were considered as significant.

RESULTS

Resting pH_i

The resting pH_i in *Calliphora* salivary glands is 7.5±0.3 (Schewe et al., 2008). To obtain initial information about any transporters that might contribute to the maintenance of the resting pH_i, we removed Na^+ from the bath solution, because alkalizing transporters (acid extruders) are predominantly Na^+ dependent. These transporters are blocked or reversed under Na^+-free conditions and an intracellular acidification is to be expected. Surprisingly, the removal of extracellular Na^+ induced an intracellular alkalinization of 0.19±0.03 pH units (N=29, one-sample t-test, P<0.0001; Fig. 1A, Fig. 2A,E). In 38% of the experiments (11 out of 29) a biphasic effect was observed: a transient acidification was followed by the intracellular alkalinization (Fig. 2A,E). Further studies revealed that the observed alkalinization was attributable to the activity of a Na^+-dependent glutamate transporter (EAAT), which will be described below.

To test whether an NHE was responsible for the transient acidification in the absence of extracellular Na^+, we used EIPA as a specific blocker for the NHE (Vigne et al., 1983). EIPA application was expected to cause an intracellular acidification if the NHE contributed to pH_i regulation in the resting gland. This effect was not observed: 50 µmol l^{-1} EIPA did not have a significant effect on

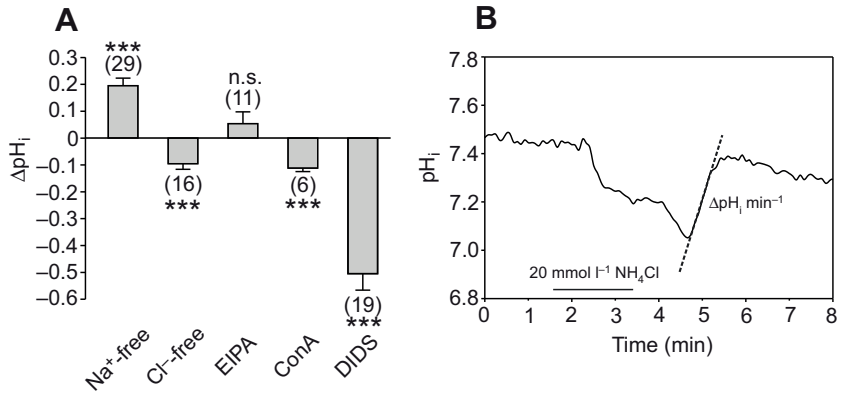

Fig. 1. (A) Summary of data from experiments in which intracellular pH (pH_i) changes were recorded upon application of Na^+- and Cl^--free bath solutions and application of the specific inhibitors EIPA, concanamycin A (ConA) and DIDS in the unstimulated salivary glands of *Calliphora vicina*. Data are means ± s.e.m.; the number of experiments is given in parentheses. A one-sample *t*-test was performed to test whether the changes shown are significantly different from zero (Na^+-free: ***P<0.0001; Cl^--free: ***P=0.0003; EIPA: P=0.2503; ConA: ***P=0.0004; DIDS: ***P<0.0001). (B) Application of 20 mmol l^{-1} NH_4Cl induced an intracellular acidification. Wash-out of NH_4Cl enhances this acidification. $\Delta pH_i\,min^{-1}$ indicates the rate of recovery from an intracellular acid load. The line under the concentration indicates the duration of the stimulus.

pH_i (0.05±0.06 pH units, N=11, one-sample *t*-test, P=0.2503; Fig. 1A, Fig. 3A). We next tested for the presence of a Na^+-dependent HCO_3^- transporter that might transport HCO_3^- into the cell in a Na^+-dependent manner. A characteristic feature of these transporters is that most (but not all) of them are inhibitable by DIDS (Boron, 2001). Application of 500 µmol l^{-1} DIDS induced a strong intracellular acidification of –0.50±0.06 pH units (N=19, one-sample *t*-test, P<0.0001; Fig. 1A, Fig. 4A). The observed acidification is indicative of an involvement of a DIDS-sensitive transporter in pH_i regulation in unstimulated salivary glands.

Further experiments addressed the dependence of pH_i on extracellular Cl^-, which is important for the activity of a Cl^-/HCO_3^- antiporter, an important acidifying transporter (acid loader), or a Cl^-/H^+ antiporter, which is considered to act as acid extruder. Under resting conditions, an AE transports HCO_3^- out of the cell and, in parallel, Cl^- into the cell. Removal of extracellular Cl^- intensifies the outward-directed Cl^- gradient and the AE should now transport HCO_3^- into the cell, which would lead to an intracellular alkalinization. However, in *C. vicina* salivary glands, an acidification of –0.10±0.02 pH units was observed under Cl^--free conditions. A characteristic feature was the slow increase of this acidification over a period of several minutes (N=16, one sample *t*-test, P=0.0003; Fig. 1A, Fig. 5A).

Another pilot experiment addressed the involvement of the apical V-ATPase in pH_i regulation in unstimulated glands. We inhibited the V-ATPase by using 1 µmol l^{-1} concanamycin A (Dröse and Altendorf, 1997; Huss et al., 2002), which is able to reach the apical membrane of the *Calliphora* salivary glands (Rein et al., 2006). Treatment with concanamycin A led to an intracellular acidification of –0.11±0.01 pH units (N=6, one sample *t*-test, P=0.0004; Fig. 1A, Fig. 4C), a result that clearly showed that the V-ATPase contributed to the maintenance of steady-state pH_i.

Taken together, this first series of screening experiments suggested that maintenance of steady-state pH_i is Na^+ and Cl^- dependent, DIDS sensitive and requires V-ATPase activity.

NH_4Cl-induced pH_i changes

Next, we changed the pH_i experimentally and studied those transporters that contributed to the recovery of resting pH_i. We changed pH_i using the NH_4Cl-prepulse technique (Boron, 2004; Roos and Boron, 1981). The basic principle of this method is to induce an intracellular acid load and to study the mechanisms that are responsible for the recovery from that acid load. Salivary gland tubules were superfused for 1.5 min with PS containing 20 mmol l^{-1} NH_4Cl. In many cells, NH_4Cl application causes an intracellular alkalinization induced by NH_3 influx and intracellular NH_3 protonation to NH_4^+. This alkalinization is usually followed by a modest fall in pH_i. NH_4Cl wash-out then induces a strong acidification (Boron, 2004; Roos and Boron, 1981). In *C. vicina* salivary glands, these typical pH_i changes were not observed. NH_4Cl application induced an immediate acidification of –0.26±0.02 pH units (N=69; Fig. 1B). This suggests that cell membranes of blowfly salivary gland cells are highly permeable for NH_4^+. Removal of NH_4Cl from the bath solution was followed by a further drop in pH_i (–0.20±0.02, N=69; Fig. 1B). Subsequently, active pH_i regulation was required to reconstitute a normal pH_i. The recovery from this acute acid load was characterized under both control and test conditions (ion substitution experiments and use of inhibitors). The rate of pH_i recovery was determined by linear regression within the first 3 min after the beginning of the recovery (Fig. 1B). The average recovery rate under control conditions was 0.32±0.03 $\Delta pH_i\,min^{-1}$ (N=69; Fig. 1B).

Na^+ dependence of active pH_i regulation

pH_i-regulating transporters that are able to counteract an intracellular acidification are, except for the V-ATPase, Na^+ dependent. Therefore, we removed Na^+ from the bath solution and applied an NH_4Cl prepulse. The amplitude of the NH_4Cl-induced acidification was significantly increased in Na^+-free PS (control: –0.18±0.03 pH units; Na^+-free: –0.39±0.05 pH units; N=6, paired *t*-test, P<0.01). The additional acidification after the removal of NH_4Cl was also increased in the absence of Na^+ (control: –0.20±0.04 pH units; Na^+-free: –0.51±0.12 pH units; N=6, paired *t*-test, P<0.05). Under Na^+-free conditions, the recovery rate, within the first 3 min after the beginning of the recovery from an intracellular acid load, became significantly faster (N=6, paired *t*-test, P<0.05; Fig. 2A,B). This result indicated that a Na^+-dependent transporter is involved in pH_i regulation. However, this transporter is neither the NHE nor an NDAE, because both of these are acid extruders; their inhibition under Na^+-free conditions should have slowed down the recovery from an acid load.

Next, the contribution of the V-ATPase was investigated by using concanamycin A as an inhibitor. Concanamycin A did not affect the kinetics or the magnitude of the NH_4Cl-induced acidification and had no significant effect on the rate of pH_i recovery from the NH_4Cl-induced acid load (N=6, paired *t*-test, P>0.05; Fig. 2C,D). Does this observation indicate that the V-ATPase is not involved in pH_i regulation after an experimentally induced acid load? To answer this question, we applied concanamycin A under Na^+-free conditions, because this should have inhibited all known pH_i-regulating transporters that could be expected to counteract an intracellular acidification. Under these experimental conditions, the acidification after the removal of NH_4Cl was again increased (control: –0.12±0.01 pH units; Na^+-free + concanamycin A:

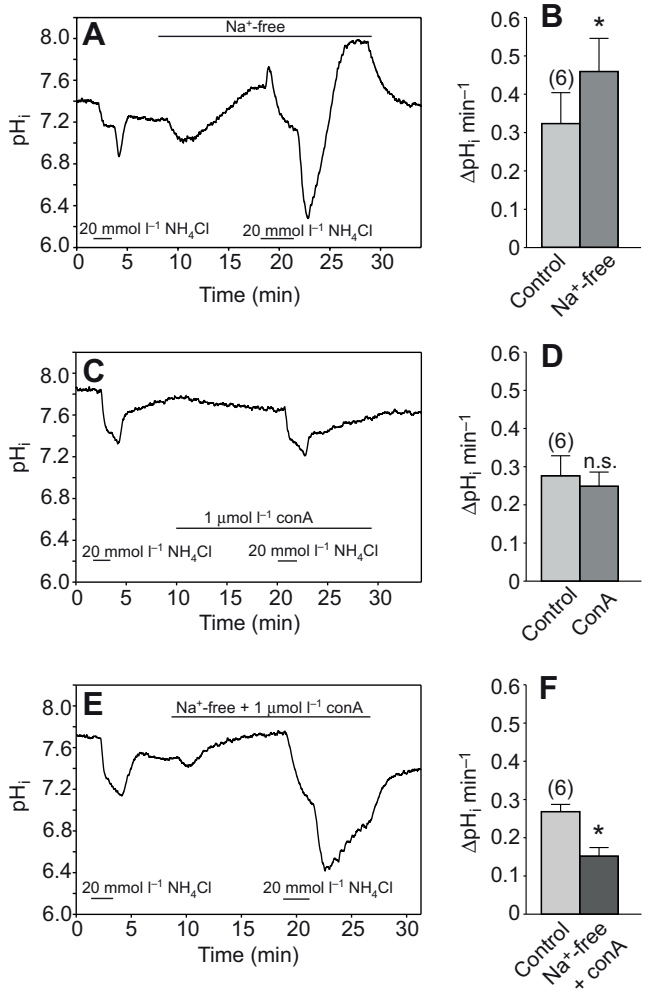

Fig. 2. Removal of Na^+ from the bath solution caused an intracellular alkalinization. (A,B) Under Na^+-free conditions, the rate of recovery from an NH_4Cl-induced intracellular acid load was enhanced (paired *t*-test, **P*<0.05). (C,D) In the continuous presence of 1 µmol l^{-1} concanamycin A (ConA), the rate of recovery from the NH_4Cl-induced acidification was not significantly affected (paired *t*-test, *P*>0.05). (E,F) Concanamycin A and Na^+-free bath solution significantly slowed the rate of recovery from an intracellular acid load (paired *t*-test, **P*<0.05). Data in B, D and F are means ± s.e.m.; the number of experiments is given in parentheses. Lines under the concentrations in A, C and E indicate the duration of the stimuli.

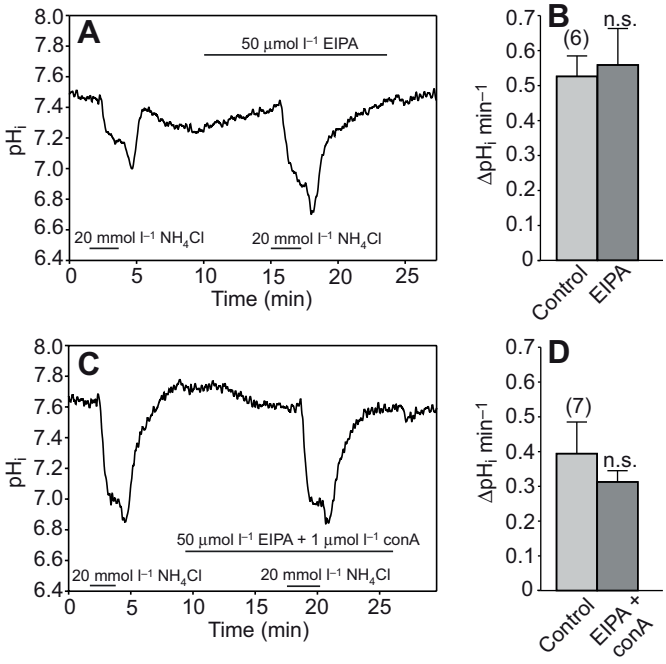

Fig. 3. (A,B) Application of 50 µmol l^{-1} EIPA had no effect on the rate of pH_i recovery from an intracellular acid load produced by NH_4Cl application (paired *t*-test, *P*>0.05). (C,D) In the continuous presence of 50 µmol l^{-1} EIPA and 1 µmol l^{-1} concanamycin A (ConA), the pH_i recovered at the same rate as under control conditions (paired *t*-test, *P*>0.05). Data in B and D are means ± s.e.m.; the number of experiments is given in parentheses. Lines under the concentrations in A and C indicate the duration of the stimuli.

–0.75±0.02 pH units; *N*=6, paired *t*-test, *P*<0.001). However, concanamycin A completely blocked and reversed the increased pH_i recovery rate in the absence of Na^+, and the pH_i recovery rate was significantly slower than that under control conditions (*N*=6, paired *t*-test, *P*<0.05; Fig. 2E,F). Together, these results indicated that at least the V-ATPase was involved in the recovery from an acute acid load whereas a Na^+-dependent mechanism seemed to slow it down; surprisingly, the absence of Na^+ accelerated the recovery. Thus the contribution of the V-ATPase becomes measurable only under Na^+-free conditions.

Na^+-dependent pH_i-regulating transporters in insects include the NHE and an NDAE, which is similar to a mammalian NDCBE (Romero et al., 2000; Sciortino et al., 2001). To distinguish between these mechanisms, we used EIPA as an inhibitor for the NHE and DIDS to block a putative NDAE. In contrast to Na^+-free PS, 50 µmol l^{-1} EIPA had no significant effect on the recovery from an NH_4Cl-induced intracellular acidification (*N*=6, paired *t*-test, *P*>0.05; Fig. 3A,B). But as seen in the absence of Na^+, EIPA significantly increased the NH_4Cl-induced acidification (control: –0.32±0.09 pH units; EIPA: –0.54±0.07 pH units; *N*=6, paired *t*-test, *P*<0.01) and the acidification after the removal of NH_4Cl (control: –0.15±0.03 pH units; EIPA: –0.30±0.03 pH units; *N*=6, paired *t*-test, *P*<0.05).

As reported above, the rate of recovery from an acid load required V-ATPase activity, but seemed to be slowed down by a Na^+-dependent mechanism that acted as an acid loader rather than as an extruder. Nevertheless, we applied 50 µmol l^{-1} EIPA in combination with 1 µmol l^{-1} concanamycin A. This also had no significant effect on the recovery rate from an intracellular acid load (*N*=7, paired *t*-test, *P*>0.05; Fig. 3C,D).

To obtain information on a possible contribution of NDAE, we applied 500 µmol l^{-1} DIDS, which itself induced a strong acidification in the resting gland so that a contribution of a DIDS-sensitive mechanism to active pH_i regulation became obvious. In addition, we found that the DIDS application reduced the rate of recovery from an NH_4Cl-induced acid load significantly (*N*=6, paired *t*-test, *P*<0.01; Fig. 4A,B). When we applied 500 µmol l^{-1} DIDS together with 1 µmol l^{-1} concanamycin A in order to inhibit additionally the V-ATPase, the rate of recovery from an intracellular acid load was almost completely blocked (*N*=5, paired *t*-test, *P*<0.05; Fig. 4C,D). Thus, in the unstimulated salivary gland of *C. vicina*, at least one DIDS-sensitive transporter and the apically located V-ATPase contribute to the recovery from an intracellular acid load.

To characterize the DIDS-sensitive component, we studied the Cl^- dependence of the rate of pH_i recovery from an acid load. Instead of applying 20 mmol l^{-1} NH_4Cl, we induced the acidification under Cl^--free conditions by 10 mmol l^{-1} ammonium sulphate [$(NH_4)_2SO_4$]

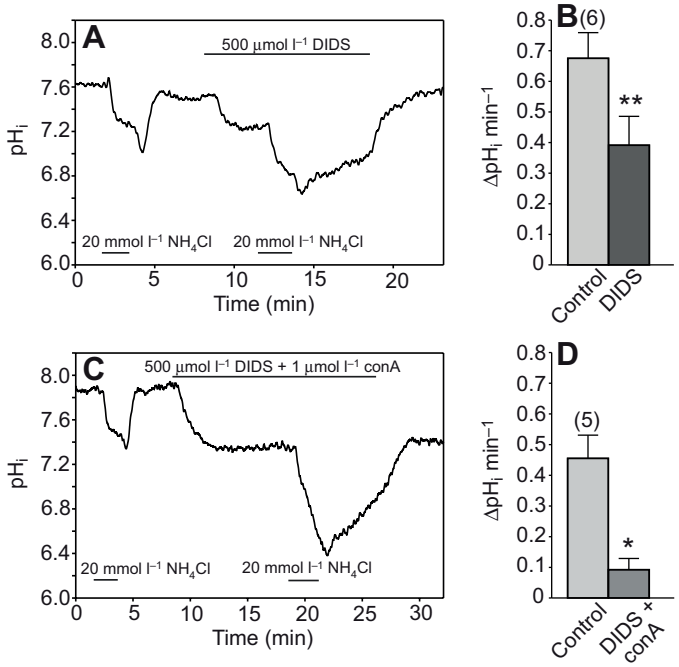

Fig. 4. Application of 500 µmol l^{-1} DIDS induced an intracellular acidification under resting conditions. (A,B) In the continuous presence of 500 µmol l^{-1} DIDS, the pH_i recovered more slowly from an NH_4Cl-induced acidification (paired *t*-test, $^{**}P<0.01$). (C,D) In the presence of 500 µmol l^{-1} DIDS and 1 µmol l^{-1} concanamycin A (ConA), the recovery from an intracellular acid load was almost completely blocked (paired *t*-test, $^{*}P<0.05$). Data in B and D are means ± s.e.m.; the number of experiments is given in parentheses. Lines under the concentrations in A and C indicate the duration of the stimuli.

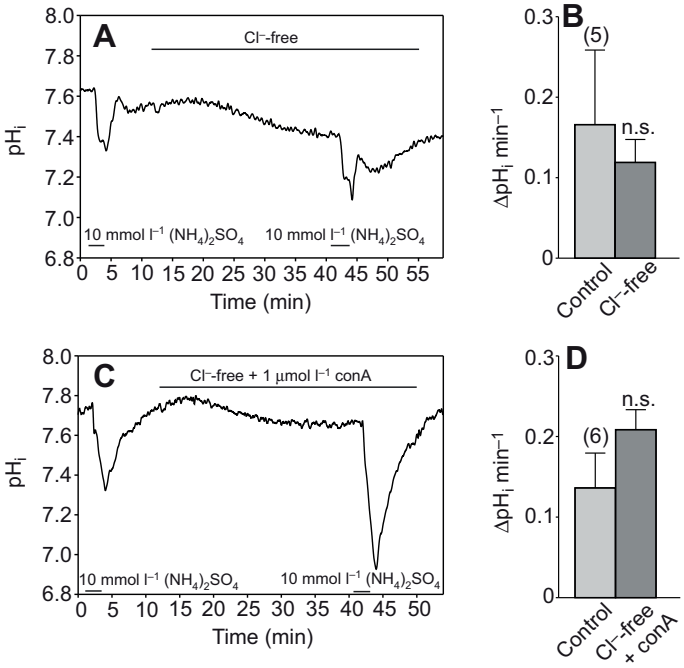

Fig. 5. Under Cl^--free conditions, a slowly increasing acidification was observed. Neither removal of Cl^- (A,B) nor the presence of 1 µmol l^{-1} concanamycin A in the absence of Cl^- (C,D) affected the recovery from an intracellular acid load (paired *t*-test, $P>0.05$). Data in B and D are means ± s.e.m.; the number of experiments is given in parentheses. Lines under the concentrations in A and C indicate the duration of the stimuli.

application. Neither under Cl^--free conditions (N=5, paired *t*-test, $P>0.05$; Fig. 5A,B) nor in the presence of concanamycin A under Cl^--free conditions (N=6, paired *t*-test, $P>0.05$; Fig. 5C,D) was the rate of pH_i recovery or the kinetics and magnitude of the NH_4Cl-induced acidification significantly affected.

Na^+-dependent glutamate transport

We have shown above that removal of extracellular Na^+ induced an intracellular alkalinization of 0.19±0.03 pH units (N=29; Fig. 1A, Fig. 2A, Fig. 6A,B). This observation was unexpected because Na^+-dependent pH_i-regulating transporters either transport H^+ out of the cell or HCO_3^- into the cell. In both cases, the application of a Na^+-free PS was expected to cause an intracellular acidification, which was seen only in 38% of the experiments, as a transient component before the alkalinization (Fig. 2A,E). In search of an interpretation for this unexpected finding, we considered a Na^+-dependent glutamate transporter (EAAT) as a likely candidate. EAAT transports equimolar amounts of glutamate and H^+ into the cell by using the energy from the inwardly directed electrochemical Na^+ gradient. Thus, after blocking the EAAT, e.g. by using a Na^+-free solution, no glutamate and fewer H^+ are transported into the cell, resulting in an intracellular alkalinization. If the activity of a Na^+-dependent glutamate transporter does contribute to steady-state pH_i, extracellular removal of glutamate should cause an intracellular alkalinization. As shown in Fig. 6B, an alkalinization (0.11±0.03 pH units, N=22) was indeed observed when a glutamate-free PS was applied. The magnitude of the pH_i changes under Na^+-free and glutamate-free conditions was not significantly different (unpaired *t*-test, $P>0.05$; Fig. 6C). These findings do not definitively establish, but are indicative of, the existence of an EAAT in *C. vicina* salivary glands.

Next, we studied the dose dependence of the glutamate-dependent pH_i (Fig. 6D). Salivary glands were first superfused in glutamate-free PS for 10 min and subsequently with PS containing increasing glutamate concentrations. The resulting acidification was measured and plotted as a function of the glutamate concentration. The dose-response curve revealed an EC_{50}=169 µmol l^{-1} for glutamate (Fig. 6D).

A molecular approach was chosen to confirm the existence of an EAAT more strongly than by the pH measurements described above. We amplified a 194 bp fragment of EAAT (Cv *EAAT*) from blowfly salivary gland cDNA. For this, we aligned the EAAT amino acid sequence of various insects and designed degenerate primers corresponding to highly conserved regions. Amino acid sequences used for a multiple sequence alignment (Fig. 7) were identified by protein:protein BLAST searches of the NCBI database (BLSTP, NCBI database) with the deduced amino acid sequence of *C. vicina* EAAT (*Cv* EAAT, 64 amino acids) as 'bait'. *Cv* EAAT displayed high similarity to corresponding sequences of *D. melanogaster* (*Dm* EAAT, 100%), *A. aegypti* (*Aa* EAAT, 81%), *T. ni* (*Tn* EAAT, 78%) and *D. punctata* (*Dp* EAAT, 75%). The expression of EAAT was determined by using reverse-transcription PCR (RT-PCR) on RNA samples from the *C. vicina* salivary glands. A fragment was amplified from DNase-treated RNA by RT-PCR. RNase treatment of the RNA preparation prior to RT-PCR abolished the amplification of any PCR product. Thus, the expression of a Na^+-dependent glutamate transporter in salivary glands of *C. vicina* was confirmed.

DISCUSSION

The steady-state pH_i in unstimulated salivary glands of *C. vicina* has been determined to be 7.5±0.3 (Schewe et al., 2008). This slighlty alkaline resting pH_i is above the pH_i expected from a passive

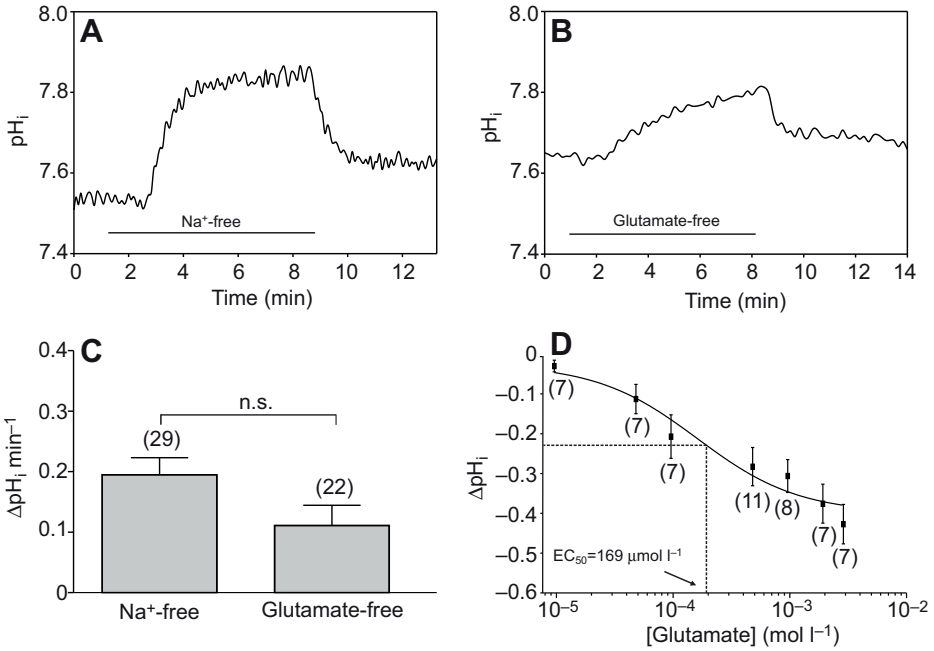

Fig. 6. (A) Removal of Na^+ from the bath solution induced an intracellular alkalinization. (B) Perfusion with a glutamate-free PS also induced an intracellular alkalinization. (C) The observed alkalinization under Na^+-free conditions was not significantly different from an alkalinization under glutamate-free conditions (unpaired *t*-test, $P>0.05$). (D) Glutamate-induced acidification for defined concentrations of glutamate were measured and plotted as a dose–response curve (nonlinear regression). An EC_{50} value of 169 μmol l^{-1} glutamate was determined. Data in C and D are means ± s.e.m.; the number of experiments is given in parentheses. Lines under the concentrations in A and B indicate the duration of the stimuli.

H^+ distribution. The driving force ($\bar{\mu}$) for H^+ can be calculated using the equation:

$$\bar{\mu} = 58\log(H^+)_o/(H^+)_i - V_m, \quad (1)$$

where H^+_o and H^+_i are the extracellular and intracellular H^+ concentrations, respectively, and V_m is the apical membrane potential. Bath pH is 7.2 in our experiments, pH_i is 7.5 (Schewe et al., 2008) and the membrane potential is –47 mV (House and Ginsborg, 1985; Prince and Berridge, 1972). With these data, the driving force for H^+ can be calculated as 64.4 mV. The positive number reveals that an influx of H^+ into the cell is favoured and that the cells have to counteract a creeping acidification. Therefore, under resting conditions, active pH_i regulation is required to maintain a normal pH_i value of 7.5. In a recent publication, we demonstrated mechanisms involved in 5-HT-induced acidification (Schewe et al., 2008), but pH_i-regulating transporters, which are responsible for the maintenance of resting pH_i and the recovery from an intracellular acid load, remained poorly characterized. In this study, we used two strategies to identify these mechanisms: ion substitution experiments and specific inhibitors.

Substitution of extracellular Na^+ inhibits or reverses acid extruders such as the NHE or the NDAE, and an intracellular acidification is expected under Na^+-free conditions. Surprisingly, in contrast to this expectation, removal of Na^+ from the bath solution causes an intracellular alkalinization in *C. vicina* salivary glands, whereas in some experiments a transient acidification was observed previous to the alkalinization. We have been able to demonstrate that this phenomenon is attributable to the activity of a Na^+-dependent glutamate transporter (EAAT). EAAT belongs to the transport family *SLC1* and not only plays a central role in the mammalian nervous system (Gegelashvili et al., 2006; Storck et al., 1992) but is also expressed in peripheral tissues (Kanai and Hediger, 2004; Berger and Hediger, 2006). In the central nervous system, members of the transport family *SLC1* maintain low glutamate levels to protect nerve cells from toxic glutamate concentrations (Castagna et al., 1997; Kanai, 1997; Robinson, 1998). In arthropods, glutamate also functions as a neurotransmitter. In addition, it is important for protein synthesis, chitin formation and energy homeostasis (McLean and Caveny, 1993).

Our discovery of a Na^+/glutamate cotransporter in *C. vicina* salivary glands provides an explanation for a 40-year-old observation. Berridge (Berridge, 1970) observed that in the absence of glutamate, the rate of saliva secretion is extremely low. Berridge postulated that the salivary gland cells take up glutamate and metabolize it within the citric acid cycle (Berridge, 1970; Rapp and Berridge, 1981). This suggestion has now been confirmed by the discovery of a Na^+-dependent glutamate transporter. Although this transporter is not a common pH_i-regulating transporter, its activity affects steady-state pH_i in *C. vicina* salivary gland cells (Fig. 8).

The nature of the transient acidification, observed in the absence of Na^+, was not further addressed in the present study, but suggests that more than one Na^+-dependent transporter is affected in the absence of Na^+. It is unlikely that an inhibition of an EIPA-sensitive NHE is responsible for this transient acidification, because in the presence of EIPA, a specific NHE inhibitor (Vigne et al., 1983), this effect was not observed. Nevertheless, a contribution of an

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Cv EAAT    1    VAALFIAQYREMSYSFGTIVAVSITATAASIGAAGIPQAGLVTMVMVLDTVGLEPKDVSLIIAV
Dm EAAT1   360  VAALFIAQYREMSYSFGTIVAVSITATAASIGAAGIPQAGLVTMVMVLDTVGLEPKDVSLIIAV
Aa EAAT    357  VAAIFIAQLRGLSLTFGNIVAISITATAASIGAAGIPQAGLVTLVMVLDTVGLPAEDVSLIIAV
Tn EAAT    360  VAAIFIAQLRKVEMSFGKIIAVSVTATAASIGAAGIPQAGLVTMVMVLDTVNLPAEDVSLILAV
Dp EAAT    360  VAAIFISQVRGMALSLGQLLAISVTATAASIGAAGIPQAGLVTMVMVLDTVGLPPEDVTLIITV
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Fig. 7. The amino acid sequence of the isolated EAAT fragment of *Calliphora vicina* (*Cv* EAAT) shows high similarity to EAAT amino acid sequences of *Drosophila melanogaster* (*Dm* EAAT, 100%, AAD47829), *Aedes aegypti* (*Aa* EAAT, 81%, AAP76304), *Trichoplusia ni* (*Tn* EAAT, 78%, AAB84380) and *Diploptera punctata* (*Dp* EAAT, 75%, AAF71701).

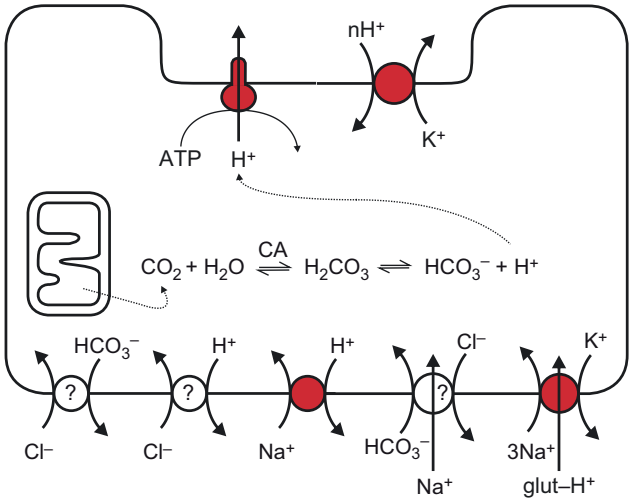

Fig. 8. Representation of transporters that we discuss in conjunction with the maintenance of steady-state pH_i in *Calliphora* salivary glands. For details, see the Discussion. Transporters for which we have strong indication are shown in red, whereas those that need further clarification are indicated with question marks. glut–H^+, 1 glutamate + 1 H^+.

EIPA-insensitive NHE and/or NHA, as described for NHE3 of *A. aegypti*, cannot be ruled out (Pullikuth et al., 2006).

Further Na^+-dependent pH_i-regulating systems include HCO_3^- transporters belonging to the transport family *SLC4* (Pushkin and Kurtz, 2006; Romero et al., 2004). The NDAE (Fig. 8) transports HCO_3^- into the cell in a Na^+-dependent manner. The inhibition of this transporter with DIDS is expected to cause an intracellular acidification. Such an acidification was indeed observed in the presence of DIDS in *C. vicina* salivary glands in the present study, although this was a long-lasting effect, not only a transient one. Nevertheless, this was initially surprising, because all experiments were performed in HCO_3^--free Tris-buffered saline. However, the nominal absence of HCO_3^- does not indicate its total absence. Nominal HCO_3^--free solutions always contain some micromoles of HCO_3^-. In an enclosed room, a CO_2 concentration of 0.05% (13.3 μmol l^{-1}) is expected because of, for example, breathing. Using the Henderson–Hasselbalch equation, we can determine that this CO_2 concentration causes a HCO_3^- concentration of 167 μmol l^{-1} in an electrolyte solution (Deitmer and Schneider, 1998). Additionally, CO_2 is produced by cellular respiration, which can be used for the production of HCO_3^-. In other systems, an involvement of HCO_3^--dependent transporters has also been observed under nominal HCO_3^--free conditions, e.g. in the glial cells of *Hirudo medicinalis* (Deitmer and Schneider, 1998), in neurons of *Schistocerca gregaria* (Schwiening and Thomas, 1992) and in ventricular myocardial muscle cells (Wu et al., 1994). However, these assumptions are somewhat speculative and have to be confirmed in further studies using HCO_3^--containing PS.

The observed DIDS-induced acidification also shows that alkalizing mechanisms predominate under resting conditions. This observation seems obvious if we take into account the fact that the cells have to counteract a creeping acidification attributable to an inwardly directed electrochemical H^+ gradient. We removed Cl^- from the bath solution to test whether, under resting conditions, a Cl^--sensitive transporter is additionally active. Under these conditions, an AE would reverse and Cl^- is transported out of the cell and HCO_3^- is transported into the cell, which would result in an intracellular alkalinization. Indeed, such an alkalinization was not observed under Cl^--free conditions. Instead, a creeping acidification was measured in the absence of Cl^-. This suggests that an alkalizing transport system is affected by Cl^- removal. The activity of the V-ATPase might be affected by Cl^- because the active H^+ transport is electrogenic. Eletrogenic V-ATPase-mediated H^+ transport requires the parallel transepithelial transport of Cl^- as a counterion. Such a Cl^- dependence has been observed in cells of the collecting ducts and proximal tubules in rabbit kidneys (Stone et al., 1983; Malnic and Geibel, 2000). In distal tubules of rat kidney, the Cl^- channel blocker NPPB enhances the luminal membrane potential and inhibits a luminal acidification mediated by V-ATPase (Fernandez et al., 1997). Additionally, in proximal tubules, the V-ATPase-mediated recovery from an intracellular acidification is blocked in Cl^--free bath solution (Wagner et al., 1998).

The situation might be similar in *C. vicina* salivary glands. The electrochemical $\bar{\mu}$ for Cl^- is 20.5 mV in the unstimulated gland, calculated with the following equation:

$$\bar{\mu} = 58\log([Cl^-]_i/[Cl^-]_o) - V_m, \quad (2)$$

where $[Cl^-]_o$ and $[Cl^-]_i$ are the extracellular and intracellular Cl^- concentrations, respectively. For this calculation, the following data were used: $[Cl^-]_o$=155 mmol l^{-1} (PS), $[Cl^-]_i$=33 mmol l^{-1} (Gupta et al., 1978) and V_m=59.5 mV (Berridge et al., 1975; Prince and Berridge, 1972).

A value of $\bar{\mu}$ >0 favours Cl^- efflux into the glandular lumen. In the absence of extracellular Cl^-, the intracellular Cl^- concentration decreases. In the case of 5 mmol l^{-1} intracellular Cl^-, the $\bar{\mu}$ is now –27 mV. This means a reversal of $\bar{\mu}$ for Cl^-, i.e. now a Cl^- influx is favoured. Consequently, the driving force for the anionic counterion is absent and V-ATPase activity decreases, following which H^+ accumulates in the cell and leads to the observed intracellular acidification.

A second possibility is the presence of a putative Cl^-/H^+ antiport activity in the basolateral membrane of the gland cells, which is also inhibitable by DIDS (Pusch et al., 2006; Wulff, 2008; Zifarelli and Pusch, 2007). An inhibition of such a transport mechanism would also block an outward-directed H^+ transport and favour H^+ accumulation within the cells, leading to an intracellular acidification, which was indeed observed. It is not possible to distinguish between these possibilities by pharmacological experiments. Therefore, molecular data are needed to support this hypothesis.

We have performed a series of experiments in order to identify transport mechanisms that reconstitute normal pH_i after an intracellular acid load experimentally produced by the NH_4Cl-prepulse technique. Bath application of NH_4Cl leads, in many cell types, initially to an intracellular alkalinization. NH_4^+ dissociates into NH_3 and H^+. NH_3 diffuses into the cell and binds H^+, which causes an intracellular alkalinization in most cells. This typical behaviour was not observed in our study. Application of 20 mmol l^{-1} NH_4Cl evoked an intracellular acidification. This suggests that NH_4^+ enters the cell. Such an acidifying effect of NH_4Cl has also been observed in the retinal glial cells of *A. mellifera* (Marcaggi et al., 1999; Marcaggi and Coles, 2000), the Malpighian tubules of *Drosophila hydei* (Bertram and Wessing, 1994), the epithelial cells of the cockroach salivary glands (Hille and Walz, 2007), the colon epithelium in rats (Ramirez et al., 1999) and *Xenopus* oocytes (Burckhardt and Frömter, 1992). The nature of the mechanism by which NH_4^+ enters the cell has not been addressed in this study. For other cell types that show the same behaviour, the $Na^+/K^+/Cl^-$ cotransporter (NKCC), the NHE, K^+-channels, a cotransporter of NH_4^+ and Cl^- and non-selective cation channels have all been

discussed as possible mechanisms (Betram and Wessing, 1994; Burckhardt and Frömter, 1992; Hille and Walz, 2007; Marcaggi et al., 1999; Marcaggi and Coles, 2000; Ramirez et al., 1999). In *C. vicina* salivary glands, the NH_4Cl pulse under Na^+-free conditions caused a transient alkalinization in some experiments (Fig. 2A), which was not seen in the presence of EIPA (Fig. 3A). These observations suggest that that NH_4^+ entry is partially Na^+ dependent, but that this Na^+ dependence does not require NHE activity.

Following the application of NH_4Cl, its removal induced an intracellular acidification, because NH_3 leaves the cells faster than NH_4^+. Active pH_i-regulating mechanisms are required to recover from such an intracellular acid load, and the transport mechanisms involved are predominantly Na^+ dependent. Under Na^+-free conditions, the recovery from an intracellular acidification was significantly enhanced. The inhibition of the apically located V-ATPase by concanamycin A had no effect on the rate of recovery from an intracellular acid load. However, the application of concanamycin A under Na^+-free conditions reduced the recovery rate significantly. This clearly shows that the enhancement of the recovery rate under Na^+-free conditions was completely abolished and reversed by concanamycin A. Our results suggest that, in *C. vicina* salivary glands, at least two transporters contribute to the recovery from an intracellular acid load: at least one Na^+-dependent mechanism and the V-ATPase. A comparable situation has been found in duct cells of the salivary glands in the cockroach *Periplaneta americana* (Hille and Walz, 2007). Other systems use only one alkalizing transporter. In mammalian colon cells and in duct cells of the salivary glands in mice, recovery from an intracellular acidification is almost completely Na^+ dependent (Chaturapanich et al., 1997; Tsuchiya et al., 2001). In contrast, in the Malpighian tubules of *Drosophila* larvae, the recovery depends on V-ATPase activity alone (Bertram and Wessing, 1994).

Among the Na^+-dependent mechanisms that might contribute to the recovery phase after an intracellular acidification, the NHE and the NDAE are likely candidates. Surprisingly, EIPA, the specific NHE inhibitor, had no effect on the rate of recovery from intracellular acidification, either alone or in combination with the V-ATPase inhibitor concanamycin A. Remarkably, an EIPA-sensitive NHE has been shown to contribute to a 5-HT-induced acidification (Schewe et al., 2008). Thus, this NHE contributes to a 5-HT-induced acidification but is not involved in the recovery from an intracellular acid load in the unstimulated gland. Furthermore, we identified a putative NHE at the mRNA level in *C. vicina* salivary glands (B.S., unpublished data). Further investigations are required to characterize this partial mRNA sequence to show which isoform was found and whether this NHE has an amiloride binding motif.

Interestingly, in the absence of Na^+ and in the presence of EIPA, the NH_4Cl-induced acidification was significantly increased. This result suggests that an EIPA-sensitive NHE is indeed important for the kinetics and the amplitude of the intracellular acid load. However, it cannot be excluded that other NHE isoforms are present within the cells, which are EIPA insensitive or located intracellularly or in the apical membrane. NHE3 from *A. aegypti* (*Ae*NHE3) is localized in the basal membrane of the Malphigian tubules and is relatively insensitive to amiloride and EIPA. Nevertheless, this transporter is able to restore resting pH_i after an intracellular acid load (Pullikuth et al., 2006). Additionally, NHE8 cloned from *A. aegypti* (*Ae*NHE8) was shown to be an intracellular cation/H^+ exchanger in renal tubules (Piermarini et al., 2009), as well as an apically located exchanger within the Malpighian tubules (Kang'ethe et al., 2007).

In contrast, our results alternatively indicate that the recovery phase was DIDS sensitive. In the presence of the V-ATPase blocker concanamycin A and DIDS, the recovery from an intracellular acid load was almost completely blocked. These findings suggest that a DIDS-sensitive NDAE or a DIDS-sensitive Cl^-/H^+ antiporter are essential for restoring resting pH_i after an intracellular acidification (Fig. 8). However, in both cases, pH_i recovery should be Cl^- sensitive, which was not the case in this study. To resolve this problem, further studies are needed to confirm the role of HCO_3^--transporting mechanisms working in the acid-extruding mode.

Additionally, one has to consider a role of the putative nH^+/K^+ antiporter in the apical membrane. Because of its H^+-transport activity, this transporter is a likely candidate to be involved in pH_i regulation (Fig. 8). Considering a stoichiometry ratio of $1\,K^+$ out and $2\,H^+$ into the cell, as already shown for the larval midgut of *Manduca sexta* (Azuma et al., 1995), this transporter is suggested to function as acid loader. Recently, for *Anopheles gambiae* and *D. melanogaster*, genes have been identified coding for an nH^+/K^+ antiporter (Day et al., 2008; Rheault et al., 2007). We already cloned a partial cDNA of the *C. vicina* nH^+/K^+ antiporter (B.S., unpublished data). However, we have to further determine its characteristics, e.g. stoichiometry, kinetics, pharmacological profile and the cellular distribution, to evaluate the contribution of this transporter to pH_i regulation in the resting gland.

Taking all aspects into account, we show that pH_i regulation in the resting gland of *C. vicina* is unexpectedly complex and some mechanisms must be further investigated.

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