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A vacuolar-type H^+-ATPase and a Na^+/H^+ exchanger contribute to intracellular pH regulation in cockroach salivary ducts

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Summary

Cells of the dopaminergically innervated salivary ducts in the cockroach *Periplaneta americana* have a vacuolar-type H^+-ATPase (V-ATPase) of unknown function in their apical membrane. We have studied whether dopamine affects intracellular pH (pH_i) in duct cells and whether and to what extent the apical V-ATPase contributes to pH_i regulation. pH_i measurements with double-barrelled pH-sensitive microelectrodes and the fluorescent dye BCECF have revealed: (1) the steady-state pH_i is 7.3±0.1; (2) dopamine induces a dose-dependent acidification up to pH 6.9±0.1 at 1 μmol l^{-1} dopamine, EC_{50} at 30 nmol l^{-1} dopamine; (3) V-ATPase inhibition with concanamycin A or Na^+-free physiological saline (PS) does not affect the steady-state pH_i; (4) concanamycin A, Na^+-free PS and Na^+/H^+ exchange inhibition with 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) each reduce the rate of pH_i recovery from a dopamine-induced acidification or an acidification induced by an NH_4Cl pulse; (5) pH_i recovery after NH_4Cl-induced acidification is almost completely blocked by concanamycin A in Na^+-free PS or by concanamycin A applied together with EIPA; (6) pH_i recovery after dopamine-induced acidification is also completely blocked by concanamycin A in Na^+-free PS but only partially blocked by concanamycin A applied together with EIPA. We therefore conclude that the apical V-ATPase and a basolateral Na^+/H^+ exchange play a minor role in steady-state pH_i regulation but contribute both to H^+ extrusion after an acute dopamine- or NH_4Cl-induced acid load.

Key words: vacuolar H^+-ATPase, V-ATPase, NHE, BCECF, intracellular pH, dopamine, biogenic amines, insect, cockroach, *Periplaneta americana*, salivary glands.

Introduction

Intracellular pH (pH_i) affects a plethora of physiological processes, such as cellular metabolism, contractility, ion channel conductivity, ion transport and cell cycle control (Madshus, 1988). Therefore, pH_i must be strictly regulated by means of several ion transport mechanisms. These mechanisms function either as acid loaders or acid extruders (Boron, 1986; Boron, 2004). One of these acid extruders is the vacuolar-type H^+-ATPase (V-ATPase), when it is localised in the plasma membrane. V-ATPases are widely used to generate electrochemical proton gradients, i.e. they energise membranes for driving other transport processes. Plasma membrane V-ATPases have been shown to be essential in processes such as acid secretion and HCO_3^- transport in the proximal tubule and collecting duct of the kidney (Brown and Breton, 1996). They also energise fluid secretion in insect Malpighian tubules and salivary glands (Wieczorek et al., 1999; Nishi and Forgac, 2002; Zimmermann et al., 2003; Beyenbach and Wieczorek, 2006). In addition, a contribution of the V-ATPase to pH_i regulation has been described in some vertebrate epithelia (Pappas and Ransom, 1993; Stankovic et al., 1997; Granger et al., 2002; Yip et al., 2002). Although the V-ATPase has been found in a number of insect tissues (Beyenbach and Wieczorek, 2006), an involvement of the V-ATPase in pH_i regulation in insects has only been suggested for *Drosophila* Malpighian tubules (Bertram and Wessing, 1994). By contrast, a Na^+/H^+ exchange (NHE) has been proposed to play a major role in pH_i regulation in *Aedes* and *Rhodnius* Malpighian tubules (Petzel, 2000; Ianowski and O'Donnell, 2006).

The epithelial cells forming the ducts in the acinar salivary glands of the cockroach *Periplaneta americana* have a V-ATPase in their highly folded apical membrane (Just and Walz, 1994a) but its functional significance is unknown. The secretory acini are innervated by dopaminergic and serotonergic fibres (Baumann et al., 2002) and secrete a NaCl-rich primary saliva upon stimulation with dopamine (DA) or serotonin (Rietdorf et al., 2003). Dopaminergic fibres also innervate the ducts, and DA has been shown to cause dramatic changes in intracellular Na^+ and K^+ concentrations in duct cells (Lang and Walz, 2001). These findings together with those

from electron probe X-ray microanalysis and capillary electrophoresis of primary and final saliva have led to the conclusion that the ducts modify primary saliva by Na^+ reabsorption and K^+ secretion (Gupta and Hall, 1983; Rietdorf et al., 2003). In insect epithelial tissues such as salivary glands, midgut and Malpighian tubules, an apical V-ATPase has been shown to energise apical K^+ secretion (Maddrell and O'Donnell, 1992; Wieczorek, 1992; Zimmermann et al., 2003). Thus, the apical V-ATPase in cockroach salivary ducts might be involved in K^+ secretion and/or in intracellular pH homeostasis.

The immediate aim of the present study has, therefore, been to study whether DA affects pH_i in duct cells and whether and to what extent the apical V-ATPase contributes to intracellular pH homeostasis. We have measured pH_i in duct cells of isolated salivary glands with double-barrelled pH-sensitive microelectrodes and microfluorometrically with the fluorescent dye BCECF and demonstrate that DA causes an intracellular acidification. We have found that V-ATPase and NHE play a minor role in steady-state pH_i regulation but contribute significantly to pH_i recovery from an acute acid load.

Materials and methods

Animals and preparation

A colony of *Periplaneta americana* L. (Blattodea, Blattidae) was reared at 27°C under a light:dark cycle of 12 h:12 h. The animals had free access to food and water. Only male adults aged between 4 and 6 weeks were used for experiments. Salivary glands were dissected in physiological saline (PS) as described previously (Just and Walz, 1996). Small pieces of the glands consisting of one lobe with its acini and ducts were used.

Solutions and chemicals

Cockroach PS contained 160 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} glucose and 10 mmol l^{-1} Tris. The pH was adjusted to 7.4 with HCl. To induce an acute acid load in the salivary glands, 20 mmol l^{-1} NH_4Cl was substituted for 20 mmol l^{-1} NaCl at pH 7.4. In Na^+-free saline, equimolar amounts of choline chloride were substituted for NaCl. BCECF/AM [2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester], concanamycin A, DA and EIPA [5-(*N*-ethyl-*N*-isopropyl)-amiloride; all from Sigma, Deisenhofen, Germany or Invitrogen, Karlsruhe, Germany] were stored as stock solutions in small aliquots at –20°C and diluted in PS immediately before an experiment. Dimethyl sulfoxide as a solvent for BCECF/AM, concanamycin A and EIPA did not affect pH_i (*N*=3; data not shown). Dimethyl-trimethylsilylamine and the H^+-ionophore I Cocktail A for pH-sensitive microelectrodes were purchased from Fluka (Buchs, Switzerland).

Microfluorometric measurements of pH_i

The pH_i in duct epithelial cells was measured microfluorometrically with the pH-sensitive fluorescent dye BCECF. Isolated gland lobes were loaded with BCECF by 10 min incubation in PS containing 0.5 μmol l^{-1} BCECF/AM at room temperature. BCECF-loaded lobes were then attached to the coverslip-bottom of a custom-built recording chamber coated with the tissue adhesive Vectabond Reagent (Axxora, Grünberg, Germany) and continuously superfused with PS. The chamber was mounted on a Zeiss Axiovert 135TV inverted microscope equipped with epifluorescence optics and a Zeiss Fluar 20/0.75 objective. For fluorescence excitation, a 75 W xenon arc lamp monochromator unit (VisiChrome, Visitron, Puchheim, Germany) was connected to the microscope by a quartz fibre-optic light guide. The epifluorescence filter block in the microscope contained a 485 nm dichroic mirror and a 515–565 nm bandpass emission filter. BCECF fluorescence was excited every 10 s or 15 s for 5–40 ms at 470 nm, and for 20–160 ms at 410 nm, depending on the BCECF concentration in the cytosol. Fluorescence images were acquired and digitised with a cooled image transfer CCD camera (CoolSnap-HQ, Roper Scientific Inc., Tucson, USA) at a 12-bit resolution. Monochromator control, image acquisition and processing were carried out using MetaFluor 6.1 software (Universal Imaging Corp., Downingtown, PA, USA). pH_i was expressed as the fluorescence ratio F_{470}/F_{410}. Background fluorescence and cell autofluorescence were negligible. Because EIPA is fluorescent in UV light, BCECF fluorescence was excited at 480 nm and 450 nm (each for 5–20 ms) and the fluorescence ratio F_{480}/F_{450} was calculated in all experiments in which EIPA was used (Lee et al., 2005). We were not able to convert the fluorescence ratio F_{470}/F_{410} into pH_i by using the classical high-K^+/nigericin method (Thomas et al., 1979) because the cells deteriorated rapidly in high-K^+/nigericin solutions. Therefore, we also measured pH_i with pH-sensitive microelectrodes in order to determine resting pH_i and the magnitude of DA-induced pH changes.

pH_i measurements with pH-sensitive microelectrodes

Recordings of pH_i with pH-sensitive microelectrodes were performed as previously described (Rein et al., 2006). In brief, the active barrel of a theta-glass double-barrelled microelectrode was silanised with dimethyl-trimethylsilylamine (Munoz et al., 1983) and its tip was filled with the H^+ sensor from behind. The sensor column was backfilled with 100 mmol l^{-1} sodium citrate (pH 6.6). The reference barrel was filled with 3 mol l^{-1} KCl. For electrical recordings, the two electrode barrels were connected to the inputs of a differential amplifier (V86; List Medical, Darmstadt, Germany). The potential recorded by the reference barrel was subtracted from that recorded by the active barrel. This differential signal, which indicates ion activity, and the voltage recorded from the reference barrel were monitored on a chart recorder and stored on a PC using TestPoint software (Keithley, Germering, Germany). The bath electrode was an Ag/AgCl pellet connected to the bath *via* a 3 mol l^{-1} KCl-agar bridge. Duct epithelial cells were impaled under optical (Zeiss Stemi SV11, Jena, Germany) and electrical control. With the electrode positioned in the cytosol, the differential voltage signal is proportional to the pH_i, and the reference barrel

records the basolateral membrane potential (PD_b). For calibration of the pH-sensitive microelectrodes, PS (pH 7.4), PS titrated to pH 7.9, and a Pipes-buffered PS (pH 6.9) were used. The pH-sensitive microelectrodes were calibrated immediately after a successful experiment following withdrawal of the microelectrode into the bath. The mean slope of the electrodes was 56±4 mV per pH unit (N=5).

Statistical analysis

Statistical comparisons were calculated by one-way ANOVA followed by Dunnett's tests or by Student's unpaired t-test. P<0.05 was considered significant. All analyses were performed using GraphPad Prism 4.01 (GraphPad Software, San Diego, USA). Results are given as mean ± s.e.m.

Results

DA-induced pH_i changes

Microfluorometric recordings of pH_i with BCECF showed that the stimulation of isolated lobes by a brief application of 1 μmol l^{-1} DA, a concentration known to stimulate fluid secretion in isolated salivary glands (Just and Walz, 1996; Rietdorf et al., 2005), caused a reversible intracellular acidification. This acidification remained stable for some minutes after DA washout, following which pH_i recovered rapidly (Fig. 1A).

The DA-induced acidification was dose dependent. In order to obtain a dose–response relationship, we applied DA twice to each specimen. The first DA application (1 μmol l^{-1} DA) served as a control stimulus and the resulting drop in pH_i was set to 100%. The DA concentration of the second stimulus was varied as illustrated in Fig. 1A. The pH_i change induced by the second DA application was then normalised to that induced by the control stimulus. As shown in Fig. 1B, the threshold for the DA-induced acidification was 3 nmol l^{-1} DA. The acidification was half-maximal at ~30 nmol l^{-1} DA and was saturated at DA concentrations ⩾100 nmol l^{-1}. The non-linear regression according to the Hill equation resulted in a Hill coefficient of n_H=1.05 indicating no cooperativity in DA binding.

Because we were not able to calibrate the BCECF signals by the high K^+/nigericin method, we also recorded steady-state pH_i and the DA-induced pH_i changes with double-barrelled pH-sensitive microelectrodes. In many of these experiments, resting pH_i appeared to be acid (pH_i<7), especially when the reference barrel of the electrode recorded poor basolateral membrane potentials (PD_b; more positive than –50 mV). This effect was more pronounced with electrodes that had a broken tip and thus, a larger tip diameter. Because we suspected that cell impalement with such poorly performing electrodes had created a H^+ leak, we discarded all recordings associated with a PD_b more positive than –50 mV. In the remaining experiments (PD_b more negative than –50 mV), the mean steady-state pH_i was 7.3±0.1, and 1 μmol l^{-1} DA induced a pH_i decrease to 6.9±0.1 (N=5; Fig. 1C). The time course of the DA-induced acidification recorded with pH-sensitive

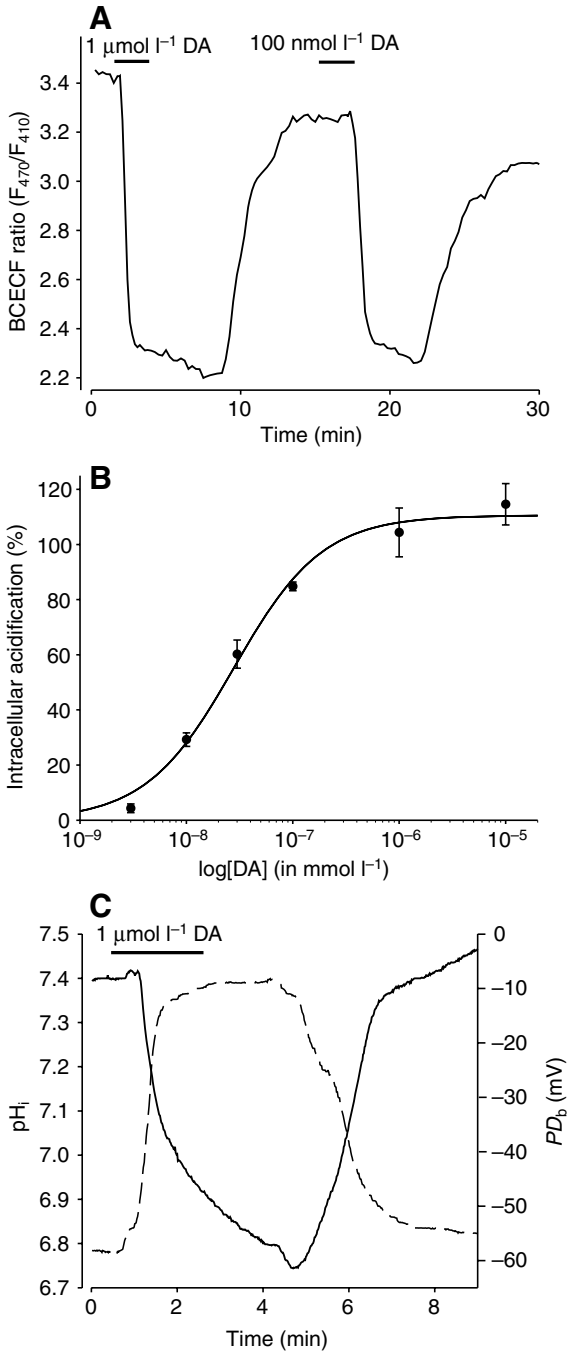

Fig. 1. Dopamine (DA)-induced changes in pH_i in duct cells of isolated salivary glands as measured with BCECF (A,B) and a double-barrelled pH-sensitive microelectrode (C). (A) Treatments with 1 μmol l^{-1} and 100 nmol l^{-1} DA induce a reversible intracellular acidification, N=5. (B) Dose–response relationship of the DA-induced intracellular acidification. Experiments were performed as in A and responses were normalised to the pH_i drop induced by a first 1 μmol l^{-1} DA control stimulus (=100%). Values are means ± s.e.m. from 4–7 experiments for each concentration. A non-linear regression according to the Hill equation was fitted through the data points. (C) Effect of DA on pH_i (solid line) and basolateral membrane potential (PD_b, broken line). This recording, with a double-barrelled pH-sensitive microelectrode, is representative of five independent experiments.

microelectrodes was almost identical to that recorded microfluorometrically with BCECF. In addition, 1 μmol l^{-1} DA induced a reversible depolarisation of PD_b from –55±3 mV to –19±3 mV (N=5; Fig. 1C) as reported previously (Lang and Walz, 2001). As shown in Fig. 1C, the onset of the pH_i decrease lagged behind the onset of the depolarisation by about 20–30 s.

Effects of V-ATPase- and Na^+-dependent acid extrusion on steady-state pH_i and pH_i recovery after DA-induced acidification

Does apical V-ATPase activity affect the DA-induced pH_i changes? In order to answer this question, we treated the salivary glands as indicated at the top of Fig. 2A. A brief application of 1 μmol l^{-1} DA induced a reversible acidification and served as a control. After the pH_i had recovered to the resting level, we superfused the preparation with concanamycin A, a specific inhibitor of the V-ATPase (Dröse et al., 1993; Dröse and Altendorf, 1997). As shown in Fig. 2A, 1 μmol l^{-1} concanamycin A did not affect resting pH_i or the kinetics and magnitude of the acidification induced by 1 μmol l^{-1} DA. However, in the presence of concanamycin A, pH_i recovered more slowly from the DA-induced acidification. We determined the rate of the initial fast phase (1–3 min) of the pH_i recovery by a linear regression (ratio-units min^{-1}) and found that concanamycin A reduced the rate of pH_i recovery significantly by ~33% (Fig. 2A,D; N=6, P<0.05).

This result suggested that the apical V-ATPase contributed to pH_i recovery but was not the sole acid extruder. Therefore, we next studied whether the duct cells had a Na^+-dependent acid extruder by testing whether the removal of extracellular Na^+ affected the rate of pH_i recovery. Because the DA-induced acidification required extracellular Na^+ (C. Hille, unpublished data), bath Na^+ was removed after DA washout (Fig. 2B). The pH_i started to recover quickly after DA washout but removal of extracellular Na^+ during this pH_i recovery reduced its rate significantly by ~70% (Fig. 2B,D; N=6, P<0.01). Re-introduction of extracellular Na^+ restored the initial fast rate of pH_i recovery (Fig. 2B).

Thus, neither concanamycin A nor the absence of extracellular Na^+ completely abolished the recovery from the DA-induced acidification. A prolonged bath application of 1 μmol l^{-1} concanamycin A in Na^+-free PS caused only a slight decrease in resting pH_i within 20–25 min (N=5, data not shown). However, when the ducts were stimulated with DA in the presence of 1 μmol l^{-1} concanamycin A and DA was

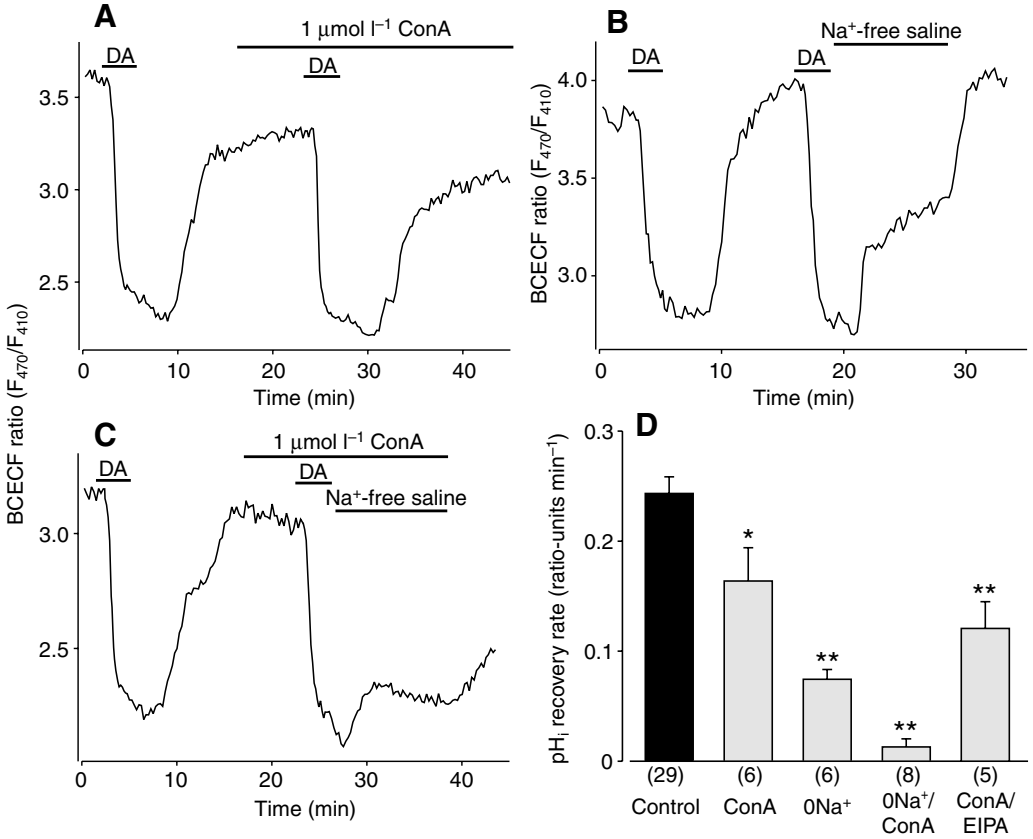

Fig. 2. Recordings of the effects of 1 μmol l^{-1} concanamycin A (A), Na^+-free ($0Na^+$) physiological saline (PS; B) and the combined application of concanamycin A (ConA) and Na^+-free PS (C) on pH changes induced by 1 μmol l^{-1} dopamine (DA). (D) Quantitative analysis of the rates of pH_i recovery in the above experiments and in an experiment in which 1 μmol l^{-1} ConA and 50 μmol l^{-1} EIPA were applied simultaneously. Values are means ± s.e.m.; the number of experiments is given under each bar; one-way ANOVA, *P<0.05, **P<0.01.

washed out with Na^+-free PS containing concanamycin A, pH_i recovery was blocked by ~95% (Fig. 2C,D; N=8, P<0.01).

In order to test, whether a NHE was responsible for the Na^+-dependent component of pH_i recovery, we studied whether EIPA, a specific inhibitor of the NHE (Petzel, 2000; Giannakou and Dow, 2001) mimicked the above effect of Na^+-free PS. Because EIPA is fluorescent under UV light, we recorded the fluorescence ratio F_{480}/F_{450} to measure pH_i. Control experiments showed that the rates of pH_i recovery were the same, independently of whether pH_i was recorded using the F_{480}/F_{450} ratio or the F_{470}/F_{410} ratio. We found that 50 μmol l^{-1} EIPA applied together with 1 μmol l^{-1} concanamycin A had no effect on resting pH_i but reduced the rate of pH_i recovery from a DA-induced acidification significantly to ~50% (Fig. 2D; N=5, P<0.01) of the control rate. Thus, the inhibition of the rate of pH_i recovery was significantly stronger than the inhibition caused by concanamycin A alone (Student's unpaired t-test, P<0.05), but less than in concanamycin A-containing Na^+-free PS.

Taken together, these data indicate that the V-ATPase and an NHE play only a minor role in the regulation of steady-state pH_i and have no effect on DA-induced acidification. The V-ATPase, an EIPA-sensitive (NHE) and an unidentified EIPA-insensitive but also Na^+-dependent acid extruder contribute to the recovery from a DA-induced acid load.

A drop in pH_i is sufficient to activate V-ATPase- and NHE-mediated acid extrusion

DA causes an increase in the intracellular Ca^{2+} concentration and possibly also in the cAMP concentration in salivary duct cells (Hille and Walz, 2006; Walz et al., 2006). Because an increase in cAMP concentration activates V-ATPase in *Calliphora* salivary glands (Dames et al., 2006), we have therefore asked whether a drop in pH_i is sufficient to stimulate V-ATPase-mediated outward H^+ pumping in *Periplaneta* salivary duct epithelial cells. We studied this question by imposing an acid load on the cells by applying a brief (1–2 min) pulse of 20 mmol l^{-1} NH_4Cl. An NH_4Cl pulse leads, in many cells, to characteristic pH_i changes (Boron and de Weer, 1976; Boron, 2004). Immediately after NH_4Cl application, pH_i increases rapidly because of an NH_3 influx and its protonation to NH_4^+. This is typically followed by a modest drop in pH_i attributable to a slow uptake of NH_4^+. The removal of NH_4Cl causes strong acidification because of the rapid NH_3 efflux from the cell, whereby H^+ ions are left behind. The pH_i then recovers from this acid load to resting pH_i *via* the activity of H^+ extruding and/or HCO_3^- importing transporters.

We found that, for duct epithelial cells, bath application of NH_4Cl induced only a rapid acidification without the typical initial alkalinisation (Fig. 3A,B,D). Removal of NH_4Cl boosted this acidification. These observations indicated that NH_4^+ entered the cells faster than NH_3. A high permeability of the basolateral membrane to NH_4^+ is not a common feature of animal plasma membranes. However, high permeabilities to NH_4^+ have been found in mouse cerebral astrocytes (Nagaraja and Brookes, 1998), in cells from the thick ascending limb of rat kidney (Bleich et al., 1995), in *Drosophila* Malpighian tubules (Bertram and Wessing, 1994) and recently in blowfly salivary glands (B. Schewe and B.W., unpublished). In these cell types, NH_4Cl induces a similar rapid decrease in pH_i to that observed in our study. Although we have not studied the pathways that mediate NH_4^+ entry, studies of other cells suggest that the $Na^+/K^+/2Cl^-$ cotransporter, an NHE or K^+ channels maybe involved (Ramirez et al., 1999; Heitzmann et al., 2000). The involvement of a Na^+-dependent entry mechanism seems likely in our preparation, because we have observed a transient alkalinisation upon NH_4Cl application in Na^+-free saline (see below, and Fig. 3B,C). Under these conditions, NH_3 entry must have been faster than that of NH_4^+. Nevertheless, after NH_4Cl washout, pH_i recovered quickly, within 2–4 min (N=23; Fig. 3). The lag of ~5 min before recovery from the DA-induced acidification began, in comparison with the immediate recovery from the NH_4Cl-induced acidification, probably reflects the more complex situation during DA stimulation, including changes in second messenger levels.

We subsequently studied whether the same transporters contributed to the recovery from an NH_4Cl- and a DA-induced acid load. As shown in Fig. 3A, bath application of 1 μmol l^{-1} concanamycin A influenced neither resting pH_i nor the NH_4Cl-induced acidification. However, concanamycin A reduced the rate of pH_i recovery significantly by ~48% of the control rate (N=5, P<0.05; Fig. 3A,E). Nevertheless, even in the presence of concanamycin A, pH_i recovered from the acid load within 10–15 min.

Because pH_i recovery was not completely abolished by concanamycin A, we next investigated whether extracellular Na^+ affected pH_i recovery. Superfusion of the preparation with Na^+-free PS did not alter resting pH_i (Fig. 3B). However, upon bath application of NH_4Cl in Na^+-free PS, a small transient intracellular alkalinisation could be observed before the pH_i decreased rapidly in the continuous presence of NH_4Cl (Fig. 3B,C). pH_i recovered completely in Na^+-free PS, but at a rate of only ~39% of the control rate (N=21, P<0.01; Fig. 3B,E).

Inhibition of the V-ATPase and removal of extracellular Na^+ by bath application of 1 μmol l^{-1} concanamycin A in Na^+-free PS inhibited pH_i recovery almost completely, by ~96% (N=6, P<0.01; Fig. 3C,E). The pH_i recovered, however, within 5–10 min after concanamycin A washout in Na^+-containing PS (Fig. 3C).

Finally, we examined whether an NHE was responsible for the Na^+-dependent component of the pH_i recovery by using EIPA as an NHE-specific inhibitor. For these experiments, we applied the acidifying NH_4Cl pulse in the presence of 1 μmol l^{-1} concanamycin A to inhibit V-ATPase-mediated outward H^+ transport. NH_4Cl washout with concanamycin A- and EIPA-containing PS (50 μmol l^{-1} EIPA) resulted in a pH_i recovery at a dramatically reduced rate (N=6, P<0.01; Fig. 3D,E). Moreover, the pH_i recovered only slowly after concanamycin A and EIPA washout (Fig. 3D).

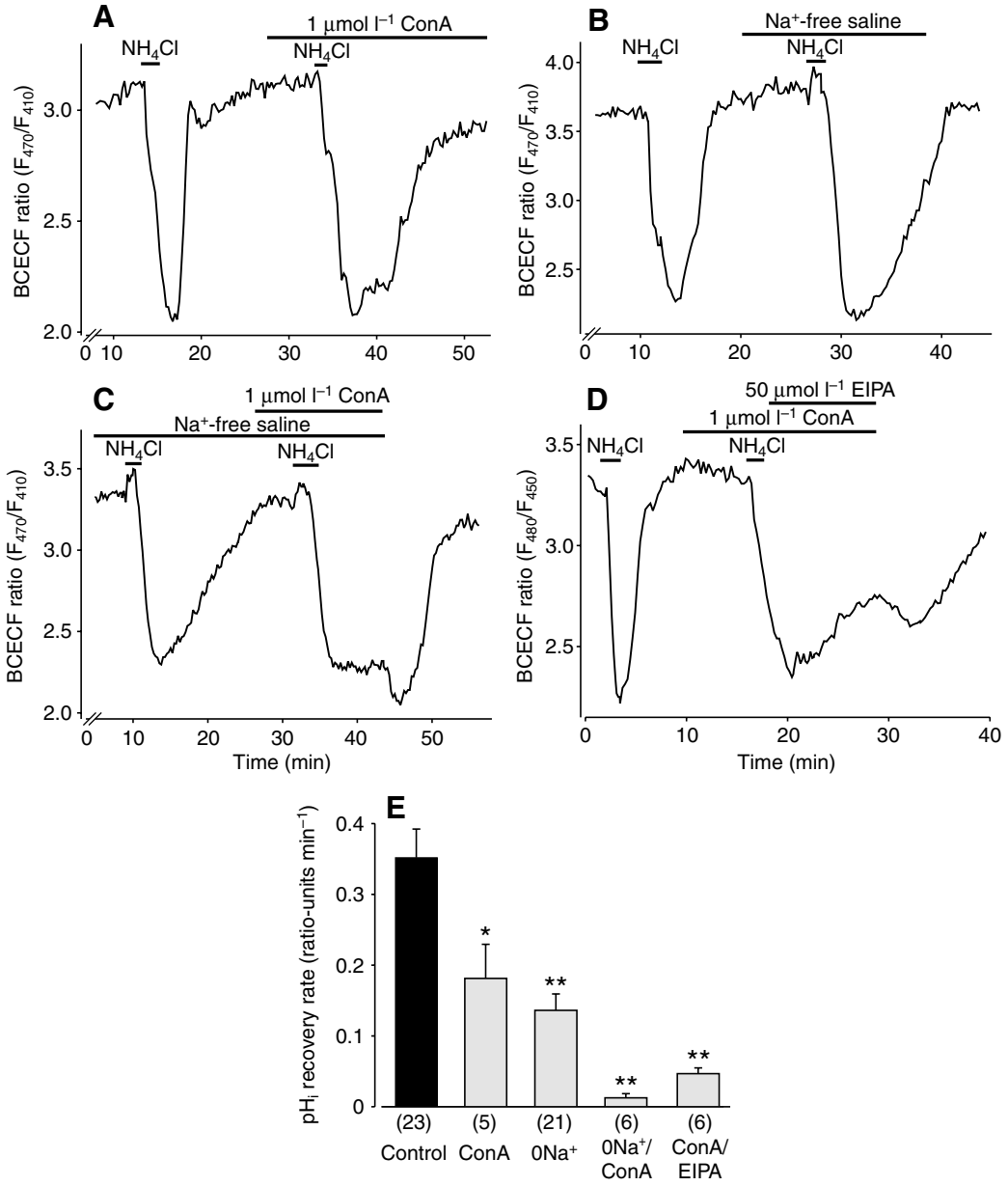

Fig. 3. Recordings of the effects of (A) 1 μmol l^{-1} concanamycin A (ConA), (B) Na^+-free physiological saline (PS), (C) 1 μmol l^{-1} ConA in Na^+-free PS (D) and 50 μmol l^{-1} EIPA together with 1 μmol l^{-1} ConA, on pH changes induced by a brief application of 20 mmol l^{-1} NH_4Cl to isolated salivary ducts. (E) Quantitative analysis of the rates of pH_i recovery in the above experiments. Values are means ± s.e.m.; the number of experiments is given under each bar; one-way ANOVA, *P<0.05, **P<0.01.

Discussion

This is the first study addressing mechanisms of intracellular pH regulation in an insect salivary gland. We are particularly interested in whether the plasma membrane V-ATPase is involved in pH_i regulation. Therefore, we have studied pH_i regulation in the dopaminergically innervated salivary ducts of *P. americana*, because the duct cells have a V-ATPase in their apical membrane (Just and Walz, 1994a; Walz et al., 2006) and its functional significance is unknown. Our major results are that the neurotransmitter DA causes an intracellular acidification and that the V-ATPase and an NHE contribute to H^+ extrusion after an acute DA- or NH_4Cl-induced acid load.

Steady-state pH_i and DA-induced pH_i changes

We have recorded a mean resting PD_b of –55±3 mV and a mean resting pH_i of 7.3±0.1 with double-barrelled pH-sensitive microelectrodes. These values indicate that H^+ is not in equilibrium across the basolateral plasma membrane. The electrochemical driving force for H^+ can be calculated by the equation:

$$DF_H = 58z^{-1}\log([H^+]_o / [H^+]_i) - PD_b ,$$

where DF_H is the electrochemical driving force for H^+, $[H^+]_o$ and $[H^+]_i$ are the extracellular and intracellular H^+ concentrations, respectively, and PD_b is the basolateral membrane potential. Positive values for DF_H favour H^+ influx. If the above values for PD_b and for $[H^+]_i$ and the known $[H^+]_o$ (=pH 7.4) are used, we obtain a strongly inwardly directed DF_H of +49.2 mV. Thus, the cells must actively regulate their pH_i even under resting conditions.

We were not able to construct our pH-sensitive microelectrodes as sharply as the double-barrelled K^+- and Na^+-sensitive microelectrodes that we used in a previous study of *Periplaneta* salivary duct cells (Lang and Walz, 2001). Therefore, we have recorded a PD_b that is ~10 mV less than that previously obtained (–65 mV) (Lang and Walz, 2001). Assuming a leak at the site of impalement and given the above calculated inwardly directed driving force for H^+, our pH_i is probably an underestimate but is sufficiently accurate as a quantitative supplement to our uncalibrated BCECF measurements.

The BCECF measurements have revealed that the inhibition of the apical V-ATPase with concanamycin A or the inhibition of the Na^+-dependent acid extruder in Na^+-free PS does not alter steady-state pH_i. If both acid extruders have a basal activity, one would have expected an acidification upon their inhibition. This result may reflect the low rate of acid loading in *Periplaneta* salivary ducts under resting conditions. Thus, V-ATPase and NHE seem to play a minor role in the regulation of steady-state pH_i but they are obviously important for pH_i recovery after acute intracellular acidification.

Bath application of the neurotransmitter DA causes the known depolarisation of the basolateral membrane (Lang and Walz, 2001) and, 20–30 s later, a reversible dose-dependent intracellular acidification of up to –0.4 pH units. The DA-induced acidification is half-maximal at ~30 nmol l^{-1} DA. This EC_{50} value compares well with previous data. Half-maximal secretory responses in salivary glands of the cockroaches *P. americana* and *Nauphoeta cinerea* have been achieved at ~110 nmol l^{-1} and ~88 nmol l^{-1} DA, respectively (House and Smith, 1978; Just and Walz, 1996), and the DA-induced hyperpolarisation of acinar cells in the salivary glands of *N. cinerea* is half-maximal at 42 nmol l^{-1} DA (Bowser-Riley and House, 1976).

We have not studied the H^+ source(s) that is(are) responsible for the DA-induced acidification. However, we know that salivary duct cells contain carbonic anhydrase activity (Just and Walz, 1994b) and display dramatic changes in intracellular Na^+, K^+ and Ca^{2+} concentrations when they are stimulated with DA (Lang and Walz, 1999; Lang and Walz, 2001; Hille and Walz, 2006). These DA-induced changes in ion concentrations may result in increased cellular respiration, concomitant CO_2 production and a metabolic acid load. Thus, the acidification is probably a result of processes involved in saliva modification in the duct system. However, preliminary experiments indicate that increased metabolism is involved but not the only basis for the acidification. Thus, a more complex scenario in duct epithelial cells is proposed, and this will be the subject of a future study.

V-ATPase and a Na^+-dependent transporter contribute to pH_i regulation after DA stimulation

A key result of the present work is that at least two acid extruders, *viz* the V-ATPase and an NHE, contribute to pH_i regulation after an acute acid load induced by a DA stimulus or an NH_4Cl pulse. The evidence for this is provided by the rate of pH_i recovery after an acid load being (1) reduced when the apical V-ATPase is specifically inhibited by bath application of the plecomacrolide antibiotic concanamycin A (Dröse et al., 1993; Dröse and Altendorf, 1997), (2) reduced in Na^+-free PS, (3) almost completely blocked when the V-ATPase is inhibited with concanamycin A in Na^+-free PS, and (4) partly reduced (after a DA-induced acidification) or almost completely reduced (after an NH_4Cl-induced acidification) when the V-ATPase is blocked with concanamycin A and the NHE is blocked simultaneously with the amiloride derivative EIPA (Petzel, 2000; Giannakou and Dow, 2001). Our observation that concanamycin A reduces the rate of pH_i recovery from a DA-induced acidification almost completely in Na^+-free PS, but much less in the presence of EIPA, indicates that the duct cells must have an additional, as yet unidentified, Na^+-dependent acid extruder that is only active after DA stimulation. Nevertheless, we cannot rule out the existence of an additional EIPA-insensitive NHE isoform activated by DA stimulation. Although NHE in *Drosophila* and *Aedes* Malpighian tubules was found to exhibit clear sensitivities to amiloride and its analogue EIPA (Petzel, 2000; Giannakou and Dow, 2001), the *Aedes* NHE3 was recently shown to be relatively insensitive to these drugs (Pullikuth et al., 2006).

The V-ATPase resides in the apical plasma membrane domain of the duct cells (Just and Walz, 1994a). Although we have applied the V-ATPase inhibitor concanamycin A to the bathing medium around the salivary gland preparation, concanamycin A nevertheless acts at the apical membrane. An effective inhibition of apical V-ATPase-mediated H^+ pumping by bath application of concanamycin A has also been shown recently for blowfly salivary gland cells (Rein et al., 2006). Our finding that Na^+-free PS mimics the pharmacological effect of EIPA indicates that the NHE is localised in the basolateral membrane of the duct cells. An exclusive basolateral localisation has also been shown for the vertebrate NHE1 isoform that is ubiquitously expressed in many epithelia (Orlowski and Grinstein, 1997; Wakabayashi et al., 1997).

The contribution of a V-ATPase to pH_i regulation has been described in several epithelia. In human eccrine sweat ducts, a V-ATPase is involved in pH_i recovery after NH_4Cl-induced acidification and probably acidifies the sweat in the duct lumen (Granger et al., 2002). An important role of V-ATPase in pH_i regulation has also been suggested for *Drosophila* Malpighian tubules (Bertram and Wessing, 1994), collecting ducts in rabbit kidneys (Yip et al., 2002), guinea pig inner ear (Stankovic et al., 1997) and rat hippocampal astrocytes (Pappas and Ransom, 1993).

NHE family members contribute to pH_i and cell volume regulation in many tissues, e.g. not only in vertebrate pancreatic ducts, colonic crypts and kidney collecting ducts, but also in

insect Malpighian tubules (Stuenkel et al., 1988; Soleimani et al., 1994; Hasselblatt et al., 2000; Petzel, 2000).

Aspects of NHE and V-ATPase activation

NHE activity is regulated by pH_i. Its activity rises with decreasing pH_i, and the existence of a H^+ binding site for the allosteric activation of NHE activity by internal H^+ has been postulated (Wakabayashi et al., 1997). Thus, in many NHE-expressing cell types, this transporter is not involved in the regulation of resting pH_i (Tønnessen et al., 1990; Brokl et al., 1998; Tsuchiya et al., 2001) but becomes active after an acute acid load. As shown in rat sublingual mucous acini, for example, the rate of pH_i recovery after an NH_4Cl-induced acid load increases linearly with decreasing pH_i as a result of differences in NHE activity (Zhang et al., 1992).

Little is known about the mechanisms that mediate between a pH_i decrease and the activation of the V-ATPase. In kidney proximal tubules and collecting ducts, chronic metabolic acidification induced by bath applications of NH_4Cl or CO_2 promotes the recruitment of V-ATPases from a cytoplasmic vesicular pool to the apical membrane thereby increasing the rate of H^+ secretion (Schwartz and Al-Awqati, 1985; Chambrey et al., 1994; Sabolic et al., 1997). However, this exocytosis-based process needs several minutes to hours to become effective and the detailed molecular mechanisms are largely unknown. Carraro-Lacroix and Malnic (Carraro-Lacroix and Malnic, 2006) have recently shown that angiotensin-II-stimulated H^+ secretion *via* V-ATPase in kidney proximal tubule cells occurs by a protein kinase A-independent mechanism but that protein kinase C and cytosolic Ca^{2+} play a critical role in this process. Our laboratory has recently demonstrated that, in *Calliphora* salivary glands, serotonin stimulates bafilomycin-sensitive V-ATPase activity, the recruitment of the V-ATPase complex V_1 to the apical membrane, the assembly of the V-ATPase V_0V_1 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; Zimmermann et al., 2003). The above effects are mediated by the serotonin-induced elevation of the intracellular cAMP concentration (Dames et al., 2006). Other studies on yeast and kidney epithelial cells have shown that V-ATPase binds directly to the glycolytic enzyme aldolase and that its activity is stimulated by glucose. This suggests a coupling of V-ATPase activity to glycolysis (Kane, 1995; Lu et al., 2001; Nakamura, 2004), perhaps to adjust V-ATPase activity to the level of intracellular acidification attributable to cellular metabolism to maintain pH_i homeostasis. Clear cells of the rat epididymis express high levels of V-ATPase at their apical pole for proton secretion that, in the vas deferens, is required for sperm maturation and storage (Brown and Breton, 1996). It has also been shown that V-ATPase accumulation is regulated by a bicarbonate-activated soluble adenylyl-cyclase-dependent increase in intracellular cAMP in response to alkaline luminal pH (Pastor-Soler et al., 2003). We have found in this study that the V-ATPase in *Periplaneta* salivary ducts is stimulated not only by the neurotransmitter DA, but also after an NH_4Cl-induced acid load. This strongly indicates that cell types are present in which a drop in pH_i is sufficient to stimulate V-ATPase-mediated H^+ extrusion. The simplest explanation for this observation is that the V-ATPase displays higher rates of transport just because of the enhanced availability of protons as substrate.

List of abbreviations

BCECF/AM	2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
DA	dopamine
EIPA	5-(*N*-ethyl-*N*-isopropyl)-amiloride
NHE	Na^+/H^+ exchange
PD_b	basolateral membrane potential
pH_i	intracellular pH
PS	physiological saline
V-ATPase	vacuolar-type H^+-ATPase

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