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Characterisation of neurotransmitter-induced electrolyte transport in cockroach salivary glands by intracellular Ca^{2+}, Na^+ and pH measurements in duct cells

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SUMMARY

Ion-transporting acinar peripheral cells in cockroach salivary glands are innervated by dopaminergic and serotonergic fibres, but saliva-modifying duct cells are innervated only by dopaminergic fibres. We used microfluorometry to record intracellular Na^+, Ca^{2+} and H^+ concentrations ($[Na^+]_i$, $[Ca^{2+}]_i$ and pH_i) in duct cells of two types of preparation, *viz* 'lobes' consisting of acini with their duct system and 'isolated ducts' without acini, in order to obtain information about the transporters involved in saliva secretion and/or modification. Our results indicate that (1) stimulation of lobes by dopamine (DA) causes a strong drop of pH_i and increases in $[Na^+]_i$ and $[Ca^{2+}]_i$ in duct cells; (2) in contrast, DA stimulation of isolated ducts produces only a small pH_i drop and no changes in $[Na^+]_i$ and $[Ca^{2+}]_i$; (3) pH_i and $[Ca^{2+}]_i$ changes are also induced in duct cells by serotonin (5-HT) stimulation of lobes, but not isolated ducts; (4) in the absence of CO_2/HCO_3^-, the DA-induced pH_i drop is strongly reduced by removal of extracellular Cl^- or inhibition of the Na^+–K^+–$2Cl^-$ cotransporter (NKCC); (5) in the presence of CO_2/HCO_3^-, the DA-induced pH_i drop is not reduced by NKCC inhibition, but rather by inhibition of the Cl^-/HCO_3^- exchanger (AE), Na^+/H^+ exchanger (NHE) or carbonic anhydrase. We suggest that DA and 5-HT act predominantly on acinar peripheral cells. Their activity (secretion of primary saliva) seems to cause changes in ion concentrations in duct cells. NKCC and/or AE/NHE activities are necessary for pH_i changes in duct cells; we consider that these transporters are involved in the secretion of the NaCl-rich primary saliva.

Key words: dopamine, serotonin, insect, salivary glands, ion transport, intracellular pH, sodium–potassium–chloride cotransporter, sodium–proton exchanger, anion exchanger.

INTRODUCTION

Mechanisms of epithelial transport in insects have been investigated extensively in Malpighian tubules (Beyenbach, 2003), midgut (Wieczorek et al., 2000) and salivary glands (House and Ginsborg, 1985). Fluid secretion by epithelial cells involves the coordinated activities of several ion transport proteins in the apical and basolateral membranes, resulting in vectorial electrolyte and water movement across the epithelium. However, the complex physiology of stimulus–secretion coupling for electrolyte and fluid transport and the involvement of the various transporters are still not completely understood in any of the above-mentioned systems. The salivary glands in the cockroach *Periplaneta americana* are a useful model system for studying cell physiological aspects of stimulus–secretion coupling (Walz et al., 2006). As discussed below, in this preparation, elementary processes of saliva formation are distributed among different cell types that are stimulated by different neurotransmitters. This allows the experimental dissection and localisation of the elementary processes of saliva secretion and modification.

Cockroach salivary glands are of the acinar type and consist of several cell types (Just and Walz, 1994a). Salivation is controlled by dopaminergic and serotonergic neurons (Baumann et al., 2002; Baumann et al., 2004). Acinar peripheral cells are specialised for electrolyte and water transport and are innervated by dopaminergic and serotonergic neurons. Acinar central cells are responsible for the production and secretion of proteins and are innervated only by serotonergic neurons. The duct cells downstream of the acini are also specialised for electrolyte transport and are innervated only by dopaminergic neurons. Because of this innervation, both biogenic amines, *viz* dopamine (DA) and serotonin (5-HT), stimulate salivation in isolated salivary glands. DA induces the secretion of a protein-free saliva, whereas stimulation with 5-HT results in the secretion of protein-rich saliva (Just and Walz, 1996). Similar to the two-stage hypothesis of salivation (Cook et al., 1994) in mammalian salivary glands, those in the cockroach secrete an isosmotic NaCl-rich primary saliva into the acinar lumen. The primary saliva is subsequently modified by Na^+ reabsorption and K^+ secretion as it passes through the ducts, resulting in a final saliva that is hyposmotic (Gupta and Hall, 1983; Rietdorf et al., 2003). Primary saliva secretion resulting from transepithelial NaCl and water transport through the peripheral cells is driven by an apical Na^+/K^+-ATPase (Gupta and Hall, 1983; Just and Walz, 1994b). A basolateral Na^+/K^+-ATPase and an apical vacuolar-type proton pump (V-H^+-ATPase) in the duct epithelial cells are thought to be important for saliva modification (Lang and Walz, 2001).

Since saliva secretion is mediated by several cell types, the coordinated activity of their ion channels and transporters is necessary. Additionally, an adjustment of ductal activity is required when the secretion of primary saliva is modulated because of different physiological requirements, e.g. brief *vs* sustained periods of saliva secretion. With regard to even more basic questions, how does a duct cell know that primary saliva is passing the ducts and that it has to be modified? The immediate aim of the present study

was therefore to obtain information about the coordinated activities of acini and ducts. One methodological problem is that the investigation of transport processes within the acini is technically challenging. The acini are compact structures with two secretory cell types; this makes it difficult to ascribe a recorded cell response to a particular cell type. Furthermore, the acinar cells are extremely difficult to load with fluorescent dyes and, even worse, they show strong autofluorescence that changes upon stimulation (K. Rietdorf and B.W., unpublished observations). However, we have measured intracellular Na^+, Ca^{2+} and H^+ concentrations in duct cells and, unexpectedly, have noted that these recordings also allow some conclusions to be drawn about ion transport activities in the acini, when recordings from preparations with or without acini are compared. In combination with pharmacological experiments, we have been able to supplement the list of ion transporters involved in DA-induced salivation. We suggest that the activity of ducts depends on acinar activity and we provide preliminary evidence that acinar peripheral cells possess multiple NaCl uptake mechanisms during primary saliva formation depending on CO_2/HCO_3^- availability.

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. Whole salivary gland complexes were dissected in physiological saline as described previously (Just and Walz, 1996). Two different types of preparation were investigated: (1) 'lobes' consisting of several acini with their corresponding duct system and (2) 'isolated ducts' consisting of a branched duct system without acini.

Solutions and chemicals

The HCO_3^--free saline 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. In Cl^--free saline, equimolar amounts of sodium isethionate were substituted for NaCl. In CO_2/HCO_3^--buffered saline, 25 mmol l^{-1} NaCl was replaced by an equimolar amount of $NaHCO_3$ and equilibrated with carbogen (5% CO_2/95% O_2) to pH 7.4. BCECF/AM (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester), Fura-2/AM, Fluo-3/AM, SBFI/AM (sodium-binding benzofuran isophthalate, acetoxymethyl ester), DA, 5-HT, bumetanide, DIDS (4,4′-diisothiocynatostilbene-2,2′-disulfonic acid), EIPA (5-(*N*-ethyl-*N*-isopropyl)amiloride) and acetazolamide (all from Sigma, Deisenhofen, Germany or Invitrogen, Karlsruhe, Germany) were stored as stock solutions in small aliquots at –20°C and diluted in saline immediately before an experiment.

Microfluorometric measurements of intracellular pH, Ca^{2+} and Na^+ in duct cells

In both preparations, *viz* lobes and isolated ducts, intracellular pH and intracellular Ca^{2+} and Na^+ concentrations (pH_i, $[Ca^{2+}]_i$ and $[Na^+]_i$) were measured in duct cells. The dye-loaded preparations were 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 saline. 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 monochromator unit including a 75 W xenon arc lamp (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. Fluorescence images were acquired and digitised with a cooled image transfer CCD camera (CoolSnap-HQ, Roper Scientific Inc., Tucson, AZ, USA) at 12-bit resolution. Monochromator control, image acquisition and processing were carried out using MetaFluor 6.1 software (Universal Imaging Corp., Downingtown, PA, USA).

For pH_i measurements in duct cells, the preparations were loaded with the pH-sensitive fluorescent dye BCECF by a 10 min incubation in saline containing 0.5 μmol l^{-1} BCECF/AM at room temperature. BCECF fluorescence was excited at 470 nm and 410 nm and the pH_i was expressed as the fluorescence ratio F_{470}/F_{410}. Because of the fluorescence of DIDS and EIPA in UV light, BCECF fluorescence was excited at 480 nm and 450 nm and the fluorescence ratio F_{480}/F_{450} was calculated in all experiments in which DIDS and EIPA were used.

For measurements of $[Ca^{2+}]_i$, in duct cells, the preparations were loaded with the Ca^{2+}-sensitive fluorescent dye Fura-2 at room temperature during a 30 min incubation in 5 μmol l^{-1} Fura-2/AM in saline. Fura-2 fluorescence was excited at 340 nm and 380 nm. Calcium signals were expressed as the fluorescence ratio F_{340}/F_{380} calculated after the subtraction of background fluorescence and of cell autofluorescence determined at the end of each experiment by quenching Fura-2 fluorescence with 20 mmol l^{-1} $MnCl_2$.

For simultaneous measurements of $[Ca^{2+}]_i$ and $[Na^+]_i$ in duct cells, the preparations were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-3 and the Na^+-sensitive fluorescent dye SBFI at room temperature during a 150–180 min incubation in 15 μmol l^{-1} SBFI/AM and 3.75 μmol l^{-1} Fluo-3/AM in saline in the presence of 0.125% Pluronic F-127 (Invitrogen, Karlsruhe, Germany). Fluorescence images were acquired at excitation wavelengths of 340 nm, 360 nm and 480 nm. Sodium signals were expressed as the fluorescence ratio F_{340}/F_{360} and calcium signals were expressed as Fluo-3 fluorescence F_{480}.

We were not able to convert the BCECF fluorescence ratio into pH_i by using the high-K^+/nigericin method (Thomas et al., 1979), because the cells deteriorated rapidly in high-K^+/nigericin solution. Because a number of difficulties are associated with both *in situ* and *in vitro* calibration procedures (Borzak et al., 1992; Boyarsky et al., 1996; Baylor and Hollingworth, 2000) and as ductal pH_i, $[Ca^{2+}]_i$ and $[Na^+]_i$ values are known (Lang and Walz, 1999; Lang and Walz, 2001; Hille and Walz, 2007), we present our data uncalibrated as ratio units or relative fluorescence for the other dyes, too.

Statistical analysis

Statistical comparisons were calculated by Student's paired or unpaired *t*-test. A *P*-value <0.05 was considered significant. All analyses were performed using GraphPad Prism 4.01 (GraphPad Software, San Diego, CA, USA). Results are given as means ± s.e.m.

RESULTS

DA-induced changes in pH_i, $[Na^+]_i$ and $[Ca^{2+}]_i$ in duct cells require the presence of acini

As shown recently (Hille and Walz, 2007), stimulation of lobes by DA caused a strong and reversible intracellular acidification in duct cells (Fig. 1A). Unexpectedly, stimulation of isolated ducts without acini by the same DA concentration resulted in only a small reversible intracellular acidification in the duct cells. Its magnitude

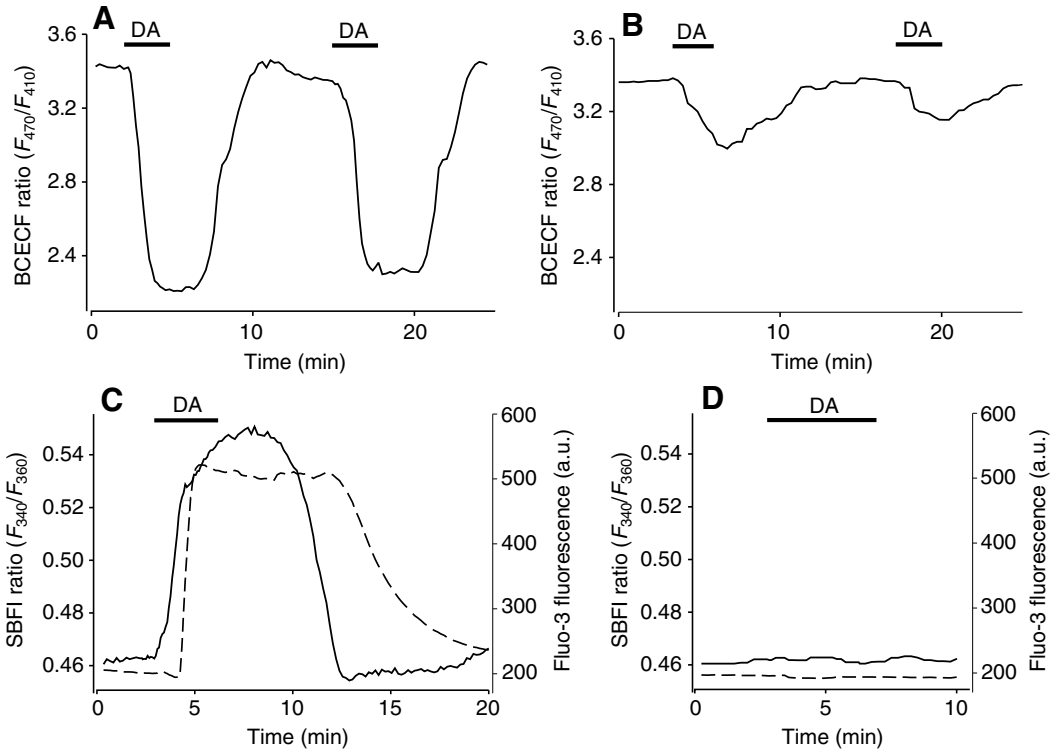

Fig. 1. Comparison of 1 μmol l^{-1} dopamine (DA)-induced effects in duct cells of the two types of preparation: lobes and isolated ducts. Changes in intracellular pH (pH_i) in duct cells as measured with BCECF in lobes (A) and isolated ducts (B). Simultaneous measurement of $[Ca^{2+}]_i$ and $[Na^+]_i$ changes in duct cells of lobes (C) and isolated ducts (D). The SBFI ratio (solid line) indicates changes in $[Na^+]_i$, whereas the Fluo-3 fluorescence (dashed line) indicates changes in $[Ca^{2+}]_i$ (a.u., arbitrary units). These original recordings are representative of at least five independent experiments.

was significantly smaller than that observed in duct cells of complete lobes (Fig. 1B; 1.07±0.06 *vs* 0.19±0.02 ratio units, N=7–9, P<0.001). Simultaneous recordings of $[Ca^{2+}]_i$ and $[Na^+]_i$ revealed that the stimulation of lobes by DA caused a $[Na^+]_i$ elevation that preceded an $[Ca^{2+}]_i$ elevation in duct epithelial cells (Hille and Walz, 2006) (Fig. 1C). Again unexpectedly, the stimulation of isolated ducts without acini by DA caused no change in $[Ca^{2+}]_i$ and $[Na^+]_i$ in the duct cells (Fig. 1D). For these experiments, isolated duct preparations and corresponding lobe preparations were dissected from the same salivary glands. Thus, the lobe preparations served as indicators for intact isolated salivary glands. In addition, the low SBFI ratio and Fluo-3 fluorescence indicated that $[Na^+]_i$ and $[Ca^{2+}]_i$ were low in duct cells of unstimulated isolated ducts, arguing against the possibility that dissection of the acini had damaged the duct cells.

5-HT-induced changes in pH_i and $[Ca^{2+}]_i$ in duct cells require the presence of acini

Previous immunofluorescence studies have demonstrated that the ducts further downstream of the acini are innervated only by dopaminergic fibres that have some release sites (Baumann et al., 2002; Baumann et al., 2004). Thus, we expected that duct cells would respond to stimulation by DA, but not by 5-HT.

We tested this expectation by recording DA- and 5-HT-induced changes in pH_i and $[Ca^{2+}]_i$ in duct cells using both types of preparation: lobes or isolated ducts. In lobe preparations, an intracellular acidification could be observed in duct cells stimulated by 1 μmol l^{-1} DA and 1 μmol l^{-1} 5-HT (Fig. 1A and Fig. 2A). The magnitude of the 5-HT-induced and the DA-induced acidifications, normalised to that after a 1 μmol l^{-1} DA control stimulation, showed no significant differences (DA: 104.4±8.8% *vs* 5-HT: 90.0±15.6%, N=5–7, P>0.05). In contrast, the stimulation of isolated ducts by 5-HT caused no changes of pH_i in the duct cells, as expected (Fig. 2B). In addition, stimulation of lobes with 5-HT induced an elevation of $[Ca^{2+}]_i$ in duct cells, as did a DA stimulation (Fig. 2C), but no $[Ca^{2+}]_i$ elevation when isolated ducts were used (Fig. 2D).

Thus, the pH_i, $[Na^+]_i$ and $[Ca^{2+}]_i$ changes in duct cells of lobes could be induced by DA and 5-HT, despite the ducts being innervated dopaminergically, but not serotonergically. Furthermore, the prominent effects of DA and 5-HT on duct cells were observed only in the presence of acini. Indeed, only the acinar peripheral cells are dopaminergically and serotonergically innervated. Consequently, we should consider the possibility that DA and 5-HT stimulate primarily the acinar peripheral cells, rather than the duct cells. In other words, the DA- and 5-HT-induced changes in pH_i, $[Na^+]_i$ and $[Ca^{2+}]_i$ in duct cells of our so-called lobe preparations are the consequence of the secretory activity of the acinar peripheral cells. If this were indeed the case, the primary saliva secreted from the acinar peripheral cells passing through the ducts must be causative, in an unidentified way, for the observed pH_i, $[Na^+]_i$ and $[Ca^{2+}]_i$ changes measurable in duct cells.

NKCC activity dominates in HCO_3^--free saline

Because the DA-induced pH_i changes in duct cells require the activity of the acinar peripheral cells, we only used lobe preparations in all the following experiments. In the first series of experiments, we recorded pH_i changes in the duct cells of lobes in standard HCO_3^--free cockroach physiological saline. We examined the Cl^- dependence of DA-induced pH_i changes in order to obtain information on the involvement of Cl^--dependent transporters, such as the Cl^-/HCO_3^- exchanger (AE) or Na^+–K^+–$2Cl^-$ cotransporter (NKCC).

We found that the DA-induced acidification in duct cells was completely abolished in the absence of extracellular Cl^- (Fig. 3A). Thus, a Cl^--dependent acid–base transporter might be active, thereby causing the DA-induced acidification. A likely candidate is the AE. We tested for the possible involvement of an AE by bath application of 500 μmol l^{-1} DIDS, a non-specific inhibitor of anion transporters in mammalian tissues (Cabantchik and Greger, 1992;

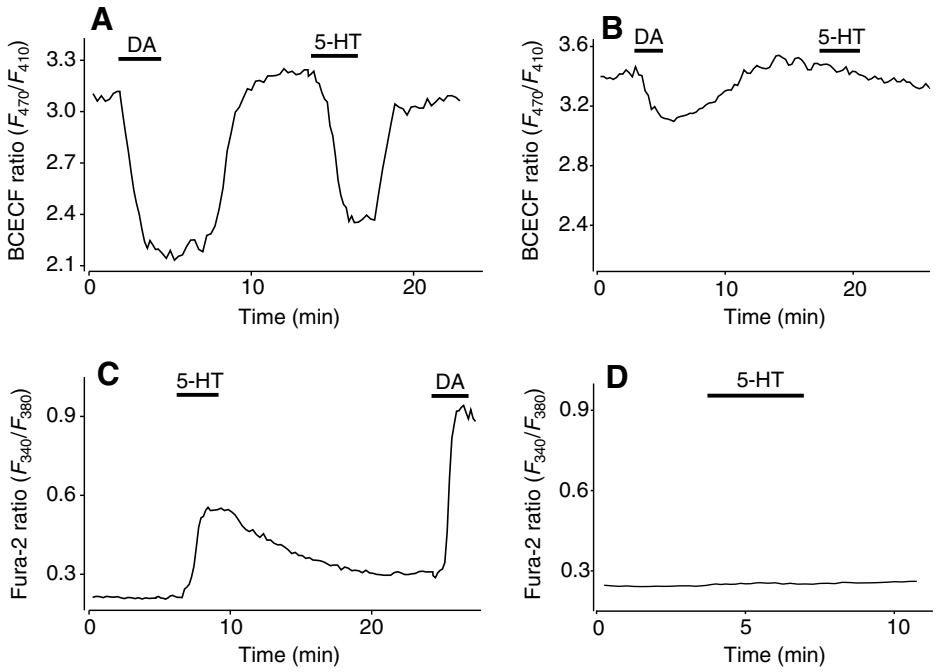

Fig. 2. Comparison of 1 μmol l^{-1} DA-induced and 1 μmol l^{-1} serotonin (5-HT)-induced effects in duct cells of both types of preparation: lobes and isolated ducts. Changes in pH_i in duct cells as measured with BCECF in lobes (A) and isolated ducts (B). Changes in $[Ca^{2+}]_i$ in duct cells as measured with Fura-2 in lobes (C) and isolated ducts (D). These original recordings are representative of at least five independent experiments.

Boron, 2001) and in insect tissues (Strange and Phillips, 1985; Boudko et al., 2001). DIDS had no effect on the DA-induced acidification in duct cells. Neither the initial rate nor the magnitude of the acidification changed significantly (Fig. 3B, N=9, P>0.05).

Previous studies have shown that an NKCC is important for saliva secretion in *Periplaneta* salivary glands (Lang and Walz, 2001; Rietdorf et al., 2003). Therefore, we considered the possibility that the Cl^- dependence of the DA-induced acidification in the duct cells could be an indirect (see Discussion) consequence of NKCC activity. We tested for this possibility by using bumetanide, a specific inhibitor of the NKCC (Russell, 2000). Bath application of 10 μmol l^{-1} bumetanide did not abolish the DA-induced acidification completely. Instead, DA in the presence of bumetanide caused only a transient acidification in duct cells, which recovered to resting pH_i in the continuous presence of DA (Fig. 3C). However, the magnitude of the acidification was significantly reduced to 56.6±10.6% of the DA-induced acidification in the absence of bumetanide (N=6, P<0.05).

We next tested the DIDS sensitivity of the transient DA-induced acidification recorded in the presence of bumetanide. We found that the combined bath application of 10 μmol l^{-1} bumetanide and 500 μmol l^{-1} DIDS resulted in a further reduction of the DA-induced acidification to 10.2±5.2% of the acidification induced by DA in the absence of bumetanide and DIDS (Fig. 3D; N=6, P<0.01). Thus, a DIDS-sensitive anion transporter, most probably the AE, is involved in generating the DA-induced acidification, even in HCO_3-free saline. This is possible because CO_2 from the ambient air equilibrates with the physiological saline producing some 100 μmol l^{-1} HCO_3^- (Deitmer and Schneider, 1998). In addition, we know that both acinar peripheral cells and duct cells contain a carbonic anhydrase, the enzyme that accelerates the hydration of CO_2 and the subsequent generation of H^+ and HCO_3^- (Just and Walz, 1994c). Thus, an inhibition of carbonic anhydrase activity by acetazolamide should reduce intracellular HCO_3^- availability for HCO_3^--dependent transport mechanisms such as the AE. We tested this prediction by the combined bath application of 10 μmol l^{-1} bumetanide and 500 μmol l^{-1} acetazolamide. This caused a reduction in the magnitude of the DA-induced acidification in duct cells to 22.1±4.7% of that of a control DA stimulus (Fig. 3F). This reduction in the DA-induced pH_i change was significantly stronger than that in the presence of bumetanide alone (N=6–8, P<0.01). Interestingly, as observed in experiments in which DIDS was applied alone (see Fig. 3B), the bath application of 500 μmol l^{-1} acetazolamide alone had no effect on the DA-induced acidification. Neither the initial rate nor the magnitude of the acidification changed significantly (Fig. 3E, N=4, P>0.05).

We conclude from these results that NKCC activity is required for the pH_i changes that we have recorded in duct cells upon DA stimulation of lobes in nominally HCO_3^--free saline. In addition, the inhibition of NKCC activity reveals an AE activity that is dependent on carbonic anhydrase activity and that causes, upon DA stimulation, only a transient acidification in duct cells, possibly because of the limited CO_2/HCO_3^- availability.

AE and Na^+/H^+ exchanger activity dominate in CO_2/HCO_3^--buffered saline

No HCO_3^--free physiological saline as used in the above experiments can mimic the situation in insect haemolymph. However, previous studies (Just and Walz, 1996; Rietdorf et al., 2003) have clearly shown neurotransmitter-induced saliva secretion in HCO_3^--free physiological saline at rates comparable with saliva secretion induced by electrical stimulation of the salivary duct nerve (Smith and House, 1977; House and Smith, 1978; Watanabe and Mizunami, 2006). This may be so, because CO_2 from the ambient air can equilibrate with the saline, producing some 100 μmol l^{-1} HCO_3^- in the nominally HCO_3^--free saline (Deitmer and Schneider, 1998), as mentioned above. Nevertheless, we next analysed pH_i changes in duct cells of lobes in CO_2/HCO_3^--buffered saline.

Addition of CO_2/HCO_3^--buffered saline caused a rapid pH_i drop in duct cells, reaching a new steady-state pH_i within 1–2 min as a result of CO_2 diffusion into the duct cells (Fig. 4A). This effect was reversible upon removal of CO_2/HCO_3^-. In the presence of CO_2/HCO_3^-, DA induced a reversible acidification (Fig. 4A). Since

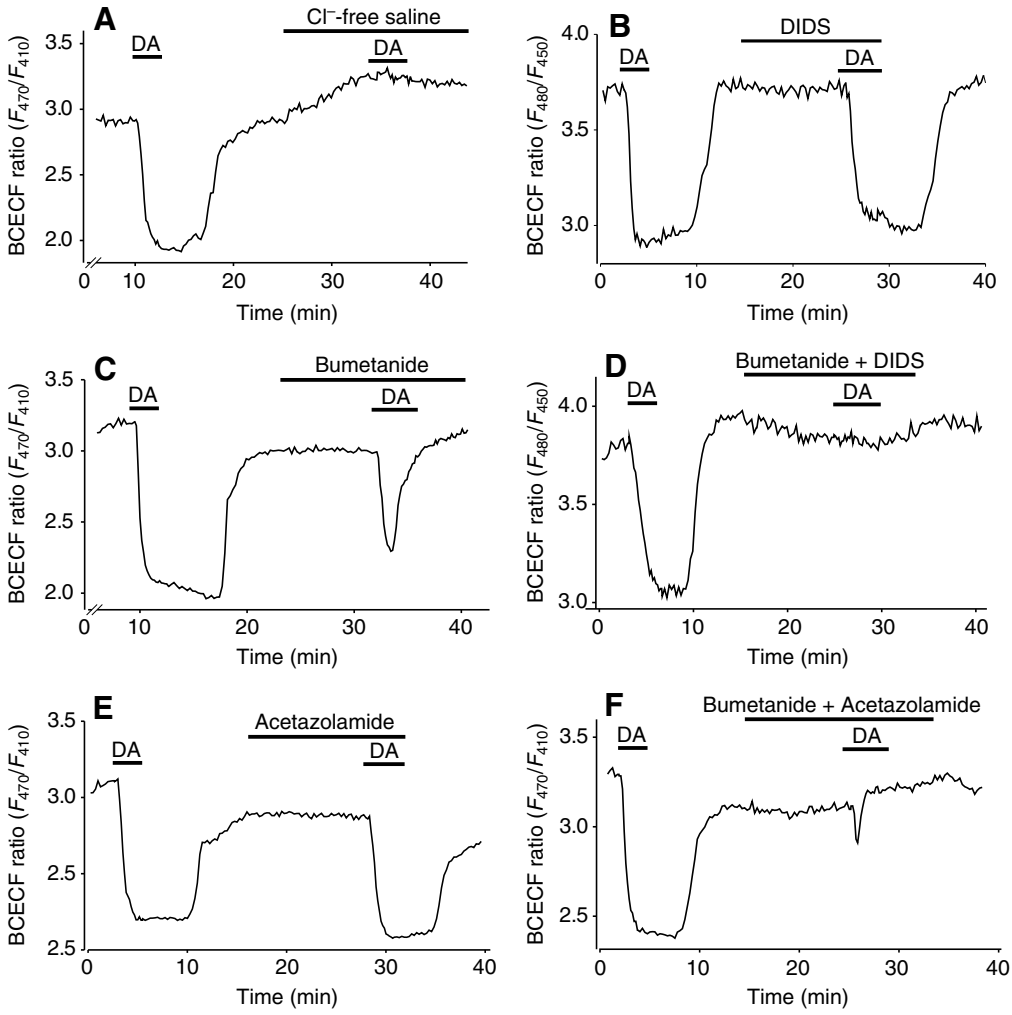

Fig. 3. Effects of various factors on intracellular acidification induced by 1 μmol l^{-1} DA in duct cells of lobe preparations in HCO_3^--free saline. (A) The acidification is abolished in Cl^-–free saline (N=4). (B) The acidification is not influenced by bath application of 500 μmol l^{-1} DIDS (N=9). (C) The acidification is transient following the bath application of 10 μmol l^{-1} bumetanide (N=6). (D) The combined bath application of 10 μmol l^{-1} bumetanide and 500 μmol l^{-1} DIDS abolishes the acidification (N=6). (E) The acidification is not influenced by bath application of 500 μmol l^{-1} acetozolamide (N=4). (F) The combined bath application of 10 μmol l^{-1} bumetanide and 500 μmol l^{-1} acetazolamide reduces the acidification dramatically (N=8).

the resting pH_i was lowered, the magnitude of this acidification was significantly reduced to 59.0±4.6% of that observed in HCO_3^--free saline (N=5, P<0.05).

Subsequently, we tested the contribution of the NKCC and AE to the generation of DA-induced pH_i changes in CO_2/HCO_3^--buffered saline pharmacologically. We found that, under these conditions, the bath application of 10 μmol l^{-1} bumetanide did not significantly affect the DA-induced acidification in duct cells, in striking contrast to its effect in HCO_3^--free saline (Fig. 4B, N=5, P>0.05). However, the combined bath application of 10 μmol l^{-1} bumetanide and 500 μmol l^{-1} DIDS resulted in a transient DA-induced acidification in duct cells, the magnitude of this acidification being significantly reduced to 28.0±8.4% of that of the DA-induced acidification under control conditions (Fig. 4C, N=6, P<0.01). The same result was observed by the combined bath application of 10 μmol l^{-1} bumetanide and 50 μmol l^{-1} EIPA, a specific inhibitor of the Na^+/H^+ exchanger (NHE) in insects (Petzel, 2000; Giannakou and Dow, 2001). Under these conditions, the DA-induced acidification was also reduced dramatically to 17.0±12.3% of that of the control stimulation (Fig. 4D, N=5, P<0.01).

Surprisingly, the inhibition of the acid loader AE by DIDS and the acid extruder NHE by EIPA resulted in the same reduction of the DA-induced acidification in duct cells. This result suggests that the activities of the AE and NHE do not directly cause the acidification in duct cells. We speculate that a coupled activity of AE and NHE is responsible for the pH_i changes in the duct cells. For the combined AE/NHE activity, intracellular HCO_3^- could be provided by carbonic anhydrase activity. This is suggested by an experiment showing that DA causes, in the presence of CO_2/HCO_3^- and the combined bath application of 10 μmol l^{-1} bumetanide and 500 μmol l^{-1} acetazolamide, only a transient acidification with a magnitude of 45.6±11.6% of that upon control stimulation (Fig. 4E, N=5, P<0.05).

DISCUSSION

Previous studies have identified a number of transport processes in the salivary gland complex of the cockroach *P. americana*. An immunocytochemical study has localised the Na^+/K^+-ATPase in the apical plasma membrane domain of acinar peripheral cells and in the basolateral plasma membrane domain of duct cells. In addition,

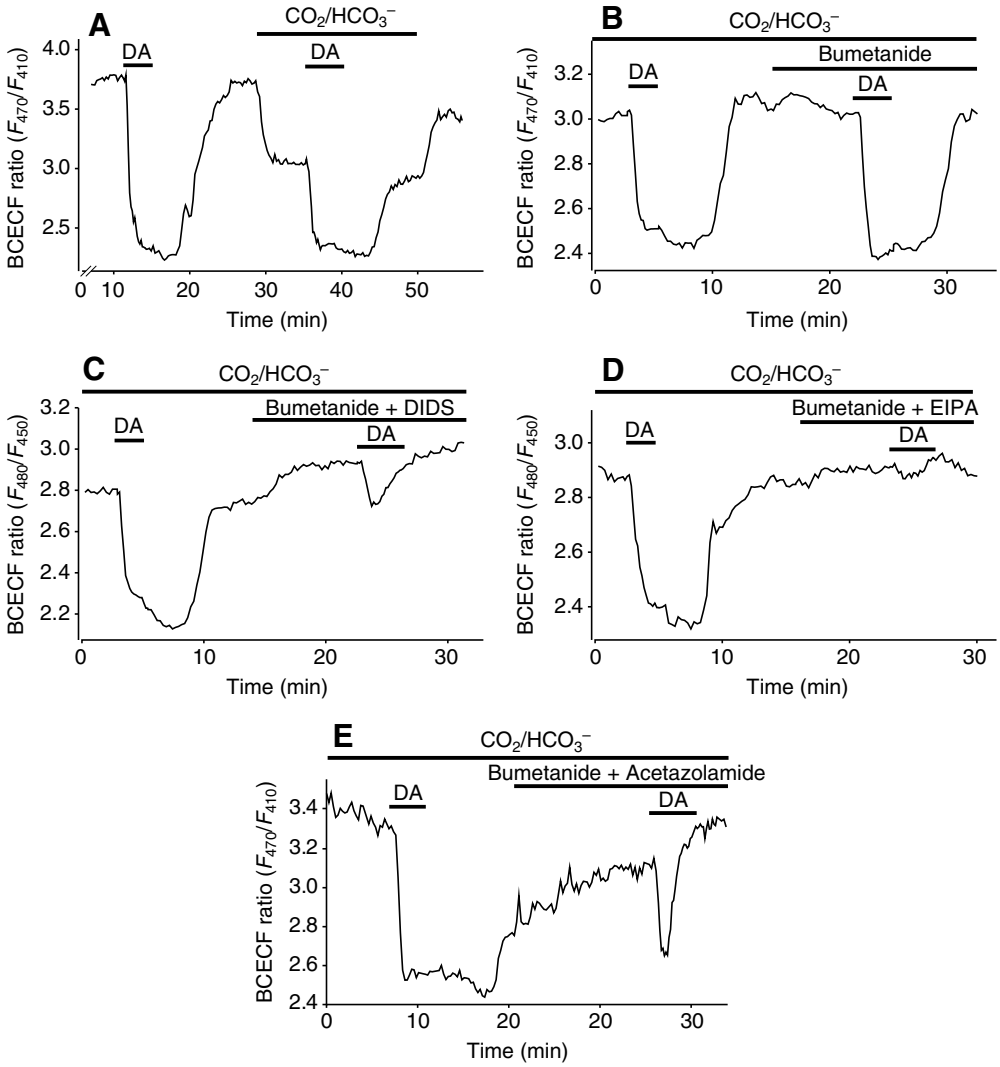

Fig. 4. Effects of various factors on intracellular acidification induced by 1 μmol l^{-1} DA in duct cells of lobe preparations in CO_2/HCO_3^--buffered saline. (A) The acidification is still present in CO_2/HCO_3^--buffered saline (*N*=5). (B) Bath application of 10 μmol l^{-1} bumetanide does not influence the acidification (*N*=5). (C) The combined bath application of 10 μmol l^{-1} bumetanide and 500 μmol l^{-1} DIDS strongly reduces the acidification (*N*=5). (D) The combined bath application of 10 μmol l^{-1} bumetanide and 50 μmol l^{-1} EIPA also reduces the acidification dramatically (*N*=5). (E) In addition, the combined bath application of 10 μmol l^{-1} bumetanide and 500 μmol l^{-1} acetozolamide decreases the acidification (*N*=5).

duct cells have been shown to have a V-H^+-ATPase in their apical membrane (Just and Walz, 1994b). Other studies have recorded intracellular pH_i, $[Na^+]_i$ and $[Ca^{2+}]_i$ in duct cells of isolated lobes (Lang and Walz, 1999; Lang and Walz, 2001; Hille and Walz, 2006). Because these lobes consist of three cell types that are stimulated by DA (duct cells), 5-HT (acinar central cells) or both neurotransmitters (acinar peripheral cells), the exact site of DA and/or 5-HT action has remained uncertain in the above studies. We have addressed this problem by comparing neurotransmitter-induced changes in pH_i, $[Na^+]_i$ and $[Ca^{2+}]_i$ in duct cells in two different preparations: lobes (consisting of acini with their ducts) and isolated ducts (without acini). Our results suggest that DA and 5-HT act predominantly on the acinar peripheral cells. Their activity (secretion of primary saliva) seems to cause changes in ion concentrations in the duct cells. Consequently, our recordings of pH_i in duct cells provide information on the transport mechanisms required for saliva secretion and on the localisation of some of these transporters.

Lobes *vs* isolated ducts

Several results from this study suggest that neurotransmitter-induced changes in pH_i, $[Na^+]_i$ and $[Ca^{2+}]_i$ in duct cells depend on acinar activity. Strong DA-induced intracellular acidification and $[Ca^{2+}]_i$ elevation in duct cells are observed only when active acini are present in the preparation (lobe preparation). In addition, a similar acidification and $[Ca^{2+}]_i$ elevation are induced in duct cells by the 5-HT stimulation of lobes, but not of isolated ducts. Because only the acinar peripheral cells are innervated dopaminergically and serotonergically (Baumann et al., 2002), the pH_i changes in duct cells are probably governed by the DA- and 5-HT-induced stimulation of the acinar peripheral cells. The key observation that DA stimulation does not dramatically affect duct cell pH_i, $[Na^+]_i$ and $[Ca^{2+}]_i$ in isolated ducts, but only in complete lobes, indicates further that DA acts on acinar peripheral cells rather than directly on the duct cells.

We exclude mechanical damage attributable to the dissection of the acini as being responsible for the observed differences in the

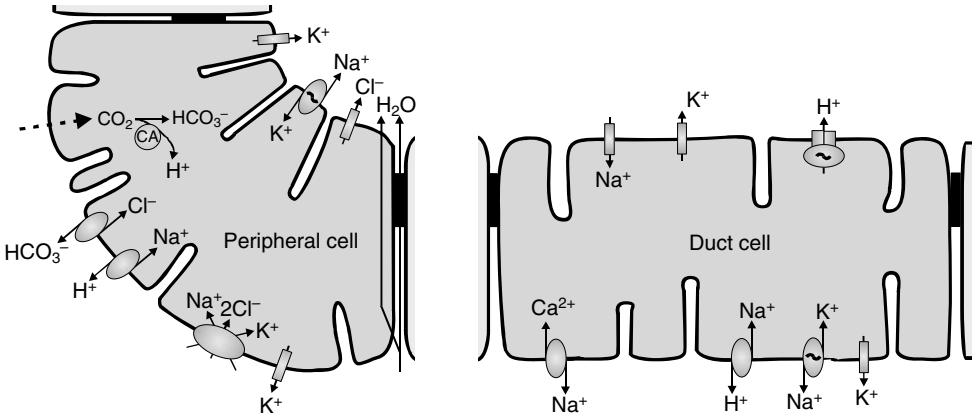

Fig. 5. Schematic diagram of proposed ion transporters in salivary glands of *Periplaneta americana*. Dopamine-induced saliva secretion involves the formation of isosmotic primary saliva in acinar peripheral cells and modification in duct cells resulting in hyposmotic final saliva. CA, carbonic anhydrase. For details, see text.

two preparations. Measurements have only been performed on cells of the isolated ducts far away from the cut edge. Moreover, the low SBFI ratio and the low Fluo-3 fluorescence (the latter is a highly sensitive indicator for a damaged cell with its damaged-induced elevated $[Ca^{2+}]_i$) suggest that the dissection of the acini does not damage duct cells in the region of our measurements. In addition, we do not think that the content of the duct lumen before the actual experiment affects our recordings, because this should be almost identical in the two preparations, *viz* lobes and isolated ducts. Indeed, the responses to a first DA stimulation are quite different for the two preparations, suggesting no dramatic influence of the 'old' duct lumenal content.

Rietdorf et al. (Rietdorf et al., 2003) have described that both neurotransmitters, *viz* DA and 5-HT, stimulate the secretion of a final saliva with similar Na^+, K^+ and Cl^- concentrations and osmolarities. This is surprising in view of the innervation pattern. Intuitively, one might have expected that the electrolyte composition of the final DA-stimulated saliva would be subjected to a stronger modification during its passage through the ducts than that of the 5-HT-stimulated saliva, because the duct cells have dopaminergic innervation. However, this unexpected observation can now be explained, providing that the activation of saliva modification by duct cells is indeed attributable to the DA- and 5-HT-induced entrance of primary saliva into the duct lumen.

Consequently, the results of this study raise the question as to how saliva modification in the ducts is activated. The simplest way would be an activation of duct cells by primary saliva flowing through the duct lumen. However, mechanosensitive flow sensors such as apical primary cilia or flow-induced circumferential stretch as shown in kidney (Liu et al., 2003; Wang, 2006) are unlikely. The cockroach salivary ducts are relatively inelastic due to a luminal cuticle and no cilia extend into the duct lumen (Just and Walz, 1994a). More complex signalling processes could be involved, since epithelial transport in glands has been shown to be influenced by certain agonists present in the luminal compartment (Forte and Currie, 1995; Sakairi et al., 1995; Leipziger, 2003; Novak, 2003). In rat submandibular salivary ducts, for instance, the activation of luminal purinergic receptors *via* ATP causes a Ca^{2+}-mediated elevation of the Cl^- conductance leading to a prolonged Cl^- reabsorption in the ducts (Lee et al., 1997; Zeng et al., 1997). In addition, Lee et al. (Lee et al., 1998) have proposed NHE-mediated Na^+ reabsorption *via* a purinergic receptor in rat submandibular salivary ducts. Moreover, ATP release into the lumen has been postulated for pancreatic acini (Sørensen and Novak, 2001), and ductal activity *via* purinergic receptors might be regulated by the secretory activity of the acini. Additionally, duct cells themselves participate in ATP release and thus would regulate saliva modification in an autocrine or paracrine fashion (Ishiguro et al., 1999). Whether the regulation of saliva modification in *Periplaneta* salivary ducts *via* receptors is indeed activated by a factor(s) released from the acini is speculative and needs further investigation.

NKCC and AE/NHE activity, dependent on HCO_3^- availability

The above considerations lead us to assume that drugs that inhibit key electrolyte transporters and affect DA- and/or 5-HT-induced pH_i changes in duct cells can do so because they inhibit these transporters in acinar peripheral cells rather than in duct cells. If this were indeed the case, we could use recordings of pH_i in duct cells to obtain information about transport processes engaged in primary saliva formation in acinar peripheral cells. According to this concept, we have been able to supplement the list of ion transport proteins in cockroach salivary glands (Fig. 5).

First, in the absence of CO_2/HCO_3^-, the DA-induced acidification in duct cells depends mainly on NKCC activity. Since the NKCC does not transport acid or base equivalents, it can induce pH_i changes only indirectly. Because we have measured, in isolated ducts, only a small DA-induced acidification, which, in addition, is not influenced by NKCC inhibition with bumetanide (data not shown), we suggest that the NKCC is localised in the acinar peripheral cells, because only this cell type, except for duct cells, is stimulated by DA. Its basolateral localisation in the acinar peripheral cells is supported by the experiment in Cl^--free saline (Fig. 5). Rietdorf et al. (Rietdorf et al., 2003) have previously shown that bumetanide reduces DA- and 5-HT-induced saliva secretion in isolated lobes by 92% and 85%, respectively. The concentrations of Na^+, K^+ and Cl^- and the osmolarities of the saliva are also dramatically reduced. Since the rate of fluid secretion is determined by the activity of peripheral cells, these cells must have a basolateral NKCC that is responsible for NaCl uptake. Indeed, DA-induced elevations in $[Na^+]_i$, $[K^+]_i$ and $[Cl^-]_i$ have been observed in peripheral cells in an X-ray microanalytical study (Gupta and Hall, 1983). Additionally, Smith and House (Smith and House, 1979) have demonstrated the dependence of fluid secretion on extracellular Na^+ in the cockroach *Nauphoeta cinerea*. Lang and Walz (Lang and Walz, 2001) have suggested the presence of the NKCC in the basolateral membrane of duct cells. However, they have shown a dramatic DA-induced NKCC-dependent $[Na^+]_i$ increase and $[K^+]_i$ decrease in the duct cells of lobes. Indeed, a large decrease in $[K^+]_i$ is hardly compatible with an active NKCC. We now assume that the $[Na^+]_i$ and $[K^+]_i$ changes in duct cells occur

during saliva modification, which has to be initiated by the presence of primary saliva in the duct lumen. The secretion of primary saliva attributable to acinar peripheral cell activity after DA-stimulation requires NKCC activity, which would explain the indirect NKCC dependence of the measured pH_i and the $[Na^+]_i$ and $[K^+]_i$ changes in duct cells (Fig. 5).

On the other hand, in the presence of CO_2/HCO_3^--buffered saline, DA-induced acidification in duct cells does not depend on NKCC activity. Under these conditions, an NHE and AE are involved, since the inhibition of these transporters reduces the DA-induced acidification in duct cells strongly. Because carbonic anhydrase inhibition also influences ductal acidification, its activity might provide intracellular HCO_3^- for AE/NHE activity. The activities of carbonic anhydrase and AE have also been observed in the absence of CO_2/HCO_3^-, since NKCC-independent transient ductal acidification can be further reduced by carbonic anhydrase or AE inhibition. However, in the absence of CO_2/HCO_3^-, the AE and NHE play only a minor role in inducing the ductal pH_i changes, perhaps because of a limited CO_2 supply. AE and NHE are most probably located in the acinar peripheral cells and induce the ductal acidification indirectly. First, inhibition of both the acid loader AE and the acid extruder NHE reduces the ductal acidification. Second, AE and NHE might compensate for the NKCC dependence of the acidification merely attributable to HCO_3^- availability, and the NKCC has been suggested to be localised in the acinar peripheral cells.

A model for DA-induced electrolyte transport

How can the results of the present study be incorporated into a model of saliva secretion? NKCC and/or AE/NHE activities are necessary for ductal pH_i changes. We suggest that these transporters are involved in the secretion of the NaCl-rich primary saliva. Located in the basolateral membrane of peripheral cells, they could function as NaCl uptake mechanisms depending on HCO_3^- availability (Fig. 5). The transepithelial NaCl and water transport in acinar peripheral cells is energised by an apical Na^+/K^+-ATPase. In addition, the apical membrane should contain a K^+ conductance for apical K^+ recycling. Transepithelial Cl^- flux could occur through Cl^- channels or paracellular transport (Gupta and Hall, 1983; Just and Walz, 1994b; Just and Walz, 1994c). This scenario finds its equivalent in mammalian salivary glands: here, NKCC activity and/or combined AE/NHE activity are also responsible for NaCl uptake within the acini (Dissing and Nauntofte, 1990; Nauntofte, 1992; Zhang et al., 1993; Cook et al., 1994; Turner and Sugiya, 2002). Additionally, Lee et al. (Lee et al., 2005) have demonstrated that the regulation of NKCC and NHE by acetylcholine in rat submandibular acini is strongly affected by the physiological level of HCO_3^-. They have found that the presence of CO_2/HCO_3^- not only enhances NHE activity but also inhibits the facilitation of NKCC. Another investigation has revealed that AE activity is increased in parotid acini from NKCC1-knockout mice suggesting a compensation of the loss of NKCC1 for salivation (Evans et al., 2000). The reason for the presence of multiple NaCl uptake mechanisms in the same gland is still not clear. However, they can coexist in the same acinar cell (Turner and George, 1988). The modulation of their relative contribution to fluid secretion might correlate with special physiological requirements, such as brief *vs* sustained salivation (Turner and Sugiya, 2002). Furthermore, fluctuations in haemolymph or blood CO_2/HCO_3^- concentrations could be much better balanced.

The primary saliva then enters the ducts, leading, through an unknown mechanism, to the activation of saliva modification by the duct cells (Fig. 5). Na^+ reabsorption by an apical Na^+ conductance is energetically possible resulting in an $[Na^+]_i$ elevation depolarising the apical membrane of the duct cells (Lang and Walz, 1999; Lang and Walz, 2001). The depolarisation would favour K^+ secretion *via* an apical K^+ conductance. The energisation of an active K^+ transport *via* the combined activities of a V-H^+-ATPase and a nH^+/K^+ exchanger, as for instance discussed for *Rhodnius* Malpighian tubules, *Manduca* midgut or *Calliphora* salivary glands (Maddrell and O'Donnell, 1992; Wieczorek, 1992; Zimmermann et al., 2003), is not required. In contrast to the situation in *Periplaneta* salivary glands, in those organs, a KCl-rich fluid is secreted against a K^+ concentration gradient. Na^+ reabsorption and K^+ secretion in *Periplaneta* salivary glands is possibly energised by the basolateral Na^+/K^+-ATPase (Rietdorf et al., 2003). The dramatic $[Na^+]_i$ elevation and the strong depolarisation of the basolateral membrane reverse the basolateral Na^+/Ca^{2+} exchanger activity into the Ca^{2+} entry mode causing an $[Ca^{2+}]_i$ elevation of still unknown function in the duct cells (Hille and Walz, 2006). The intracellular acidification during saliva modification could be the result of increased cellular metabolism. Although the proton source(s) for the acidification has/have not yet been identified, the apical V-H^+-ATPase and a basolateral NHE contribute to pH_i recovery (Hille and Walz, 2007).

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