

RESEARCH ARTICLE

Development of apical membrane organization and V-ATPase regulation in blowfly salivary glands

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SUMMARY

Secretory cells in blowfly salivary gland are specialized via morphological and physiological attributes in order to serve their main function, i.e. the transport of solutes at a high rate in response to a hormonal stimulus, namely serotonin (5-HT). This study examines the way that 5-HT-insensitive precursor cells differentiate into morphologically complex 5-HT-responsive secretory cells. By means of immunofluorescence microscopy, immunoblotting and measurements of the transepithelial potential changes, we show the following. (1) The apical membrane of the secretory cells becomes organized into an elaborate system of canaliculi and is folded into pleats during the last pupal day and the first day of adulthood. (2) The structural reorganization of the apical membrane is accompanied by an enrichment of actin filaments and phosphorylated ERM protein (phospho-moesin) at this membrane domain and by the deployment of the membrane-integral part of vacuolar-type H⁺-ATPase (V-ATPase). These findings suggest a role for phospho-moesin, a linker between actin filaments and membrane components, in apical membrane morphogenesis. (3) The assembly and activation of V-ATPase can be induced immediately after eclosion by way of 8-CPT-cAMP, a membrane-permeant cAMP analogue. (4) 5-HT, however, produces the assembly and activation of V-ATPase only in flies aged for at least 2 h after eclosion, indicating that, at eclosion, the 5-HT receptor/adenylyl cyclase/cAMP signalling pathway is inoperative upstream of cAMP. (5) 5-HT activates both the Ca²⁺ signalling pathway and the cAMP signalling cascade in fully differentiated secretory cells. However, the functionality of these signalling cascades does not seem to be established in a tightly coordinated manner during cell differentiation.

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INTRODUCTION

Salivary glands of the blowfly *Calliphora vicina* have for decades been a model system for the analysis of intracellular Ca²⁺ and cAMP signalling, and of the modes and regulation of transcellular ion transport (Berridge and Patel, 1968; Prince et al., 1975; Zimmermann and Walz, 1997; Berridge, 2005; Baumann and Walz, 2012). These glands are specialized for the production of copious amounts of salivary fluid upon demand, i.e. exposure to the neurohormone serotonin (5-hydroxytryptamine, 5-HT) (Berridge and Patel, 1968). To serve this task, the plasma membrane of the secretory cells is enormously expanded, providing the area for the positioning of an enormous number of ion transporters and channels (Oschman and Berridge, 1970; Baumann and Walz, 2012). Whereas the basolateral domain of the plasma membrane is folded into a basal labyrinth, the apical membrane forms an extensive system of branching canaliculi that are covered by long and closely stacked pleats of membrane, termed microplicae (Oschman and Berridge, 1970; Zimmermann et al., 2003). These secretory cells with their elaborate architecture arise from simple undifferentiated epithelial cells during the pupal and early adult stage (Berridge et al., 1976). The process of cell differentiation, however, has not yet been explored in detail.

The salivary glands are a pair of tubules that extend through the entire abdomen and the thorax and unite and open into the oesophagus (Oschman and Berridge, 1970; Rotte et al., 2008). The

abdominal region of the salivary gland, i.e. the portion that has been subject to numerous physiological studies, is a monolayer of homotypic cells that produce a KCl-rich primary saliva in a 5-HT-dependent mode (Berridge and Patel, 1968; Baumann and Walz, 2012). The secretory cells express two different 5-HT receptors, one of them (Cv5-HT_{2α}) activating the phospholipase C/inositol 1,4,5-trisphosphate (InsP₃)/Ca²⁺ signalling cascade, with the other (Cv5-HT₇) activating the adenylyl cyclase/cAMP/protein kinase A (PKA) signalling pathway (Berridge and Heslop, 1981; Röser et al., 2012). 5-HT thus evokes a parallel rise in both [cAMP] and [Ca²⁺] within the secretory cells (Berridge, 1980a; Heslop and Berridge, 1980; Zimmermann and Walz, 1997). The 5-HT-induced increase in intracellular [cAMP] elicits, via PKA, the assembly and activation of vacuolar-type proton pumps (V-ATPase) in the apical membrane (Dames et al., 2006; Rein et al., 2006; Rein et al., 2008). This ion pump is largely disassembled into a membrane-integral V₀ domain and a cytosolic V₁ domain, and thus is non-functional in unstimulated secretory cells. Upon 5-HT-induced translocation of V₁ domain from the cytosol to the membrane and assembly of V₀V₁ holoenzymes, V-ATPase is active and establishes an electrochemical proton gradient that energizes a cation/proton antiporter for the extrusion of K⁺ into the lumen of the gland (Baumann and Walz, 2012). The 5-HT-induced rise in cytosolic [Ca²⁺] stimulates transepithelial Cl⁻ transport into the lumen of the gland (Berridge et al., 1975; Berridge, 1980b).

Berridge et al. (Berridge et al., 1976) have analysed the development of the adult salivary gland during the larval and pupal stages. A few progenitor cells at the junction between the secretory region and the duct of the larval salivary gland generate the adult salivary gland. During the larval stages and the first half of pupal life, these imaginal cells generate ~20,000 cells by repetitive cell divisions (Berridge et al., 1976). During the last half of pupal life, mitosis seems to have ceased and the salivary glands increase in length and diameter by cell growth. Up to shortly before eclosion of the imagines, the cells remain relatively undifferentiated with the apical and basal surfaces being flat. At the time of eclosion, the apical membrane expands and folds into microplicae. At this stage, however, the glands are not yet able to produce saliva in a 5-HT-dependent mode. By 2 h post-eclosion (p.e.), 5-HT evokes salivation, with a maximal secretion rate being induced in animals aged to 4 h (Berridge et al., 1976). The reasons for the 5-HT insensitivity of salivary glands at adult emergence are unknown, although likely possibilities are incomplete secretory canaliculi, inoperative V-ATPase molecules or a deficient signalling pathway for V-ATPase activation at this developmental stage.

The present study examines the structural differentiation of the adult salivary glands in the period after eclosion, with a particular focus on the organization of the apical membrane domain. We further examine the developmental expression pattern of some proteins associated with the apical membrane, including the V-ATPase that is of key importance for saliva secretion. Finally, we analyse the development of the functionality of the cAMP/PKA signalling pathway that mediates the 5-HT-induced activation of the V-ATPase.

MATERIALS AND METHODS

Animals and preparation

Blowflies (*Calliphora vicina* Robineau-Desvoidy) were reared in a 12h:12h light:dark cycle at 25°C and a relative humidity of 60%. In flies aged for defined periods, the salivary glands were dissected in physiological saline containing 128 mmol l⁻¹ NaCl, 10 mmol l⁻¹ KCl, 2 mmol l⁻¹ MgCl₂, 2 mmol l⁻¹ CaCl₂, 3 mmol l⁻¹ sodium glutamate, 2.8 mmol l⁻¹ maleic acid, 10 mmol l⁻¹ D-glucose and 10 mmol l⁻¹ Tris-HCl (pH 7.2).

Reagents

Antisera against the following proteins were used: bovine V-ATPase subunit A and subunit E (Roussa et al., 1998), *Culex quinquefasciatus* V-ATPase subunit B (Filippova et al., 1998), *Manduca sexta* V-ATPase subunits d, C and D (Merzendorfer et al., 2000; Tiburcy et al., 2013) and *Drosophila melanogaster* PKA catalytic subunit and regulatory subunit II (Lane and Calderon, 1993; Skoulakis et al., 1993). Cross-reactivity of these antibodies with the homologous proteins in *C. vicina* has been demonstrated previously (Zimmermann et al., 2003; Dames et al., 2006; Voss et al., 2007; Voss et al., 2009; Baumann and Walz, 2012). Monoclonal anti-actin (clone C4) was obtained from Merck (Darmstadt, Germany), rabbit antiserum (cat. no. 3141) and monoclonal antibody (cat. no. 3149) against phosphorylated ezrin/radixin/moesin (pERM) and rabbit antiserum (cat. no. 3142) against non-phosphorylated ezrin/radixin/moesin (ERM) were from Cell Signaling Technology (Danvers, MA, USA). Cy3-tagged secondary antibodies and AlexaFluor488-phalloidin were acquired from Dianova (Hamburg, Germany) and Life Technologies (Darmstadt, Germany). 5-HT was from Sigma-Aldrich Chemie (Munich, Germany) and 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphate (8-CPT-cAMP) was from Biolog Life Science Institute (Bremen, Germany).

Immunofluorescence microscopy

In the case of experimental treatment of the glands, one of the pair of the salivary glands of each fly was kept as an unstimulated control, the other gland being incubated for 5 min in physiological saline with 30 nmol l⁻¹ 5-HT or for 10 min in physiological saline with 50 μmol l⁻¹ 8-CPT-cAMP. Subsequently, glands were chemically fixed, cryosectioned and immunostained as described in detail previously (Dames et al., 2006; Rotte et al., 2008). Fluorescence images were recorded with a Zeiss LSM 510 or a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss Microscopy, Jena, Germany). For a quantitative analysis, fluorescence intensities were determined in randomly selected areas of ~1.6 μm² over the canalicular system (identified by intense labelling for F-actin) and in the adjacent cytoplasm by use of the Zeiss ZEN software. Five measurements were made for either compartment in each section, averaged and corrected for background fluorescence that was determined in tissue-free areas next to the gland.

Biochemical techniques

Salivary glands of staged flies were isolated, homogenized in reducing sample buffer (Carl Roth, Karlsruhe, Germany) and heated to 60°C for 5 min. Equal amounts of protein were loaded per lane on 10 or 14% polyacrylamide gels, separated by gel electrophoresis and electrotransferred onto polyvinylidene fluoride membranes. Western blotting was performed as described in detail previously (Zimmermann et al., 2003).

Measurements of the transepithelial potential

The transepithelial potential was recorded by the oil gap method (Berridge and Prince, 1972), as described previously (Schmidt et al., 2008).

RESULTS

Structural differentiation of the apical plasma membrane

Shortly before eclosion of the imagines, the prospective secretory cells in the salivary gland have a simple architecture. The basal and apical domains of the plasma membrane are relatively flat (Berridge et al., 1976). To determine the structural reorganization of the apical membrane domain during cell differentiation, cryosections through salivary glands at various developmental stages were labelled with fluorophore-tagged phalloidin. Since microplicae have actin filaments in their interior, staining with the F-actin probe phalloidin visualizes the entire apical membrane of fully differentiated secretory cells in the adult salivary gland (Zimmermann, 2000; Zimmermann et al., 2003; Rotte et al., 2008).

At ~90% pupal development (p.d.), salivary glands have an outer diameter of 38±7 μm (mean ± s.d.; N=23). The gland tubules consist of a thin monolayer of epithelial cells that surround a large lumen. Only weak labelling for F-actin is associated with the entire plasma membrane of the secretory cells at this developmental stage (Fig. 1A,B). By the time of eclosion, the gland diameter has decreased to 34±3 μm (N=22) and the secretory cells have adopted a pyramidal shape. The nuclear area of the cell bulges towards the centre of the gland, thus confining the gland lumen to a thin layer (Fig. 1C,D). The apicolateral region of the plasma membrane is intensely stained for F-actin and forms short branching canaliculi that extend radially towards the basal pole of the secretory cells. During the subsequent 4 h, the secretory cells grow in height (Fig. 1E–H), resulting in an enlargement of the outer diameter of the glands to 39±6 μm (N=16). With increasing diameter, the canaliculi become longer, their tips keeping a distance of 1–2 μm from the basal domain of the plasma membrane. The final tubule

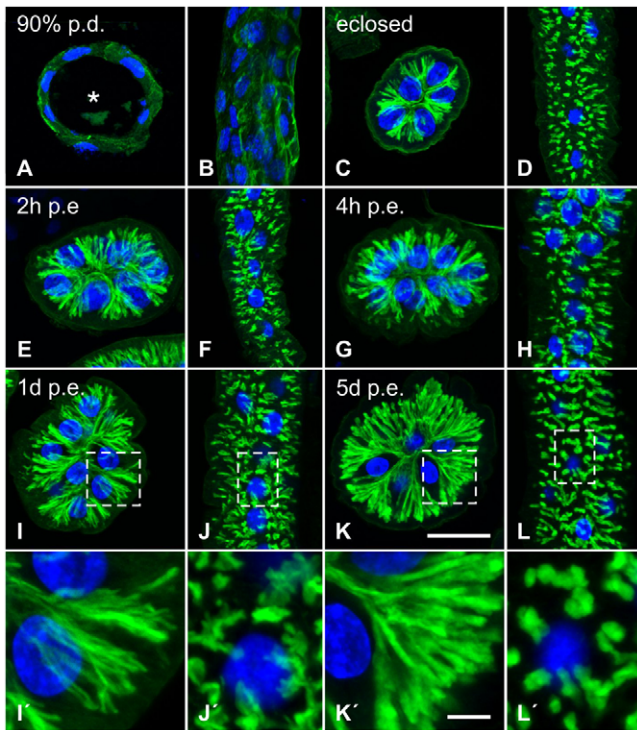


Fig. 1. Differentiation of the apical membrane in salivary glands of the blowfly *Calliphora vicina*. Cryosections through salivary glands were stained with AlexaFluor488-phalloidin (green) and DAPI (blue). Images show maximum intensity projections of image stacks representing 2.6 μm in cross-sections (A,C,E,G,I,K) and 5.6 μm in longitudinal sections (B,D,F,H,J,L). The boxed areas in I–L are shown at higher magnification in I'–L'. (A,B) At 90% pupal development (p.d.), the glands have a large lumen (*) and the apical membrane of the epithelial cells is devoid of canaliculi. (C,D) In newly eclosed flies, the apical membrane is folded into canaliculi. (E–H) After eclosion (p.e.), the canalicular system becomes more elaborate and reaches its full extent by 1 day p.e. (I–L') Between 1 and 5 days p.e., the cross-sectional area of the canaliculi increases. Scale bars: (A–L) 25 μm ; (I'–L') 5 μm .

diameter of $52 \pm 8 \mu\text{m}$ and the final extent of the canalicular system is reached by 1 day after eclosion (Fig. 1I,J). Subsequently, the canaliculi increase in cross-sectional area (Fig. 1K,L), probably reflecting a growth in microplacae length.

Enrichment of phosphorylated moesin at the apical plasma membrane

Members of the ERM (ezrin/radixin/moesin) protein family play a crucial role in the morphogenesis and organization of the apical domain of epithelial cells (Bretscher et al., 1997; Fehon et al., 2010), including the larval salivary gland of *Drosophila* (Xu et al., 2011). Typically, ERM proteins consist of an N-terminal domain that binds to phospholipids and membrane proteins, an α -helical domain, and a C-terminal F-actin-binding domain. The binding of ERM proteins to F-actin and other binding partners is regulated by the phosphorylation of a conserved Thr residue within the F-actin-binding site (Fehon et al., 2010). In vertebrates there are three members of the ERM protein family, ezrin, radixin and moesin, whereas only one member has been identified in *Drosophila* and other invertebrates (Polesello et al., 2002). In *Drosophila*, this ERM protein is named either moesin or Dmoesin (Polesello et al., 2002; Karagiannis and Ready, 2004).

Since ERM proteins have been identified as key organizers of the apical membrane domain, we have examined the developmental distribution of activated phosphorylated ERM in the salivary glands. Fig. 2A demonstrates that antibodies against non-phosphorylated and phosphorylated ERM proteins (anti-ERM; anti-pERM) identify, in the salivary gland, a single protein of $\sim 75 \text{ kDa}$ corresponding well to the molecular mass of *Drosophila* moesin (Chorna-Ornan et al., 2005). These antibodies are directed against a peptide in the F-actin-binding site that is highly conserved between vertebrate ERM members and *Drosophila* moesin and that contains the Thr for phosphorylation (Polesello et al., 2002). By immunofluorescence on salivary glands of several-day-old adult flies, anti-ERM produced a weak labelling throughout the cytoplasm, whereas staining with anti-pERM was restricted to the apical membrane domain including the canaliculi, colocalizing with F-actin (Fig. 2B–E). Thus by western blotting and subcellular localization, anti-pERM identifies in blowfly salivary glands activated membrane-associated ERM protein that we termed phospho-moesin, analogous to the *Drosophila* ERM protein.

To determine whether the amount of moesin and phospho-moesin changes during the morphogenesis of the secretory cells, salivary glands of staged flies were probed by western blotting with anti-actin, anti-ERM and anti-pERM, with identical amounts of total protein being loaded per lane (Fig. 2F). The relative amount of unphosphorylated moesin remained quite constant from eclosion to day 5 of adulthood. Phospho-moesin, in contrast, increased during this period and in parallel with the amount of actin, reaching a maximum at day 5. By immunofluorescence microscopy, phospho-moesin was localized to the apical and basal domain of the plasma membrane in secretory cells at 90% p.d. (Fig. 2G,M). During the first 2 h after eclosion, anti-pERM labelling on the basal domain faded, vesicular structures in the cytoplasm were pERM positive, and staining of the apical membrane increased in intensity (Fig. 2H,I,N,O). In the subsequent period up to day 5 of adulthood, cytoplasmic labelling disappeared and labelling at the apical domain increased further (Fig. 2J–L,P–R). In summary, the amounts of phospho-moesin and F-actin at the apical membrane seem to rise in parallel during cell morphogenesis.

Temporal expression pattern of V-ATPase

V-ATPase is composed of an ATP-hydrolysing cytosolic V_1 domain and a membrane-integral proton-conducting V_0 domain (Drory and Nelson, 2006; Forgac, 2007). To determine the expression pattern of V-ATPase components during secretory cell morphogenesis, salivary glands of staged flies were probed by western blotting with antibodies against the V_0 subunit d and the V_1 subunits A, B, C, D and E. Fig. 3A demonstrates that all V-ATPase subunits could be detected in salivary glands of freshly eclosed flies, and that the relative amount of the various subunits increased in parallel and to a similar extent as actin and phospho-moesin, reaching a maximum at day 5 of adulthood.

To determine the developmental pattern in the subcellular distribution of V-ATPase V_0 domain, cryostat sections were probed by immunofluorescence microscopy with anti-subunit d. At 90% p.d., labelling for subunit d was present on vesicular structures distributed throughout the cytoplasm but was non-detectable on the plasma membrane of the future secretory cells (Fig. 3B,H). By eclosion, subunit d was not only highly enriched on the apical membrane, but also detected on vesicular structures within the cytoplasm. No obvious change occurred in the subcellular distribution of subunit d while the eclosed flies aged, except for a growth of the subunit-d-positive canalicular system

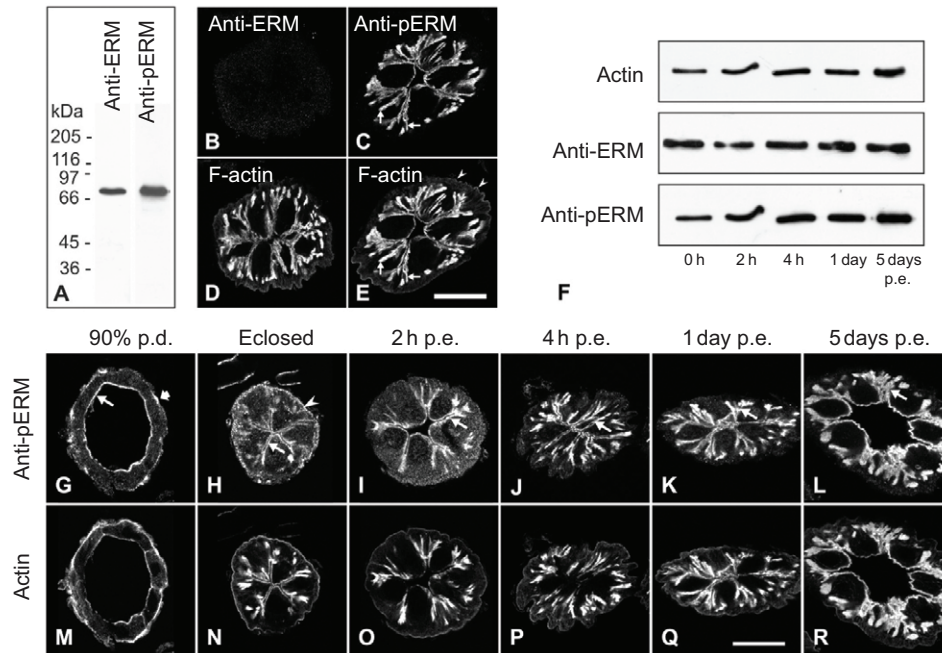


Fig. 2. Developmental distribution of phospho-moesin. (A) Western blot analysis of blowfly salivary glands with antibodies against unphosphorylated (anti-ERM) and phosphorylated (anti-pERM) ezrin/radixin/moesin. Both antibodies label a band of ~75 kDa. (B–E) Immunofluorescence labelling of cryostat sections with anti-ERM and anti-pERM. Anti-pERM labelling is highly enriched at the apical membrane but absent on the basolateral membrane domain (arrowhead in E). Weak staining with anti-ERM is diffusely distributed in the cytoplasm. (F) Developmental analysis of the relative amount of actin and moesin in salivary glands. Identical amounts of protein for each developmental stage (p.e.) were separated by SDS-polyacrylamide gel electrophoresis and probed with antibodies against actin, ERM and pERM. Note that the amount of anti-ERM-immunoreactive protein (=non-phosphorylated moesin) remains constant, whereas the amount of anti-pERM-positive protein (=phospho-moesin) increases in parallel with actin. (G–R) Phospho-moesin becomes enriched at the apical membrane domain during cell morphogenesis in adult salivary glands. Phospho-moesin is detected at both the apical (thin arrow) and basal (thick arrow) domain of the plasma membrane in secretory cells at 90% p.d. At 0 and 2 h after eclosion, phospho-moesin is present at the apical membrane (arrow) and on vesicular structures (arrowhead) in the cytoplasm. By 4 h p.e., phospho-moesin is essentially restricted to the apical membrane (arrows). Scale bars, 25 µm.

(Fig. 3C–G, I–M). These results suggest that at least the V_0 domain of V-ATPase becomes positioned to and enriched on the apical membrane just before eclosion of the flies, alongside the reconstruction of this membrane domain into the canalicular system.

Developmental analysis of cAMP-dependent V-ATPase assembly and activation

The neurohormone 5-HT induces, *via* the cAMP/PKA signalling pathway, a reversible assembly of functional V_1V_0 holoenzymes on the apical membrane of the secretory cells and thus boosts V-ATPase activity and H^+ transport in adult salivary glands (Dames et al., 2006; Rein et al., 2008; Baumann and Walz, 2012). To determine whether developmental differences exist in the ability of secretory cells to assemble V_1V_0 holoenzymes in a cAMP-dependent mode, we first determined the developmental expression pattern of the catalytic (PKA-C) and regulatory (PKA-RII) subunit of PKA. Fig. 4 demonstrates that PKA-RII and the major PKA-C isoform expressed in the salivary glands, namely PKA-C1 corresponding to the intense 41 kDa band on the anti-PKA-C blot (Voss et al., 2009), were expressed at a similar level in newly eclosed salivary glands and at latter developmental stages.

To determine whether an increase in intracellular cAMP level was able to elicit an assembly of V_1V_0 holoenzymes at various developmental stages, salivary glands were incubated with the cell-permeant cAMP analogue 8-CPT-cAMP and then probed for the subcellular distribution of subunits B and D, used as tracers for the

V_1 domain (Fig. 5). In non-stimulated controls at all developmental stages, subunits B and D were distributed throughout the cytoplasm, but not in the nucleus. The canalicular system of the apical membrane was labelled at a similar intensity as the cytoplasm (supplementary material Fig. S1). V_0 component subunit d and V_1 components subunits B and D thus have a different distribution pattern under these conditions, suggesting that most V-ATPase molecules are disassembled into the inactive V_1 and V_0 complexes (Dames et al., 2006). Upon exposure to 8-CPT-cAMP, both V_1 subunits became enriched on the apical membrane in the salivary glands of freshly eclosed flies and in older imagines. This cAMP-induced translocation of V_1 components to the V_0 -holding apical membrane is indicative of an assembly of functional V_1V_0 holoenzymes (Dames et al., 2006; Baumann and Walz, 2012). However, 8-CPT-cAMP-induced translocation of V_1 complexes to the apical membrane seems to become more efficient with aging, as indicated by the increase in the ratio of the labelling intensities between the apical membrane and the cytoplasm for subunits B and D, respectively (supplementary material Fig. S1). These results suggest that salivary glands in freshly eclosed flies have the ability to assemble V_1V_0 holoenzymes in a cAMP-dependent mode, although this mechanism may not yet be as effective as in older flies.

To determine whether cAMP-dependent V-ATPase activity depended on the developmental stage, we measured the transepithelial potential (TEP) in salivary glands. As previously shown, the lumen of the salivary glands of imagines of several days

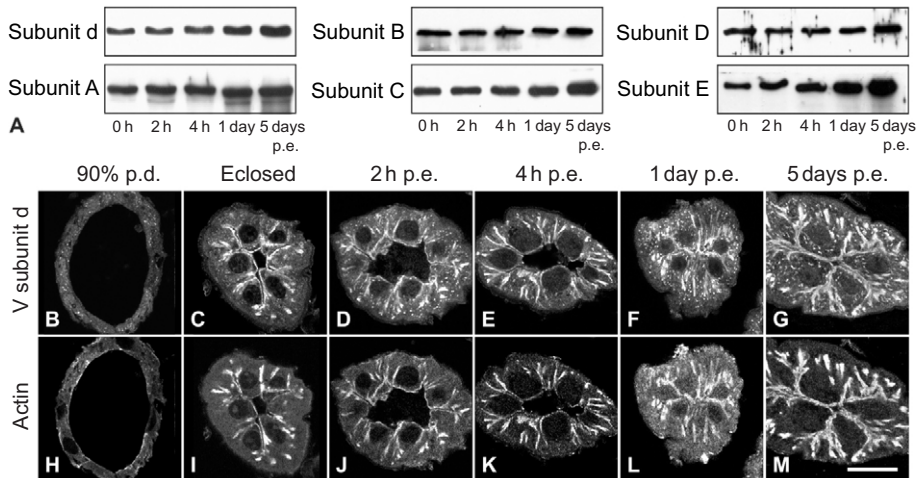


Fig. 3. Developmental analysis of the expression and distribution of V-ATPase components in blowfly salivary glands. (A) The relative amount of V-ATPase in salivary glands. Identical amounts of protein for each developmental stage (p.e.) were separated by SDS-PAGE and probed with antibodies against V_0 component subunit d and V_1 components subunits A, B, C, D and E. (B–M) Immunofluorescence localization of V-ATPase subunit d and actin. Subunit d is detected in vesicular structures at all developmental stages but is present on the apical plasma membrane only after eclosion. Scale bar, 25 μ m.

of age becomes positive by several tens of millivolts when cAMP or cAMP analogues are applied (Berridge and Prince, 1972; Prince and Berridge, 1972; Rein et al., 2008). This electrophysiological response is reversible and reflects a hyperpolarization of the apical membrane by the enhanced activity of the electrogenic V-ATPase (Prince and Berridge, 1972; Baumann and Walz, 2012). Fig. 6A,F shows that the application of $50 \mu\text{mol l}^{-1}$ 8-CPT-cAMP for 5 min induced an increase in luminal positivity by $\sim 18 \text{ mV}$ in salivary glands of newly eclosed flies. The TEP rose slowly, reaching the maximal amplitude about 10 min after onset of 8-CPT-cAMP treatment. As the flies aged, 8-CPT-cAMP produced a TEP response with a faster rising phase and an increasing amplitude, reaching a maximal change of $\sim 45 \text{ mV}$ in 4- to 5-day-old flies. Moreover, in many preparations aged for 4 h and longer, the TEP showed regular or irregular oscillations of variable amplitudes during application of 8-CPT-cAMP, as described previously for cAMP (Berridge and Prince, 1972; Prince and Berridge, 1972). As these TEP oscillations were abolished in Cl^- -free saline (data not presented), we suppose that they result from transient activation of transepithelial Cl^- transport.

Development of 5-HT-induced V-ATPase assembly and activation

Since 8-CPT-cAMP can induce an assembly and activation of V-ATPase (above results) but because 5-HT does not elicit salivation in the salivary glands of newly eclosed flies (Berridge et al., 1976), we have also examined whether developmental differences are found in the ability of 5-HT to induce the assembly of V_1V_0 holoenzymes and the activation of cation transport. In salivary glands of newly emerged flies, 30 nmol l^{-1} 5-HT (5 min) did not induce an obvious

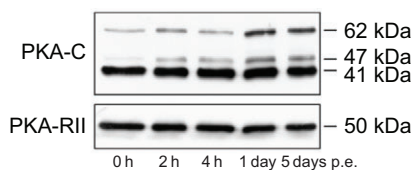


Fig. 4. Comparative analysis of the relative amount of the catalytic (PKA-C) and regulatory subunit (PKA-RII) of protein kinase A in salivary glands at various times after eclosion (p.e.). The intense 41 kDa band represents PKA-C1, the major PKA-C isoform in the salivary glands, whereas the faint bands at 47 and 62 kDa might represent PKA-C isoforms PKA-C2 and PKA-C3 (Voss et al., 2009).

change in the subcellular distribution of V_1 subunits B and D (Fig. 5C,F, supplementary material Fig. S1). Both subunits remained distributed throughout the cytoplasm, similar to the situation in unstimulated controls (Fig. 5A,D). In salivary glands of flies aged for 2 h, 5-HT led to an enrichment of V_1 subunits B and D on the apical membrane to a variable extent, with a large fraction of these proteins retaining a cytoplasmic localization (Fig. 5I,L, supplementary material Fig. S1). At later developmental stages, 5-HT-induced translocation of V_1 subunits was highly effective, with only weak residual labelling in the cytoplasm (Fig. 5O,R,U,X).

5-HT was also ineffective in producing an electrical response in TEP measurements from salivary glands of newly eclosed flies (Fig. 7A,F). A TEP response to 5-HT was observed in 2-h-old salivary glands, although amplitudes and shapes were variable between the preparations. In some cases, such as that in Fig. 7G, 5-HT induced a transient monophasic positive-going electrical response. In other cases, either positive-going or negative-going changes occurred in the TEP, with slow kinetics and amplitudes of only a few millivolts during exposure to 5-HT; upon 5-HT washout, a fast and transient positive-going TEP change was apparent (Fig. 7B). Similar TEP responses to 5-HT were observed in 4-h-old salivary glands, although the amplitudes were generally larger than in 2-h-old glands (Fig. 7C,F). In salivary glands of flies aged for at least 1 day, 5-HT produced the typical biphasic TEP response, with a negative phase during 5-HT exposure and a positive phase after 5-HT washout (Fig. 7D–F). The negative phase reflects Ca^{2+} -induced Cl^- transport into the gland lumen, whereas the positive phase is attributable to cAMP-regulated transepithelial cation transport into the lumen; this persists for some time after stimulus offset (Berridge and Prince, 1972).

Salivary glands in newly eclosed flies are delicate and the 5-HT insensitivity of the glands at this developmental stage might thus have been attributable to the adverse effects of dissection. However, when the glands were isolated and repetitively stimulated with 5-HT pulses during aging within the perfusion chamber, a 5-HT-induced electrical response appeared by about 1–2 h (Fig. 7A–C). Moreover, 8-CPT-cAMP produced an electrical response in 5-HT-insensitive glands of newly emerged flies (Fig. 7G). These results demonstrate that the 5-HT insensitivity of salivary glands of newly emerged flies is not caused by cell damage.

DISCUSSION

The secretory cells of the blowfly salivary gland are specialized for the transepithelial transport of ions and water at a high rate

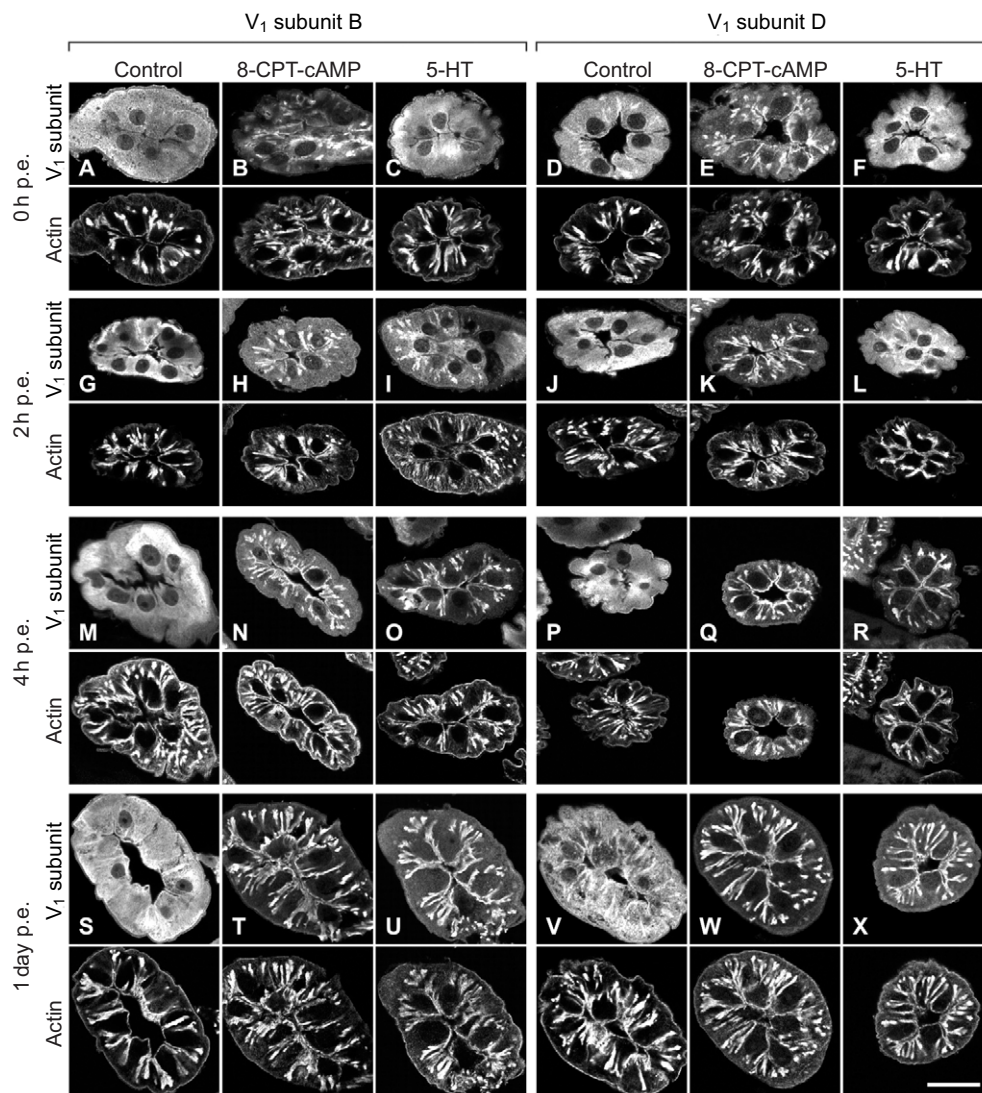


Fig. 5. 8-CPT-cAMP but not 5-HT provokes a translocation of V-ATPase V_1 components to the apical membrane in newly emerged flies. Salivary glands of staged flies were incubated with $50 \mu\text{mol l}^{-1}$ 8-CPT-cAMP (10 min) or 30nmol l^{-1} 5-HT (5 min) in physiological saline, fixed and co-labelled with antibodies against V-ATPase subunits B or D, respectively, and fluorophore-tagged phalloidin (actin). Note that 8-CPT-cAMP induces a translocation of V_1 subunits to the apical membrane after eclosion, whereas 5-HT leads to a translocation only after aging for at least 2 h. See supplementary material Fig. S1 for a quantitative analysis. Scale bar, $25 \mu\text{m}$.

upon demand. This study has examined the morphological and physiological differentiation of the secretory cells. The major findings are that: (1) the apical membrane becomes organized into an elaborate system of canaliculi just before eclosion of the imagines; (2) the reorganization of the apical membrane is accompanied by an enrichment of actin filaments and phosphorylated ERM protein (phospho-moesin) at this membrane domain and the deployment of V_0 , the membrane-integral part of the V-ATPase; (3) cAMP-dependent assembly and activation of V-ATPase can be induced even in the salivary glands of freshly eclosed flies; and (4) 5-HT induces the assembly and activation of V-ATPase only at a later developmental stage, i.e. by 2 h after eclosion.

Morphogenesis of the secretory cells

In late pupal development, the prospective secretory cells in the abdominal portion of the salivary glands are cuboidal and have a flat plasma membrane on both their apical and basal sides (Berridge et al., 1976). Conversely, in fully differentiated secretory cells of adult flies, the apical membrane forms a canalicular system that is further enlarged by being folded into tightly packed microplicae, resulting in an enlargement of the surface area by a factor of approximately 250 (Oschman and Berridge, 1970). This remarkable

reorganization and elaboration of the apical membrane has not yet been examined in detail. By use of phalloidin as a probe for membrane-associated actin, we have been able to image (indirectly) the three-dimensional layout of the apical membrane during this morphogenetic event. Our results suggest that this developmental process can be subdivided into three steps. (1) In late pupal development, the salivary glands shrink in diameter, providing a surplus of apical membrane for folding into canaliculi. (2) During the first day after eclosion, the canaliculi grow in length, while the secretory cells become taller and the gland diameter increases again. This expansion of the membrane is probably fostered by the *de novo* synthesis of membrane components. Indeed, images obtained by transmission electron microscopy show numerous vesicular structures close to the apical membrane in secretory cells of eclosed flies (Berridge et al., 1976). (3) The final length of the canalicular system is reached by about 24 h after adult emergence. During the following days, substantial increases continue to occur in the amount of integral membrane proteins (V_0 subunit d) and membrane-associated proteins (actin, phospho-moesin), as demonstrated by western blotting. Because these and probably other proteins become localized to the apical domain of the plasma membrane, further growth presumably takes place in the apical surface. This additional membrane probably leads to an increase in microplicae length, as

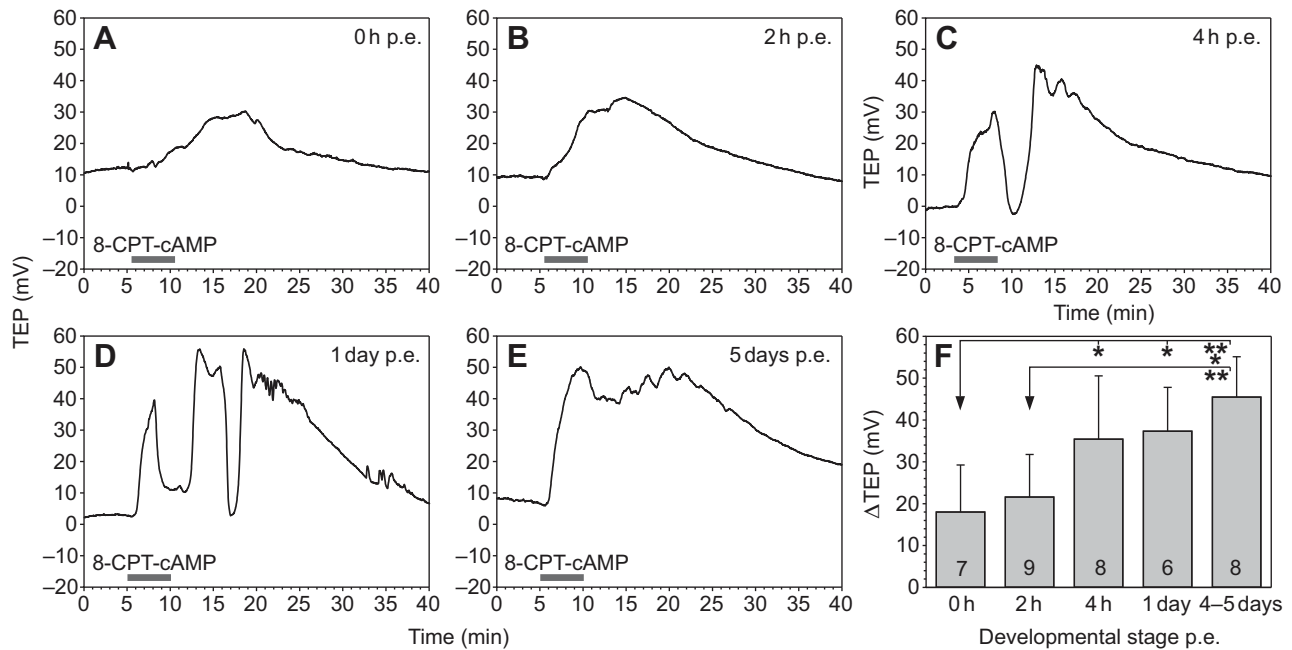


Fig. 6. 8-CPT-cAMP-induced changes in the transepithelial potential (TEP) of salivary glands. Salivary glands were dissected from flies that were aged for the time indicated and were transiently superfused with physiological saline containing $50 \mu\text{mol l}^{-1}$ 8-CPT-cAMP (grey bars). (A–E) Upon exposure to 8-CPT-cAMP, the gland lumen becomes reversibly positive at all developmental stages. (F) Maximal amplitude of the 8-CPT-cAMP-induced TEP changes. Data are means \pm s.d.; numbers within bars represent the number (N) of experiments. Statistical analysis by the Holm–Sidak method (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

suggested by the increase in cross-sectional area of the canaliculi during this developmental phase.

The reorganization and growth of the apical membrane domain in the secretory cells is accompanied by an increase in the amount of phospho-moesin and the association of this protein with the apical membrane. This finding implies that activated moesin is involved with the development of this membrane domain, as in other insect epithelial cells with prominent F-actin-containing membrane projections on their apical surface. In *Drosophila* photoreceptors, for instance, mutations in the ERM-protein-encoding gene *Moesin* impair the morphogenesis of the rhabdomere, an array of densely packed microvilli on the apical membrane (Karagiosis and Ready, 2004). This function of ERM proteins in the organization of membrane domains and surface projections depends on the ability of the activated ERM proteins to crosslink actin filaments with lipids and integral membrane proteins (Bretscher et al., 2002; Fehon et al., 2010). In vertebrate systems, several interaction partners of ERM proteins have been identified, including adhesion proteins, signalling proteins and scaffolding proteins (Bretscher et al., 2002). In the last-mentioned case, the PDZ protein NHE-RF (Na^+/H^+ exchanger regulatory factor) seems to be particularly interesting, since it has the ability to bind not only to ERM proteins, but also to ion-transporting proteins of which homologues are present in the apical membrane of the secretory cells, i.e. cation/proton transporters and V-ATPase (Breton et al., 2000; Pushkin et al., 2003; Shenolikar et al., 2004). According to the mRNA expression databank FlyAtlas (Chintapalli et al., 2007), Sip1, the *Drosophila* homologue of NHE-RF, is highly expressed in the *Drosophila* adult salivary gland. Recent experiments have demonstrated that Sip1 can form a complex with moesin and is required for moesin phosphorylation (Hughes et al., 2010). Whether Sip1 interacts with V-ATPase or the cation/proton antiporter at the apical membrane of salivary

glands and whether it anchors these proteins to actin filaments within the microplacae remains to be determined.

Newly synthesized ERM proteins must become activated in order to associate with the apical membrane and filamentous actin. In the inactive form, ERM proteins are folded and their N-terminal and C-terminal domains interact with each other, thus masking protein binding sites and the F-actin binding site in the head and tail domains, respectively (Bretscher et al., 2002; Fehon et al., 2010). Conversion to the active conformation occurs in a sequential mode by binding of the head domain to phosphatidylinositol 4,5-bisphosphate, unfolding and phosphorylation of a conserved threonine in the tail domain (Fievet et al., 2004; Ben-Aissa et al., 2012). In *Drosophila*, the latter step is mediated by the sterile 20 family kinase Slik (Hipfner et al., 2004). It has been proposed that activated *Drosophila* moesin influences the organization of the cortical cytoskeleton by acting antagonistically to the Rho signalling pathway (Speck et al., 2003; Xu et al., 2011). Notably, Slik and Rho signalling components seem to be expressed at a high level in the adult *Drosophila* salivary gland (Chintapalli et al., 2007).

ERM proteins are not only involved in the structural organization of the apical domain of epithelial cells by linking membrane components to filamentous actin, but also contribute to the coordination of signalling events by binding, either directly or via scaffolding proteins, to molecular components of various signal transduction pathways (Fehon et al., 2010; Neisch and Fehon, 2011). In this respect, the ability of ERM proteins to associate with the cAMP effector protein PKA could be of particular relevance for the physiology of the salivary gland (Sun et al., 2000; Semenova et al., 2009; Hochbaum et al., 2011). Phospho-moesin at the apical membrane of the secretory cells may thus immobilize a fraction of PKA molecules on this membrane domain. Compartmentalization of PKA together with its target molecule V-ATPase (Voss et al., 2007; Rein et al., 2008; Baumann and Walz, 2012) within the narrow

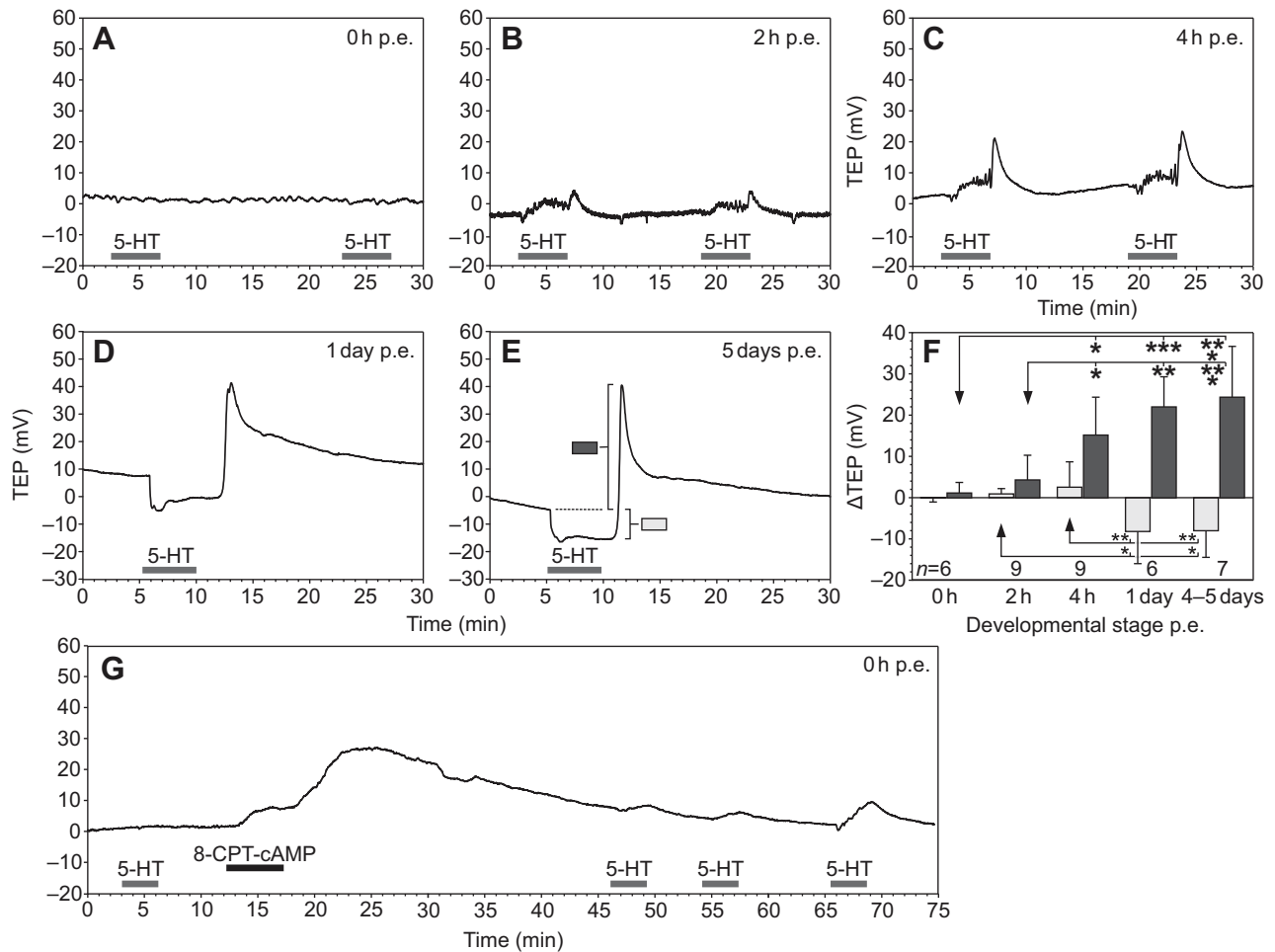


Fig. 7. 5-HT-induced changes in the TEP of salivary glands at various developmental stages. Salivary glands were dissected and exposed to 30 nmol l^{-1} 5-HT (grey bars). (A) Directly after eclosion, 5-HT does not induce an electrical response in the salivary glands. (B,C) At 2–4 h after emergence (p.e.), the salivary glands become sensitive to 5-HT, although TEP changes have a relatively small amplitude. Note that A–C were recorded from the same salivary gland, suggesting that insensitivity of the gland at 0 h p.e. was not attributable to cell damage. (D,E) In salivary glands of flies aged for at least 1 day p.e., 5-HT induces a biphasic TEP response, first negative and, after washout of 5-HT, transiently positive. (F) Quantitative analysis of the 5-HT-induced TEP changes. Bars in light grey represent the TEP change at the end of the 5-HT stimulus; bars in dark grey represent the maximal amplitude of the positive TEP phase after exposure to 5-HT (see E). Data are means \pm s.d.; numbers below the bars represent the number N of experiments. Statistical analysis by the Holm–Sidak method (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (G) TEP measurement from a salivary gland of a newly emerged fly, demonstrating insensitivity to 5-HT but sensitivity to 8-CPT-cAMP. Sensitivity to 5-HT arises after aging for ~ 1 h.

space of the microplacae may enhance the speed and efficiency of V-ATPase regulation.

Functional differentiation of the secretory cells

Berridge et al. (Berridge et al., 1976) have suggested that salivary glands at the time of eclosion are not yet functional as they are unable to secrete fluid when exposed to 5-HT. Our TEP measurements corroborate this conclusion and demonstrate that 5-HT at a concentration that is saturating at later developmental stages does not induce an electrical response of the secretory cells. Since a response can be elicited in the same salivary glands by different means (8-CPT-cAMP) or after aging for several hours, the unresponsiveness to 5-HT is not attributable to any impairment of the delicate organs during their preparation, but probably reflects the situation *in situ*. Similarly, it has been reported that mosquito Malpighian tubules are insensitive to external stimuli for some period after adult emergence (Schepel et al., 2010).

Transepithelial ion transport of the salivary glands is energized by the activity of V-ATPase in the apical membrane. Thus an as

yet non-functional V-ATPase might provide an explanation for the incapacity to produce saliva at this developmental stage. This possibility, however, can be excluded for several reasons. First, we have not noticed a dramatic difference in the expression level of various V-ATPase subunits in the salivary glands between eclosion and 4 h p.e., the stage at which 5-HT-induced secretion reaches maximal rate. Notably, this also applies to subunit C, which seems to play a key role in regulating V-ATPase assembly and activity in the salivary glands (Voss et al., 2007; Baumann and Walz, 2012). Although we do not have antibodies against all V-ATPase subunits, we consider that any subunits not detected in our assay have a similar temporal expression pattern. Indeed, the developmental expression of V-ATPase subunits is coordinated in the moth *Manduca sexta* (Reineke et al., 2002). Second, and importantly, our results suggest that V-ATPase is operative even in the salivary glands of eclosed flies, because superfusion with 8-CPT-cAMP induces V_1 translocation to the apical membrane, indicative of the assembly of V-ATPase holoenzymes. This interpretation is supported by the results of electrical measurements, demonstrating a 8-CPT-cAMP-

induced positive-going TEP change, as is to be expected by the activation of cation transport into the gland lumen. Based on this latter finding, we can further exclude that the inability of the salivary glands at eclosion to produce saliva is attributable to metabolic reasons, i.e. a low ATP level.

Although cAMP-mediated V-ATPase assembly can be elicited in the salivary glands of eclosed flies, this process seems to be not as effective as that in fully differentiated secretory cells. Similarly, the 8-CPT-cAMP-induced TEP change has a slower rising phase and a lower amplitude at 0 h p.e. than at 4 h p.e. These kinetic differences might be attributable to morphological constraints on V-ATPase translocation and assembly provided by the numerous vesicular structures in the cytoplasm at eclosion. Alternatively or additionally, the translocation of V₁ domains to the apical membrane might occur by diffusion at the time of emergence, but might be promoted by an active transport mechanism involving cytoskeletal elements on the full differentiation of the secretory cells. Evidence for an interaction of V₁ subunits with actin filaments has been presented (Holliday et al., 2000; Vitavska et al., 2003; Vitavska et al., 2005). Moreover, interaction of the V₁ domain with actin filaments may not be static but modulated by intracellular signalling events (Chen et al., 2004). Whether V₁ components are actively transported towards the V₀-holding apical membrane for holoenzyme assembly is still enigmatic.

Why can 5-HT not elicit the activation of V-ATPase in the salivary glands at the time of eclosion? As PKA is expressed and as PKA activation by 8-CPT-cAMP induces V-ATPase assembly and activation, 5-HT signalling to V-ATPase must be inoperative at this developmental stage at a step upstream of PKA. In this respect, the 5-HT receptor, G-protein and adenylyl cyclase(s) are possible candidates. Whether these signalling elements are expressed and functional at the time of eclosion remains to be determined by future experiments.

The 5-HT-induced TEP responses consist of two components: a negative phase attributable to Ca²⁺-dependent Cl⁻ fluxes across the epithelium, and a positive phase attributable to cAMP-dependent activation of cation transport. Thus TEP responses provide information not only on the functionality of the 5-HT₇ receptor/cAMP/PKA signalling cascade, but also on the 5-HT_{2α} receptor/phospholipase C/Ca²⁺ signalling pathway at the various developmental stages. The complex and variable shape and time course of the TEP responses at 2–4 h p.e. suggest that the relative contribution of both components during 5-HT exposure differs between specimens and changes by aging. Notably, 5-HT elicits a positive-going TEP change without a prior negative-going response in some cases. Development of the functionality of the Ca²⁺ signalling pathway and of the cAMP signalling pathway may thus not be tightly correlated.

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
8-CPT-cAMP	8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphate
cAMP	cyclic adenosine 3',5'-monophosphate
ERM	ezrin/radixin/moesin
InsP ₃	inositol 1,4,5-trisphosphate
NHE-RF	Na ⁺ /H ⁺ exchanger regulatory factor
p.d.	pupal development
p.e.	post-eclosion
pERM	phosphorylated ezrin/radixin/moesin
PKA	protein kinase A
PKA-C	catalytic subunit of protein kinase A
PKA-RII	regulatory subunit II of protein kinase A
TEP	transepithelial potential
V-ATPase	vacuolar-type H ⁺ -ATPase

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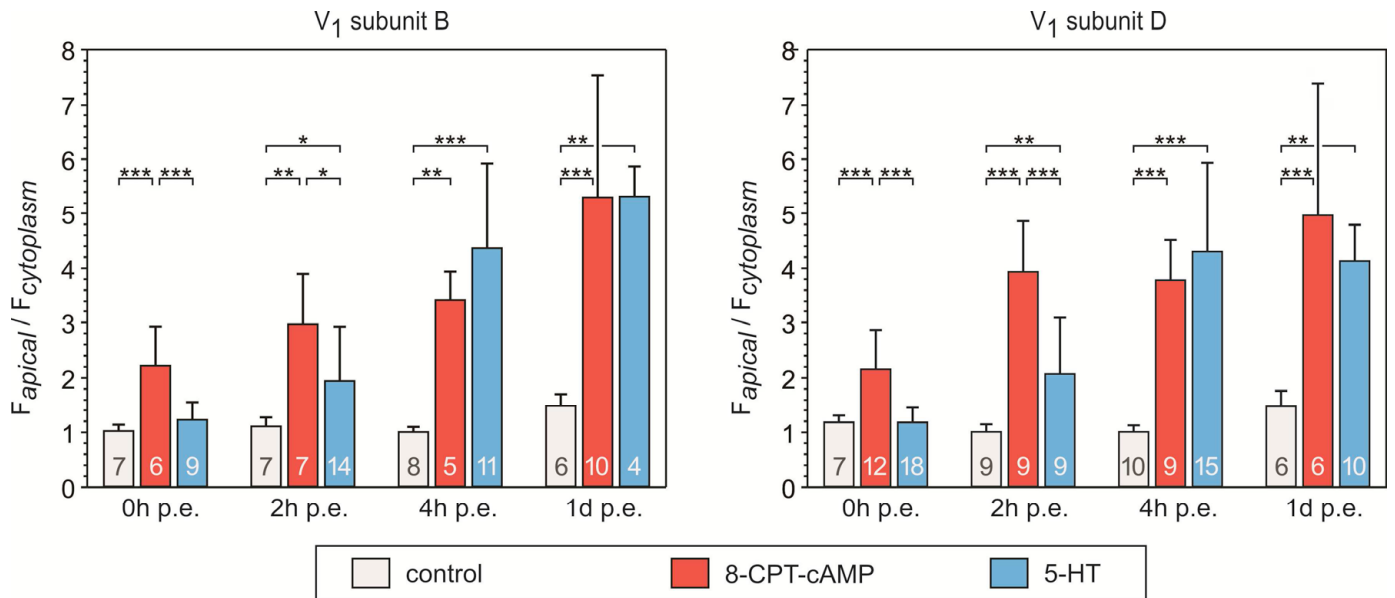


Fig. S1. Quantitative analysis of the distribution of immunofluorescence labelling for V1 subunits B and D. Data represent the ratio values between the fluorescence intensity of the apical membrane (F_{apical}) and the fluorescence intensity of the cytoplasm ($F_{\text{cytoplasm}}$). In the non-stimulated control (white) at all developmental stages (p.e., post-eclosion), immunofluorescence labelling for V1 subunits B and D was quite evenly distributed between the apical membrane and the cytoplasm, as indicated by a ratio of ~ 1 . 8-CPT-cAMP induced an enrichment of V1 subunits to the apical membrane at all developmental stages, but translocation to the apical membrane became more efficient by increasing age. 5-HT induced an enrichment of V1 subunits on the apical membrane only in salivary glands aged for at least 2 h after eclosion. Data are means \pm s.d.; numbers below the bars represent the number (N) of sections analysed. Statistical analysis by the Holm–Sidak method (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).