Protein kinase A dependent and independent activation of the V-ATPase in Malpighian tubules of *Aedes aegypti*.

Felix Tiburcy[1], Klaus W. Beyenbach[2] and Helmut Wieczorek[1]*

[1]Department of Biology/Chemistry, Division of Animal Physiology, University of Osnabrück, 49069 Osnabrück, Germany and [2]Department of Biomedical Sciences, VRT 8004, Cornell University, Ithaca, NY 14853, USA

*Author for correspondence (e-mail: Wieczorek@biologie.uni-osnabrueck.de)

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SUMMARY

Transepithelial ion transport in insect Malpighian tubules is energized by an apical V-ATPase. In hematophagous insects, a blood meal during which the animal ingests huge amounts of salt and water stimulates transepithelial transport processes linked to V-ATPase activation, but how this is accomplished is still unclear. Here we report that membrane-permeant derivatives of cAMP increase the bafilomycin-sensitive ATPase activity in Malpighian tubules of *Aedes aegypti* twofold and activate ATP-dependent transport processes. In parallel, membrane-association of the V_1 subunits C and D increases, consistent with the assembly of the holoenzyme. The protein kinase A inhibitor H-89 abolishes all cAMP-induced effects, consistent with PKA being involved in V-ATPase activation. Metabolic inhibition induced by KCN, azide and 2,4-dinitrophenol, respectively, also induces assembly of functional V-ATPases at the membrane without protein kinase A involvement, indicating a phosphorylation independent activation mechanism.

INTRODUCTION

The five Malpighian tubules of the yellow fever mosquito *Aedes aegypti* are the main organs for the excretion of solutes and water, thereby regulating and maintaining extracellular fluid homeostasis (Beyenbach and Piermarini, 2011). The homeostasis is particularly challenged by a blood meal when the mosquito ingests a volume of about twice her own body mass. Excess of salts and water that is taken up has to be removed from the animal rapidly in order to reduce the flight payload. Accordingly, Malpighian tubules commence a diuresis while the mosquito is still feeding on blood (Beyenbach, 2003).

Since Malpighian tubules are not innervated, the blood meal induced diuresis is under hormonal control. In *A. aegypti*, several diuretic peptides have been found that are released to the haemolymph by the central nervous system (Jagge and Pietrantonio, 2008; Beyenbach et al., 2010). Shortly after the onset of a blood meal the natriuretic peptide appears in the haemolymph (Petzel et al., 1985; Wheelock et al., 1988), followed by further peptides that have a nonselective diuretic effect (Coast, 2009). The natriuretic peptide, previously referred to as mosquito natriuretic peptide (MNP) (Petzel et al., 1985) and now known to be the calcitonin-like peptide Anoga-DH_{31}, increases the secretion of NaCl-rich fluid in isolated tubules of *A. aegypti* severalfold (Petzel et al., 1985; Coast et al., 2005). MNP acts *via* the second messenger cyclic AMP, and its diuretic effects can be duplicated by membrane-permeant cAMP analogues (Petzel et al., 1987; Coast et al., 2005; Beyenbach et al., 2009). In blood-fed mosquitoes an elevated level of MNP was found in the haemolymph, along with an increased cAMP concentration in the Malpighian tubules (Petzel et al., 1987; Wheelock et al., 1988). In contrast to the selective activation of transepithelial Na^+ secretion by MNP, kinin diuretic peptides selectively activate the transepithelial secretion of Cl^-, thereby increasing the transepithelial secretion of NaCl, KCl and water (Pannabecker et al., 1993; Schepel et al., 2010). This process was demonstrated to depend on the second messenger Ca^{2+} which induces the rapid opening of a shunt pathway located outside of the principal cells (Yu and Beyenbach, 2002).

Active ion transport in the Malpighian tubules of *A. aegypti* is accomplished mainly by the principal cells, whose apical brush border membrane is densely occupied by V-ATPases that energize the apical and basolateral membrane as well as the paracellular pathway, thereby driving the transepithelial secretion of KCl, NaCl and probably other solutes (Beyenbach, 2001). Due to its predominant role in fluid secretion the V-ATPase is a likely target for regulators of diuresis. Of the various mechanisms for V-ATPase regulation identified so far, the best understood is the reversible dissociation of the V_1 complex from the membrane

bound V_O complex, which was first demonstrated in the midgut of *Manduca sexta* and in *Saccharomyces cerevisiae* (Kane, 1995; Sumner et al., 1995).

In yeast, the cAMP/PKA pathway is most likely involved in the reversible dissociation of V-ATPases, but whether an active PKA enhances the (re)assembly or prevents the dissociation of the V-ATPase is still unknown (Wieczorek et al., 2009). In salivary glands of *Calliphora vicina*, the reversible assembly and activation of the V-ATPase was also shown to depend on cAMP and the activation of protein kinase A in response to the hormone serotonin (Dames et al., 2006; Rein et al., 2008). The V_1 subunit C is one likely target of the PKA in salivary glands since it was shown to be phosphorylated in a cAMP dependent manner (Voss et al., 2007).

PKA is also involved in the reversible insertion of fully assembled V-ATPases into the apical membrane of renal intercalated cells and epididymal clear cells of rats (Alzamora et al., 2010; Gong et al., 2010). In clear cells an alkaline pH in the epididymal lumen is sensed by a bicarbonate-sensitive adenylate cyclase, which increases the cellular level of cAMP. This increase activates PKA, finally leading to the fusion of V-ATPase containing vesicles with the apical membrane (Pastor-Soler et al., 2003).

Little is known about the activity and regulation of the V-ATPase in Malpighian tubules. It is well known that cAMP stimulates the secretion of fluid in a variety of Malpighian tubules, which has been linked to increased V-ATPase activity (Williams and Beyenbach, 1983; Coast et al., 2001). However, the molecular mechanisms for activating the V-ATPase in renal epithelia of insects are largely unknown. In *Drosophila melanogaster*, subtle mobilization of the V_1 subunit H to the apical membrane was observed in tubules stimulated with the diuretic Capa-1, which the authors consider a minor regulatory mechanism of the V-ATPase controlled by the availability of ATP (Terhzaz et al., 2006).

In the present study we try to unravel the involvement of the V-ATPase in the hormone-induced diuresis of *A. aegypti* and the mechanisms that lead to V-ATPase activation.

MATERIALS AND METHODS

Chemicals

All chemicals were used in the highest commercially available purity. Bafilomycin A_1 was the kind gift of Stephanie Grond (Institute of Organic Chemistry, University of Tübingen, Germany).

Mosquitoes and Malpighian tubules

Aedes aegypti was reared at 27°C, a relative humidity of 70% and in a 12 : 12 h (light : dark) cycle. Larvae were kept in autoclaved tap-water and fed finely ground TetraMin flakes. Adult

mosquitoes had access to autoclaved tap water and to 10% sucrose. Only female mosquitoes 3-9 days posteclosion without access to sucrose for at least 24 h were used in the experiments, in order to assure a common nutritional state. Mosquitoes were cold anesthetized and decapitated. Using fine forceps, the Malpighian tubules were removed at room temperature under Ringer solution (150 mM NaCl, 25 mM HEPES, 3.4 mM KCl, 1.8 mM $NaHCO_3$, 1.0 mM $MgSO_4$, 1.7 mM $CaCl_2$, and 5.0 mM glucose, adjusted to pH 7.1 with NaOH) by pulling on the rectum, while the abdomen was fixed. The Malpighian tubules were detached from hindgut and midgut and transferred into low-binding reaction tubes for further experiments.

ATPase activity assays

Malpighian tubules were dissected and transferred into low-binding reaction tubes. Excess Ringer solution was carefully removed and the tubules were frozen in liquid nitrogen. To compare the effect of test substances on ATPase activity in intact Malpighian tubules, only Malpighian tubules of the same animals were compared. Excess Ringer solution was exchanged for Ringer solution with or without the test substance and the tubules were then incubated at room temperature according to Table 1. Thereafter, Ringer solution was aspirated, leaving just enough to cover the Malpighian tubules, before the samples were frozen in liquid nitrogen and stored at -80°C until usage.

On the day of the ATPase assay, the frozen tubules were homogenized on ice with a micro pestle (Eppendorf AG, Hamburg, Germany) in 100 µl of lysis buffer (5 mM Na-HEPES, pH 7.1, 2 mM EGTA, 10 mM β-mercaptoethanol, Protease Inhibitor Cocktail Set I (Calbiochem, Merck KGaA, Darmstadt, Germany)) and then centrifuged (6 x 10^6 g x min, 4°C). To remove endogenous phosphate, the crude membrane pellet was washed once more with 100 µl lysis buffer and centrifuged again. The final crude membrane pellet was resuspended in lysis buffer without protease inhibitor to yield appropriate final concentrations. Comparative assays of V-ATPase activity were performed in duplicates, using an equivalent of 0.5 Malpighian tubules in 160 µl of a solution consisting of 50 mM Tris-MES, pH 6.9, 3.75 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 20 mM KCl, 0.5 mM NaN_3, 5 mM Tris-HCl, 2.5 mM β-mercaptoethanol, 1 mM di-Tris-ATP and 6.25% DMSO, with or without 0.3 µM bafilomycin A_1. Samples were preincubated for 8 min at 30°C, and the reaction was started by the addition of ATP. The reaction was stopped after 45-60 min by freezing the samples in liquid nitrogen. Linearity of ATPase activities under these conditions was confirmed.

In order to determine the ratio of Na^+/K^+-ATPase and V-ATPase activities, an equivalent of 0.625 Malpighian tubules were incubated in 160 µl of a solution consisting of 50 mM Tris-MES, pH 6.9, 3.75 mM $MgCl_2$, 20 mM KCl, 120 mM NaCl, 5 mM Tris-HCl, 2.5 mM β-

mercaptoethanol, 2 mM di-Tris-ATP and 6.25% DMSO. In order to inhibit F- and P-ATPase activities, 0.5 mM NaN_3 (Mitchell and Moyle, 1971) and 0.1 mM sodium orthovanadate (Macara, 1980) were applied. Specific inhibition of V-ATPase activity was achieved by 0.3 µM bafilomycin A_1 (Bowman et al., 1988), and of Na^+/K^+-ATPase activity by adding 1 mM ouabain (Robinson and Flashner, 1979), in both cases in the absence of azide and vanadate. Samples were preincubated for 8 min at 30°C, and the reaction was started by the addition of ATP. The reaction was stopped after 20 min by freezing the samples in liquid nitrogen.

As a measure of ATPase activity the produced inorganic phosphate was determined as described previously (Wieczorek et al., 1990). Activities were normalized to the average protein content of the crude membrane pellet of Malpighian tubules, which was found to be 0.45 ± 0.08 µg per tubule (± s.d.; n = 43). The protein content of tubule crude extract was determined to be 0.73 ± 0.09 µg per tubule (± s.d.; n = 35), which is comparable to values obtained elsewhere (Weng et al., 2003).

cAMP-determination

The concentration of cAMP in Malpighian tubules was determined as previously described (Beyenbach et al., 2009). Briefly, 20 Malpighian tubules were incubated in Ringer solution supplemented with 0.5 mM IBMX for 15 min at room temperature. Tubules were then incubated for 2 min with either 1 µM Anoga-DH_{31}, 1 µM aedeskinin-III or Ringer solution, and subsequently frozen in liquid nitrogen and stored at -80°C.

The extraction of cyclic nucleotide was accomplished by homogenization of the frozen tubules in 200 µl of ice-cold ethanol, supplemented with 0.5 mM IBMX, on ice. The tubules were further homogenized by sonication for 90 s and were then centrifuged at 3,000 x g at 4°C for 10 min. The pellet was used to determine the protein concentration, while the supernatant was evaporated in a concentrator (Concentrator plus, Eppendorf AG, Hamburg, Germany) at 60°C. The resulting residue was resuspended in 100 µl 0.1 M HCl, and the cyclic nucleotide concentration was determined with the "Direct cAMP Correlate-EIA Kit" (Assay Designs, Ann Arbor, MI, USA) according to the manufacturer's protocols.

Three electrode voltage clamp studies

Two-electrode voltage clamp (TEVC) experiments were performed as described previously (Masia et al., 2000; Schepel et al., 2010). In addition, we used a third microelectrode which impaled the tubule lumen for measurements of the transepithelial voltage (V_t). The headstage of the third microelectrode (HS-2-1L; Axon instruments, Molecular Devices, Sunnyvale, CA, USA) was coupled to the same ground electrode as the TEVC-electrodes. V_t was measured with an additional Geneclamp 500 amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). Digital data were acquired using a Digidata 1322A (Molecular

Devices, Sunnyvale, CA, USA) controlled *via* the Clampex module of the pCLAMP software package (version 9.2; Molecular Devices, Sunnyvale, CA, USA). In the typical experiment, one current and one voltage electrode impaled a single principal cell of the tubule for the measurement of the basolateral membrane voltage (V_{bl}) and the cell input resistance (R_{in}). The third electrode impaled the tubule lumen by passing through an adjacent stellate cell, allowing the measurement of V_t. The voltage across the apical membrane (V_a) of the impaled principal cell was calculated as the difference V_t-V_{bl}. After recording stable voltages, the test substances were added to the peritubular bath (Volume 0.5 ml) from stock solutions to yield the desired final concentrations (Table 1). To measure KCN-induced depolarization of voltages, the bath was flushed with 1 mM KCN in Ringer solution at a rate of 3 ml/min.

Immunocytochemistry

Malpighian tubules were fixed in Lawdowsky's fixative (ethanol/formalin/acetic acid/water = 50:10:4:40) for 90 min and subsequently washed three times in PBS for 10 min (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4). The tubules were then washed twice in 10% sucrose in PBS for 15 min, once in 20% sucrose PBS and finally stored at 4°C overnight in 30% sucrose in PBS. The Malpighian tubules were embedded in tissue freezing medium (Jung, Leica Microsystems, Wetzlar, Germany) and frozen in melting isopentane. A cryostat (Leica CM1900, Leica Microsystems, Wetzlar, Germany) was used to cut 8 µm sections at -20°C, which were collected on SuperFrost Plus microscope slides (Gerhard Menzel GmbH, Braunschweig, Germany) and fixed at 40°C on a hotplate. For immunofluorescence staining, specimens were washed three times in PBS, blocked for 1 h with 2% BSA in 0.1% Triton X-100 in PBS and incubated with the monospecific primary antibody 488-1 to subunit C (1:2000) (Merzendorfer et al., 2000). After removal of unbound antibody by three washes with PBS, specimens were incubated in the dark with Cy3-conjugated anti-guinea pig IgG (H+L) antibody (1:10000) (Jackson ImmunoResearch Laboratories, Suffolk, UK) for 1 h. Finally, the specimens were washed three times in PBS, mounted in Vectashield (Vector Laboratories, Inc, Burlingame, CA, USA) and examined using an Olympus IX70 fluorescence microscope (Excitation maximum 545 nm; emission maximum 605 nm).

Quantitative slot blots

After isolating 30 Malpighian tubules, 15 tubules were used as control, while 15 tubules were incubated with the test substances listed in Table 1 for the times shown. Thereafter, excess Ringer solution was removed and the tubules were frozen in liquid nitrogen. On the day of the assay, the frozen tubules were homogenized in 125 µl lysis buffer and centrifuged (6 x 10^6 g x min, 4°C). The supernatant (cytosolic fraction) was removed and stored, and the pellet was

resuspended in 125 µl lysis puffer (crude membrane pellet). All samples were then transferred equally onto four nitrocellulose membranes *via* the slot-blot technique and transfer was verified by Ponceau S staining. The four membranes were blocked with 5% milk in TBSN-Tween (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.02% NaN_3, 0.05% Tween-20) for 1 h at room temperature. Three of the four identical slot blots were now exposed for 1 h to different primary antibodies recognizing subunit a (818-2; 1:2000; blot 1) (Merzendorfer et al., 2000), subunit C (mc-2; 1:3000; blot 2) (Vitavska et al., 2005) or subunit D (1026-5; 1:500; blot 3) (M. Huss and H.W., unpublished) in 2.5% milk in TBSN-T, while the fourth blot was used as negative control. The blots were washed three times for 5 min with TBSN-T and then incubated for 1 h in the dark with an IRDye800-conjugated anti-guinea pig antibody (1:5000; Rockland Immunochemicals Inc., Gilbertsville, PA, USA) in 2.5% milk in TBSN-T. After final washing three times for 10 min with TBSN-T, the blots were analysed using the Odyssey infrared imaging system (Li-Cor, Lincoln, NE, USA), and the software provided by the supplier. Fluorescence intensities were quantified using the 1D gel analysis module of the ImageQuant TL software (GE Healthcare, Little Chalfont, UK) and were corrected for the values of the negative control.

Phospho-PKA substrate blot

Isolated Malpighian tubules were incubated with the agent of interest for 1 min at the concentrations given in Table 1, before being frozen in liquid nitrogen. The samples (15 Malpighian tubules each) were homogenized on ice in 15 µl 2 x Laemmli, supplemented with Protease Inhibitor Cocktail Set I and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Samples were heated to 98°C for 1 min, subjected to SDS-PAGE and subsequently transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with 5% milk in TBSN-Tween, washed three times and incubated with the Phospho-PKA Substrate (RRXS*/T*; * indicating a phosphorylation) Rabbit monoclonal antibody (1:1000; Cell Signaling Technologies, Danvers, MA, USA) in 5% BSA in TBSN-T. The blot was washed three times 5 min with TBSN-T and then incubated for 1 h with an alkaline phosphatase-conjugated anti-rabbit IgG (whole molecule) antibody (1:10000; Sigma-Aldrich, St. Louis, MO, USA). After final washing, the immunoreactive proteins were visualized with NBT/BCIP.

Statistical evaluation of data

If not stated otherwise, data are summarized as means ± standard deviations. Statistical significance was calculated be applying the two-tailed Student *t*-test. To check normality of distribution the Kolmogorov-Smirnov test was used.

Other methods

Protein concentrations were determined *via* the amido black method (Wieczorek et al., 1990). SDS-PAGE and Western blotting was performed as described previously (Schweikl et al., 1989; Wieczorek et al., 1991).

RESULTS

V-ATPase activities in Malpighian tubules

We determined the azide-, vanadate- and bafilomycin-sensitive ATPase activity, representing the sum of activities produced by F-, P-, and V-ATPases, in the crude membrane pellet of Malpighian tubules to be approximately 0.3 µmol P_i min^{-1} mg^{-1}. Roughly 60% of this activity originated from the bafilomycin A_1 sensitive V-ATPase, while about 28% could be attributed to the ouabain-sensitive Na^+/K^+-ATPase (Fig. 1A). V-ATPase activities of unstimulated Malpighian tubules differed greatly between tubules from different animals and were found to be in the range from 0.01 to 0.30 µmol P_i min^{-1} mg^{-1} (Fig. 1B). This correlates with the highly variable secretion rates of individual tubules under control conditions ranging from 0.19 - 2.6 nl/min (Williams and Beyenbach, 1983; Petzel et al., 1985; Petzel et al., 1999).

The V-ATPase is the driving force of ion transport in the Malpighian tubules and we therefore set out to investigate its role in the diuresis induced by a blood meal. Since blood meal-induced diuresis is known to involve elevation of the intracellular cAMP level (Petzel et al., 1987), we analysed the effect of membrane-permeant cAMP analogues on the V-ATPase activity. To exclude the variability in the data from different mosquitoes, tubules from only one mosquito were studied in any one test. Two tubules served as a control, two tubules served the experiment, and the fifth tubule was not used. When comparing two pairs of tubules from one animal under control conditions, the average V-ATPase activities in both samples did not differ (Fig. 1C). Incubation of tubules for 10 min with 0.1 mM of the cAMP analogues 6-MB-cAMP and cBIMPS significantly increased membrane-associated V-ATPase activity by 170 and 135, respectively, whereas 1 µM aedeskinin-III did not significantly affect V-ATPase activity (Fig. 1C).

The natriuretic peptide Anoga-DH_{31} increases cytoplasmic cAMP level

To test whether Anoga-DH_{31}, like MNP (Petzel et al., 1987), increases the intracellular cAMP concentration in *A. aegypti* Malpighian tubules, we first measured cAMP in 12 unstimulated tubules and found a value of 14 pmol per mg protein, corresponding to approximately 11 fmol per tubule (Fig. 2). Incubating Malpighian tubules with 1 µM Anoga-DH_{31} for 2 min significantly increased the intracellular cAMP concentration tenfold to 140 pmol per mg of protein. Since the cytoplasmic volume of one *A. aegypti* Malpighian tubule is on average

27 nl (Massaro et al., 2004), the basal cytosolic cAMP concentration was approximately 0.4 μM, a level which is in the normal physiological range of eukaryotic cells, and rose to almost 4 μM after stimulation with Anoga-DH_{31}. Incubation of the tubules in 1 μM aedeskinin-III for 2 min did not affect the cAMP level, which was not unexpected since kinins are known to utilize Ca^{2+} as second messenger.

cAMP induced activation of ATP-consumers correlates with stimulation of V-ATPase activity

We have developed a method for assessing transport-related ATP consumption in intact Malpighian tubules. Since the apical membrane voltage (V_a) derives primarily from the ATP-dependent transport activity of the V-ATPase, V_a is largely an active transport potential rather than a diffusion potential. As shown in Fig. 3A, V_a oscillated in the vicinity of 130 mV under control conditions when the cell input resistance was 228 kΩ. V_a did not change appreciably in the presence of cBIMPS, a membrane-permeable analogue of cAMP. However, like cAMP (Sawyer and Beyenbach, 1985), cBIMPS significantly depolarized the basolateral membrane voltage (V_{bl}) and significantly hyperpolarized the transepithelial voltage with the same magnitude and time course while R_{in} decreased to 182 kΩ.

In spite of the large changes in V_{bl} and V_t, as well as the known increase in transepithelial NaCl secretion (Williams and Beyenbach, 1983; Beyenbach, 2003) V_a remained constant in the presence of cBIMPS, consistent with mitochondrial ATP synthesis keeping up with ATP utilization by the V-ATPase. However, when mitochondrial ATP synthesis was inhibited by KCN (Fig. 3A), V_a, V_{bl} and V_t all depolarized towards zero in parallel because of the electrical coupling. Importantly, V_a depolarized in the presence of KCN which is known to reduce intracellular ATP concentrations in *A. aegypti* Malpighian tubules (Wu and Beyenbach, 2003). Accordingly, the rate of V_a depolarization reflected the run-down of the intracellular ATP pool when ATP was no longer produced in the presence of KCN. As shown in Fig. 3B, the run-down of the intracellular ATP pool was slowest in the presence of the V-ATPase inhibitor bafilomycin A_1, was fastest in the presence of the V-ATPase activator cBIMPS, and was intermediate in control, unstimulated Malpighian tubules. Inhibiting the V-ATPase with bafilomycin A_1 was shown to slowly depolarize all membrane potentials in a previous study (Beyenbach et al., 2000). Similarly, preincubation with bafilomycin A_1 slowly depolarized V_a to approximately 70 mV in the present study before KCN was added. The velocity of the run-down of the intracellular ATP pool in the presence of bafilomycin A_1 decreased with the time the tubules were exposed to the V-ATPase inhibitor (Fig. 3C). This confirms that the V-ATPase is one of the main ATP-consumers in Malpighian tubules of *A. aegypti*.

Fig. 3D shows that the slope of V_a depolarization (ATP run-down) in cBIMPS- and 6-MB-cAMP-stimulated tubules was almost tenfold higher compared to control tubules. In contrast, aedeskinin-III had no effect on the rate of V_a depolarization. To verify that Ca^{2+} induced diuresis does not activate ATP-dependent secretion, we also used thapsigargin which elevates intracellular Ca^{2+} concentration by inhibition of SERCA, the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase. Like aedeskinin-III, thapsigargin also did not stimulate ATP consumption.

Membrane-association of V_1 subunits increases in stimulated Malpighian tubules

The serotonin-mediated assembly of V_1 and V_O complexes was thoroughly documented in salivary glands of *C. vicina* (Dames et al., 2006). To explore whether this mechanism also applies for Malpighian tubules of *A. aegypti*, we first analysed the location of subunit C as a representative of the V_1 complex immunohistochemically in cryosections of Malpighian tubules. In nearly all of 327 examined tubules from around 120 mosquitoes antibodies derived against the *M. sexta* subunit C (488-1) almost exclusively labelled the apical membrane in Malpighian tubules stimulated with Anoga-DH_{31} or membrane-permeant cAMP analogues, while the labelling in 135 control tubules from 45 mosquitoes was rather ambiguous, ranging from dominant labelling of the apical membrane to strong labelling of the cytosol (Fig. 4A). This variability between different sets of tubules suggests differences in the amount of fully assembled V-ATPases under control conditions, which again resembles the variability observed for the V-ATPase activity in unstimulated tubules. However, there can be little doubt that subunit C is translocated to the apical membrane after natriuretic stimulation.

In order to quantify the assembly of cytosolic V_1 and membrane-embedded V_O complexes, we analysed, in a second step, the distribution of the V_O subunit a and of the V_1 subunits C and D in unstimulated and stimulated tubules by quantitative Western blots (Fig. 4B). In unstimulated tubules over 90% of the V_O subunit a was recovered in the crude membrane pellet. The location of subunit a did not change significantly after incubating tubules with the test substances (Fig. 4C-F). In contrast, cAMP analogues significantly affected the association of the V_1 subunits C and D with the membrane. Under control conditions, around 60% of subunit C and 73% of subunit D were recovered in the crude membrane pellet of tubules. Membrane-association of the V_1 subunits significantly increased in tubules stimulated with 6-MB-cAMP or cBIMPS by up to 18% (Fig. 4C-D). This suggests that at least part of the increased V-ATPase activity is due to V_1V_O assembly.

The incubation of *A. aegypti* Malpighian tubules with bafilomycin A_1 did not alter the distribution of V-ATPase subunits (Fig 4E). Accordingly, the inhibition of the V-ATPase did not lead to dissociation of the holoenzyme in *A. aegypti* Malpighian tubules. This result was

not unexpected, since in yeast V-ATPase inhibition is known to prevent dissociation (Parra and Kane, 1998). Aedeskinin-III did not trigger membrane localization of the V_1-complex (Fig. 4F). This observation is consistent with its failure to affect ATPase activity of the proton pump (Fig. 1C).

Metabolic inhibitors trigger membrane-association of the V_1-complex and increase V-ATPase activity

Under control conditions the V-ATPase was found to be predominantly assembled and active. In order to maximize the response to substances which cause an increase in V-ATPase activity, we set out to induce V-ATPase disassembly by reducing the ATP/ADP ratio, as had been demonstrated *in vitro* for the *M. sexta* V-ATPase (Huss and Wieczorek, 2007). Therefore, we incubated the Malpighian tubules with the metabolic inhibitor KCN which reduces intracellular ATP concentration rapidly in a reversible manner (Wu and Beyenbach, 2003).

Unexpectedly, in tubules incubated with 1 mM KCN for 10 min the membrane-association of V_1 subunits C and D significantly increased by 18% and 23%, respectively (Fig. 5A). In addition, V-ATPase activity in the crude membrane pellet of KCN-incubated tubules doubled (Fig. 5B). V-ATPase activity was found to be approximately twofold increased also in the crude membrane pellet of tubules incubated with other metabolic inhibitors (Fig. 5B). Incubation with 10 mM of the ATP synthase inhibitor azide for 15 min or with 0.5 mM of the uncoupler 2,4-dinitrophenol for 5 min both significantly increased V-ATPase activity by 91% and 169%, respectively. The degree of activation was comparable to that induced by cBIMPS and evidently involved the same pool of V-ATPases (Fig. 5C). These results suggest that activation of the V-ATPase during cAMP-induced diuresis shares a common signal with the metabolic inhibitor-induced assembly of functional V-ATPases at the membrane.

V-ATPase activation can occur *via* PKA dependent and PKA independent pathways

The V-ATPase is a well-known downstream target of protein kinases in a variety of systems (Voss et al., 2007; Hallows et al., 2009; Alzamora et al., 2010), and the involvement of cAMP in diuresis suggests that this is also the case in Malpighian tubules of *A. aegypti* (Williams and Beyenbach, 1983; Sawyer and Beyenbach, 1985).

We used an antibody against phosphorylated PKA substrates to examine protein kinase activation in response to diuretic stimulation. The Western blot in Fig. 6A confirms protein kinase activation after stimulation with the cAMP analogues 6-MB-cAMP and cBIMPS, but not by KCN or by aedeskinin-III. Since KCN induces the functional assembly of the V-ATPase at the membrane, this result indicates that the activation of V-ATPases can also occur in a protein kinase independent way.

To confirm the involvement of protein kinase A in the cAMP-dependent pathway that activates the V-ATPase and stimulates natriuresis, we incubated Malpighian tubules with the PKA-inhibitor H-89. Preincubation with 0.1 mM H-89 for 20 min - the concentration needed to prevent phosphorylations as monitored by Western blots (not shown) - abolished the cAMP-induced depolarization of V_{bl} and hyperpolarization of V_t. KCN-induced depolarization of V_a revealed that in those tubules 6-MB-cAMP failed to activate ATP-dependent transport (Fig. 6B). Consequently, preincubation with H-89 also significantly impeded the cAMP-induced V-ATPase assembly at the membrane (Fig. 6C) and the cAMP-induced stimulation of V-ATPase activity in the crude membrane pellet, while having no significant effect on basal V-ATPase activity (Fig. 6D). Due to the high concentration of inhibitor used, cytotoxic effects that impede tubular function had to be excluded. We therefore analysed whether preincubation with H-89 also prevents KCN-induced V-ATPase activation and found that H-89 does not interfere with this activation mechanism (Fig. 6D), supporting the notion that this type of V-ATPase activation in Malpighian tubules of *A. aegypti* does not rely on PKA-dependent phosphorylation.

DISCUSSION

Basal V-ATPase activity in unstimulated Malpighian tubules

The V-ATPase in unstimulated Malpighian tubules of female *Aedes aegypti* exhibits highly variable rates of enzyme activity, in line with highly variable rates of secretion, which are also found in other species (Williams and Beyenbach, 1983; Petzel et al., 1985; Dow et al., 1994; Petzel et al., 1999). Nevertheless, the V-ATPase appears to be the main energizer in Malpighian tubules since 60% of the total ATPase activity can be assigned to the V-ATPase. With 28% of the total ATPase activity the Na^+/K^+-ATPase also appears to play a major role in membrane energization, indicating that it may be more important for Malpighian tubules function than generally thought (Beyenbach, 2001; Patrick et al., 2006; Beyenbach and Piermarini, 2011). The Na^+/K^+-ATPase might be involved in cell housekeeping functions and in addition determine the Na^+/K^+ ratio of the fluid secreted by Malpighian tubules (Grieco and Lopes, 1997). Still, the main ATP consumer is the V-ATPase as confirmed by the slow KCN-induced V_a depolarization in the presence of bafilomycin (Fig. 3B and C). Our results bear resemblance to those of Terhzaz and colleagues (2006) who showed that incubation with bafilomycin almost doubles the ATP content in Malpighian tubules of *Drosophila melanogaster* (Terhzaz et al., 2006).

Besides their essential role after a blood meal, Malpighian tubules are involved in other vital tasks such as osmoregulation, the excretion of nitrogenous wastes, the elimination of xenobiotics, and immunity (Dow and Davies, 2006; O'Donnell, 2009; Beyenbach et al.,

2010). Some of these processes depend on active transport, which most likely will also rely on the energization by the V-ATPase, consistent with its high basal activity in unstimulated tubules that can be further increased upon diuretic requirement. Therefore, the Malpighian tubules can be seen as an intermediate model for studies of V-ATPase regulation. The two other well established insect models are the larval midgut of *Manduca sexta* and the salivary glands of the blowfly *Calliphora vicina*. In the *M. sexta* midgut, the "default" state is a high basal V-ATPase activity, and the disassembly of the holoenzyme occurs only under conditions that do not demand the activity of this proton pump as during moult, when feeding and digestion are suspended (Sumner et al., 1995). In *C. vicina* salivary glands, the V-ATPase is inactive most of the time. The proton pump is only assembled and activated when needed (Dames et al., 2006). In contrast to *M. sexta* and *C. vicina*, the assembly, ATP hydrolysis and transport activity of Malpighian tubules of *A. aegypti*, is highly variable even under control conditions, with a large functional reserve under conditions of diuretic stimulation.

Diuresis, the V-ATPase and protein kinase A

Fluid secretion in *A. aegypti* Malpighian tubules increases threefold after stimulation with mosquito natriuretic peptide via cAMP as second messenger (Beyenbach, 2003), leading to the activation of V-ATPase driven cation transport processes. By contrast, kinins double fluid secretion rates at best *via* the elevation of Ca^{2+} levels which results in an elevated Cl^- permeability of the tubules (Beyenbach and Piermarini, 2011). The present study clearly shows that the Ca^{2+}-mediated diuresis does not require the assembly and activation of the V-ATPase (Figs. 1 and 4).

Our observations closely resemble those in the salivary glands of the blowfly. Here, serotonin increases salivary fluid secretion up to 60-fold (Baumann and Walz, 2012). Importantly, serotonin activates two parallel signalling pathways *via* two different receptors in the same cell (Berridge and Heslop, 1981). Binding to one receptor, serotonin raises intracellular Ca^{2+} concentration which stimulates transepithelial Cl^- transport to the lumen by increasing the Cl^- permeability of both apical and basolateral membranes of the cell (Prince et al., 1973; Zimmermann and Walz, 1997). Binding to the other receptor, serotonin elevates intracellular cAMP thereby activating an electrogenic K^+ transport mechanism in the apical membrane of the secretory cells consisting of a V-ATPase and a K^+/nH^+ antiporter (Baumann and Walz, 2012). This active transport mechanism is not K^+ selective, but can also facilitate Na^+-transport (Berridge et al., 1976; Gupta et al., 1978; Berridge and Heslop, 1981).

In salivary glands of the blowfly the V-ATPase is one of the downstream targets of serotonin signalling. In unstimulated glands only 25-40% of the V_1 subunits are associated with the apical membrane (Dames et al., 2006), whereas in unstimulated Malpighian tubules

of *A. aegypti* on average 40-73% of the V_1 subunits were found to be membrane-associated (Figs. 4 and 6). Nevertheless, reassembly of V_1 and V_O complexes was also observed in Malpighian tubules of *A. aegypti* upon stimulation with cAMP analogues. Due to the already high abundance of membrane-associated V_1 complexes the relative increase in assembled holoenzyme was lower than in blowfly salivary glands.

V-ATPase assembly and activation is mediated by cAMP in salivary glands and does not rely on Ca^{2+} signalling (Baumann and Walz, 2012). The cAMP effect is implemented by protein kinase A, and inhibitors of PKA abolish the response of the V-ATPase to stimulation with serotonin even though intracellular cAMP and Ca^{2+} levels do increase (Rein et al., 2008; Voss et al., 2010). Congruently, only subtle changes in subunit distribution were observed in *Drosophila* Malpighian tubules that had been diuretically stimulated with the Capa-1 neuropeptide, known to act *via* Ca^{2+} dependent pathways (Terhzaz et al., 2006). In this study the authors suggested that Ca^{2+} rather regulates mitochondrial ATP-production and thereby indirectly influences V-ATPase activity *via* the ATP supply (Terhzaz et al., 2006). Direct PKA-dependent regulation of the V-ATPase has been verified in the salivary glands of *C. vicina*, since the V_1 subunit C was found to be phosphorylated in intact salivary glands in response to serotonin (Voss et al., 2007). Our studies clearly demonstrate the involvement of PKA in the assembly and activation of the V-ATPase in Malpighian tubules of A. aegypti upon natriuretic stimulation. However, preliminary observations do not indicate the phosphorylation of V-ATPase subunits by PKA in Malpighian tubules (unpublished results). These preliminary observations may indicate a regulatory role of PKA independent of or in addition to the phosphorylation of V-ATPase subunits.

Metabolic inhibition – a hint to pH as the cellular signal?

We found that metabolic inhibition induces the assembly of V_1 and V_O complexes to the same extent as membrane-permeant cAMP analogues, but without PKA activation. This raises the question whether these different cellular events share a common signal that leads to the initiation of V-ATPase assembly. Such a common signal could be intracellular acidification which takes place in principal cells stimulated with cAMP (Petzel et al., 1999) and which is also associated with metabolic inhibition in a variety of tissues, *e.g.* the larval Malpighian tubules of *Drosophila hydei* (Bertram and Wessing, 1994) or the larval midgut of *M. sexta* (Zeiske et al., 2002). Indeed, intracellular acidification has also been demonstrated in salivary glands of *C. vicina* upon stimulation with serotonin, cAMP, forskolin or IBMX (Schewe et al., 2008), stimuli that induce assembly and activation of the V-ATPase in this tissue. Preliminary data from intracellular pH measurements using BCECF-AM performed by us show that cAMP analogues as well as metabolic inhibitors acidify principal cells in

Malpighian tubules of adult *A. aegypti*, while elevating intracellular Ca^{2+} concentration does not alter the pH. In *Periplaneta americana* salivary ducts the V-ATPase is activated after an NH_4Cl-induced acid-load, suggesting that in some cell types of the salivary duct a drop in intracellular pH is sufficient to stimulate V-ATPase activity (Hille and Walz, 2007). PKA is most definitely involved in the signalling pathway that leads to activation of the V-ATPase, since its inhibitor Rp-cAMPS abolishes the increase in fluid secretion and, in support of our hypothesis, also abolishes the intracellular acidification induced by cAMP (Petzel et al., 1999). Although we cannot fully exclude that activation of the V-ATPase in Malpighian tubules of *A. aegypti* depends on direct phosphorylation of the enzyme, another activation mechanism appears conceivable. A potential target of PKA could be the apical Na^+/H^+ exchanger NHA, recently identified in larval *A. gambiae* and adult *D. melanogaster* Malpighian tubules (Rheault et al., 2007; Day et al., 2008), which upon phosphorylation might increase its activity, thereby contributing to an acidification of the cytosol in principal cells. This pH drop could then induce the assembly of V_1 and V_O complexes and hence increase transepithelial ion secretion. Since cAMP also activates Na^+ channels and a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter in the basolateral membrane of Malpighian tubules (Hegarty et al., 1991; Beyenbach, 2003), the competitive status of intracellular Na^+ for elimination *via* the apical NHA increases, resulting in natriuresis.

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FIGURE LEGENDS

Fig. 1. ATPase activities in the crude membrane pellet of unstimulated Malpighian tubules and the impact of diuretic effectors on V-ATPase activity. (A) Comparison of bafilomycin-sensitive and ouabain-sensitive ATPase activity in the crude membrane pellet. Total activity represents that of F-ATPases (inhibited by azide), of P-ATPases (inhibited by vanadate) and of V-ATPases (inhibited by bafilomycin A_1). V-ATPase activity was found to be approximately 0.18 µmol P_i min^{-1} mg^{-1}, while Na^+/K^+-ATPase activity accounted for 0.08 µmol P_i min^{-1} mg^{-1} (n = 29). (B) Distribution of V-ATPase activities in 145 crude membrane pellets, showing the high degree of variability in unstimulated control tubules. Enzyme activities were normally distributed. (C) The cAMP analogues 6-MB-cAMP and cBIMPS significantly increased membrane-associated V-ATPase activity from 0.043 ± 0.022 to 0.098 ± 0.034 µmol P_i min^{-1} mg^{-1} and 0.064 ± 0.033 to 0.120 ± 0.065 µmol P_i min^{-1} mg^{-1}, respectively. Aedeskinin-III had no effect on V-ATPase activity. Percental changes in means ± s.e.m. *** = $p < 0.001$, n.s. = not significant.

Fig. 2. Effect of diuretic peptides on the intracellular levels of cAMP. Anoga-DH_{31} significantly increased intracellular cAMP concentration, while aedeskinin-III did not affect the cAMP level. *** = $p < 0.001$.

Fig. 3. Electrophysiological assay of ATP consumption in Malpighian tubules in the presence of KCN. (A) General experimental approach: the apical membrane voltage V_a is assumed to reflect the transport activity of the V-ATPase located at that membrane. V_a remains constant in the absence and presence of 0.1 mM cBIMPS. In the presence of cBIMPS and 1 mM KCN, V_a rapidly collapses reflecting the rate of ATP utilization in the absence of ATP synthesis. (B) Average time course of KCN-induced V_a depolarization (addition of KCN until the maximal effect) in control and cBIMPS preincubated tubules and tubules that were preincubated with 20 µM bafilomycin A_1 for 15 min (means ± s.e.m). C: Time dependence of KCN-induced depolarization of V_a in tubules preincubated with 20 µM bafilomycin. D: Rates of KCN-induced V_a depolarization reflecting rates of ATP consumption in control tubules and tubules incubated with 0.1 mM cBIMPS, 0.1 mM 6-MB-cAMP, 1 µM aedeskinin-III, and 1 µM thapsigargin. KCN was added when the maximal effect of the preincubation was observed. * = $p < 0.05$; *** = $p < 0.001$.

Fig. 4. Assembly of the V_1V_O-complex induced by diuretic stimulation of the Malpighian tubules. (A) Cross-sections showing the location of subunit C in three unstimulated and one

stimulated tubule by immunelabelling using the monospecific antibody 488-1. Calibration bar: 50 µm. (B) Representative quantitative slot blot. (C-F) Corrected fluorescence intensities were used to calculate the percental membrane-association of V-ATPase subunits a, C and D in stimulated *versus* unstimulated Malpighian tubules of the same animals. * = $p < 0.05$; *** = $p < 0.001$.

Fig. 5. Metabolic inhibitors duplicate the effects of cAMP. (A) Membrane-association of subunits C and D significantly increased in tubules incubated with KCN. (B) V-ATPase activity in the crude membrane pellet of tubules incubated for 10 min in 1mM KCN increased from 0.095 ± 0.052 to 0.158 ± 0.071 µmol P_i min^{-1} mg^{-1}. Azide (15 min, 10 mM) and 2,4-dinitrophenol (5 min, 0.5 mM) both significantly increased V-ATPase activity from 0.127 ± 0.070 to 0.213 ± 0.071 µmol P_i min^{-1} mg^{-1} and 0.104 ± 0.070 to 0.213 ± 0.096 µmol P_i min^{-1} mg^{-1}, respectively. Percental changes in means ± s.e.m. (C) cAMP and metabolic inhibitors activate the same pool of V-ATPases. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; n.s. = not significant.

Fig. 6. Involvement of protein kinase A in the cAMP-induced activation of the V-ATPase. (A) Protein kinase activation detected by a phosphorylation-sensitive antibody. (B) Effect of the protein kinase A inhibitor H-89 on the 6-MB-cAMP-induced activation of ATP consumers. (C) Effect of H-89 on V-ATPase assembly at the membrane after stimulation with 6-MB-cAMP. (D) Effect of H-89 on V-ATPase activity in response to 6-MB-cAMP stimulation. Percental changes in means ± s.e.m. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 1: Test substances and conditions applied in the experiments.

Test substance	Final concentration	Incubation time [min]
2,4-dinitrophenol	0.5 mM	5
6-MB-cAMP	0.1 mM	10
Aedeskinin-III	1 µM	2
Anoga-DH_{31}	1 µM	2
Bafilomycin A_1	20 µM (0.2% DMSO)	5 - 20
H-89 (preincubation)	0.1 mM	20
H-89/6-MB-cAMP	0.1 mM each	10
H-89/KCN	0.1 mM / 1mM	10
KCN	1 mM	10
NaN_3	10 mM	15
Sp-5,6-DCl-cBIMPS	0.1 mM	10
Thapsigargin	1 µM	5

Figure 1

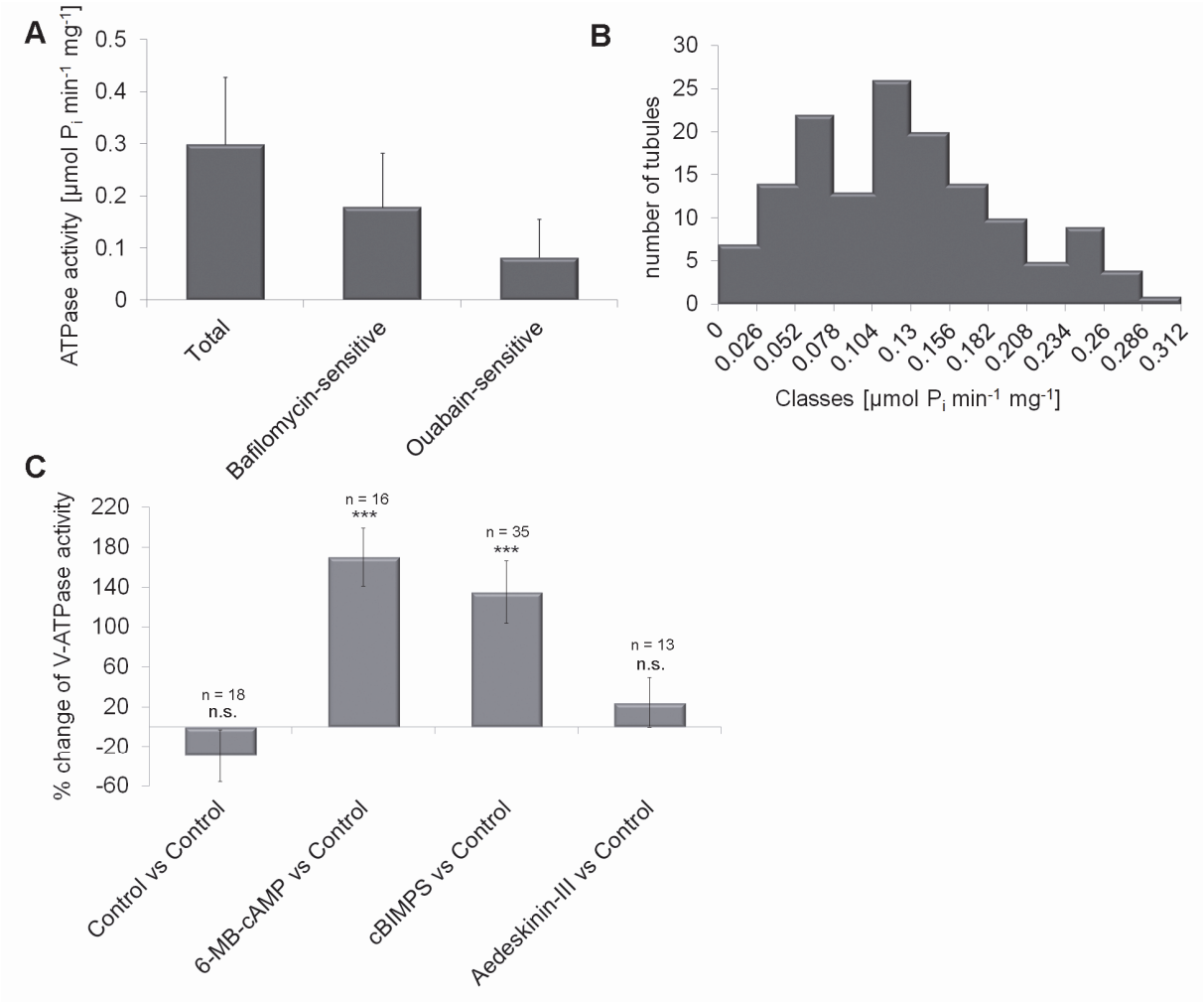

Figure 2

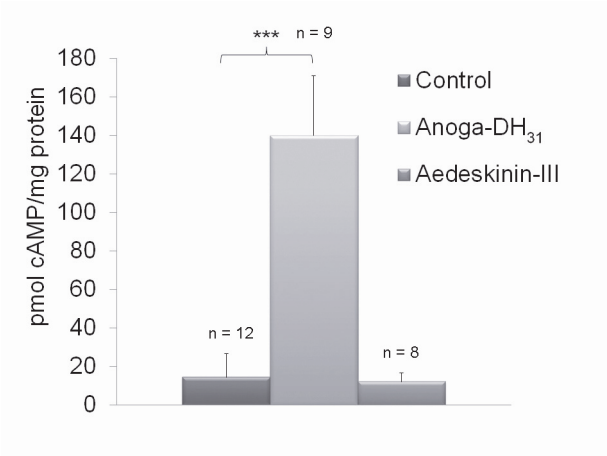

Figure 3

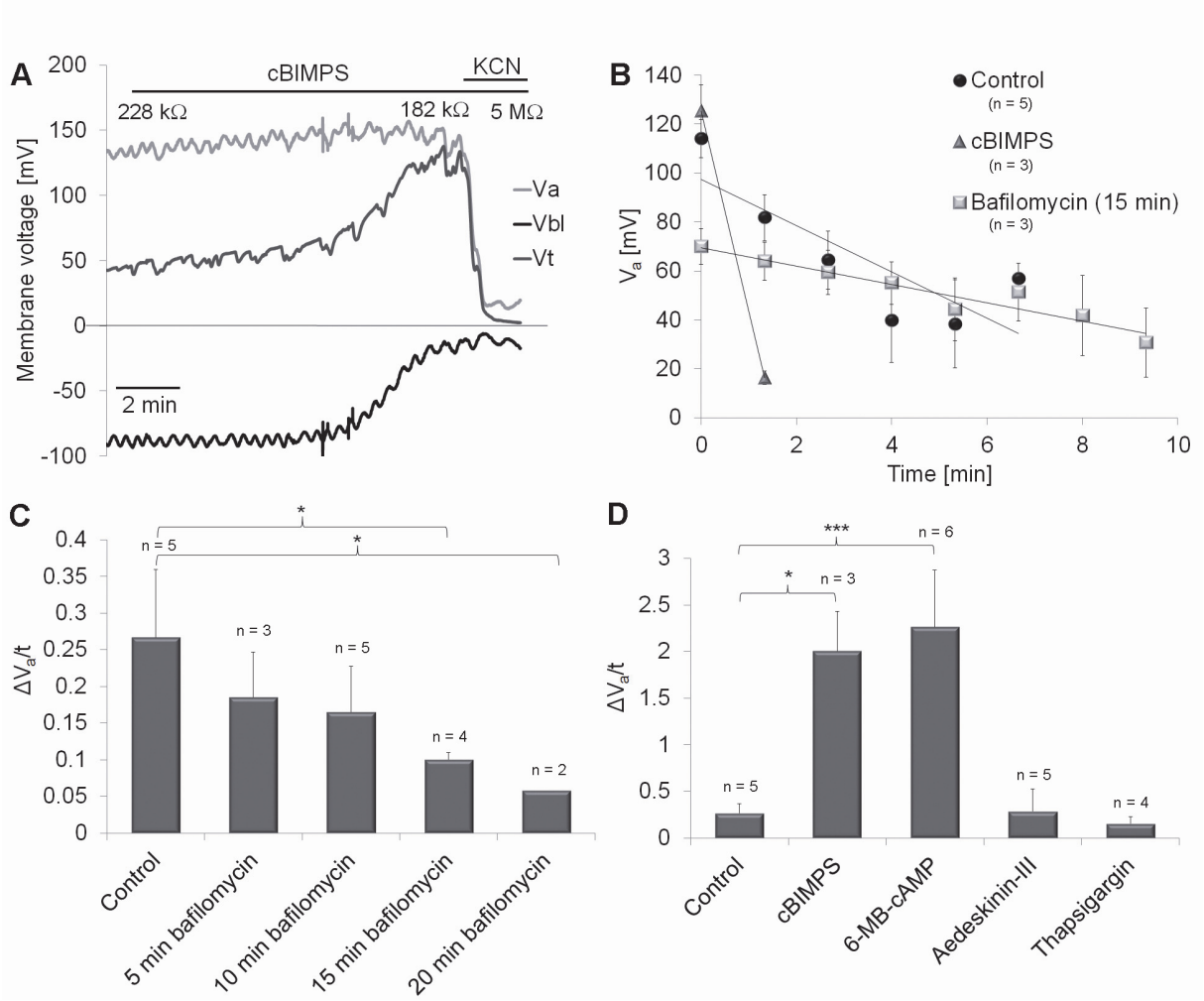

Figure 4

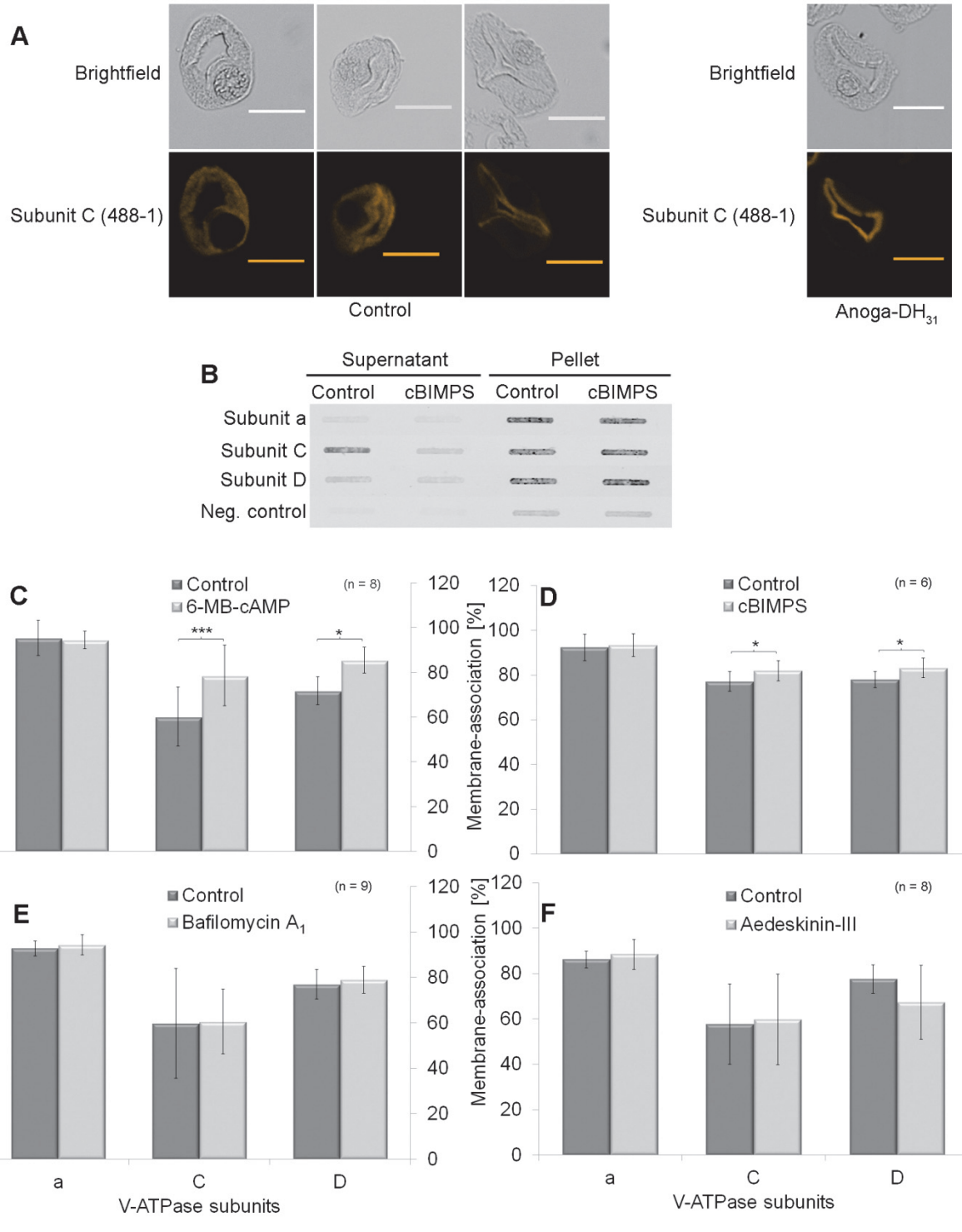

Figure 5

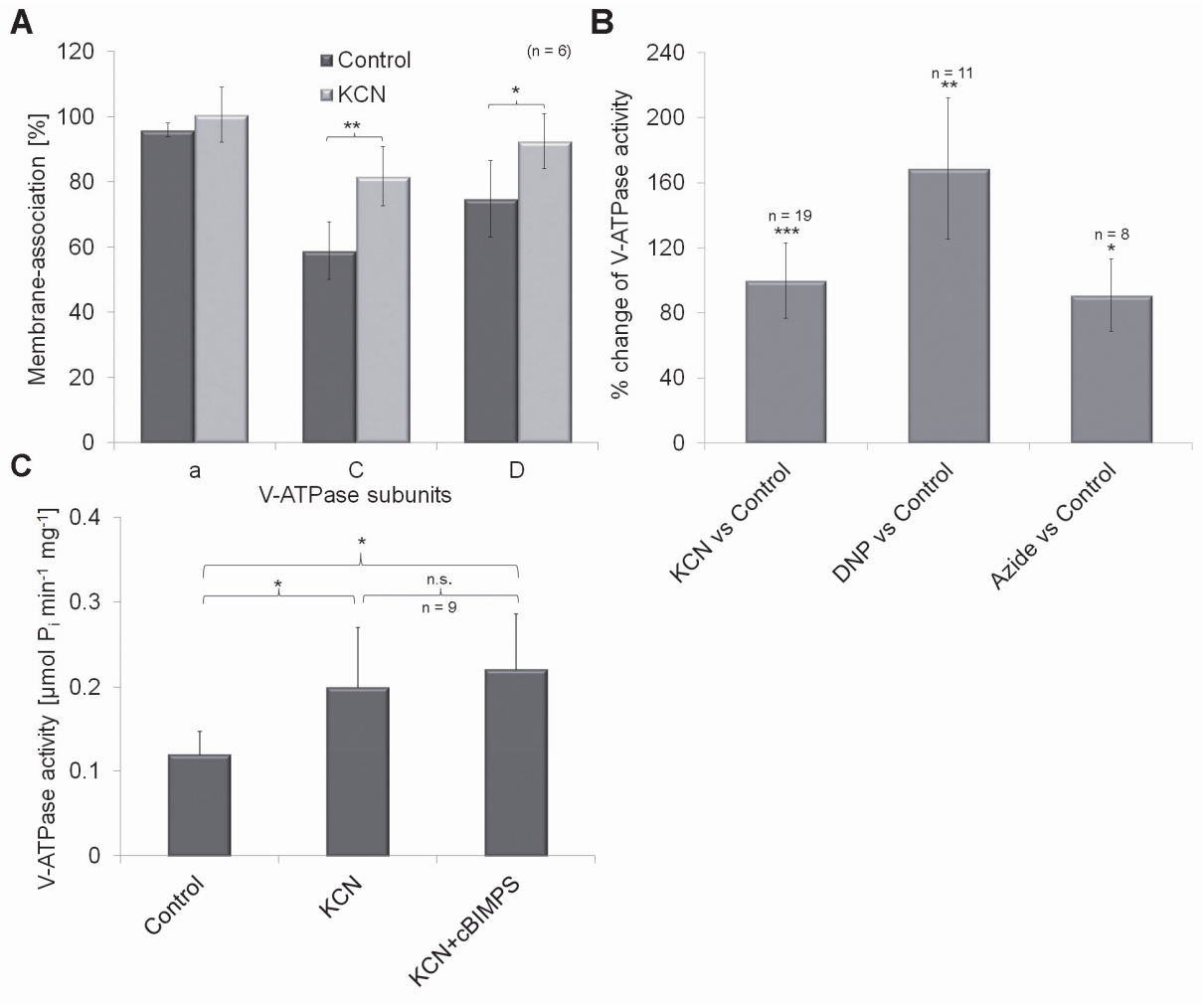

Figure 6