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RESEARCH ARTICLE

First evidence of epithelial transport in tardigrades: a comparative investigation of organic anion transport

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SUMMARY

We investigated transport of the organic anion Chlorophenol Red (CPR) in the tardigrade *Halobiotus crispae* using a new method for quantifying non-fluorescent dyes. We compared the results acquired from the tardigrade with CPR transport data obtained from Malpighian tubules of the desert locust *Schistocerca gregaria*. CPR accumulated in the midgut lumen of *H. crispae*, indicating that organic anion transport takes place here. Our results show that CPR transport is inhibited by the mitochondrial un-coupler DNP (1 mmol l^{-1}; 81% reduction), the Na^+/K^+-ATPase inhibitor ouabain (10 mmol l^{-1}; 21% reduction) and the vacuolar H^+-ATPase inhibitor bafilomycin (5 μmol l^{-1}; 21% reduction), and by the organic anions PAH (10 mmol l^{-1}; 44% reduction) and probenecid (10 mmol l^{-1}; 61% reduction, concentration-dependent inhibition). Transport by locust Malpighian tubules exhibits a similar pharmacological profile, albeit with markedly higher concentrations of CPR being reached in *S. gregaria*. Immunolocalization of the Na^+/K^+-ATPase α-subunit in *S. gregaria* revealed that this transporter is abundantly expressed and localized to the basal cell membranes. Immunolocalization data could not be obtained from *H. crispae*. Our results indicate that organic anion secretion by the tardigrade midgut is transporter mediated with likely candidates for the basolateral entry step being members of the Oat and/or Oatp transporter families. From our results, we cautiously suggest that apical H^+ and possibly basal Na^+/K^+ pumps provide the driving force for the transport; the exact coupling between electrochemical gradients generated by the pumps and transport of ions, as well as the nature of the apical exit step, are unknown. This study is, to our knowledge, the first to show active epithelial transport in tardigrades.

Key words: organic anion transport, Chlorophenol Red, 2,4-dinitrophenol, ouabain, bafilomycin, probenecid, *para*-aminohippuric acid, tardigrade, insect, V-type H^+-ATPase, Na^+/K^+-ATPase, Malpighian tubule.

INTRODUCTION

The ability to excrete metabolic waste products as well as environmental toxins (xenobiotics) is a fundamental prerequisite for animal life. One of the earliest identified systems involved in such an excretion is the classic organic anion transport system (Marshall and Vickers, 1923). In vertebrates, this system is (especially) well known in the proximal tubule where it rids the organism of various xenobiotic and endobiotic compounds, through an ATP-dependent, net transepithelial secretory pathway (reviewed by Dantzler, 2002; Burckardt and Burckardt, 2003; Russel, 2010). The importance and understanding of similar transport activities in various invertebrate phyla, however, is extremely limited. In this study, we expand on the current knowledge by investigating organic anion transport in the tardigrade *Halobiotus crispae* (Tardigrada), as compared with the desert locust *Schistocerca gregaria* (Arthropoda).

The phylum Tardigrada consists of a group of minute, eight-legged, multicellular animals that, like arthropods and nematodes, belong to the invertebrate superclade Ecdyzosoa (Aguinaldo et al., 1997). They are considered essential to our understanding of early metazoan evolution, yet fundamental questions concerning their basic biology remain unanswered (Møbjerg et al., 2011). In spite of their small size (~50–1200 μm), tardigrades are relatively complex animals; they are composed of >1000 cells, and possess a well-developed musculature and nervous system, as well as a complex alimentary canal and specialized reproductive and excretory organs (Dewel and Dewel, 1979; Rebecchi and Bertolani, 1994; Møbjerg and Dahl, 1996; Schmidt-Raesa and Kulessa, 2007; Pelzer et al., 2007; Zantke et al., 2008; Halberg et al., 2009a; Møbjerg et al., 2011; Rost-Roszkowska et al., 2011). The alimentary canal can be divided into several morphologically distinct regions i.e. bucco-pharyngeal apparatus, oesophagus, midgut and hindgut, with the Malpighian tubules (MTs) of eutardigrades positioned at the junction between these last two sections. Interestingly, the same basic organizational pattern is found in insects, and has been used as a strong argument for the homology of these two organ systems (Greven, 1982; Møbjerg and Dahl, 1996). Among multi-cellular animals, tardigrades exhibit an extraordinary ability to resist environmental extremes, and are known to survive conditions greatly exceeding those encountered in their natural habitat – even in space (Jönsson et al., 2008; Rebecchi et al., 2008; Persson et al., 2011). The biochemical and physiological mechanisms mediating this unique tolerance, however, remain largely unidentified. Previously, we have shown that the marine eutardigrade *H. crispae* tolerates large changes in external salinity surviving periods of osmotic stress by maintaining haemolymph osmotic pressure above that of the external medium (Halberg et al., 2009b); an adaptive mechanism likely present in all eutardigrades (Møbjerg et al., 2011). Here, we identify organs involved in transepithelial transport of organic anions and investigate the transport characteristics with the aim of providing a better understanding of the unique biology in these animals.

Organic anion transport has previously been described in other groups of invertebrates (George et al., 1999; Torrie et al., 2004; Faria et al., 2010). Notably, the alimentary canal and MTs of insects, which collectively form the functional analogue of the vertebrate kidney, have been studied (reviewed by Phillips, 1981; O'Donnell et al., 2003; Dow and Davies, 2006). As in vertebrates, the excretory organs of insects transport a wide range of organic solutes and exogenous toxins through organic anion transporters (Oats), organic anion-transporting peptides (Oatps), P-glycoproteins (Mdr/P-gp) as well as multidrug resistance-associated proteins (Mrps) (Maddrell et al., 1974; Bresler et al., 1990; Lanning et al., 1996; Linton and O'Donnell, 2000; Torrie et al., 2004; Neufeld et al., 2005; Leader and O'Donnell, 2005; O'Donnell and Leader, 2006; Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). Organic anions are divided into type I and type II organic anions, on the basis of structural and chemical properties (e.g. Wright and Dantzler, 2004). The different groups of transporters vary in their transport mechanisms, but overlap in their substrate specificity, as they transport carboxylates and sulphonates interchangeably (Neufeld et al., 2005; Chahine and O'Donnell, 2009). Oats and Oatps (solute carriers belonging to the SLC22 and SLC21/SLCO family) transport both small (<400–500 Da) hydrophilic type I organic anions (Oats) and large (>450 Da) hydrophobic type II organic anions (Oatps), whereas Mdr/P-gp and Mrps (ABC transporters, ABCB and ABCC subfamilies) generally transport large (>500 Da) polyvalent type II organic anions (Wright and Dantzler, 2004; Russel, 2010). From the insect's perspective, the clearance of exogenous toxins is of particular interest, as insects often live in environments with high xenobiotic exposure, potentially at harmful or lethal concentrations. Accordingly, they are forced to process naturally occurring plant toxins (Torrie et al., 2004; Neufeld et al., 2005), as well as anthropogenic contaminants, such as insecticides (Lanning et al., 1996; Neufeld et al., 2005; Buss and Callaghan, 2008). The physiological importance of this detoxification system – and implicitly the MTs – is emphasized by the fact that transcripts for these transporters are enriched in the transcriptome of the MTs (Wang et al., 2004), and because the expression of several Oatp and Mdr transporters is significantly upregulated upon dietary exposure to Oatp and Mdr substrates (Mulenga et al., 2008; Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). Indeed, it has been suggested that organic solute excretion is the most significant function of the insect MT (Dow and Davies, 2006). Nevertheless, in spite of significant efforts in the past decades, the mechanisms underlying transepithelial transport of organic anions is far from understood, and our knowledge is limited to relatively few taxa.

In this study we examined epithelial transport in tardigrades. Using a comparative approach, we investigated the sites, characteristics and pharmacological profile of the net transepithelial transport of Chlorophenol Red (CPR; 3′,3″-dichlorophenol-sulphone-phthalein), a pH indicator and a prototypic substrate of the classic organic anion secretory pathway, in the tardigrade *H. crispae* and the desert locust *S. gregaria*. Our results show that the tardigrade midgut is the principal site of CPR transport, and that this transport is active and transporter mediated. Additionally, our data show that the pharmacological profiles of CPR transport in the tardigrade midgut and locust MT are surprisingly similar.

MATERIALS AND METHODS

Quantification of CPR accumulation

The quantification of CPR accumulation was performed by introducing a new method for quantifying non-fluorescent dyes. The method exploits the optical properties of CPR by relating relative changes in spectral light to that of dye concentration (Fig. 1). As CPR exhibits a pH-dependent shift in colour (from yellow to violet) between pH 4.6 and 7.0, and a secondary shift (to purple) at pH>10, a standard curve was constructed at three discrete colours relevant to our investigation (red, violet and purple corresponding to pH 6–11). This was done in order to correct for potential pH dependent effects on CPR quantification caused by the tissue. The standard curves were constructed from optical analysis of micrographs taken of samples (40 µl) with known dye concentrations (ranging from 0.1 to 5 mmol l^{-1}) at each respective pH value. The optical measurements provided relative, arbitrary values for the light intensities of the red, green and blue spectrum of spectral light from each sample, and were acquired using the image analysis and visualization software Imaris 6.4 (Bitplane, Zurich, Switzerland). The standard curves are expressed as percentage difference in the red spectrum/green spectrum relationship as a function of CPR concentration (Fig. 1). The curves were fitted by regression analysis using OriginPro 7.5 (OriginLab, Northampton, MA, USA) with a third order polynomial providing the best fit with r^2 values close to unity. The CPR concentration in a given tissue was subsequently calculated by employing the appropriate standard curve. The standard curve was chosen by visually comparing the colour of the accumulated CPR with that of the respective standard curves.

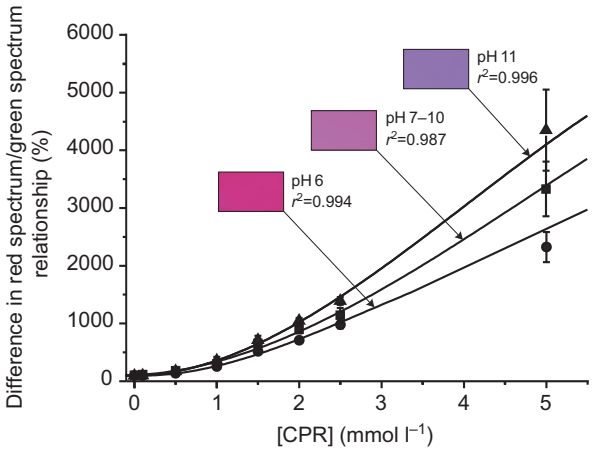

Fig. 1. Standard curves showing the percentage difference in the red spectrum/green spectrum relationship as a function of Chlorophenol Red (CPR) concentration at different pH values (pH 6, 7–10 and 11). The curves were fitted by regression analysis using OriginPro 7.5 with a third order polynomial providing the best fit with r^2 values close to unity.

Contrary to our expectations, the midgut of *H. crispae* was the only organ in which CPR accumulation was clearly visualized. No accumulation was observed in the tardigrade MTs. As such, quantification of dye accumulation was only performed from the tardigrade midgut, and compared with the accumulation in the MTs of *S. gregaria*. Specifically, 6–12 regions from areas of the tardigrade midgut containing the highest dye intensity were selected arbitrarily, in addition to a similar number from the adjacent haemolymph, while four different regions were selected from each insect MT. Each region selected represented a circle with a diameter of 5 µm for *H. crispae* and 25 µm for *S. gregaria*. The average light intensity within these circles was measured by the Imaris program. Averaging the measured intensity of all selected regions provided an overall average intensity for the red, green and blue colour spectrum within each investigated organ following each exposure. As CPR predominantly appears red (to violet) in the midgut of *H. crispae* and in the MTs of *S. gregaria*,

the red spectrum/green spectrum relationship offered an estimate of dye accumulation, as calculated from the appropriate standard curve. The final CPR concentration was normalized according to the background light intensity, i.e. the tardigrade haemolymph or untreated (control) insect MTs. For tardigrades, only regions without gut content were selected for quantification, in order to avoid the influence of gut content on the wavelength of captured light. Deviations in animal depth did not influence CPR quantification within the variation encountered in this study. The MTs of *S. gregaria* consist of three structurally distinct regions, i.e. proximal, middle and distal relative to the gut (Garret et al., 1988). Dye accumulation was estimated from the proximal region and parts of the middle region within 5 mm of the junction with the gut, as dye accumulation was highest here.

Test solutions

The haemolymph osmolality of *H. crispae*, kept at a salinity of 20 p.p.t., was previously measured by nanolitre osmometry to ~950 mOsm kg^{-1} (Halberg et al., 2009b). At present we do not know the composition of tardigrade extracellular fluids. As such, the experimental solution (control solution) was prepared from evaporative reduction of seawater (SW; salinity 20 p.p.t., pH 8) collected at the locality, and 35 mmol l^{-1} glucose was added to alleviate potential variation in experiments caused by differences in nutrient availability. This yielded a final measured osmolality of 950±3 mOsm kg^{-1} (N=3). For *S. gregaria* an insect saline (control solution) was prepared containing (in mmol l^{-1}): 130 NaCl, 10 KCl, 4 $NaHCO_3$, 2 $MgSO_4$, 2 $CaCl_2$, 6 NaH_2PO_4, 35 glucose and 5 Hepes, titrated to pH 7.2, with a measured osmolality of 336±2 mOsm kg^{-1} (N=3).

In order to explore the kinetics of CPR transport by MTs of *S. gregaria*, a concentration–response curve was constructed over a 5000-fold range of CPR concentrations (1 µmol l^{-1} to 5 mmol l^{-1}) (Fig. 2). These data revealed that CPR transport is saturated at an external CPR concentration of ~1.6 mmol l^{-1} (V_{max}=1.58 mmol l^{-1}, K_m=81.8 µmol l^{-1}; Fig. 2). However, the MTs are unable to concentrate the dye at high concentrations of CPR (>1 mmol l^{-1}; Fig. 2, inset). Experiments, on both animals, were performed at a concentration of 1 mmol l^{-1} CPR.

Test solutions were prepared from the two control solutions containing 1 mmol l^{-1} CPR, and one of the following inhibitors: 2,4-dinitrophenol (DNP, 1 mmol l^{-1}), ouabain (10 mmol l^{-1}), bafilomycin A_1 (5 µmol l^{-1}), *para*-aminohippuric acid (PAH; 10 mmol l^{-1}) or probenecid (0.1–10 mmol l^{-1}). The final osmolality of the solutions was measured on a Vapro 5520 vapour pressure osmometer (Wescor, Logan, UT, USA). All solutions were titrated with NaOH to pH 8 for *H. crispae* and pH 7.2 for *S. gregaria*.

Experimental animals

Specimens of *H. crispae* Kristensen 1982 were collected on 11 February 2008 and 17 January 2010 at Vellerup Vig, Isefjord, Denmark (55°44.206′N, 11°51.258′E) (see Fig. 3A). Bottom samples were collected with a mini van Veen grab at a depth of 1–2 m (salinity ~20 p.p.t., pH 8). Rocks, algae and sediment collected with the grab were freshwater shocked. The debris was decanted into a conical net (mesh size 63 µm) and subsequently transferred to SW from the locality and kept at 4°C. Animals in the active stage (see Kristensen, 1982; Møbjerg et al., 2007; Halberg et al., 2009b) were identified using a dissection microscope. The tardigrades were kept for a period of up to 6 months at 4°C in SW (salinity 20 p.p.t., pH 8) and regularly supplied with fresh substrate, consisting mainly of sediment, organic debris, filamentous algae and diatoms.

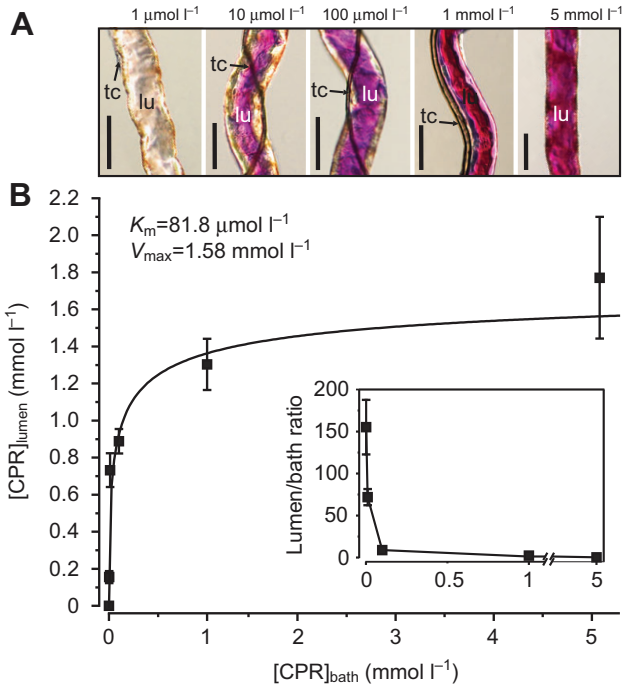

Fig. 2. Accumulation of CPR as a function of external CPR concentration in Malpighian tubules (MTs) of *Schistocerca gregaria*. (A) Representative light micrographs of the MTs following exposure to different concentrations (1 µmol l^{-1}, 10 µmol l^{-1}, 100 µmol l^{-1}, 1 mmol l^{-1} and 5 mmol l^{-1}) of CPR. Scale bars, 100 µm. lu, lumen; tc, trachea. (B) Luminal CPR concentration as a function of bath CPR concentration, revealing the kinetic parameters K_m and V_{max} for CPR transport. Each point shows the mean ± s.d. for N=4–6 animals with 3–5 MTs providing the estimate for each animal. The solid line represents the fit to the Michaelis–Menten equation by non-linear regression analysis (using error as weight). Insert shows the lumen/bath ratio of CPR as a function of external CPR concentration.

Specimens of *S. gregaria* Forskål 1775 were acquired from a specialized animal shop (www.exopark.dk), and kept at room temperature (RT) with light:dark periods of 16 h:8 h and fed annual meadow grass (*Poa annua*) *ad libitum*.

Exposure to test solutions

Halobiotus crispae

Evaluation of CPR transport by tardigrade epithelia was performed on whole animals immersed in the dye solution. Data obtained from 82 animals were used for the study – no distinction was made between male and female specimens. Initial observations revealed that the animals did not take up dye through the mouth or cuticle (CPR non-punctured, Fig. 3) and test solutions were therefore introduced to the haemolymph of single specimens (length 300–500 µm) through a small hole made in the cuticle in the anterior part of the animal. The animal was incubated for a period of 60 min at RT in the respective test solution, and quickly washed in SW prior to photography. Light micrographs of the specimens were taken in bright-field at a ×40 magnification, using an Olympus DP20 camera mounted on an Olympus BX50 microscope (Olympus, Hamburg, Germany). Additionally, in order to investigate whether the test solutions were ingested during the experimental period, intact non-punctured animals were incubated in the CPR test solution for a corresponding period. The animals were washed and photographed as described above.

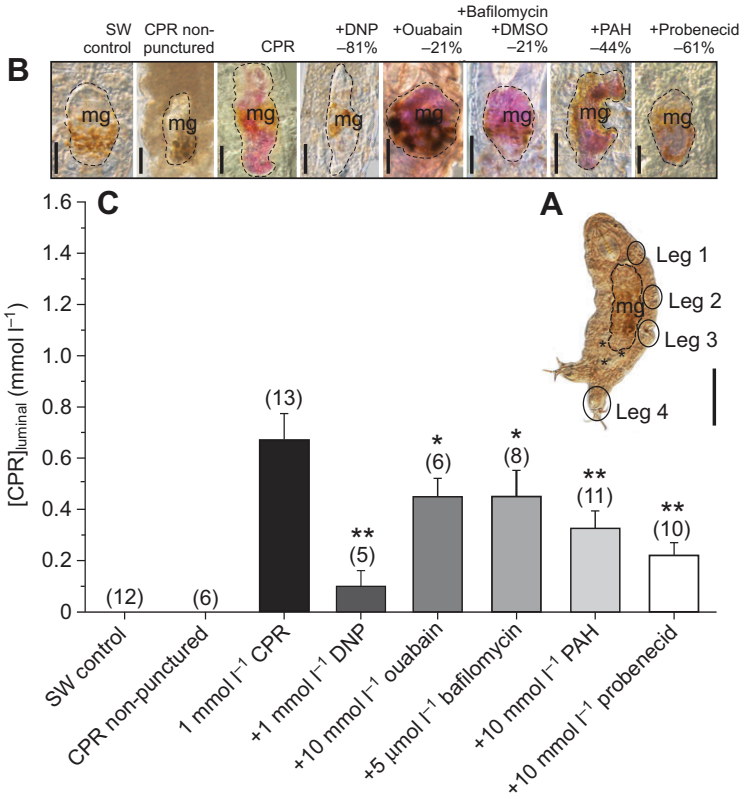

Fig. 3. CPR accumulation in the gut lumen of *Halobiotus crispae*, and the effect of inhibitors on dye accumulation. (A) Light micrograph of *H. crispae* from a ventral view showing the animal's basic morphology. mg, midgut. Asterisks indicate MTs. Scale bar, 100 µm. (B) Representative light micrographs of the midgut (mg) following exposure to the various test solutions. Seawater control shows the midgut of a punctured animal following immersion in seawater, while CPR non-punctured shows the midgut of an intact animal after immersion in CPR solutions. Estimations of luminal CPR concentration were performed solely from areas devoid of gut content, i.e. brown material in the gut. The percentage change in CPR concentration, compared with experiments on CPR alone, is noted. Scale bars, 50 µm. (C) Corresponding luminal concentrations of CPR. Data are depicted as means ± s.d. Asterisks refer to a significant difference from CPR alone (**P*<0.05, significant; ***P*<0.01, highly significant). Numbers in parentheses indicate the number of animals tested.

A separate experiment was performed to evaluate whole-organism activity of *H. crispae* following puncture of the cuticle and exposure to a pure SW control and the various test solutions, following the procedure described above. The animals were scored with a numerical value according to their activity, which was defined as: 0, no movement discerned; +1, small movement of leg or claw; +2, clear movement of body and extremities. Thirty specimens were used for each test solution, and a cumulative score was given for each group (see Fig. 4).

Schistocerca gregaria

The entire alimentary canal including ~233 MTs (Garret el al., 1988) was dissected from adult specimens (see Fig. 5A) (imago females and males; 2–10 days post-fifth instar nymph) under insect saline; 94 animals were used in total. Prior to transfer into the test solutions, several MTs were randomly chosen and removed, and micrographs were taken in order to acquire an estimate of the background light intensity for each animal. The background light intensity was later used for normalizing the estimate of the CPR concentration (see above). The gut systems were incubated in the respective test solutions for 60 min at RT. Following a quick rinse in insect saline, individual MTs representative of overall dye intensity were subsequently isolated under microscope (Zeiss Stemi 200-CS, Carl Zeiss International, Oberkochen, Germany), and prepared for photography. MTs obviously damaged during dissection (i.e. that did not accumulate CPR) were avoided. Details pertaining to the method of photography were as described above. Five to 11 locusts with 3–5 MTs per specimen were used in estimations of the CPR concentration for each exposure; a minimum of 20 and a maximum of 40 MTs were analysed in total for each test solution.

Midgut and MT structure and immunostaining

Whole animals of *H. crispae* and MTs dissected from *S. gregaria* were prepared for structural investigation and immunostaining.

MTs of *S. gregaria* were fixed for 60 min at RT in an aldehyde fixative containing: 1.2% glutaraldehyde, 1% paraformaldehyde, 0.05 mol l^{-1} sucrose and 0.05 mol l^{-1} sodium cacodylate buffer (pH 7.4) and subsequently rinsed and stored in 0.05 mol l^{-1} sodium cacodylate buffer with 0.05 mol l^{-1} sucrose. Following 1 h post-fixation in 2% OsO_4 with 0.1 mol l^{-1} sodium cacodylate, tubules were dehydrated through a graded series of ethanol and propylenoxide and embedded in Araldite®. Semi-thin sections were cut on a Leica ultramicrotome EM UC6 (Leica,

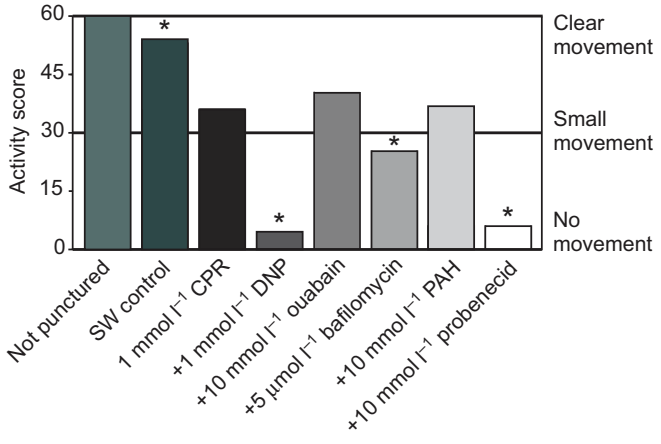

Fig. 4. Animal activity of *H. crispae* (with punctured cuticle) following exposure to test solutions. Thirty animals were tested for each exposure. Each animal was scored with a numerical value according to their activity, defined as: 0, no movement discerned; +1, small movement of leg or claw; +2, clear movement of body and extremities. A cumulative score was given for each group. The statistical significance of differences in activity score was tested using Mann–Whitney's *U*-test. Asterisks refer to significant difference from 1 mmol l^{-1} CPR (no asterisk, *P*>0.05, not significant; **P*<0.05, significant).

Microsystems, Wetzlar, Germany) with glass knives and subsequently stained with Toluidine Blue.

For immunocytochemistry, whole animals of *H. crispae* and MTs of *S. gregaria* were fixed in 3% paraformaldehyde in 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.4) for 60 min and subsequently transferred to 0.1 mol l^{-1} sodium cacodylate buffer. The tissue was then dehydrated through a graded series of ethanol and xylene, embedded in paraffin and sectioned into ~10 µm sections, or transferred to PBS and used as whole mounts for immunostaining. Paraffin sections were deparaffinized through a graded series of xylene and alcohol, washed in saline (control solution; see 'Test solutions' above) and blocked with 10% normal goat serum (Invitrogen, Carlsbad, CA, USA) for 30 min, prior to incubation with primary antibody. Paraffin sections, as well as whole mounts, were incubated overnight at 4°C in insect saline (MTs) or PBS (tardigrades) containing 10% normal goat serum, 0.1% Triton-X and primary antibody. The Na^+/K^+-ATPase α-subunit monoclonal mouse primary antibody α5-IgG (10 µg ml^{-1}) was developed by D. M. Famborough, and obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA). This antibody has been used to identify the Na^+/K^+-ATPase in numerous excretory tissues, including MTs of *Drosophila melanogaster* (Lebovitz et al., 1989; Torrie et al., 2004), the gills of the blue crab *Callinectes sapidus* (Towle et al., 2001) and the pronephros of *Ambystoma mexicanum* (Haugen et al., 2010). Following an extensive wash in saline, the tissue was incubated with (anti-mouse) Alexa Fluor 594 (1:100) secondary antibody (Invitrogen) overnight at 4°C. The tissue was rinsed with saline then counterstained with Alexa Fluor 488-conjugated phalloidin (luminal marker; 1:40; Invitrogen) and DAPI (50 µg ml^{-1}; Invitrogen) for 2 h, washed and mounted on glass coverslips in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Images were acquired using a Leica DM RXE 6 TL microscope equipped with a Leica TCS SP2 AOBS confocal laser scanning unit (Leica Microsystems, Wetzlar, Germany). The tissue was scanned employing sequential scanning (setting: between frames) using the 488 nm line of an argon/krypton laser and the 594 nm line of a helium laser, in addition to the 405 nm UV laser line. The image series was processed and edited using Imaris software. Confocal images are based on 240 optical sections of a *Z*-series performed at intervals of 0.7 µm. Experiments were conducted multiple times with corresponding results. All control preparations without primary antibody were negative for immunostaining.

Chemicals

All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA). Bafilomycin was dissolved and stored in dimethyl sulphoxide (DMSO). Inhibitors were allowed to dissolve in the CPR solution overnight prior to use.

Statistics

Data are expressed as means ± s.d. unless otherwise stated. The statistical significance of differences between the various exposures was tested using one-way ANOVA followed by a Tukey multiple comparisons of means. The statistical tests were performed using the data analysis program OriginPro 7.5 (OriginLab). Significance levels were $P>0.05$ (not significant), $P<0.05$ (significant, *) and $P<0.01$ (highly significant, **).

RESULTS

CPR accumulation

During our initial observations on CPR transport in punctured *H. crispae*, dye accumulation was almost exclusively observed in the tardigrade midgut, and for this reason subsequent analyses were performed solely on this structure (slight residual coloration was occasionally observed in the haemolymph). These results were compared with the accumulation in *S. gregaria* MTs. The luminal concentration of CPR in the gut of *H. crispae* and in the MT of *S. gregaria* when exposed to a 1 mmol l^{-1} CPR solution, in the presence or absence of inhibitors, is shown in Figs 3 and 5. Activity of *H. crispae* during the corresponding exposures is depicted in Fig. 4.

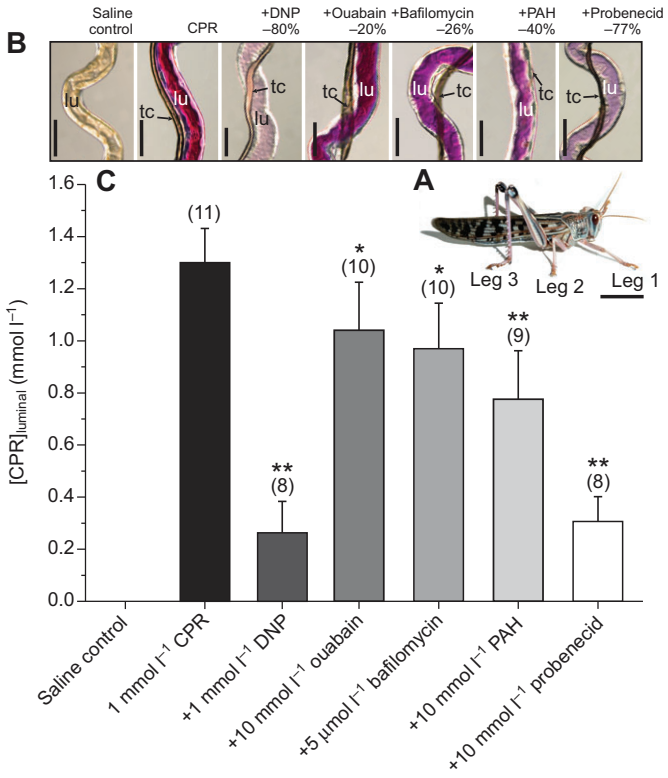

Fig. 5. CPR accumulation in MTs of *S. gregaria*, and the effect of inhibitors on dye accumulation. (A) Photo of *S. gregaria*. Scale bar, 1 cm. (B) Representative light micrographs of the MTs from exposure to the various test solutions. Control saline shows an MT prior to immersion in CPR solution. The percentage change in CPR concentration, compared with experiments on CPR alone, is noted. Scale bars, 100 µm. (C) Corresponding luminal concentration of CPR in the MTs. Asterisks refer to significant difference from 1 mmol l^{-1} CPR alone (*$P<0.05$, significant; **$P<0.01$, highly significant). Numbers in parentheses indicate the number of animals tested. For *S. gregaria*, measurements on 3–5 MTs provided a mean for each animal.

In *H. crispae*, areas void of gut contents appear transparent, whereas those with gut contents are a light to dark brown colour (Fig. 3B, SW control). Only areas devoid of gut content were used for quantification of dye accumulation. In order to expose internal epithelia to the test solutions a small hole was made in the cuticle in the anterior part of the animal. Although clearly affected by the punctured cuticle compared with untreated specimens, animal activity was surprisingly high under control conditions, even 60 min after the small hole in the cuticle was made (Fig. 4). This implies that the osmolality as well as the ionic composition of the used SW are within tolerable limits of extracellular fluid conditions.

When intact, non-punctured animals were exposed to the 1 mmol l^{-1} CPR solution, no internal CPR accumulation was evident, showing that the surrounding medium is not ingested by the animals within the experimental period (Fig. 3B). However, following exposure of punctured animals to the same 1 mmol l^{-1} CPR solution,

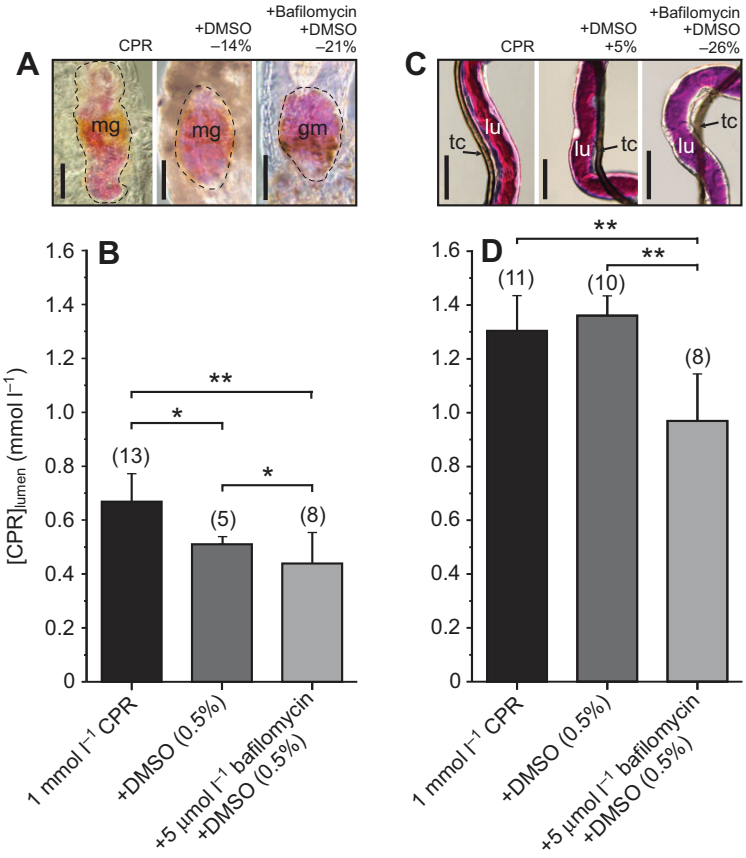

Fig. 6. The effect of bafilomycin and dimethylsulphoxide (DMSO) on CPR accumulation. (A) Representative light micrographs of the tardigrade midgut (mg) following exposure to the test solutions. The percentage change in CPR concentration, compared with experiments with CPR alone, is noted. Scale bars, 50 µm. (B) Corresponding luminal concentration of CPR in midgut lumen. (C) Representative light micrographs of locust MTs following exposure to the test solutions. The percentage change in CPR concentration, compared with experiments on CPR alone, is noted. Scale bars, 100 µm. (D) Corresponding luminal concentration of CPR in the MTs. Asterisks refer to significant difference from 1 mmol l^{-1} CPR alone (*P<0.05, significant; **P<0.01, highly significant). Numbers in parentheses indicate the number of animals tested. For *S. gregaria*, measurements on 3–5 MTs provided a mean for each animal.

dye accumulation was readily visible in the midgut lumen, suggesting that organic anion transport is mediated by the midgut epithelium; CPR concentration in the midgut lumen reached a mean of 0.68±0.1 mmol l^{-1} (Fig. 3B,C). Similarly, dye accumulation was evident in the lumen of the insect MTs, reaching a mean CPR concentration of 1.3±0.13 mmol l^{-1} (Fig. 5B,C).

DNP is a mitochondrial un-coupler that inhibits mitochondrial ATP production. For *H. crispae*, ~81% reduction in the mean CPR concentration was detected in experiments using DNP, resulting in a midgut CPR concentration of only 0.1±0.07 mmol l^{-1} (Fig. 3). The tardigrades almost all became passive (Fig. 4), indicating that a range of tissues was affected by the DNP application. In comparison, a similar reduction of ~80% was observed in the MTs of *S. gregaria*, with CPR concentrations of 0.26±0.12 mmol l^{-1} (Fig. 5). This finding shows that CPR accumulation is ATP dependent. The Na^+/K^+-ATPase and vacuolar H^+-ATPase are likely ATP-consuming candidates, which could energize transepithelial dye transport. We therefore investigated CPR transport during applications of known pump inhibitors.

Solutions containing ouabain, a plant alkaloid that binds to and inhibits the Na^+/K^+-ATPase, revealed a significant effect on CPR transport in both *H. crispae* and *S. gregaria*. The mean CPR concentration was 0.44±0.11 mmol l^{-1} (Fig. 3) in *H. crispae*, which is a 21% reduction in luminal CPR concentration, and 1.04±0.19 mmol l^{-1} (Fig. 5) in *S. gregaria*, corresponding to ~20% reduction. Activity of *H. crispae* was not affected by this test solution (Fig. 4).

The vacuolar H^+-ATPase inhibitor bafilomycin A_1 similarly affected CPR transport significantly, showing a mean CPR concentration of 0.44±0.13 mmol l^{-1} (Fig. 3) in *H. crispae*, which is a 21% reduction compared with animals treated with CPR alone (note, bafilomycin was dissolved in DMSO, and a control experiment showed that the observed inhibition is partly due to DMSO – see below). Although the mean CPR concentration in the presence of bafiolmycin A_1 was higher in *S. gregaria*, i.e. 0.97±0.18 mmol l^{-1} (Fig. 5), a corresponding 26% reduction was observed. The activity of *H. crispae* was markedly affected by bafilomycin A_1, with most animals showing movements only following tactile stimuli (Fig. 4). Notably, visual colour changes to CPR were observed (i.e. the dye turned purple) in some specimens immediately after transfer to SW, and in all animals 10 min post-exposure, suggesting an alkalization of the midgut content, which is consistent with the specific inhibition of an apical H^+-ATPase. Because bafilomycin was dissolved in DMSO, we tested whether the used concentrations of DMSO (0.5%) had an effect on CPR accumulation in both animals. In *S. gregaria*, no significant change in CPR accumulation was detected (Fig. 6C,D), but in *H. crispae*, a significant change was observed in the presence of 0.5% DMSO (Fig. 6A,B). A significant difference between 0.5% DMSO-treated animals and 0.5% DMSO + bafilomycin-treated specimens, however, was also detected (Fig. 6A,B), revealing that the observed inhibition is only partly caused by bafilomycin in *H. crispae*. The observation that DMSO has an effect on CPR transport in tardigrades is in contrast to previous reports from insects, stating that a concentration of DMSO below 1% has no effect on the fluid secretion rate in the MTs of *Drosophila* (Linton and O'Donnell, 2000).

Furthermore, we investigated the impact of two well-known substrates of organic anion transporters, PAH and probenecid, on the ability to accumulate CPR. Both PAH and probenecid are type I organic anions (carboxylates), which appeared to reduce the CPR concentration in our pilot experiments on both *H. crispae* and *S. gregaria*. Addition of 10 mmol l^{-1} PAH caused a reduction of ~44% in luminal CPR concentration in *H. crispae* and ~40% in *S.*

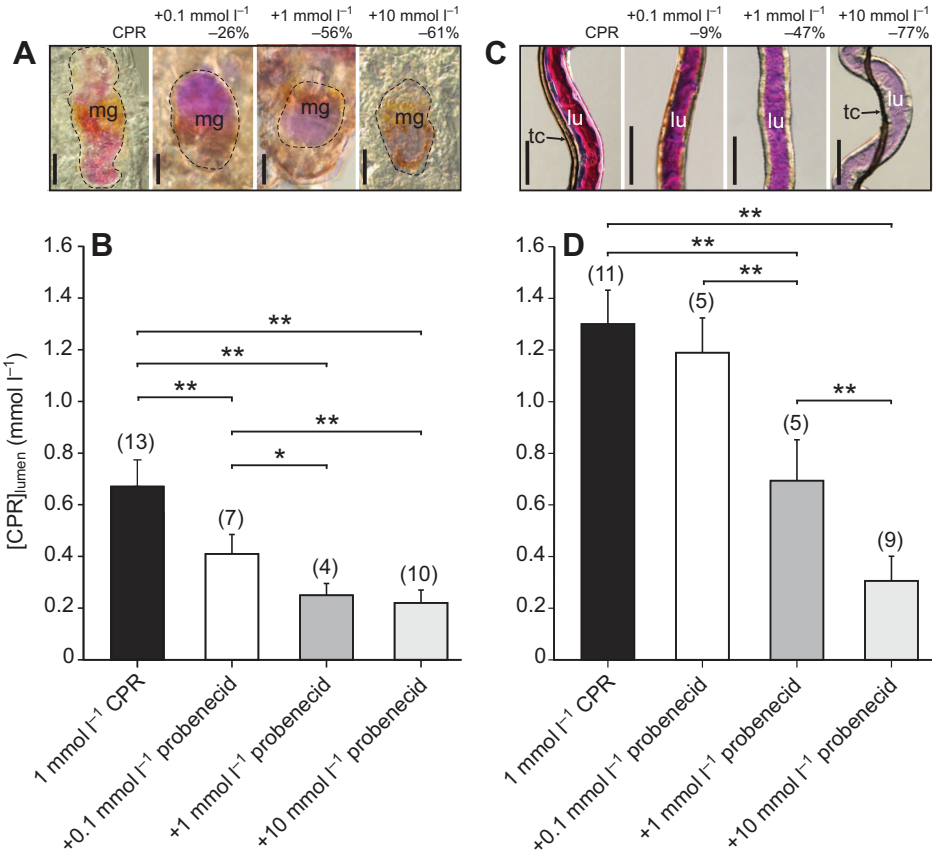

Fig. 7. Titration of the inhibitory effect of probenecid. (A) Representative light micrographs of the tardigrade midgut (mg) following exposure to the different concentrations (0.1, 1 and 10 mmol l^{-1}) of probenecid. The percentage change in CPR concentration, compared with experiments with CPR alone, is noted. Scale bars, 50 μm. (B) Corresponding luminal concentration of CPR in midgut lumen. (C) Representative light micrographs of the insect MTs following exposure to the different concentrations (0.1, 1 and 10 mmol l^{-1}) of probenecid. The percentage change in CPR concentration, compared with experiments on CPR alone, is noted. Scale bars, 100 μm. (D) Corresponding luminal concentration of CPR in the MTs. Asterisks refer to significant difference from 1 mmol l^{-1} CPR alone (*P<0.05, significant; **P<0.01, highly significant). The numbers in parentheses indicate the number of animals tested. For *S. gregaria*, measurements on 3–5 MTs provided a mean for each animal.

gregaria, with a mean concentration of 0.31±0.07 mmol l^{-1} (Fig. 3) and 0.78±0.19 mmol l^{-1} (Fig. 5), respectively. In solutions containing 10 mmol l^{-1} probenecid, dye accumulation was drastically reduced in *H. crispae* by ~61% compared with solutions containing CPR only, averaging merely 0.21±0.05 mmol l^{-1} in luminal concentration (Fig. 3). Dye accumulation was even more reduced in *S. gregaria* (by ~77%), averaging 0.31±0.1 mmol l^{-1} in luminal concentration of probenecid-exposed tubules (Fig. 5). *Halobiotus crispae* became noticeably passive during probenecid exposure (Fig. 4), indicating that, at the high concentration given, probenecid affects processes in addition to organic anion transport. Indeed, there is evidence that probenecid inhibits cellular oxidative metabolism at concentrations of >1 mmol l^{-1} (Masereeuw et al., 2000). Consequently, we titrated the effect of probenecid through successive dilution of the inhibitor, using 0.1 and 1 mmol l^{-1} concentrations, in addition to 10 mmol l^{-1} (Fig. 7). These results show an inverse relationship between

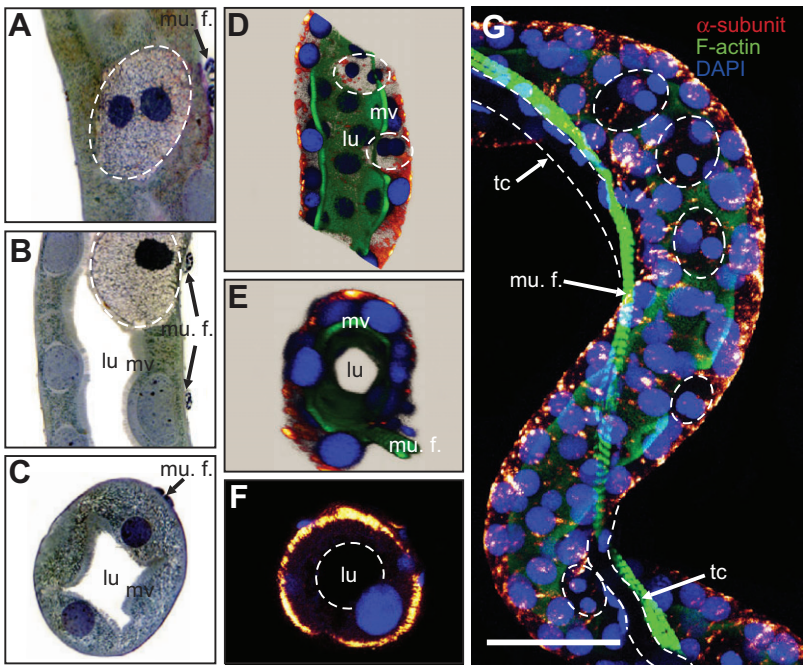

Fig. 8. MT structure and subcellular localization of the α-subunit of the Na^+/K^+-ATPase in *S. gregaria*. (A–C) Sections (2 μm Araldite®) stained with Toluidine Blue. A prominent brushborder is present, as well as several transverse muscle fibers; contraction creates peristalsis, which aids in luminal clearance (visual observation). Mononucleated principal-like cells and double-nucleated cells (dashed circles) were observed. (D) Imaris reconstruction (shadow projection mode) of a partial confocal laser scanning microscopy stack displaying a median, longitudinal cross-section. (E) Imaris reconstruction (shadow projection mode) displaying the tubular lumen. Basal localization of the α-subunit is evident. (F) Immunohistochemistry on paraffin sections confirms the basal localization of the pump. (G) Imaris three-dimensional reconstruction of an entire stack, again showing that the Na^+/K^+-ATPase localizes to the basal plasma cell membranes. A longitudinal muscle fiber (mu. f.) runs the length of the MT. lu, lumen; mv, microvilli. Scale bar, 75 μm.

probenecid concentration and CPR accumulation (and activity; data not shown). At a concentration of 0.1 mmol l^{-1} probenecid, CPR concentration was reduced by 26% with a luminal concentration of 0.42±0.07 mmol l^{-1} in *H. crispae*, and by 9% corresponding to a concentration of 1.2±0.14 mmol l^{-1} in *S. gregaria*. When applied at a concentration of 1 mmol l^{-1}, probenecid reduced CPR concentration by 56% corresponding to a luminal concentration of 0.25±0.05 mmol l^{-1} in *H. crispae*, and by 47% equivalent to 0.7±0.16 mmol l^{-1} in *S. gregaria* (Fig. 7).

Midgut and MT structure and immunostaining

Our transport studies indicate that CPR transport is active and transporter mediated in the midgut of *H. crispae* as well as the MTs of *S. gregaria*. It is well established that the vacuolar H^+-ATPase is important for energizing transepithelial transport in insects, whereas (and in contrast to vertebrate literature) the role of the Na^+/K^+-ATPase is controversial (e.g. Torrie et al., 2004; Beyenbach et al., 2010). The data obtained in the present study using ouabain would suggest that the Na^+/K^+-ATPase is important for CPR transport in both animals. Conversely, ouabain could act as a competitive inhibitor, being transported by the same transporters as CPR. We therefore investigated whether the Na^+/K^+ pump is expressed in the epithelia.

Unfortunately, our attempt to localize Na^+/K^+-ATPase in tardigrades was unsuccessful, both on whole mounts and on paraffin sections, and also in attempts at antigen retrieval.

Our observations on the MTs of *S. gregaria* generally correspond with those previously described (Garret et al., 1988), when focusing on the proximal region and parts of the middle region used in the present study. In general, we observed a prominent brushborder (Fig. 8B,C) and at least two cell types. One type, a principal-like cell, has a single nucleus, while the other is a very large double-nucleated cell (dashed circles in Fig. 8). Cross-striated muscle fibers are present in both the transverse (Fig. 8A,B) and longitudinal direction (Fig. 8E,G). The transverse muscle fibers were only observed in the proximal-most region of the MTs, whereas the longitudinal muscle extended the entire length of the tubule. Visual observations revealed that the transverse muscles aid the clearance of luminal content into the midgut through peristaltic contractions. Our immunocytochemical analysis shows that the Na^+/K^+-ATPase is expressed in the MTs of *S. gregaria*, and is localized to the basal plasma membranes (Fig. 8D–G), although to a lesser extent in the double-nucleated cells (Fig. 8D,G). The localization was confirmed by counterstaining of nuclei by DAPI and staining apical microvilli with phalloidin. This is, to our knowledge, the first study showing immunolocalization of the Na^+/K^+-ATPase in *S. gregaria* MTs. Interestingly, the localization of the transporter substantiates previously published data on isolated basal membrane fractions from MTs and hindgut from *S. gregaria*, which show a prominent Na^+/K^+-ATPase activity (Al-Fifi, 2007).

DISCUSSION

Our study is significant in two main aspects: (i) we are the first to provide evidence for active epithelial transport in tardigrades, and (ii) our data show that tardigrades possess an organic anion transport system. Consequently, our study is important for understanding the evolution of transport systems.

CPR is categorized as a sulphonate, but is known to compete with substrates for both solute carriers (SLC22 and SLC21/SLCO) and ABC transporters in a number of organisms (Pritchard et al., 1999; Linton and O'Donnell, 2000; Chahine and O'Donnell, 2009). In *H. crispae*, CPR transport is ATP dependent (strong inhibition by DNP), probably energized by both the Na^+/K^+-ATPase and the V-type H^+-ATPase (bafilomycin and ouabain reduce CPR accumulation), and possibly transporter mediated (inhibited by the prototypic organic anions PAH and probenecid). The latter suggests that CPR transport is (at least partly) transcellular. Comparing the transport characteristics of CPR transport between the tardigrade midgut and the insect MT reveals a surprisingly similar overall pharmacological profile of the investigated tissues, albeit with markedly higher concentrations of CPR observed in the insect MT. In addition to transcellular transport of the dye, fluid secretion may augment transport of organic anions by convective secretion through the paracellular pathway, and/or reduce diffusive back-flux of organic anions from the tubule lumen to the haemolymph (O'Donnell and Leader, 2006; Chahine and O'Donnell, 2010). Consequently, without a measure of fluid secretion rates, and thereby a measure of net CPR secreted, we cannot conclude whether the difference in relative concentration of CPR in the gut lumen of *H. crispae*, compared with the MTs of *S. gregaria*, is a consequence of a lower dye transport capacity, or whether it reflects differences in fluid transport rates. Also, we cannot be certain that the concentration of CPR in the haemolymph of *H. crispae* is exactly the same as that of the surrounding bath – although, the relatively high dye concentration used along with the small diffusion distances involved, the relatively long exposure time (60 min) and the animal movements that facilitate fluid exchange, would make potential concentration differences negligible.

A vast number of papers have investigated various aspects of organic anion transport in insect MTs. In the present study we observed a mean CPR concentration of 1.3±0.13 mmol l^{-1} when no inhibitors were added. This concentration is elevated above the bath concentration (1 mmol l^{-1}), albeit by a much smaller factor than that reported from other insects (Maddrell et al., 1974; Bresler et al., 1990; Linton and O'Donnell, 2000; Leader and O'Donnell, 2005). However, when exposed to external CPR concentrations of 1, 10 and 100 µmol l^{-1}, the mean luminal CPR concentrations were 0.16±0.03, 0.73±0.09 and 0.89±0.07 mmol l^{-1}, respectively, which is a factor of ~160, 70 and 9 above bath concentrations (Fig. 2). When exposed to a bath concentration of 5 mmol l^{-1} CPR, the mean luminal concentration was 1.79±0.33 mmol l^{-1}, a factor of 2.8 below external concentrations. Consequently, the luminal CPR concentration is maximally elevated (~160-fold) above that in the bathing medium when the latter contains CPR at a concentration of 1 µmol l^{-1}. This concentrative ability is among the highest measured for insects, and is an additional confirmation of active transport of CPR by the insect MTs.

Ouabain, a well-characterized, potent inhibitor of the Na^+/K^+-ATPase, reduced the CPR concentration in both the tardigrade midgut and the insect MTs by ~20–21%. A similar 23% reduction in PAH secretion in the presence of 1 mmol l^{-1} ouabain was reported from MTs of *D. melanogaster* (Linton and O'Donnell, 2000), while fluorescein uptake was reduced in MTs of *Blaberus giganteus* by 30% and in MTs of *Locusta migratoria* by 20%, when ouabain was used at 1 and 0.1 mmol l^{-1}, respectively (Bresler et al., 1990). Our immunocytochemical investigation on the MTs of *S. gregaria* revealed expression of the Na^+/K^+-ATPase in the basal cell membranes, although to a lesser extent in the double-nucleated cells. A basal localization of the Na^+/K^+-ATPase in principal cells of MTs of *D. melanogaster* has previously been reported (Torrie et al., 2004). The fact that there is expression of the Na^+/K^+-ATPase in the basal membranes, and that CPR transport is ouabain sensitive, suggests that the pump is important for CPR transport. Alternatively, it could be argued that the inhibitory effects of ouabain are due to competitive inhibition, rather than to non-competitive inhibition, given the fact that ouabain is actively transported by members of the SLC21/SLCO

subfamily (Oatps) in *D. melanogaster* (Torrie et al., 2004). Whether this is ubiquitous in insects is at present not known; however, several members of the Oatp family are known to transport ouabain in human tissue (see Hagenbuch and Gui, 2008). Competition studies using fluorescently labeled ouabain would help in clarifying this matter. Considering the striking similarities between the pharmacological profiles of the tardigrade midgut and insect MT, it seems reasonable to assume that the Na^+/K^+-ATPase is similarly present in the basal cell membranes of the tardigrade midgut (in spite of failed attempts to localize this transporter). In support of this interpretation is the fact that transcripts for the β-subunit of the Na^+/K^+-ATPase were found in the expressed sequence tags (EST) library from *Hybsibius dujardini* Doyère, 1840 (TardiBASE cluster ID: HDC01733 TardiBASE; http://xyala.cap.ed.ac.uk/research/tardigrades/tardibase.shtml).

Bafilomycin is a specific inhibitor of the V-type H^+-ATPase and was found to reduce CPR accumulation in both *H. crispae* and *S. gregaria*. This observation is consistent with the fact that the V-type H^+-ATPase is viewed as being central to the transport activities of the MT in insects (Weng et al., 2003; Beyenbach et al., 2010) and perhaps also for transport in tardigrades; the B-, C-, D-, E-, G- and H-subunits of the V-type H^+-ATPase were found in the EST library of *H. dujardini* (TardiBASE). Indeed, visual changes to CPR colour were observed in the midgut of *H. crispae* (CPR became purple; data not shown) 10 min post-incubation, suggesting an alkalization of the midgut content. This observation is in accordance with specific inhibition of an apical V-type H^+-ATPase in the tardigrade midgut.

PAH is a prototypical substrate of the classic organic anion transport system (i.e. the SLC22 subfamily) and was shown to be an effective inhibitor of CPR transport in both *H. crispae* and *S. gregaria* (~40% reduction in both animals). This finding is a strong indication that PAH (carboxylate) and CPR (sulphonate) transport are mediated by a common transporter, or alternatively through two separate transport systems overlapping in affinity, in both investigated epithelia. Whether carboxylates (e.g. PAH and probenecid) and sulphonates (e.g. CPR) are handled by a common transporter (Bresler et al., 1990), or by two separate transport systems (Maddrell et al., 1974; Linton and O'Donnell, 2000; Chahine and O'Donnell, 2009), appears to be highly variable and/or species specific. Indeed, transport of PAH and probenecid (carboxylates) and CPR and methotrexate (MTX; sulphonates) are all mediated by one tranporter (OAT1) in humans, but not in rats (Burckardt and Burckardt, 2003), whereas MTX uptake is competitively inhibited by CPR and probenecid, but not PAH in *D. melongaster* (Cahine and O'Donnell, 2009).

Probenecid is implicitly regarded as a competitive inhibitor of organic anion transport, and has been shown to compete with both solute carrier and ABC transporter substrates (Bresler et al., 1990; Linton and O'Donnell, 2000; Neufeld et al., 2005; Leader and O'Donnell, 2005). It is typically applied in a concentration of 1 mmol l^{-1}; here, we examined the effects of probenecid on CPR transport in the range 0.1–10 mmol l^{-1}. In *H. crispae*, probenecid reduced CPR accumulation by 26%, 56% and 61% at a concentration of 0.1, 1 and 10 mmol l^{-1}, respectively, whereas the corresponding reduction in CPR concentration was 9%, 47% and 77% in *S. gregaria*. In the presence of very high probenecid concentrations (10 mmol l^{-1}), we observed an almost total loss of motility in *H. crispae*. Indeed, the level of animal movement was comparable to that in treatments containing DNP. This observation suggests that the drug affected processes in addition to CPR transport at the given concentration. In fact, when applied at concentrations of 1 mmol l^{-1} or above, probenecid is reported to induce a range of non-specific effects presumably initiated by the uncoupling of mitochondrial oxidative phosphorylation (Masereeuw et al., 2000). It is therefore possible that the reduction in CPR transport is non-specific when the drug is applied in concentrations ≥1 mmol l^{-1}. At lower concentrations of probenecid (<1 mmol l^{-1}), however, animal motility was regained (in a concentration-dependent manner), and was not significantly different from that in solutions containing CPR only (data not shown). Noticeably, the relative inhibition of CPR transport by probenecid was of a greater magnitude in the midgut of *H. crispae* than in the MT of *S. gregaria* at the lower (specific) concentrations of probenecid. This could indicate a higher specificity for probenecid by the tardigrade transporter(s) compared with that of *S. gregaria*.

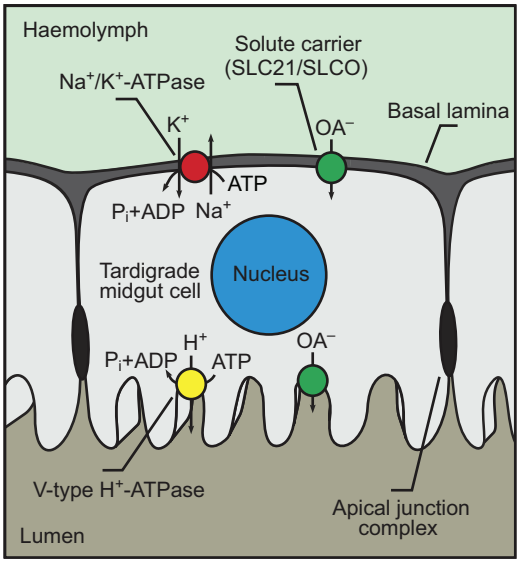

Fig. 9. Tentative model of the tardigrade midgut cell derived from the current study on *H. crispae*. Based on the pharmacological profile of the tardigrade midgut epithelium, both the Na^+/K^+-ATPase and the V-type H^+-ATPase are potential candidates for providing an electrochemical driving force for the transepithelial movement of organic anions. Transport characteristics and the presence in tardigrade EST libraries suggest that a member of the SLC21/SLCO transporter family may mediate the basolateral entry of organic anions in tardigrades. The exact coupling between electrochemical gradients generated by the pumps and transport of ions, as well as the nature of the apical exit step, are not known.

The tardigrade midgut cell transports organic anions

The observed CPR transport by the tardigrade midgut and insect MT presumably takes place because CPR is structurally and chemically similar to compounds encountered in the natural habitat of these animals. In terrestrial insects, organic anion transport seems especially important for excreting xenobiotics such as plant toxins and pesticides (Lanning et al., 1996; Torrie et al., 2004; Neufeld et al., 2005; Buss and Callaghan, 2008); however, a similar physiological requirement is not as readily apparent in tardigrades. The handling of endogenous toxins derived from metabolism, in contrast, is a ubiquitous prerequisite for all organisms, and it is likely that this is the primary function of the organic anion transport system in tardigrades.

Because of the broad overlap in substrate specificity between the different groups of organic anion transporters, we cannot definitively identify, on the basis of the data presented here, the type of transporter(s) responsible for the observed CPR transport. However, searching the EST library of *H. dujardini* (TardiBASE) revealed that putative genes of Oatp (cluster ID: HDC00927) (SLC21/SLCO protein family), P-gp (cluster ID: HDC01698) and Mrps (cluster

ID: HDC00004, HDC02687 and HDC03352) (ABCB and ABCC protein families) are present and expressed in tardigrades – just as they are in insects (Maddrell et al., 1974; Lanning et al., 1996; Bresler et al., 1990; Linton and O'Donnell, 2000; Torrie et al., 2004; Neufeld et al., 2005; Leader and O'Donnell, 2005; O'Donnell and Leader, 2006; Chahine and O'Donnell, 2009). Comparing the transport characteristics of the different groups of transporters reveals that it is unlikely that members of the ABC transporter family (i.e. P-gps and Mrps) mediate the basolateral entry of CPR in *H. crispae* and *S. gregaria*. This assumption is based on the fact that P-gp transporters predominantly recognize large cationic species (Russel, 2010), and because Mrp transporters mainly transport large (>500 Da) polyvalent type II organic anions (Wright and Dantzler, 2004; Russel, 2010) – the apical transporter MRP2, however, was shown to transport PAH in humans (see below). In addition, PAH does not compete with MTX (an Mrp substrate) in *D. melongaster*, suggesting distinct transport systems for these compounds in insects (Chahine and O'Donnell, 2009). In contrast, both members of the SLC22 and SLC21/SLCO protein families (Oats and Oatps) have been reported to transport all the investigated organic anions, including probenecid, PAH and CPR (Pritchard et al., 1999; Lee and Kim, 2004; Torrie et al., 2004). Therefore, it seems probable that an Oat or Oatp homologue mediates the basolateral entry of CPR in the tardigrade midgut cell as well as the insect MTs (Fig. 9). At present, our data do not allow us to make assumptions on the nature of the luminal exit. Evidence suggests that MRP2 is involved in the efflux of organic anions across the brush-border membrane in the human kidney proximal tubule (Leier et al., 2000), and a similar situation could exist in tardigrades and insects. Our data tentatively suggest that the V-type H^+-ATPase, and perhaps also the Na^+/K^+-ATPase, provide the driving force for the transepithelial transport of organic anions in both *H. crispae* and *S. gregaria* (Fig. 9). It is likely that a large lumen-positive transepithelial potential, generated by an apical H^+-ATPase provides a significant driving force for the accumulation of anions in the lumen. However, the exact coupling between electrochemical gradients generated by the pumps and transport of the ions is not known.

In future studies, it would be of interest to investigate whether substrates of Mrps (e.g. Texas Red and MTX) also accumulate in the midgut of tardigrades, and whether transport of these anions occurs *via* a separate or a common transporter to CPR. Similarly, an understanding of the electrophysiological properties of the midgut epithelium would be highly relevant in our ongoing struggle to understand the complex biology of these amazing animals.

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