A novel role for a *Drosophila* homologue of cGMP-specific phosphodiesterase in the active transport of cGMP

Jonathan P. DAY*, Miles D. HOUSLAY† and Shireen-A. DAVIES*

*Institute of Biomedical and Life Sciences, Division of Molecular Genetics, University of Glasgow, Glasgow G11 6NU, U.K., and †Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow G11 6NU, U.K.

cGMP was first discovered in urine, demonstrating that kidney cells extrude this cyclic nucleotide. *Drosophila* Malpighian tubules provide a model renal system in which a homologue of mammalian PDE (phosphodiesterase) 6 is expressed. In humans, this cG-PDE (cGMP-specific PDE) is specifically expressed in the retinal system, where it controls visual signal transduction. In order to gain insight into the functional role of *DmPDE6* (*Drosophila* PDE6-like enzyme) in epithelial function, we generated transgenic animals with targeted expression of *DmPDE6* to tubule Type I (principal) cells. This revealed localization of *DmPDE6* primarily at the apical membranes. As expected, overexpression of *DmPDE6* resulted in elevated cG-PDE activity and decreased tubule cGMP content. However, such targeted overexpression of *DmPDE6* creates a novel phenotype that manifests itself in inhibition of the active transport and efflux of cGMP by tubules. This effect is specific to *DmPDE6* action, as no effect on cGMP

transport is observed in tubules from a bovine PDE5 transgenic line which display reduced rates of fluid secretion, an effect not seen in *DmPDE6* transgenic animals. Specific ablation of *DmPDE6* in tubule principal cells, via expression of a targeted *DmPDE6* RNAi (RNA interference) transgene, conferred increased active transport of cGMP, confirming a direct role for *DmPDE6* in regulating cGMP transport in tubule principal cells. Pharmacological inhibition of *DmPDE6* in wild-type tubules using the cG-PDE inhibitor, zaprinast, similarly results in stimulated cGMP transport. We provide the first demonstration of a novel role for a cG-PDE in modulating cGMP transport and efflux.

Key words: cGMP, *Drosophila*, phosphodiesterase 6 (PDE6), renal epithelium, RNA interference (RNAi).

INTRODUCTION

The maintenance of intracellular levels of the second messenger cGMP consists of its synthesis by guanylate cyclases, hydrolysis by cyclic nucleotide PDEs (phosphodiesterases) and the excretion (efflux) of cGMP. The existence of unaltered cGMP in urine as a result of efflux led to its discovery and identification [1]; furthermore, the excretion of cGMP may be important in health and disease [2,3]. Studies of cGMP efflux have shown that this is an active transport process that is dependent on ATP and involves membrane proteins [4,5]. Candidates for such transport proteins include members of the MRP (multidrug-resistance protein) family and also OAT1, a member of the organic anion transporter family [4].

The use of genetic model organisms (e.g. *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus*) has proved a powerful tool in the demonstration of *in vivo* roles for cGMP signalling [6–10]. Furthermore, the recent characterization of novel PDEs from organisms such as *Trypanosoma brucei* has stimulated interest in the design of novel selective therapeutics [11,12]. Given that relatively little is known about *in vivo* roles for cG-PDEs (cGMP-specific PDEs), work in invertebrate model organisms provides a novel means of assessing functional roles.

The *Drosophila* Malpighian (renal) tubule, a fluid-transporting, osmoregulatory and xenobiotic-transporting tissue is modulated by cGMP [10]. The transgenic tools available to engineer *Drosophila* [13] allow a sophisticated system for ectopic expression or disruption of genes of choice in particular cell types or tissues in the intact animal and scan for phenotype change.

The *Drosophila* genome encodes several close homologues of vertebrate cG-PDEs, including a homologue of PDE6, encoded by the gene *CG8279* [14]. Alignments of *Drosophila* and human PDEs suggest that the *Drosophila* PDE6-like enzyme encoded by *CG8279* is closely related to both PDE5 and PDE6, although a specific prenylation motif at the N-terminal present in both human PDE6 β and *Drosophila* PDE6 (but not PDE5), suggest that the *Drosophila* enzyme is not a PDE5 homologue [14]. Therefore *CG8279* was assigned as a PDE6-encoding gene [14]. Biochemical characterization of *DmPDE6* (*Drosophila melanogaster* PDE6) shows that this is a cG-PDE, exhibits a high K_m for cGMP and is sensitive to inhibition by both zaprinast and sildenafil [14].

Functional roles for mammalian PDE6 have been most studied in the context of phototransduction [15–17]. However, the *DmPDE6* homologue is expressed in both the head and tubules [14]. Insect phototransduction does not rely on the cGMP cascade and, as such, *DmPDE6* expressed in the head is probably associated with other physiological functions; the expression of *DmPDE6* in tubules implies a functional role(s) for PDE6-like enzymes outside the eye. Vertebrate PDE6 expression has been documented in chick pineal gland [18] and in mouse F9 embryonic stem cells [19]. Thus it is clear that functional non-retinal PDE6 in vertebrate systems does occur, but its importance is not known.

The tubule provides a powerful *in vivo* model in which to assess function of novel cG-PDEs via targeted expression and ablation. We exploit this in the present study to reveal that *DmPDE6* has a novel role in achieving the specific regulation of active transport of cGMP in the renal tubule.

Abbreviations used: ABC, ATP-binding cassette; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; MRP, multidrug-resistance protein; OAT, organic anion transporter; PDE, phosphodiesterase; cA-PDE, cAMP-dependent PDE; cG-PDE, cGMP-specific PDE; *DmPDE6*, *Drosophila melanogaster* PDE6; PKG, cGMP-dependent protein kinase; RNAi, RNA interference; UAS, upstream activating sequence; UTR, untranslated region.

¹ To whom correspondence should be addressed (email s.a.davies@bio.gla.ac.uk).

EXPERIMENTAL

In situ hybridizations

The protocol adopted for *in situ* hybridization of *CG8279* in Malpighian tubules was based on that described in [20]. The 3'-UTR (untranslated region) of *CG8279* [14] was cloned into the pCR2.1 vector (Invitrogen) and digoxigenin-labelled probes generated by *in vitro* transcription. Intact tubules from 7-day-old adult flies were incubated with appropriate reagents in Eppendorf tubes, processed on to glass slides under coverslips and viewed with a Zeiss Axiocam imaging system.

Confocal microscopy

Tubules expressing GFP (green fluorescent protein)-tagged *DmPDE6* in principal cells were dissected in Schneider's medium (Invitrogen) and mounted on poly(L-lysine)-coated slides. Tubules were fixed and permeabilized as described previously [21] and were incubated for 4 h in 1 μ M Texas Red-conjugated phalloidin (Sigma), an F-actin stain indicative of tubule apical membrane [22], in PBS with 0.3 % (v/v) Triton X-100. Tubules were then stained with DAPI (4,6-diamidino-2-phenylindole) to allow visualization of nuclei [23]. Slides were mounted in Vectashield (Vector Laboratories). Samples were imaged using a Zeiss Pascal confocal system using a helium/neon 543 nm laser with a 561–625 nm band pass filter for Texas Red-conjugated phalloidin, and an argon 488 nm laser with a 505–530 nm band pass filter for GFP fluorescence. For visualization of DAPI, a pseudo-DAPI technique involving excitation by a UV source and image capture by confocal photomultipliers was used. DAPI images were merged with the other channels retrospectively, using Adobe Photoshop 7.0. A 63 $\times$ objective was used in all cases.

Generation of transgenic lines

Full-length *CG8279* was cloned as described previously [14] and sub-cloned into pP{EGFP-UAST} vector [24], which results in UAS (upstream activating sequence)–GFP transgene upon GAL4-driven expression. For generation of RNAi (RNA interference) *DmPDE6* flies, an inverted repeat construct directed against *CG8279* was made. A forward 900 bp fragment was amplified from the 3'-UTR of the *CG8279* transcript with PCR primers PDE6iFEco and PDE6iRBglA (see Supplementary Table 1 at <http://www.BiochemJ.org/bj/393/bj3930481add.htm>) encoding EcoRI and BglII restriction sites. The reverse 500 bp fragment, identical with the first 500 bp of the forward fragment, was amplified using PCR primers PDE6iFNot and PDE6iRBglB (see Supplementary Table 1 at <http://www.BiochemJ.org/bj/393/bj3930481add.htm>). These fragments were then subcloned sequentially into pP{UAST} [13]. Constructs were used to transform *Drosophila* embryos according to standard techniques as described previously [23].

Drosophila stocks

All strains were maintained on standard *Drosophila* diet at 25 °C and 55 % humidity, on a 12:12 photoperiod. *Drosophila* lines used in this study were: Oregon R, wild-type strain; *w¹¹¹⁸* (parental strain used for transformation of embryos); UAS-GFP:PDE6; UAS-RNAi PDE6 (transgenic lines bearing either overexpression or RNAi constructs for *CG8279*, *DmPDE6*, generated for the present study); UAS-PDE5 (transgenic line for targeted expression of bovine PDE5 [23]); c42 (GAL4 enhancer trap line, used to drive expression of UAS constructs in only tubule principal cells [23,25]); P{GAL4-da.G32} (daG32 *daughterless* ubiquitous GAL4 driver; <http://flybase.net/bin/fbidq.html?FBtp0001168>); P{Act5c-GAL4} (actin-GAL4 ubiquitous GAL4 driver; <http://flybase.net/bin/fbidq.html?FBtp0018505>).

While both daG32 and actin-GAL4 drive ubiquitous expression, adult emergence rates differ between the progeny of crosses from these drivers and UAS lines, and so, where required, actin-GAL4 was utilized.

The following lines were also used, which were the progeny of crosses between c42 and specific UAS transgenic lines: c42/UAS-GFP:PDE6; c42/UAS-RNAi PDE6; c42/UAS-PDE5 [23]. Additionally, daG32/UAS-GFP:PDE6 was also used, derived from crosses between daG32 and UAS-GFP:PDE6; as well as actin-GAL4/UAS-RNAi PDE6, derived from crosses between actin-GAL4 and UAS-RNAi PDE6. Various balancer lines were also used to establish chromosomal location of transgenes in UAS lines generated (results not shown). Adults were used at 7 days post-emergence for all experiments.

Western blotting

Tubule samples were homogenized, on ice, in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 % (v/v) Igepal CA-630 [octylphenyl-poly(ethylene glycol)] (Sigma) and 1 μ g/ml PMSF/protease inhibitors obtained as protease inhibitor cocktail (Sigma). Protein samples (20 μ g/lane) were separated by SDS/PAGE (10 % gels) and Western blotting was carried out using the ECL[®] (enhanced luminescence) system (GE Healthcare) according to standard protocols. A 1:5000 dilution of anti-GFP antibody (Invitrogen) followed by a 1:5000 dilution of anti-mouse secondary antibody (GE Healthcare) was used.

PDE assays

Assays for either cG-PDE or cA-PDE (cAMP-specific PDE) in whole flies and in tubules were performed essentially as described previously [14,26] using five whole flies or 200 tubules (approx. 50 μ g of protein) for each sample, homogenized and assayed in 37 MBq/ml of tritiated cGMP or cAMP (GE Healthcare) in 100 μ l KHEM buffer [50 mM KCl, 10 mM EGTA, 1.92 mM MgCl₂, 1 mM DTT (dithiothreitol), 50 mM Hepes, pH 7.21, 1 μ l of Sigma P8340 protease inhibitor cocktail]. A substrate concentration of 10 μ M of either cGMP or cAMP, as required, was used in reactions, as endogenous *Drosophila* cG-PDEs are high-*K_m* enzymes [14]. PDE activity was expressed as pmol of cGMP or cAMP/min per mg of protein.

cGMP-dependent protein kinase assays

Assays for *Drosophila* tubules were carried out essentially as described previously [26,27] using the following assay reaction mixture, prepared with and without the addition of 10 μ M cGMP: 20 mM Tris/HCl, pH 7.5, 10 mM magnesium acetate, 1 mM EDTA, 2 mM EGTA, 0.2 μ g/ml Glasstide {RKRSRAE, a heptapeptide PKG (cGMP-dependent protein kinase)-specific substrate (Calbiochem) [28]}, 20 μ M ATP, 0.5–2 μ l of [α -³²P]ATP (370 MBq/ μ l, to an approximate specific activity of 4000 c.p.m./pmol; GE Healthcare), 1 nM PKI (protein kinase A inhibitor: TYADFIASGRTGRRNAI-NH₂), 1 mM zaprinast and 1 mM DTT.

Fluid transport assays

Intact Malpighian tubules were isolated from parental lines (c42), c42/UAS-GFP:PDE6 transgenic lines or wild-type Oregon R, into 10 μ l drops of Schneider's medium under liquid paraffin. Fluid transport rates were measured in tubules as detailed previously [29]. Basal rates of fluid transport were measured for 30 min, tubules were treated with cGMP (0.1 mM), and stimulated secretion rates were measured for 40 min. cGMP enters

Malpighian tubules via specific transporters [30] and is a known stimulant of fluid transport [31]; 0.1 mM cGMP elicits maximum fluid transport responses compared with controls [32]. Data are expressed as means $\pm$ S.E.M. fluid transport rates (nl/min).

cGMP transport assays

Malpighian tubules from 7-day-old adult flies were set up as for a standard fluid transport assay, as described previously [29]. Preliminary cGMP transport experiments, based on the methodology for studying ouabain transport [33], indicated that active transport of cGMP occurs at 1 or 100 μ M cGMP (transport ratios of 1.98 ± 0.29 for 1 μ M cGMP; 1.88 ± 0.32 for 100 μ M cGMP) in wild-type (Oregon R) tubules. All further experiments were carried out at 100 μ M cGMP in the presence of 0.2 μ Ci of [3 H]cGMP (GE Healthcare). Tubules were allowed to secrete for 1 h, and diameters of the secreted droplets were measured. Secreted droplets, and a 1 μ l aliquot of each bathing droplet, were then removed to Eppendorf tubes containing scintillation fluid. The rate of cGMP transport, in fmol/min, was calculated by using the equation: Rate = $S[\text{substrate}]/Bt$; where S is counts in the secreted drop (c.p.m.), $[\text{substrate}]$ is cGMP concentration (μ M), B is counts in the bathing drop (c.p.m.), and t is time (min). The transport ratio was calculated by dividing the concentration of [3 H]cGMP in the secreted droplet by the concentration of [3 H]cGMP in the bathing droplet. cGMP is an uncharged molecule in solution, so a transport ratio greater than 1 is indicative of active transport of cGMP by the tubule. However, if active transport of cGMP across the tubule epithelium did not occur, the transport ratio would be equal to or less than 1. These experiments were performed on tubules from UAS-GFP:PDE6 and c42/UAS-GFP:PDE6, UAS-PDE5 and c42/UAS-PDE5 [23], UAS-RNAi PDE6 and c42/UAS-RNAi PDE6, and Oregon R. Results are expressed as mean units $\pm$ S.E.M. ($n = 12$) of either the rate of transport (fmol cGMP/min) or the transport ratio.

Immunoprecipitations

For each experiment, 15 flies were homogenized in 10% (v/v) glycerol, 1% (v/v) Triton X-100, 150 mM NaCl and 50 mM Hepes, pH 7.5, with 10 μ l protease inhibitor cocktail (Sigma), and debris was removed by centrifugation at 15 000 g for 10 min at 4°C. Protein was quantified and adjusted to 900 μ g per sample. DmPDE6 was immunoprecipitated using a polyclonal antibody raised against a 15-amino-acid peptide sequence from the non-conserved C-terminal region of the protein [14] using 30 μ g of antibody per immunoprecipitation reaction, allowing complete immunoprecipitation of DmPDE6 as described previously [14]. cG-PDE activity was assayed at 10 μ M cGMP [14]. Background PDE activity was established via immunoprecipitated assays with pre-immune serum and subtracted from sample values.

Statistics

Where appropriate, statistical significance was assessed using Student's t test for unpaired samples, taking $P < 0.05$ as the critical value.

RESULTS

DmPDE6 is localized to the apical membrane tubule principal cells

The *D. melanogaster* Malpighian tubule comprises a number of genetically and functionally distinct domains (Figure 1A, panel i). *In situ* hybridization was used to show that endogenous CG8279-encoded PDE6 is expressed in all cells in the main segment in

Drosophila tubules (Figure 1A, panel ii), whereas an antisense probe showed no signal (Figure 1A, panel iii). In order to determine the function of this PDE6-like enzyme *in vivo*, the *Drosophila* Malpighian (renal) tubules were utilized as an organotypic screen.

Transgenic flies expressing GFP-tagged DmPDE6 under UAS control were generated, which allowed targeted expression of DmPDE6 in specific cell types via GAL4 enhancer trap drivers. Specific expression of DmPDE6 in principal cells in the tubule main segment is effectively achieved using the c42 GAL4 driver [23,25].

Localization of DmPDE6 *in vivo* was assessed by confocal imaging of c42/UAS-GFP:PDE6 tubules (Figure 1B). DmPDE6 is localized mainly at the apical plasma membrane, indicated by phalloidin staining of F-actin, an established apical membrane marker [22], with fainter staining at the basolateral membrane.

The GFP-tagged DmPDE6 transgene is effective *in vivo*

Generation of transgenic lines for tagged DmPDE6 (UAS-GFP:PDE6) allowed cell-specific targeted expression of these enzymes in tubules under GAL4 control. However, in order to establish that the GFP-tagged DmPDE6 transgenes encoded a functional cG-PDE, the UAS-GFP:PDE6 flies were crossed with the ubiquitous GAL4 driver, daG32. Whole adult flies were then assayed for cG-PDE and cA-PDE activity. Results in Figure 2(A) show that cG-PDE activity of daG32/UAS-GFP:PDE6 flies increased 3.5-fold compared with parental lines (cG-PDE activity of parental daG32 and UAS-GFP:PDE6 lines was approx. 450 pmol of cGMP/mg of protein per min; cG-PDE activity of daG32/UAS-GFP:PDE6 flies was approx. 1500 pmol of cGMP/mg of protein per min). No significant change in cAMP-PDE activity was observed in flies expressing DmPDE6 (Figure 2B), consistent with the role of DmPDE6 as a cG-PDE [14]. As the DmPDE6 transgene was functional as a cGMP-specific PDE in the whole fly, the function of GFP-tagged DmPDE6 was assessed in tubule principal cells using the c42 GAL4 driver. Tubules expressing targeted GFP-tagged DmPDE6 (inset, Figure 2C) displayed an approx. 20-fold increase in cG-PDE activity at approx. 1000 pmol of cGMP/min per mg of protein (Figure 2C) compared with parental lines.

Overexpression of DmPDE6 in tubule principal cells results in an approx. 25% reduction in tubule cGMP content (Figure 2D). Thus targeted expression of the DmPDE6 transgene *in vivo* results in increased cG-PDE activity and resultant reduction in cGMP content in tubules.

In order to rule out effects of DmPDE6 overexpression on other components of the cGMP pathway that are known to be operational in tubules, for example PKG [27], measurements of endogenous PKG activity were made on tubules from c42 and UAS-PDE6 parental lines, and on c42/UAS-PDE6 tubules. Results in Figure 2(E) show that PKG activity is unaffected by overexpression of DmPDE6.

Exogenous cGMP enters principal cells of the Malpighian tubule via cyclic nucleotide transporter(s) [30] and results in stimulated fluid transport [34]. Furthermore, modulation of PDE activity either by genetic or pharmacological intervention or via ectopic expression of bovine PDE5 in tubule principal cells has impact on fluid transport rates in these cells [23,26,35]. Thus in order to ascertain whether *Drosophila* PDE6 also plays a role in the regulation of fluid transport, the basal and cGMP-stimulated secretion rate of tubules overexpressing DmPDE6 in the principal cells was measured. In spite of markedly increased cG-PDE activity and decreased cGMP content in c42/UAS-PDE6 tubules, no significant change in either basal or stimulated

Figure 1 *DmPDE6* is localized to the main segment apical membrane in a polarized epithelium

(A) Panels i–iii: *in situ* hybridization of *CG8279* in intact tubules. Panel i. Diagrammatic representation of the *Drosophila* tubule, indicating regions and cell types from [42]. Numbers of cells within each region are indicated. Panel ii. Tubules treated with anti-sense RNA probe, showing region-specific expression of *DmPDE6* in the main segment of the tubule. Panel iii. No staining was observed in control tubules treated with control sense RNA probe. Tubule diameter is $35\ \mu\text{m}$ in all images. (B) Transgenic flies expressing wild-type GFP-tagged *DmPDE6* under UAS control were generated and crossed with the GAL4 driver *c42*, which directs transgene expression to principal cells in the main segment. Tubules from adult *c42/UAS-GFP:PDE6* progeny were dissected, stained for the apical membrane marker F-actin using phalloidin, and counterstained with the nuclear stain DAPI, which allows principal and stellate cells to be distinguished, based on nuclear size [23]. Scale bar, $10\ \mu\text{m}$; tubule diameter can be taken as $35\ \mu\text{m}$. In the image shown, green fluorescence denotes *DmPDE6*, F-actin is stained red, and nuclei are stained blue. No GFP signal was observed in parental *c42* tubules, or in parental UAS-GFP:PDE6 (results not shown).

fluid secretion rates was observed when compared with wild-type tubules (Figure 2F), thus *DmPDE6* does not modulate fluid transport.

Overexpression of *DmPDE6* inhibits cGMP transport across the tubule

The maintenance of regulatory intracellular cyclic nucleotide levels in renal cells is, presumably, achieved by a combination of both cellular efflux and hydrolysis by PDEs. Extruded cyclic nucleotides are not rapidly metabolized into their cognate 5'-mononucleotides, and thus the stability of cGMP transported by the kidney has allowed monitoring of plasma and urine cGMP levels to be utilized as a biomarker of disease [2,3]. This process is conserved in *Drosophila*: cGMP, but not cAMP, has been found to be one of the major components of fluid secreted by Malpighian tubules, occurring at an estimated concentration of $8.3\ \mu\text{M}$ [36].

To test whether *DmPDE6* activity in tubule principal cells regulates the magnitude of active transport of cGMP, cGMP transport assays were performed on parental UAS-GFP:PDE6 tubules and on *c42/UAS-GFP:PDE6* tubules. Targeted expression of *DmPDE6* in principal cells results in a reduction in cGMP transport rates to $32 \pm 5\%$ of parental UAS-GFP:PDE6 tubules (Figure 3A). This is reflected by the inhibition of active transport, whereby the transport ratio is reduced from 1.54 ± 0.16 by UAS-GFP:PDE6 tubules to 0.39 ± 0.03 by *c42/UAS-GFP:PDE6* tubules (Figure 3B).

Increased bovine PDE5 activity in tubule principal cells, via ectopic expression of a PDE5 transgene, has been shown to severely reduce cGMP content and to modulate fluid transport [23]. However, the results in Figures 3(C) and 3(D) show that PDE5 overexpression does not affect either cGMP transport rates or transport ratios compared with parental UAS-PDE5 tubules. This indicates that the effects of *DmPDE6* on cGMP transport are

Figure 2 Targeted expression of *DmPDE6* increases cG-PDE activity but does not affect PKG activity, or fluid transport rates

DmPDE6 was overexpressed throughout the fly using the ubiquitous GAL4 driver *daG32*, and homogenates of adults were assayed for PDE activity at $10 \mu\text{M}$ substrate concentration. (A) cG-PDE activity; (B) cA-PDE activity. Data for parental lines, *daG32* and *UAS-GFP:PDE6*, and progeny of crosses (*daG32/UAS-GFP:PDE6*) are shown. (C) cG-PDE activity was also determined in tubules overexpressing *DmPDE6* (*c42/UAS-GFP:PDE6*), as well as in parental lines. Inset, Western blot using anti-GFP antibody of parental *UAS-GFP:PDE6* tubules (lane A) and *DmPDE6*-overexpressing tubules (*c42/UAS-GFP:PDE6*) (lane B), showing a specific band of approx. 160 kDa for *DmPDE6* [14] only in *c42/UAS-GFP:PDE6* tubules. For (A)–(C), enzyme activity is expressed as means $\pm$ S.E.M. (pmol of cGMP or cAMP hydrolysed/mg of protein

Figure 3 *DmPDE6* modulates active transport of cGMP

(A) cGMP transport rates were measured in *UAS-GFP:PDE6* and *c42/UAS-GFP:PDE6* tubules, and are means $\pm$ S.E.M. expressed as fmol of cGMP/min ($n = 12$). (C) Mean rates $\pm$ S.E.M. of cGMP transport for *UAS-PDE5* and *c42/UAS-PDE5* tubules, expressed as fmol of cGMP/min ($n = 12$). (B, D) In order to establish that the cGMP transport observed was due to active transport, measurements of transport ratio were made for tubules from the lines above. Transport ratios less than 1 are not considered to be due to active transport. Results are means $\pm$ S.E.M., expressed as units of transport ratio ($n = 12$).

not due to a general action of cG-PDEs, but, rather, are specific to *DmPDE6*.

Ablation of *DmPDE6* reverses the cGMP transport phenotype

Overexpression of *DmPDE6* results in a transport ratio of < 1 and so eliminates active transport of cGMP across the Malpighian tubule. In order to verify that the modulation of cGMP transport is specifically due to *DmPDE6*, transport assays were conducted using tubules from a transgenic RNAi *DmPDE6* line. Results shown in Figures 4(A) and 4(B) show that successful knock-down of PDE6 activity occurs as a result of targeted expression of a *DmPDE6* RNAi construct. Both immunoprecipitation of *DmPDE6* from the whole fly using anti-*DmPDE6* antibody [14], (Figure 4A); and assay for cG-PDE activity in tubules [23], (Figure 4B) demonstrate a significant reduction in *DmPDE6* activity in the RNAi line. Given that cG-PDE activity is reduced in tubules to the same extent as *DmPDE6* activity is inhibited in the whole fly (Figure 4A), it is unlikely that other cGMP-hydrolysing PDEs are affected by the ablation of *DmPDE6*. While this RNAi line does not constitute a complete knockdown of *DmPDE6*, this specific reduction in *DmPDE6* activity is nevertheless sufficient

per min) ($n = 3$). (D) cGMP content in *UAS-GFP:PDE6* and *c42/UAS-GFP:PDE6* tubules. cGMP content was measured by RIA using a commercial kit (GE Healthcare) as described previously [31]. Results are means $\pm$ S.E.M. (fmol of cGMP/20 tubules) ($n = 5$). (E) PKG activity was measured in tubules from parental *UAS-GFP:PDE6* and *c42*, and *c42/UAS-GFP:PDE6*. Results are means $\pm$ S.E.M. PKG activity (pmol of ATP/mg of protein per min) ($n = 6$). (F) Fluid transport rates of Malpighian tubules expressing *DmPDE6* in principal cells (*c42/UAS-GFP:PDE6*) and parental tubules (*UAS-GFP:PDE6*) were monitored before and after addition of 0.1 mM cGMP [29]. Fluid transport rates are means $\pm$ S.E.M. expressed as nl/min ($n = 6$ –8). For all panels, significance was verified by Student's *t* test (unpaired samples) comparing each *c42/UAS* line with appropriate parental *UAS* lines (*, $P < 0.05$).

Figure 4 Ablation of *DmPDE6* activity stimulates cGMP transport

(A, B) Successful ablation of *DmPDE6* *in vivo* using RNAi. (A) Immunoprecipitated *DmPDE6* activity in whole actin-GAL4/UAS-RNAi PDE6 flies; (B) cG-PDE activity in c42/UAS-RNAi PDE6 tubules. Owing to the amounts of starting material required for immunoprecipitation (IP) experiments, IP studies were only carried out on whole fly preparations, in which RNAi expression is driven by the ubiquitous actin-GAL4 driver. Results are means $\pm$ S.E.M. of cG-PDE activity (pmol of cGMP/mg of protein per min) ($n = 6$). (C, D) cGMP transport assays were performed on UAS-RNAi PDE6 and c42/UAS-RNAi PDE6 tubules. (C) Mean $\pm$ S.E.M. transport rates (fmol of cGMP/min) ($n = 19$). (D) Mean $\pm$ S.E.M. transport ratios (units of transport ratio) ($n = 12$). (E, F) cGMP transport assays were performed on wild-type (Oregon R) tubules in the presence of 5 μ M zaprinast. This concentration of zaprinast inhibits more than 80% of PDE6 activity, whereas approx. 50% of PDE11 cG-PDE activity and less than 10% of PDE1 cG-PDE activity are inhibited [14]. (E) Mean $\pm$ S.E.M. transport rates (fmol of cGMP/min) ($n = 19$). (F) Mean $\pm$ S.E.M. transport ratios (units of transport ratio) ($n = 12$). (G) Mean maximum fluid transport rates (fluid transport rates measured for 40 min) of Oregon R tubules in the absence and presence of 5 μ M zaprinast. Fluid transport rates are means $\pm$ S.E.M., expressed as nl/min ($n = 6$).

to stimulate both transport rates and active transport of cGMP (Figures 4C and 4D).

The effect of *DmPDE6* knockdown on cGMP transport was phenocopied by treating wild-type tubules with the pharmacological inhibitor of PDE6, zaprinast [14]. Results in Figures 4(E) and 4(F) show that zaprinast potentiates both cGMP transport rate and active transport in wild-type tubules. Importantly, treatment of tubules with this concentration of zaprinast did not alter the fluid secretion rate, compared with untreated tubules (Figure 4G).

DISCUSSION

Sequence comparisons and biochemical analysis show CG8279-encoded PDE6 to be the closest *Drosophila* homologue of mammalian PDE5, and a close homologue of mammalian retinal PDE6 β [14]. Although CG8279 may encode an orthologue of mammalian PDE5/PDE6, the presence of a prenylation motif in both *DmPDE6* and in mammalian PDE6, suggests that the *Drosophila* enzyme encoded by CG8279 is a PDE6 homologue. Also, *DmPDE6* is a prenylated enzyme *in vivo*, and interacts with the *Drosophila* small protein prenyl-binding protein, PDE δ [36a], which may suggest a similar mechanism of regulation to mammalian PDE6 β [37], but not PDE5.

Mammalian PDE6 is expressed only in the visual system; however, in other vertebrates, PDE6 has been suggested to have roles in circadian rhythms in avian pineal gland [18] and is in-

involved with *wnt* signalling and zebrafish development [19]. Thus vertebrate PDE6 may have further, yet unexplored, roles in cellular physiology.

In order to determine the function of *DmPDE6* *in vivo*, transgenic *Drosophila* lines were generated, allowing overexpression or ablation of *DmPDE6* either globally in the fly, or precise targeting within the principal cells of the Malpighian tubule. Previous work has shown that function of the *Drosophila* Malpighian (renal) tubule is modulated by cGMP. As such, this tissue is an excellent organotypic screen for elements of the cGMP signalling pathway. What are the consequences of *DmPDE6* overexpression in tubules? It has been established that tubule fluid transport via principal cells is modulated by alterations in cGMP levels [31,35]. Given that cG-PDE activity is substantially increased in transgenic tubules in which *DmPDE6* overexpression is targeted to principal cells, it was surprising that fluid transport rates were not altered. However, only a modest change in cGMP content was measured in tubules that overexpress *DmPDE6*, and it may be that such small changes in cGMP levels are insufficient to modulate fluid transport. Also, this may suggest that other endogenous *Drosophila* PDEs, for example the PDE1c and PDE11 homologues [14], may modulate fluid transport *in vivo*.

However, as cGMP is the major compound in fluid excreted by the tubule principal cells [36], we investigated the process of cGMP transport in the *DmPDE6* transgenic lines and showed that targeted overexpression of *DmPDE6* in principal cells inhibits active transport of cGMP. Interestingly, ectopic expression of bovine PDE5 in tubules [23] does not lead to inhibition of cGMP

transport, suggesting that, in tubules, *DmPDE6* is associated with a pool of cGMP distinct from that recognized by PDE5. This may also explain the modest effect of *DmPDE6* overexpression on tubule cGMP content, compared with that observed with overexpression of bovine PDE5 [23], where a 50 % reduction in cGMP content occurs.

Genetic and pharmacological ablation of *DmPDE6* in principal cells reverses the cGMP transport phenotype and confirms that endogenous *DmPDE6*, and not other endogenous cG-PDEs in tubule, modulates active transport of cGMP.

It is likely that cGMP efflux is modulated by cGMP levels. Although we show that overexpression of *DmPDE6* leads to only a modest reduction in cGMP levels, this can be explained by the fact that measurements of tubule cGMP content reflect whole tubule content, and thus do not reflect localized cGMP levels. Therefore direct regulation of cGMP concentration by either *DmPDE6* overexpression or *DmPDE6* ablation may markedly affect the concentration of cGMP in localized pools associated with cGMP transporter(s), thus affecting their activity and rates of cGMP efflux. However, the only current possibility for direct testing of the 'cGMP pool' hypotheses lies in the use of targeted reporters for cGMP [38], which will be of great use if achievable in living tissues using transgenesis.

The tubule is highly enriched in several classes of broad-specificity transporters including OATs, ABC (ATP-binding cassette) MRPs and a multidrug efflux transporter [39]. Six *Drosophila* OATs are expressed in the Malpighian tubule [33], yet none of these are close homologues of the mammalian cGMP transporter, OAT1 [4]. However, seven *Drosophila* ABC MRPs exist, of which only five are expressed in the Malpighian tubule: *CG7806*, *CG11897*, *CG9270*, *CG4562* and *CG6214*. *CG9270* is most highly expressed in tubule (expression levels are 22-fold enriched compared with the whole fly [39]) and therefore may represent the best candidate *Drosophila* cGMP transporter in tubule. The vertebrate cyclic nucleotide transporters, including MRP4 and MRP5, have been shown to have K_m values for cGMP in the micromolar range [40]. Estimates of neuropeptide-stimulated cGMP levels in the tubule principal cell suggest that these can be as high as 10 μ M at least. Given that the *Drosophila* cG-PDEs, including *DmPDE6*, are high- K_m (micromolar range) enzymes and that the concentration of cGMP in secreted fluid by the tubule has been shown to be 8.3 μ M [36], it is possible that the cGMP transporter in tubule has a similar K_m to that of vertebrate cGMP transporters.

Vertebrate MRP transporters are inhibited by cG-PDE inhibitors, for, e.g., zaprinast [4]; in contrast, the present study suggests that *Drosophila* cGMP transporter(s) in tubules is stimulated by zaprinast. Thus, although expected similarities occur in structure and function between vertebrate and *Drosophila* ABC MRP transporters, important differences must exist between key residues which confer pharmacological sensitivity. Interestingly, although recent work has shown that the vertebrate MRP proteins MRP4 and MRP5 are low-affinity transporters for cGMP [41], it seems that the roles of these proteins are not critical for maintenance of intracellular levels of cGMP. Therefore, in some cells, cGMP efflux may represent instead a mechanism of regulation of autocrine and paracrine signalling by cGMP [17,41].

Our studies using transgenic animals have allowed us to ascribe a novel effect of *DmPDE6* on active transport of cGMP in *Drosophila* tubules. Given the close functional and structural homology of vertebrate and *Drosophila* PDEs [14], this first demonstration of cG-PDE regulation of transepithelial cGMP transport in *Drosophila* suggests that specific cyclic nucleotide PDEs may provide important control mechanisms for regulating cellular efflux of cGMP in vertebrates.

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