Ectopic Expression of Bovine Type 5 Phosphodiesterase Confers a Renal Phenotype in *Drosophila*

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cGMP signaling regulates epithelial fluid transport by *Drosophila* Malpighian (renal) tubules. In order to directly evaluate the importance of cGMP-degrading phosphodiesterases (PDEs) in epithelial transport, bovine PDE5 (a *bona fide* cG-PDE), was ectopically expressed in vivo. Transgenic UAS-PDE5 *Drosophila* were generated, and PDE5 expression was driven in specified tubule cells in vivo by cell-specific GAL4 drivers. Targeted expression was verified by PCR and Western blotting. Immunolocalization of PDE5 in tubule confirmed specificity of expression and demonstrated localization to the apical plasma membrane. GAL4/UAS-PDE5 tubules exhibit increased cG-PDE activity and reduced basal cGMP levels compared with control lines. We show that wild-type and control tubules are sensitive to the PDE5-specific inhibitor sildenafil and that GAL4/UAS-PDE5 tubules display enhanced sensitivity to sildenafil, compared with controls. cGMP content in GAL4/UAS-PDE5 tubules is restored to control levels by treatment with sildenafil. Thus bovine PDE5 retains cGMP-degrading activity and inhibitor sensitivity when expressed in *Drosophila*. Expression of PDE5 in tubule principal cells results in an epithelial phenotype, reducing rates of basal and cGMP-stimulated fluid transport rates to control levels. However, corticotrophin releasing factor-like-stimulated transport, which is activated by cAMP signaling, was unaffected, confirming that only cGMP-stimulated signaling events in tubule are compromised by overexpression of PDE5. Successful ectopic expression of a vertebrate cG-PDE in *Drosophila* has shown that cG-PDE has a critical role in tubule function in vivo and that cG-PDE function is conserved across evolution. The transgene also provides a generic tool for the analysis of cGMP signaling in *Drosophila*.

An important role of cGMP in epithelial fluid transport has been demonstrated in the insect equivalent of the vertebrate renal system, the Malpighian tubules (1). Malpighian tubules are fluid-transporting, osmoregulatory organs that are critical for insect life (2). *Drosophila* tubules, which constitute an important genetic model for transporting epithelia (3), display elevated rates of fluid transport when stimulated by either exogenous cGMP, which enter tubule cells via a cyclic nucleotide transporter (4), nitric oxide, or neuropeptide-generated nitric oxide/cGMP (5–8). Components of the cGMP signaling pathway, including cGMP-dependent protein kinases (cGK) (9) and guanylate cyclase (2) have been described previously in tubules. Furthermore, genetic modulation of cGK activity (2) and pharmacological inhibition of cGMP-specific phosphodiesterase (cG-PDE) activity (5) both result in an epithelial phenotype. cG-PDE has also been shown to be important in regulating physiological responses of the tubule to transgenic modulation of NO production (10).

NO/GMP signaling is compartmentalized to principal cells in the main fluid-secreting segment of tubules, containing the electrogenic vacuolar H⁺-ATPase (V-ATPase) pump (11), which energizes fluid transport. Furthermore, electrophysiological studies suggest that cGMP signaling modulates V-ATPase activity (6), suggesting that cGMP signaling may regulate ion transport in tubules. A role for cGMP signaling in ion transport processes in vertebrate kidney has also been shown, where NO inhibits sodium/potassium/chloride co-transport as well as sodium/potassium V-ATPase activity in the macula densa (12). cGMP has also been shown to influence insertion of aquaporins in vertebrate kidney cells (13).

Given the importance of cGMP in fluid transport, homeostasis of cGMP must be critical to tubule function. Previous work (7, 8) has shown that inhibition of soluble guanylate cyclase reduces cGMP-stimulated fluid transport and that cG-PDE activity modulates fluid transport (5). However, as the biochemical characteristics of *Drosophila* PDEs have not yet been established, and we wished to investigate the role of cGMP-specific PDEs in tubule function, we used a transgenic method to ectopically express a well characterized vertebrate cG-PDE, bovine PDE5, in *Drosophila*. The recent annotation of the *Drosophila* genome shows that PDE5 is not encoded (flybase.bio.indiana.edu/genes/). As such, the targeting of a *bona fide* cG-PDE to *Drosophila* may be a valuable approach.

PDE5 is a 100-kDa cGMP-binding and cGMP-specific PDE, which acts as a homodimer. cGMP binds with high specificity at two allosteric cGMP-binding sites found in the regulatory region of the enzyme (14). PDE5 is expressed in lung epithelial tissue (15) and has also been implicated in modulating NO- induced vasopressin release in rat small intestine (16). PDE5

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‡ The abbreviations used are: cGK, cGMP-dependent protein kinases; PDE, phosphodiesterase; CRF, corticotrophin releasing factor; RT-PCR, reverse transcriptase-PCR; DAPI, 4,6-diamidino-2-phenylindole hydrochloride; PBS, phosphate-buffered saline; cG-PDE, cGMP-specific phosphodiesterase; V-ATPase, vacuolar H⁺-ATPase; UAS, upstream activating sequence.

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2 V. P. Pollock, J. A. T. Dow, and S. A. Davies, unpublished data.
Overexpression of PDE5 in Drosophila

has also been found in kidney, where cG-PDE activity is high in glomeruli and in tubules (17). In particular, PDE5 activity is the dominant cG-PDE in glomeruli; increased PDE5 activity in this region of the kidney is associated with pathogenicity in rat kidney (18), which suggests the importance of cG-PDE in renal tissue.

Only one PDE5 gene is known so far, PDE5A, from which there are two major splice variants, PDE5A1 and PDE5A2 (19, 20). PDE5 is selectively inhibited by zaprinast, dipyridamole, and sildenafil. Sildenafil acts to occupy the same sites within the catalytic domain as cGMP and is the most potent inhibitor of PDE5 (21).

In order to investigate the in vivo roles of specific gene products, targeted expression of transgenes is a useful approach. Targeting genes of choice to designated regions and to cells in vivo is achieved in Drosophila by using the GAL4/UAS enhancer trap system (22). To target expression of the PDE5 gene to different cell types of the tubule, two cell type-specific P(GAL4) enhancer trap lines (23) were used to direct cell-specific expression of the GAL4-responsive PDE5 transgene. In tubules, successful targeting of aquorin and green fluorescent protein transgenes (3, 24) and vertebrate receptors (25) to specified cell types of the main segment in tubules (23), and the c724 GAL4 line, which targets expression to tubule stellate cells (23). Here we describe the targeting of bovine PDE5A to Drosophila tubules and show that expression of functional vertebrate cG-PDE occurs in specified tubule cells. Furthermore, modulation of cGMP signaling via PDE5 results in a transport phenotype. Given the functionality of a vertebrate cG-PDE in Drosophila cells, this demonstrates a conserved role for cGMP signaling in renal function.

MATERIALS AND METHODS

Generation of PDE5 Transgenic Lines—The bovine PDE5A gene was provided as a 2.6-kb cDNA cloned into between the Apal and XbaI sites of pcdNA3.1(−Zeocin).—This was a kind gift of J. Corbin, Vanderbilt University School of Medicine.

In order to sub-clone PDE5A into the Drosophila p[UAST] vector, a PCR mutagenesis strategy was used to amplify a 2.6-kb fragment from the cDNA clone. This resulted in the PDE5A open reading frame with a 5′ NotI site upstream of the ATG and the removal of a NotI site 30 bp downstream of the ATG. The PCR product was gel-purified and sub-cloned into the TOPO TA cloning vector pCR2.1 (Invitrogen). The cloned fragment was sequenced before being cloned between the 5′ XbaI and 3′ NotI sites in p[PIUAST] and then sequenced again to confirm directional cloning of the open reading frame (Fig. 1). p[UAST] containing the PDE5A open reading frame was used to transform Drosophila embryos.

Germ Line Transformation of Drosophila—This was performed according to standard protocols using an Eppendorf FemtoJet pressure modulator. Briefly, more than 30 embryos from dechorionated eggs from 500 3°-w1118® females were transferred to a 0.78 mm (inner diameter) x 0.1 mM NaH2PO4, 5 mM KCl, pH 7.8). Embryos were viewed under a Zeiss inverted stage microscope.

Slides containing injected embryos were placed on a fresh grape juice agar plate. The embryos were left to recover for 1–7 days at 18 °C and any hatched larvae transferred to standard food. Surviving adults were mated individually back to the host strain (w1118) and the progeny scored for eye color transformation. Red-eyed transformants were crossed again to w1118, and transformed progeny of this cross were mated to siblings. Selection against flies with white eyes through generation in most cases allowed homozygous lines to be generated for each P-element insertion.

Resultant transgenic lines were designated UAS-PDE5. The chromosomal location of each insertion was determined by standard crossing schemes.

Drosophila Stocks—All strains were maintained on standard Drosophila diet at 25 °C and 55% humidity, on a 12:12 photoperiod. The following lines were used in this study as follows: w1118; c24, GAL4 enhancer trap line used to drive expression of UAS constructs in only tubule principal cells (24); c724, GAL4 enhancer trap line (23) used to drive expression of UAS constructs in only tubule stellate cells (26, 27); c42/UAS-PDE5, progeny of crosses between c42 and UAS-PDE5; and c724/UAS-PDE5, progeny of crosses between c724 and UAS-PDE5. Various balancer lines were also used to establish chromosomal location of transgene in UAS-PDE5 lines (not shown).

Reverse-transcriptase PCR—Expression of bovine PDE5A was determined by performing reverse transcriptase-PCR (RT-PCR) on control and transgenic fly tubule cDNA preparations and from progeny of GAL4/UAS-PDE5, in which expression of the gene should occur. Twenty tubules were dissected, and poly(A)+ RNA was extracted (Dynabead mRNA direct kit) and reverse-transcribed with Superscript Plus (Invitrogen) as described previously (5). 1 μl of the reverse transcription reaction, corresponding to cDNAs derived from 1 tubule (∼160 cells), was used as a template for PCR containing PDE5a gene-specific primer pairs based on published sequences. Additionally, to control against genomic contamination in cDNA preparations, primers were used that had been designed around intron/exon boundaries of genes known to be expressed in tubule, for example PK53e (28). Use of such primers verified the cDNA quality used in PCRs. Sequences of primers used are as follows: BovPDE5 Forward, 5′-GTCGAGAACAGAGAAATCAG-3′, and BovPDE5 Reverse, 5′-CATCCTTGCGTGGTAAAGCC-3′, PK53e Forward, 5′- AGACACAAAGATCTCGCCGC-3′, and PK53e Reverse, 5′-GCCGTTATGGCCTGGAC-3′.

PCR cycle conditions were as follows: 94 °C (1 min), 36 cycles of 94 °C (30 s), 58 °C (30 s), 72 °C (1 min), 72 °C (1 min). PCR products obtained from such RT-PCR experiments were cloned using the Invitrogen topoisomerase (TOPO TA Cloning) system. Cloned plasmids were purified using Qiagen kits and sequenced using U. S. Biological Corp. Sequences kits to confirm their identity. The cloned PCR products shared 100% sequence identity with PDE5A (data not shown).

Western Blot Analysis—Protein samples were prepared from tubules from each line (described under “Drosophila Stocks”) homogenized in ice-cold Tris/lys/buffer (2% w/v SDS, 70 mM Tris, pH 6.8, containing protease inhibitor mixture) and centrifuged at 12,000 × g for 5 min to remove debris. Supernatants were assayed for protein concentration (Bradford), and 10–30 μg of protein was run on 4–15% pre-cast gels (Bio-Rad). Western blots using rabbit anti-PDE5 antibody (Calbiochem) (1:1000 dilution) were performed according to standard protocols, using the Bio-Rad Mini-Protein blotting system. Loading uniformity was assessed by Ponceau S staining after blotting. Immunoblotting was visualized using ECL (Amersham Biosciences) after treatment with horseradish peroxidase-labeled secondary antibody (Diagnostic Scotland).

Immunocytochemistry—Experiments were performed using standard protocols as described previously (29) on fixed, intact tubules from control and GAL4/UAS-PDE5 tubules, as described in Fig. 2 legend. Antibodies used were as follows: rabbit polyclonal anti-PDE5 antibody (Calbiochem), 1:100 dilution; fluorescent-labeled goat anti-rabbit antibody (Diagnostic Scotland), 1:250 dilution.

Counterstaining of stellate cells in tubule stained for PDE5 was achieved by visualization of tubule cell nuclei, as differently sized nuclei achieved by visualization of tubule cell nuclei, as differently sized nuclei were used as cell-specific markers (23). Staining of nuclei was achieved using 4′,6-diamino-2-phenylindole (DAPI) (1 μg/ml) and observed under phase contrast (Bradford), and 10–30 μg of protein was run on 4–15% pre-cast gels (Bio-Rad). Western blots using rabbit anti-PDE5 antibody (Calbiochem), 1:1000 dilution; fluorescent-labeled goat anti-rabbit antibody (Diagnostic Scotland), 1:250 dilution.

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Assay for Tubule cG-PDE Activity—Assays for cG-PDE activity in tubules were performed essentially as described for tubule cPDE assays (30). For each sample, 50 tubules (20–30 μg of protein) from each line, treated as defined in Fig. 3 legend, were dissected into 50 μl of PBS, pH 7.4, containing protease inhibitor mixture (Sigma) and homogenized. 50 μl of tritiated cGMP working stock (0.185 kBq/ml in 10 mM Tris, 5 mM MgCl2, pH 7.4) was added to each sample on ice. Final cGMP

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substrate) concentrations used in assays as shown in Fig. 3 were derived by adjusting the concentration of unlabeled cGMP in the working stock. Blank samples were prepared using 50 μl of PBS and 50 μl of working stock; positive control was made as blanks except that PBS was replaced by bovine PDE5-transformed cell lysates (31).

The samples were incubated at 30 °C for 10 min, and reactions were terminated by boiling for 2 min. Samples were chilled on ice and incubated for 10 min with 25 μl of 1 mg/ml Crotalus atrox 5′-nucleotid-
Fig. 2. **PDE5 is expressed at the apical membrane in principal cells of Drosophila tubules.** Anti-bovPDE5 antibody was used to examine localization of PDE5 in intact c42/UAS-PDE5 tubules, as described. Tubules were viewed under UV light (Zeiss) and images captured with an AxioCam imaging system. In all pictures, tubule diameter can be taken as 35 μm. A, PDE5 is expressed in principal cells in the main fluid-transporting segment in tubules (main/initial boundary indicated by yellow line; m, main segment; i, initial segment (23); image ×5 magnification). B, UAS-PDE5 (control) line showing autofluorescence, ×10 magnification. Similar images were obtained at ×20 magnification (data not shown). C, strong staining of only principal cells in c42/UAS-PDE5 tubules, ×10 magnification. Yellow arrows indicate unstained stellate cells. D, apical staining in principal cells of c42/UAS-PDE5 tubules, viewed under ×20 magnification. Blue arrows indicate apical localization. Note unstained stellate cells (yellow arrows). E, tubule preparation as in D but counterstained with DAPI (10). Note occurrence of large principal cell nuclei at regular intervals (red arrows) and smaller stellate cell nucleus in unstained stellate cells. Yellow line indicates apical localization observed in D is due to different focal plane required for stained nuclei for image in E.

*Overexpression of PDE5 in Drosophila*
molecular weight products are observed in all samples, which are probably because of nonspecific interactions of the anti-PDE5 antibody in *Drosophila* homogenates. It is unlikely that these low molecular weight bands are due to recognition of endogenous *Drosophila* PDEs by the anti-PDE antibody because analysis of the closest homologue of vertebrate FIG. 3.

**A** cG-PDE activity in tubules transgenic for PDE5. A, cG-PDE activity in tubules from control, UAS-PDE5 (open bars), and c42/UAS-PDE5 (black bars) assayed at different cGMP substrate concentrations as shown. Data are expressed as nanomoles of GMP/min/mg protein ± S.E., n = 5. Statistically significant differences from control line indicated by * (p < 0.05). Insets, substrate velocity curves plotted using data shown in A for UAS-PDE5 (upper) and c42/UAS-PDE5 (lower). Data expressed as A, except that velocity of reaction at 20 μM cGMP (0.318 ± 0.072 nmol of GMP/min/mg of protein) was plotted for UAS-PDE5 to enable calculation of $K_m$ values using Prism4 software. B, cG-PDE activity in tubules from parental/control lines: w1118 (shaded bars), UAS-PDE5 (open bars); and c42/UAS-PDE5 (black bars) assayed at 5 μM cGMP in the absence (−) and presence (+) of 10−6 M sildenafil. Data are expressed as nanomoles of GMP/min/mg of protein ± S.E., n = 4–6. Statistically significant differences between untreated and sildenafil-treated tubules are indicated by * (p < 0.05). C, dose-response curve of sildenafil effects on cG-PDE activity in UAS-PDE5 (open squares) and c42/UAS-PDE5 (black squares) tubules: C-i, assayed at 20 and 2.5 μM cGMP, respectively, and C-ii, assayed at 5 μM cGMP. The concentrations used for sildenafil as shown in C-ii (10−7 to 10−6 M) were based on effects of sildenafil at those concentrations in C-i. Data are expressed as % of cG-PDE activity compared with untreated tubules from each line; ± S.E., n = 4. (Errors for c42/UAS-PDE5 (black squares) are too low to appear in C-i; errors for both lines (open and black squares) are similarly low in C-ii.) Data were calculated as (cG-PDE activity in the presence of sildenafil (nmol of GMP/min/mg of protein)/cG-PDE activity in untreated tubules (nmol of GMP/min/mg of protein)) × 100%.
PDE5, *Drosophila* PDE6,\(^3\) shows a significantly higher molecular weight compared with the bands in question. Furthermore, the likelihood that the antibody recognizes nonspecific products is low, given the specific staining observed in c42/UAS-PDE5 compared with UAS-PDE5, in the immunocytochemical evidence shown in Fig. 2.

Expressed protein was not detected in c724/UAS-PDE5 tubule preparations. As there are only 22 stellate cells in tubule main segment (23), expression of PDE protein in such a small number of cells is not detectable by Western analysis, with transcript expression being only barely detectable (see above).

**PDE5 Is Localized to the Apical Membrane in Tubule Principal Cells**—Immunocytochemistry of whole mount tubules using commercial anti-PDE5 antibody was used to resolve the spatial and cellular localization of GAL4-driven PDE5A expression in tubules. Fig. 2 shows that the control UAS-PDE5 line does not express PDE5 protein in tubules (B), as data shown in Fig. 1 would lead us to expect. By contrast, the c42 GAL4 driver, which confers expression only in principal cells in tubule main segment, confers spatial expression of PDE5 in this tubule region (Fig. 2A) and in only principal cells (Fig. 2, C–E).

Targeted expression of PDE5 in only the principal cells is supported by the use of the c42 GAL4 driver in previous work (24), in which an aequorin transgene was targeted to specified cells in tubules. Furthermore, counterstaining of cell nuclei with DAPI (Fig. 2E) clearly shows the presence of a small nucleus in a black, unstained stellate cell (see yellow arrow in Fig. 2E and compare with the larger principal cell nucleus as indicated by red arrows). Note also unstained stellates, yellow arrows, in C and D). Cell nuclei are known markers for stellate cells and have been documented previously (23) as being smaller than the polypliod nuclei of principal cells.

Importantly, we demonstrate that bovine PDE5 is expressed at apical membranes in principal cells (blue arrows (Fig. 2, D and E). This site of expression is identical to that marked by expression of the vacuolar H\(^+\)-ATPase subunit, vhaSFD (10), which is known to be targeted to the tubule apical membrane. Thus, targeting of PDE5 to specified membranes in polarized tubule cells occurs *in vivo*.

**Increased cG-PDE Activity Is Associated with PDE5 Expression in Tubules**—In order to determine the effects of ectopic expression of PDE5 in tubules, cG-PDE activity was measured at a range of substrate concentrations in tubule preparations, from control (UAS-PDE5) and c42/UAS-PDE5 lines. Results presented in Fig. 3A show that in control lines, maximum cG-PDE was observed at 10 \(\mu\)M cGMP concentration. Measurements of cG-PDE activity at higher substrate concentrations were prone to large errors, as radioactive counts associated with GMP obtained under these assay conditions were very low. By contrast, very low levels of cG-PDE activity were measured at and between 0.5 and 2.5 \(\mu\)M substrate concentrations, which are normal substrate concentrations used to measure vertebrate cG-PDE activity. As such, it is likely that endogenous cG-PDE activity in tubules consists of a high \(K_m\) enzyme.

This is supported by analysis of the data (substrate velocity curve, Fig. 3, upper inset). Calculations show that the apparent \(K_m\) value of native cG-PDE activity is 15.88 ± 10.2 \(\mu\)M, with a linear Lineweaver-Burke plot. The large S.E. reflects the unavoidable errors in cG-PDE activity measurements at high substrate concentrations.

Significantly higher cG-PDE activity is observable between 0.5 and 10 \(\mu\)M cGMP in c42/UAS-PDE5 tubules, compared with the control line. In particular, cG-PDE activity in the PDE5 transgenic tubules are significantly increased to similar levels between 2.5 and 10 \(\mu\)M, reflecting those substrate concentrations that represent the \(K_m\) of PDE5 (1–5 \(\mu\)M). Analysis of these data by plotting a substrate velocity curve (Fig. 3, lower inset) reveals a \(K_m\) of 5.08 ± 3.14 \(\mu\)M, which is within the established \(K_m\) for PDE5.

Similar experiments were performed on c724/UAS-PDE5 tubules. No significant detectable differences in PDE activity were measurable in these tubules compared with controls, when assayed at 2.5 \(\mu\)M cGMP (0.148 ± 0.027 nmol of GMP/min/mg), due possibly to the very small number of cells that were expressing PDE5. Given both the lack of expression of PDE5 and of increased cG-PDE activity in c724/UAS-PDE5 tubules, all further experiments using the transgenic tubules were carried out on c42/UAS-PDE5.

**Sensitivity of Tubule cG-PDE Activity to Inhibition by Sildenafil**—Inhibitors of vertebrate cG-PDEs are effective in *Drosophila* tubules; inhibition of cG-PDE activity is observed in *w*\(^{1118}\) and control UAS-PDE5 tubules using sildenafil, a specific inhibitor of PDE5 (Fig. 3B). Furthermore, although cG-PDE activity is significantly higher in c42/UAS-PDE5 tubules than in controls (see also Fig. 3A), the levels of cG-PDE activity remaining in sildenafil-treated c42/UAS-PDE5 tubules are comparable with those in sildenafil-treated parental and control UAS lines. Tubules from an established wild-type line, Oregon R, show similar cG-PDE sensitivity to sildenafil as *w*\(^{1118}\) and UAS-PDE5 (data not shown). Thus, we demonstrate that a greater proportion of cG-PDE activity in c42/UAS-PDE5 tubules is sildenafil-sensitive, compared with wild-type/controls.

This is confirmed by the results shown in Fig. 3C–i, where cG-PDE activity was measured over a range of sildenafil concentrations in UAS-PDE5 and c42/UAS-PDE5 tubules. In order to measure high \(K_m\) endogenous cG-PDE activity and lower \(K_m\) PDE5 activity, we assayed cG-PDE activity at two different substrate concentrations (20 \(\mu\)M for UAS-PDE5 and 2.5 \(\mu\)M for c42/UAS-PDE5). These substrate concentrations are close to the \(K_m\) value for the PDE5 and the endogenous PDE, respectively, and will ensure that each enzyme is ~50% saturated.

\(^{3}\) J. P. Day, M. D. Houslay, and S. A. Davies, unpublished observations.
with substrate. Therefore, results obtained from the different preparations will be directly comparable; any difference in sensitivity of the native and PDE5 transgenic tubule preparations to sildenafil will be due to intrinsic kinetic factors distinct for either the endogenous or PDE5 enzyme. Fig. 3C-i shows that compared with control tubules, c42/UAS-PDE5 tubules are more sensitive to sildenafil, especially at low concentration. At $10^{-5}$, $10^{-6}$, and $10^{-7}$ M sildenafil, cG-PDE activity is almost completely inhibited compared with untreated tubules ($4.5 \pm 1.2$, $7.1 \pm 0.9$, and $5.8 \pm 1.4$% activity remaining at respective concentrations of sildenafil). At higher concentrations of sildenafil, $-11-12$% of cG-PDE activity remains, significantly dif-
The data are expressed as either mean basal or maximum fluid secretion levels (nl/min) or transgenic c42/UAS-PDE5 (black bars) were assayed for transport phenotype in the absence or presence of sildenafil (10⁻⁶ M) for 10 min (A and B) and were either left unstimulated (A) or stimulated with cGMP (10⁻⁴ M) (B) for 30 min as in Fig. 5. A shows the mean basal rates of fluid transport of tubules treated as shown, although B shows the maximum fluid secretion rate measured from stimulated tubules. Data are expressed as either mean basal or maximum fluid secretion levels (nl/min) ± S.E., n = 8. * indicates data that is statistically significant from control (p < 0.05).

Figure 6. Inhibition of PDE5 restores basal and stimulated fluid transport rates to normal levels. Tubules from either parental control UAS-PDE5 (open bars) or transgenic c42/UAS-PDE5 (black bars) were assayed for transport phenotype in the absence or presence of sildenafil (10⁻⁶ M) for 10 min (A and B) and were either left unstimulated (A) or stimulated with cGMP (10⁻⁴ M) (B) for 30 min as in Fig. 5. A shows the mean basal rates of fluid transport of tubules treated as shown, although B shows the maximum fluid secretion rate measured from stimulated tubules. * indicates data that is statistically significant from control (p < 0.05).

A different from that at low sildenafil concentration. By contrast, UAS-PDE5 tubules maintain 30–50% of cG-PDE activity at all concentrations of sildenafil.

In order to further examine sildenafil inhibition of endogenous cG-PDE and PDE5 encoded by the transgene, the experiment in Fig. 3C-i was repeated for both lines at 5 μM cGMP, using those concentrations of sildenafil shown to give significant inhibition of cG-PDE activity (Fig. 3C-ii). The data in Fig. 3C-ii show that in the presence of sildenafil, native tubules maintain 60–70% of cG-PDE activity at low concentrations of cGMP, compared with 30–50% of cG-PDE activity observed at high substrate concentrations (Fig. 3C-i), suggesting that the sildenafil-inhibited cG-PDE activity of native tubules may be due to a high Kₘ enzyme. By contrast, the response of the transgenic c42/UAS-PDE5 tubules to sildenafil is not significantly different at either 2.5 μM cGMP (Fig. 3C-i) or 5 μM (Fig. 3C-ii), where almost complete inhibition of cG-PDE activity is observed.

These results demonstrate that Drosophila tubules are sensitive to sildenafil, a PDE5-specific inhibitor. However, ectopic expression of PDE5 in tubules increases sensitivity to sildenafil. This increased sensitivity suggests that the increased cG-PDE activity observed in PDE5 transgenic tubules can be ascribed to functional PDE5. Thus, ectopically expressed PDE5 in Drosophila tubules maintain both activity and inhibitor sensitivity in vivo.

Sildenafil Reverses the PDE5-induced Reduction in cGMP Content of Transgenic Tubules—Fig. 4 shows that intracellular cGMP levels in vivo are modulated by ectopic expression of PDE5 in tubules.

Expression of the bovine PDE5A transgene in tubule principal cells reduces resting cGMP content (Fig. 4). Data in Fig. 3 showed that sildenafil inhibited cG-PDE activity in c42/UAS-PDE5 tubules. Use of sildenafil to inhibit PDE5 activity in c42/UAS-PDE5 tubules restores cGMP content to wild-type levels (Fig. 4).

Ectopic Expression of Bovine Lung PDE5 Results in an Epithelial Phenotype in Drosophila—Activated cGMP signaling in tubule principal cells increases fluid transport (5, 7). Accordingly, we investigated the possible impact of expression of a bona fide cG-PDE in tubules on epithelial fluid transport. We show in Fig. 5 that expression of PDE5 in principal cells results in a transport phenotype. Fig. 5, A–C, shows that basal rates of fluid transport are significantly reduced in c42/UAS-PDE5 tubules compared with the parental UAS-PDE5 control line.

Furthermore, stimulated fluid transport by either exogenous cGMP (5) or the nitridergic neuropeptide CAP 2b (6) is reduced in the transgenic line. However, c42/UAS-PDE5 tubules do respond in a similar manner to control tubules to application of a cAMP-mobilizing neuropeptide, Drosophila CRF-like peptide (30), despite the lower basal transport levels. Whereas CRF-stimulated transport at 70 min diverges significantly between c42/UAS-PDE5 and UAS-PDE5 lines, the overall increase in transport rates between basal and CRF-stimulated conditions in both lines is similar: −300% increase upon CRF stimulation in UAS-PDE5 and 290% in c42/UAS-PDE5 tubules. Hence, ectopic expression of PDE5 affects cGMP- and cAMP-associated signaling events in the tubule.

Sildenafil Restores Basal and Stimulated Fluid Transport Levels in Transgenic Tubules—The previous data have shown that inhibition of PDE5 by sildenafil restores resting cGMP content to wild-type levels. In Fig. 6A, we show that treatment of c42/UAS-PDE5 tubules with sildenafil results in a restoration of basal rates of fluid transport. Furthermore, sildenafil treatment prevents the observed reductions in cGMP-stimulated fluid transport by c42/UAS-PDE5 tubules seen in Fig. 5A (Fig. 6B). Thus, direct effects of ectopically expressed bovine PDE5 action on cGMP content and cGMP signaling result in the tubule transport phenotype in Drosophila.

DISCUSSION

Although much is known about cAMP and cA-PDEs in cellular and tissue function, roles for cGMP have been slower to emerge. More recently, however, physiological roles for cGMP...
epithelia. Given the conservation of cGMP signaling across evolution (1, 36), this also suggests the importance of cG-PDEs in both vertebrate and insect renal function.

As such, a direct role of cG-PDE in fluid transport was investigated, using ectopic expression of a well characterized cGMP-specific PDE, bovine PDE5, targeted to specified cells in Malpighian tubules.

We have achieved precise targeting of functional PDE5 in principal cells of Drosophila tubules using the GAL4-UAS system. We show that a vertebrate cG-PDE is localized to apical membranes in principal cells. PDE5 maintains enzymatic activity, and substrate specificities in vivo, when expressed in Drosophila tubules.

Ectopic expression of PDE5 confers increased sensitivity to sildenafil at nanomolar concentrations, which is very different from the micromolar sensitivities observed for other PDEs to sildenafil (37). Thus, this increased sildenafil sensitivity is specifically due to expression of bovine PDE5. The nanomolar sensitivity observed may also be a consequence of still undocumented post-translational modification of PDE5 in tubules. However, recent work (38) on the crystal structure of PDE5 shows that the $K_d$ for sildenafil is between 0.5 and 6.6 nM, which is within the nanomolar concentration range of sildenafil action observed in Drosophila.

We also provide the first demonstration that wild-type insect tubules are sensitive to sildenafil. Although only around 50–70% of the cG-PDE activity in wild-type tubules is inhibited by sildenafil under conditions of high substrate, its sildenafil sensitivity is exquisite, with inhibitory effects in the nanomolar range. However, at low substrate concentration around the $K_m$ of PDE5, the proportion of sildenafil-sensitive enzyme in native tubules is decreased. This suggests that native tubules contain a high $K_m$, sildenafil-sensitive enzyme. Recent annotation of PDEs in the Drosophila genome shows that Drosophila do not encode PDE5 but a close homologue, PDE6 (flybase.bio.indiana.edu/). Furthermore, in vitro studies show that PDE6, which is expressed in tubules, is a high $K_m$, sildenafil-sensitive enzyme.3 Thus, it is possible that the sildenafil sensitivity in native tubules is a consequence of PDE6 expression.

Targeted expression of PDE5 to tubule principal cells in the main segment affects epithelial fluid transport by modulating basal transport levels and also cGMP- and CAP2b-stimulated transport levels. Previous work has shown that the cG-PDE inhibitor, zaprinast, modulates fluid transport and cGMP content in wild-type tubules. Treatment of tubules with zaprinast also causes increased cGMP content at the apical membranes of principal cells (10), suggesting localization of native cG-PDE at this site. The localization of PDE5 to principal cell apical membranes (Fig. 2) suggests that activation of cGMP signaling is compartmentalized in the vicinity of the major ion transport proteins in this cell type (Fig. 7), and so modulates fluid transport rates. Interestingly, PDE5 is targeted to the same cellular region, which shows that in the differentiated tubule correct targeting of a vertebrate gene product occurs. Furthermore, this implies conservation of targeting sequences between vertebrate and invertebrate cG-PDE.

PDE5 expression reduces tubule cGMP content, which is associated with the transport phenotype. Furthermore, inhibition of PDE5 activity by the specific inhibitor, sildenafil, restores cGMP content to wild-type levels and also restores basal and cGMP-stimulated rates of fluid transport. Targeted overexpression of PDE5 provides a generic tool to reduce cGMP levels in cells of choice under GAL4/UAS control. This will be useful not just in the study of renal function but of other physiological processes, for example brain and behavior.
Finally, the ectopic expression of vertebrate transgenes in *Drosophila* will help unravel the complexities of cellular signaling in an organotypic context and will also allow inferences regarding the roles of specific genes in vivo.

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Ectopic Expression of Bovine Type 5 Phosphodiesterase Confers a Renal Phenotype in Drosophila

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