The Journal of Experimental Biology 207, 2769-2776
Published by The Company of Biologists 2004
doi:10.1242/jeb.01086

The *dg2 (for)* gene confers a renal phenotype in *Drosophila* by modulation of cGMP-specific phosphodiesterase

Matthew R. MacPherson[1,*], Kate E. Broderick[1,*], Shirley Graham[1], Jonathan P. Day[1], Miles D. Houslay[2], Julian A. T. Dow[1] and Shireen A. Davies[1,†]

Institute of Biomedical and Life Sciences, Divisions of [1]Molecular Genetics and *[2]Biochemistry and Molecular Biology, University of Glasgow, Glasgow G11 6NU, UK*

*These authors contributed equally to this work
†Author for correspondence (e-mail: s.a.davies@bio.gla.ac.uk)

Accepted 12 May 2004

Summary

Fluid transport in *Drosophila melanogaster* tubules is regulated by guanosine 3′,5′-cyclic monophosphate (cGMP) signalling. Here we compare the functional effects on tubules of different alleles of the *dg2* (*foraging* or *for*) gene encoding a cGMP-dependent protein kinase (cGK), and show that the *for^s* allele confers an epithelial phenotype. This manifests itself as hypersensitivity of epithelial fluid transport to the nitridergic neuropeptide, capa-1, which acts through nitric oxide and cGMP. However, there was no significant difference in tubule cGK activity between *for^s* and *for^R* adults. Nonetheless, *for^s* tubules contained higher levels of cGMP-specific phosphodiesterase (cG-PDE) activity compared to *for^R*. This increase in cGMP-PDE activity sufficed to decrease cGMP content in *for^s* tubules compared to *for^R*. Challenge of tubules with capa-1 increases cGMP content in both *for^s* and *for^R* tubules, although the increase from resting cGMP levels is greater in *for^s* tubules. Capa-1 stimulation of tubules reveals a potent inhibition of cG-PDE in both lines, although this is greater in *for^s*; and is sufficient to explain the hypersensitive transport phenotype observed.

Thus, polymorphisms at the *dg2* locus do indeed confer a cGMP-dependent transport phenotype, but this can best be ascribed to an indirect modulation of cG-PDE activity, and thence cGMP homeostasis, rather than a direct effect on cGK levels.

Key words: Malpighian tubule, cyclic nucleotide, capa-1, epithelial transport, *Drosophila melanogaster*, *dg2* locus.

Introduction

An important role of guanosine 3′,5′-cyclic monophosphate (cGMP) in epithelial fluid transport has been demonstrated in the insect equivalent of the vertebrate renal system, the Malpighian tubules (Davies, 2000). Malpighian tubules are fluid transporting, osmoregulatory organs that are critical for insect life (Dow and Davies, 2001). *Drosophila melanogaster* tubules, which constitute an important genetic model for transporting epithelia (Dow and Davies, 2003), display elevated rates of fluid transport when stimulated by either exogenous cGMP, nitric oxide or neuropeptide-generated nitric oxide/cGMP (Davies et al., 1995, 1997; Dow et al., 1994b; Kean et al., 2002).

An autocrine role for NO/cGMP has been proposed for tubule principal cells (Broderick et al., 2003), with NO/GMP signalling being compartmentalised to principal cells in the main, fluid-secreting segment of tubules. These cells contain the electrogenic vacuolar H^+-ATPase (V-ATPase) pump (Dow, 1999), which energises fluid transport. Furthermore, electrophysiological studies show that cGMP signalling modulates V-ATPase activity (Davies et al., 1995), suggesting that cGMP signalling may regulate ion transport in tubules. Major effectors of cGMP signalling, including cGMP-dependent protein kinases (cGK) (Vaandrager and de Jonge, 1996) have previously been described in tubules. Furthermore, pharmacological and transgenic modulation of cGMP-specific phosphodiesterase (cG-PDE) activity (Broderick et al., 2004, 2003; Dow et al., 1994b) both result in an epithelial phenotype.

In *Drosophila*, cGK is encoded by two genes, *dg1* (Foster et al., 1996) and *dg2*. Both genes are expressed by Malpighian tubules (Dow et al., 1994b). *dg2* was isolated and characterised during a search for cAMP-dependent kinase genes (Kalderon and Rubin, 1989) and the putative cGK shown to be transcribed into three major RNA species of different size and several minor RNA species. These main transcripts (T1, T2 and T3) collectively code for at least three (de Belle et al., 1993), and possibly more, different polypeptides. The DG2 protein shares 64% overall homology with the prototypical bovine lung cGK, with 64% and 75% sequence identity to the cGMP-binding and kinase domains, respectively.

Studies in *Drosophila* have revealed *in vivo* roles for *dg2*

and cGK. The naturally occurring rover/sitter *foraging* polymorphism in *Drosophila*, which defines larval food search strategies, has been mapped to the *dg2* gene (de Belle et al., 1989, 1993). Rovers (*for*^{R}) have significantly longer path lengths than sitters (*for*^{s}) in a nutritive environment, although both travel similar distances when food is absent. Similarly, adult *for*^{s} animals travel shorter distances around nutrients (Pereira and Sokolowski, 1993). Phosphorylation studies performed on samples from adult heads showed that *for*^{s} contained slightly (10%) reduced cGK enzyme activity compared to *for*^{R}. Also, northern and western analysis showed a small reduction in RNA and protein levels in *for*^{s} compared to *for*^{R} (Osborne et al., 1997). It has therefore been suggested that a reduction in amounts of cGK transcript and protein, together with reduced cGK activity, may account for the *for*^{s} phenotype in larvae. Thus, the *foraging* polymorphism points to the possibility that subtle alterations in cGK levels can have profound effects on the whole animal.

Fluid transport assays performed on tubules from adult *for* lines has demonstrated that tubules from *for*^{s} flies exhibit hypersensitivity to exogenously applied cGMP in comparison to *for*^{R} or wild-type flies (Dow and Davies, 2001). However, stimulation of fluid transport by leucokinin, which stimulates fluid secretion *via* a calcium signal in the stellate cells, is unaltered in the *for* alleles (Dow and Davies, 2001), suggesting that effects of alterations in cGK are confined to principal cells.

We show here that the *for*^{s} allele results in hypersensitivity of tubule fluid transport (compared with *for*^{R}) in response to the neurohormone, capa-1. Intriguingly, the *for*^{s} mutation does not appear to affect cGK activity in tubules; rather it impacts on cGMP content, and on cG-PDE activity. Capa-1 inhibits cG-PDE, which results in increased cGMP content, and the transport phenotype observed; this also demonstrates modulation of cG-PDE activity by a neurohormone in insects, for the first time.

Materials and methods

Drosophila stocks

All strains were maintained at 22°C on standard *Drosophila* diet over a 12 h:12 h photoperiod at 55% humidity. Lines used in this study were *for*^{R} and *for*^{s} (naturally occurring polymorphisms of *dg2*; a kind gift of M. Sokolowski, University of Toronto at Missisauga, Canada).

Materials

Schneider's medium (Gibco) was obtained from Invitrogen (Renfrew, UK). The nitridergic neuropeptide capa-1 was used in this study (GANMGLYAFPRVamide) because of its identical mode of action but slightly greater potency than capa-2 (Kean et al., 2002) and was synthesised by Research Genetics, Inc., now Invitrogen. Radiochemicals were obtained from Amersham Biosciences (Chalfont St Giles, UK). All other chemicals were obtained from Sigma-Aldrich (Gillingham, UK) unless stated otherwise.

Locomotion activity monitoring

Fly lines were assessed by monitoring activity of adult flies using the *Drosophila* locomotor activity monitor IV, (TriKinetics Inc., Waltham, MA, USA) in order to verify previously published adult behavioural phenotypes ascribed to *for* alleles.

Flies were maintained at 22°C on standard *Drosophila* diet over a 12 h:12 h photoperiod, with lights on at 11.30 am. Tubes were made from 7.5 cm lengths of Tygon (Charny, France) clear flexible plastic tubing (R3603, i.d. 5/32", o.d. 7/32", wall 1/32"), plugged at one end with normal fly food and sealed with clear tape. 7-day-old male flies were anaesthetized and placed singly into each tube. Ends of tubes were then plugged with cotton wool. Tubes were placed in the monitor and flies allowed to recover overnight prior to monitoring. Readings were taken every 30 min over a period of 7 days.

Transport assays

Flies were used 1 week post-emergence, cooled on ice, then decapitated before dissection to isolate whole Malpighian tubules. Tubules were isolated into 10 ml drops of Schneider's medium under liquid paraffin and fluid secretion rates measured in tubules as detailed elsewhere (Dow et al., 1994a) under various conditions, as described in the text. Basal rates of fluid transport were measured for 30 min, and capa-1 (10^{-7} mol l^{-1}; Kean et al., 2002) added as indicated, after which transport rates were measured for a further 30 min.

Assay for tubule cGK activity

The protocol, based on quantification of ^{32}P-labelled phosphopeptide under conditions where cGK is active, has been adapted from the SignaTECT^{TM} cyclic AMP-dependent protein kinase assay system (Promega, Southampton, UK) and from Osborne et al. (1997).

Approximately 80 tubules from either *for*^{R} or *for*^{s} 1-week-old adults were dissected and placed in 20 µl buffer (25 mmol l^{-1} Tris, pH 7.4, 150 mmol l^{-1} sucrose, 2 mmol l^{-1} EDTA, 100 mmol l^{-1} NaCl, 50 mmol l^{-1} β-mercaptoethanol, 2 mg ml^{-1} leupeptin, 5 mg ml^{-1} aprotinin, 1 mg ml^{-1} phenylmethylsulphonyl fluoride). Tubules were either treated with hormone for 10 min, or left untreated, before being homogenised and centrifuged for 5 min at 13 000 ***g***. Protein concentration of tubule homogenates was determined by the Lowry assay and homogenates adjusted to equivalent protein concentrations for use in cGK assays. cGK activity was assayed with and without cGMP for each tubule preparation.

Two reaction mixes were prepared with and without the addition of 1 µmol l^{-1} cGMP, containing 25 mmol l^{-1} Tris, pH 7.4, 7 mmol l^{-1} magnesium acetate, 1 mmol l^{-1} EDTA, 2 mmol l^{-1} EGTA, 0.2 mg ml^{-1} GLASStide [RKRSRAE, a heptapeptide cGK-specific substrate; Calbiochem, Beeston, UK (Hall et al., 1999)], 20 µmol l^{-1} ATP, 0.5–2 ml [γ-^{32}P]dATP (370 MBq µl^{-1}, to an approximate specific activity of 4000 c.p.m. pmol^{-1} ATP), 1 nmol l^{-1} PKI (PKA inhibitor, TYADFIASGRTGRRNAI-NH_2) and 1 mmol l^{-1} dithiothreitol (DTT).

For each reaction, 40 μl reaction buffer was added to 5 μl (approximately 30 μg protein) tubule sample. This was done with both cGMP-containing (+cGMP) and cGMP-absent (–cGMP) buffer. Each tubule preparation was assayed as 2–4 separate reactions within the experiment. However, all cGK experiments were carried out on several separate biological replicates in order to obtain statistically sound data. Sample blanks were generated using 40 μl reaction buffer and 5 μl of homogenisation buffer. Reactions were incubated for 30 min at 30°C, after which 35 μl of each sample was spotted onto individual squares of P81 paper (Whatman, Maidstone, Kent). These squares of paper are referred to as reaction samples. In order to determine the specific activity of the radiolabelled ATP at the end of the reaction, several reactions were chosen randomly and 5 μl samples (representative of 1/9 of total counts) of each spotted onto individual squares of P81 paper ('total count'), allowed to dry and set aside.

The reaction samples were washed for 3× 5 min in 75 mmol l^{-1} phosphoric acid, then washed once for 15–20 s in ethanol and allowed to dry. All squares of paper, including the total count samples, were then transferred to scintillation vials, with the addition of 3 ml scintillation fluid and counted in a scintillation counter (Beckman, High Wycombe, UK) for 60 s.

Specific activity of [γ-^{32}P]ATP was calculated (9× mean c.p.m. of total count squares/[ATP] in reaction) and used to calculate protein kinase activity (pmol ATP min^{-1} μg^{-1} protein), as follows: (sample c.p.m. – sample blanks / sample volume on filter × reaction time × protein amount × specific activity). Values for 'sample c.p.m.' were based on those obtained by subtracting mean –cGMP values from mean +cGMP values for each set of replicate reactions.

cG-PDE activity assays

Assays for cG-PDE activity in tubules were performed essentially as previously described (Broderick et al., 2003) using 50 tubules (20–30 μg protein) for each sample, assayed in 0.185 kBq ml^{-1} ^{3}H-cGMP in 20 μmol l^{-1} cGMP, 10 mmol l^{-1} Tris, 5 mmol l^{-1} $MgCl_2$, pH 7.4. For cG-PDE assays in heads, six heads from each line were dissected into 100 μl KHEM buffer (50 mmol l^{-1} KCl, 10 mmol l^{-1} EGTA, 1.92 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} DTT, 50 mmol l^{-1} Hepes, pH 7.21, 1 μl Sigma P8340 protease inhibitor cocktail), disrupted with a pestle, sonicated for 10 s and centrifuged at 15 000 ***g*** for 5 min at 4°C. Supernatants were assayed for protein concentration, and 50 μl samples (containing 10 μg protein) assayed for cG-PDE activity as for tubule samples. A final substrate concentration of 10 μmol l^{-1} cGMP was used in reactions, as endogenous *Drosophila* cG-PDEs are enzymes with high K_m (Broderick et al., 2004; Day et al., 2003). Final activity was expressed per mg protein. Protein concentrations were assayed according to standard protocols (Lowry Assay).

Tubule cyclic GMP assays

Cyclic GMP levels were measured in pooled samples of 20 tubules by radioimmunoassay (Amersham Biotrak Amerlex M), as previously described (Dow et al., 1994b). Tubules were pre-incubated with 10^{-8} mol l^{-1} of the cG-PDE inhibitor Zaprinast (Calbiochem, Beeston, UK) for 10 min. Where required, capa-1 (10^{-7} mol l^{-1}) was added to tubules for a further 10 min. Incubations were terminated with ice-cold ethanol and homogenised. Samples were dried down and dissolved in 0.05 mol l^{-1} sodium acetate buffer (Amersham) and processed for cGMP content according to the manufacturer's protocol.

Statistics

Data are presented as mean ± S.E.M. Where appropriate, the significance of differences between data points was analysed using Student's *t*-test for unpaired samples, taking $P<0.05$ as the critical level.

Results

for^R *and* for^s *adults show distinct locomotor patterns*

Published work on the *for* alleles show distinct behavioural differences between *for^R* and *for^s* adults on nutritive substances (Pereira and Sokolowski, 1993). We investigated locomotor function of *for^R* and *for^s* adults in small food-containing tubes to determine the behavioural phenotype of the lines in our hands. Flies were assessed using a Trikinetics activity monitor over a 7-day period. Fig. 1 shows the results of such analysis, on day 1 and day 3 of trials, and pooled data for each day. Activity in all lines tested (including wild-type Oregon R flies, data not shown) display an overall reduction in activity during the course of the trial. *for^R* animals are significantly more active compared to *for^s* animals, on two different days of testing (Fig. 1Ai,ii, Bi,ii). Furthermore, these differences were observed throughout the 7-day trial period (data not shown). Although cGK has been shown to be involved with resetting the circadian clock in mammals (Oster et al., 2003), the data shown in Fig. 1 do not indicate any shift in the phase of activity but only in the amplitude: both lines showed similar cycling of activity at all times tested. Finally, analysis of the peak area shows that on both days, *for^R* flies are significantly more active than *for^s* (Fig. 1Aiii, Biii). Thus, *for^s* display the known 'sitter' phenotype in our hands.

Basal and capa-1 stimulated fluid transport rates are modulated in a dg2 *allele*

A significant increase in neuropeptide-induced transport is observed at 50 and 60 min in tubules from *for^s* animals, compared to those from *for^R* (Fig. 2). This is especially pronounced at 60 min, when maximum fluid transport rates are approximately 2 nl min^{-1} in *for^s* tubules, and approximately 1 nl min^{-1} in *for^R* tubules. However, no differences are observed in basal transport rates between the two lines.

cGK activity is not significantly perturbed in for^s *tissue*

It has been previously shown (Osborne et al., 1997) that cGK activity in the heads of *for^s* mutants is slightly downregulated. In order to test the effect of the *for^s* mutation

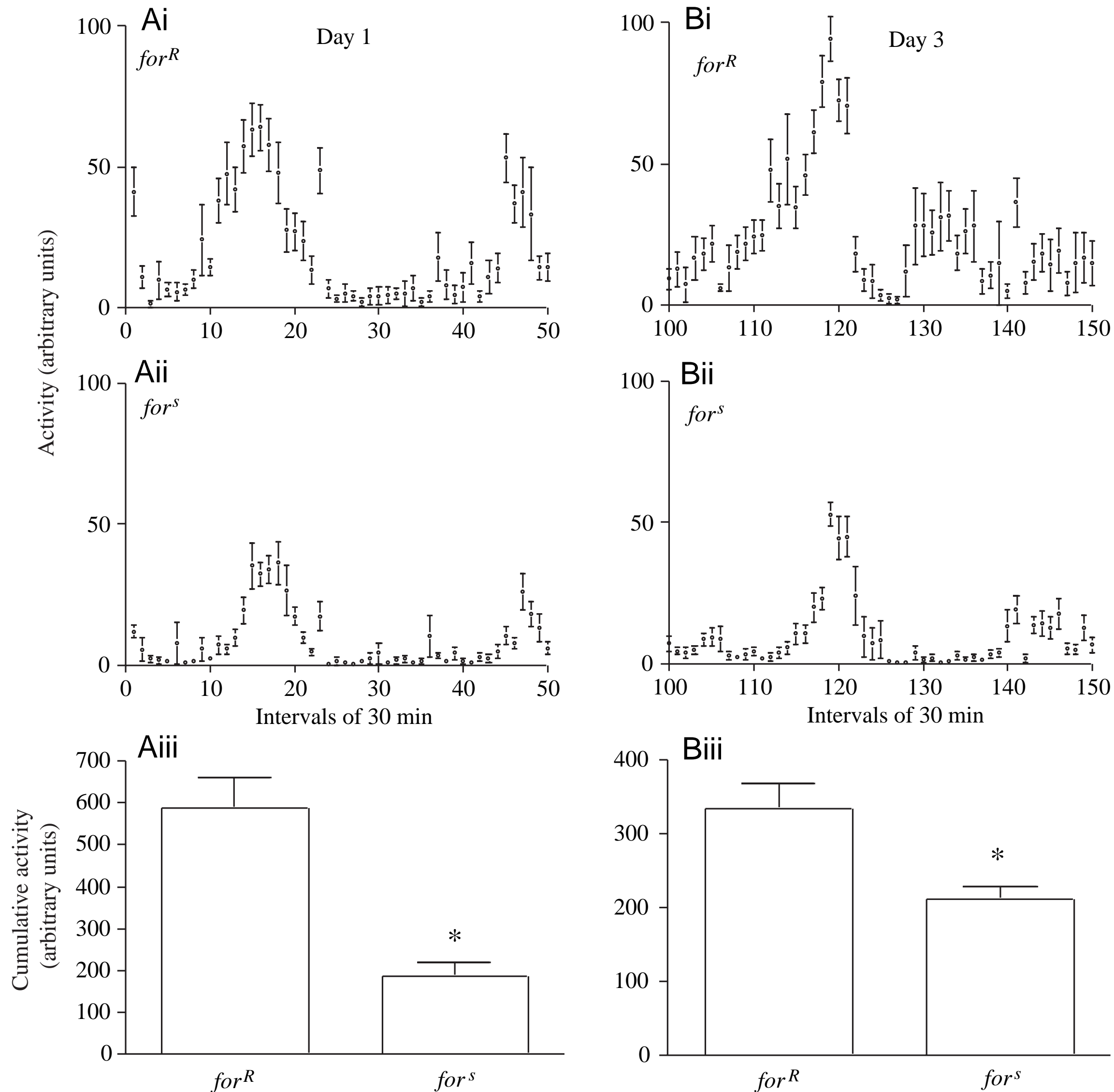

Fig. 1. Adult *for*^{R} and *for*^{s} display distinct locomotor activities. Adult flies were put through activity monitor trials as described. The results show activity over the course of the first day (A) after the initial rest period, of *for*^{R} (Ai) and *for*^{s} (Aii) flies, and on day 3 (Bi,ii). Results of analysis of the area under the major peaks (data points 10–25 on day 1; 110–125 on day 3) are shown in (Aiii, Biii). Values (*y* axis) are arbitary units ± S.E.M. (N=9–10). *P<0.05.

in tubules, we assayed tubule cGK activity, as well as that from head and body. Measurements of tubule cGK activity using a cGK-specific phosphorylation substrate showed this to be unchanged in *for*^{s} tubules compared to *for*^{R} (Fig. 3, P=0.109, unpaired *t*-test). Similar analyses of bodies also failed to show any difference in cGK activity (Fig. 3, P=0.577, unpaired *t*-test); however, whilst analyses of head extract appear to show a slight decrease in cGK activity (consistent with published results), this proved not to be statistically significant (Fig. 3, P=0.148, unpaired *t*-test).

Given the similarities in cGK activity between *for*^{R} and *for*^{s} tubules, the epithelial phenotype characterised in the studies shown in Fig. 2 would appear to be due to modulation of other regulatory components.

The for^s *allele impacts on cG-PDE activity*

We have previously shown that the degradation of cGMP by cG-PDE activity is critical in the regulation of fluid transport by the tubule (Broderick et al., 2003, 2004). Given this, we set out to assess any impact of the *for*^{s} allele on cG-PDE activity in both tubules and heads.

We identified a small, but nevertheless significant, increase in the cG-PDE activity of tubules from *for*^{s} compared to *for*^{R} flies (Fig. 4). In contrast to this, there is no significant difference in cG-PDE activity between heads from the *for*^{R} and *for*^{s} lines (P=0.73, unpaired *t*-test).

for^s *tubules contain reduced cGMP*

cGMP is a direct modulator of fluid transport by Malpighian

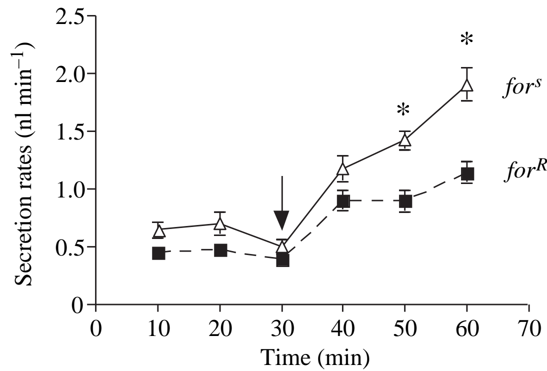

Fig. 2. The *for*^{s} allele results in a transport phenotype in tubules. Fluid secretion assays were performed on intact tubules from 7-day-old adult *for*^{R} and *for*^{s} flies. Basal rates of secretion were measured and capa-1 (10^{-7} mol l^{-1}) (Kean et al., 2002) added at 30 min (arrow). Secretion rates were monitored for a further 30 min. Fluid secretion rate (nl min^{-1}) are means ± S.E.M. (N=8) for *for*^{R} tubules (broken line) and *for*^{s} tubules (solid line). *P<0.05, using t-tests on experimental *versus* control at each time point separately.

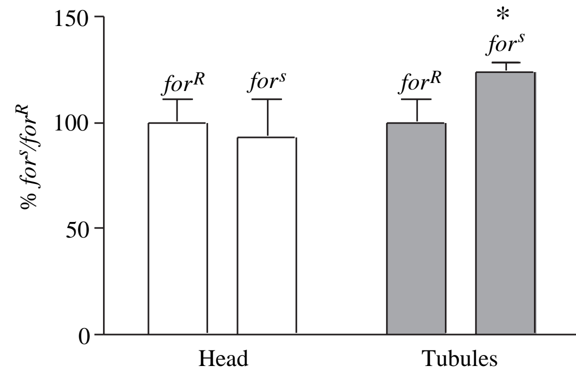

Fig. 4. *for*^{s} tubules display increased basal cGMP-phosphodiesterase (cG-PDE) activity. cG-PDE activity was assayed in preparations from head (open bars) and tubule (grey bars) from *for*^{R} and *for*^{s} animals as described. To aid comparison between head and tubule samples, cG-PDE activity in *for*^{R} is taken as 100%, and *for*^{s} activity normalised against this, for each tissue (means ± S.E.M., N=4–6), *P<0.05. *for*^{R} cG-PDE activities: head, 211±23 pmol min^{-1} mg^{-1}; tubule, 642±72 pmol min^{-1} mg^{-1} protein.

tubules (Dow et al., 1994b), where the use of selective inhibitors has identified cG-PDE activity as playing a key regulatory role by manipulating cGMP levels in tubules. Here we identify differences in cG-PDE activity in *for*^{s} flies (Fig. 4). To determine if this could influence resting cGMP levels, we assessed the cGMP content in tubules from both *for*^{R} and *for*^{s} lines (Fig. 5). Intriguingly, basal cGMP levels are significantly reduced in *for*^{s} (28±6 fmol 20^{-1} tubules) compared to *for*^{R} tubules (40±4 fmol 20^{-1} tubules). By contrast, cGMP levels are elevated to the same levels in both lines upon stimulation by the nitridergic peptide, capa-1 (*for*^{R}: 76±11; *for*^{s}: 69±12 pmol 20^{-1} tubules, Fig. 5). This suggests that in *for*^{s} tubules there is a greater increase in cGMP content compared to that in *for*^{R} tubules, in response to capa-1 (*for*^{R}: approx 187% stimulation; *for*^{s}: approx. 250% stimulation). Also, the *for*^{s} allele does not compromise the ability of these tubules to synthesise cGMP upon hormonal stimulation.

Capa modulation of fluid transport via *cGMP: downregulating cG-PDE*

The novel transport phenotype identified here in *for*^{s} tubules is observed upon stimulation with exogenously added cGMP (Dow and Davies, 2001) and also with capa-1 (Fig. 2). We thus assayed capa-1 stimulated cGK and cG-PDE activity to determine if a change in their activities may play a role in the phenotype. Fig. 6 shows a small reduction in cGK activity

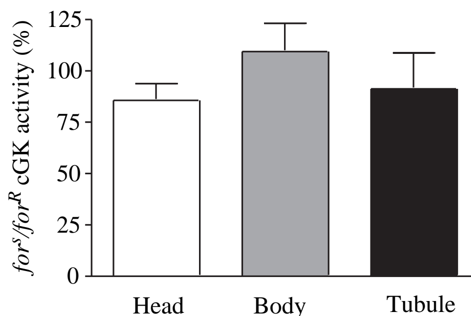

Fig. 3. Cyclic-GMP dependent kinase (cGK) activity in *for*^{s} tissue. cGK activity was assayed in head, body and tubule preparations from *for*^{R} and *for*^{s} 7-day-old adults, as described, in the absence and presence of cGMP. Data was corrected for specific cGMP-dependent activity, and *for*^{s} data expressed as a % of *for*^{R}. Values are means ± S.E.M., N=8–12. Mean cGMP-dependent activities (pmol ATP min^{-1} mg^{-1} protein) for *for*^{R} flies were: 15.4±1.3 (heads), 1.1±0.1 (bodies), 12.9±1.5 (tubules); and *for*^{s} flies: 12.84±1.1 (heads), 1.2±0.1 (bodies), 13.57±2.6 (tubules). In all experiments, cGK activity (pmol ATP min^{-1} mg^{-1} protein) was consistently lower in bodies than in either head or tubules.

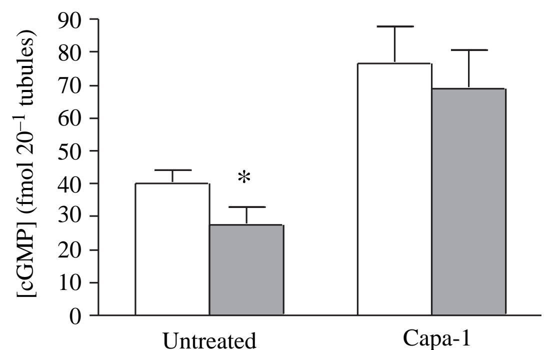

Fig. 5. Resting cGMP content is reduced in *for*^{s} tubules. cGMP content of *for*^{R} (unshaded bars) and *for*^{s} (grey bars) tubules were assayed (20 per sample) as described. Tubule samples were either untreated or treated with capa-1 (10^{-7} mol l^{-1}) (Kean et al., 2002) for 10 min prior to terminating the reaction. Data are expressed as cGMP content (fmol 20^{-1} tubules) mean ± S.E.M., N=4. *Statistically significant data between *for*^{R} and *for*^{S} (P<0.05).

in capa-1-stimulated *for*^{R} tubules (Fig. 6A). Interestingly, however, no change in capa-1-stimulated cGK activity is observed in *for*^{s} flies (Fig. 6A). Thus, capa-1 modulation of cGK activity in *for*^{s} tubules is not measurable.

In contrast to this, capa-1 treatment results in a significant reduction in cG-PDE activity in both *for*^{R} (approx. 37% reduction, Fig. 6B) and *for*^{s} (approx. 70% reduction, Fig. 6B). cG-PDE activity was also reduced in wild-type Oregon R tubules to a similar extent as *for*^{R} (data not shown). The extent of inhibition of cG-PDE activity was greater in *for*^{s} tubules compared to *for*^{R} (Fig. 6B; cG-PDE activities, expressed as pmol GMP min^{-1} mg^{-1} protein: *for*^{s} control, 798±31; *for*^{s} capa-1 treated, 238±21; *for*^{R} control, 642±72; *for*^{R} capa-1 treated, 407±64) Under such conditions, reduced cG-PDE activity can be expected to result in maintenance of high intracellular cGMP levels (Broderick et al., 2003), ultimately resulting in elevated fluid transport rates upon capa-1 stimulation in *for*^{s}.

Discussion

Previous work has shown that cGMP signalling, and the action of cG-PDE, is critical for epithelial transport (Broderick et al., 2003, 2004; Davies et al., 1995, 1997; Dow et al., 1994b). Work in vertebrates has also shown that cGKII is necessary for the correct physiological function of several epithelial tissues (French et al., 1995; Gambaryan et al., 1996; Pfeifer et al., 1996). Thus, in order to further define the role of cGMP signalling in epithelial transport, we compared two naturally occurring polymorphic alleles of *Drosophila dg2*, *for*^{R} and *for*^{s} (de Belle et al., 1989; Sokolowski and Hansell, 1992) – as a 10% reduction in head cGK activity in *for*^{s} animals has been noted by others and suggested as being associated with a behavioural phenotype (Osborne et al., 1997).

While we did observe a small inhibition of cGK in the heads of *for*^{s} adults (and despite phenotypic confirmation of the 'sitter' phenotype), the reduction was not, however, statistically significant (Fig. 2). Nevertheless, this result does not necessarily exclude that changes in *dg2* underlie the *foraging* polymorphism. It is possible that subtle environmental effects could lead to small differences in cGK assessments in different laboratories, or that the difference in cGK activity associated with the polymorphism is relatively modest. Furthermore, compartmentalisation of cAMP and cGMP signalling pathways (Edwards and Scott, 2000; Schlossmann et al., 2000), results in changes in phosphorylation status of proteins associated with such 'pools'. Therefore, measurements of bulk, as opposed to localised, cGK activity, may not be sufficient to monitor subtle changes in cGK. Perhaps most obviously, there are two cGK genes in *Drosophila*, and so even substantial changes in *dg2* levels might be undetectable against a high background of *dg1* protein.

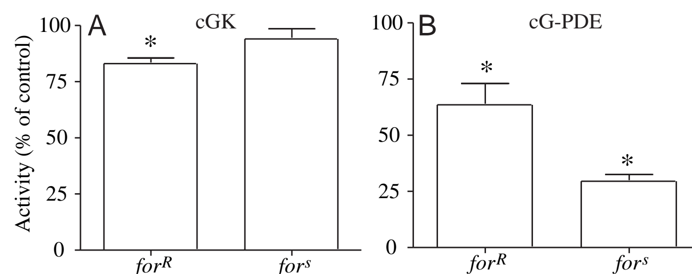

Fig. 6. cG-PDE activity is significantly inhibited by capa-1 peptide. cGK (A) and cG-PDE (B) activities were assayed in tubule preparations from *for*^{R} and *for*^{s} animals as described, under control and capa-1-stimulated conditions. cGK activity was assessed in the presence of cGMP. Tubules were pre-treated with capa-1 (10^{-7} mol l^{-1}) (Kean et al., 2002) for 10 min, prior to homogenisation and sample preparation. In order to aid comparison, data for both cGK and cG-PDE activity in the presence of capa-1 are expressed as % of untreated activity, mean ± S.E.M. (N=4–6). *Statistically significant data between controls (100%) and capa-1-treated samples (P<0.05).

Notwithstanding this, our data indicated that cGK activity was unchanged in tubule and so clearly did not seem to form the basis of the *dg2* phenotype observed in tubules. As cGMP signalling has been clearly shown to play a pivotal role in tubule functioning, we reasoned that other components of the cGMP pathway might be involved. In this regard, cG-PDE provides the sole route for the degradation of the second messenger, cGMP, and as such, is poised to play a key regulatory role in controlling cGMP signalling in cells. Indeed, we have shown this to be the case in tubules (Broderick et al., 2004, 2003; Dow et al., 1994b), prompting us to probe for any role of cG-PDE activity in this tubule phenotype.

Analysis of cG-PDE activity in *for*^{R} and *for*^{s} adults showed that cG-PDE activity is affected in several ways in the *dg2* mutation. Firstly, in *for*^{s} animals, basal cG-PDE activity is increased in tubules, although not in head, which results in decreased cGMP levels. This is consistent with the role of cG-PDE in maintaining cGMP homeostasis. Secondly, capa-1 stimulated cGMP levels are increased to similar amounts in both lines, suggesting that the increase in cGMP is greater in *for*^{s} compared to *for*^{R}, which may implicate differential regulation of cG-PDE activity by capa-1 in *for*^{s} tubules. This is confirmed by the data on capa-1 modulation of cGK and cG-PDE (Fig. 6). cG-PDE activity is depressed by capa-1, resulting in increased cGMP content. This occurs to a greater extent in *for*^{s}, however, resulting in the greater fold-stimulation in cGMP content. This would appear to provide a rationale for the transport phenotype (hypersensitivity to capa-1) observed in *for*^{s} tubules. The effects of capa-1 on cG-PDE activity are consistent with its role in stimulated fluid transport, and show that the mode of action of this peptide is not merely to stimulate cGMP production *via* soluble guanylate cyclase (Kean et al., 2002), but also to act *via* the potent inhibition of cGMP breakdown. *Manduca sexta* CAP_{2b} (Davies et al., 1995), another member of the capa family (Kean et al., 2002), also acts to inhibit cG-PDE in Oregon R, *for*^{R} and *for*^{s} tubules (data not shown). Thus, the inhibition of cG-PDE may be a general mechanism of action by the capa family of nitridergic peptides. Previous work shows that

regulation of cGMP breakdown *via* cG-PDE, as opposed to cGMP synthesis, is a powerful modulator of fluid transport in tubules (Broderick et al., 2003), suggesting that cG-PDE(s) have a central role in epithelial transport and are thus candidate targets for nitridergic peptide action. An analogous situation exists for cAMP signalling in tubules. A cAMP-mobilising hormone, Corticotrophin-like Releasing Factor (CRF), has been shown to modulate cAMP-specific PDE activity in tubules (Cabrero et al., 2002). Thus in insects, as in vertebrates, modulation of PDEs by specific hormones is an effective signalling mechanism (Dousa, 1999).

No measurable change in cGK activity was observed in capa-stimulated for^s tubules, which suggests that the neuropeptide-stimulated epithelial phenotype in for^s tubules is entirely due to cG-PDE. Modulation of cGK activity is not implicated in this process. However, in for^R, a small but significant decrease in tubule cGK activity is observed upon capa-1 stimulation, for which there is currently no explanation.

How can polymorphism at the *for* locus act on a functionally related, but physically remote, gene? The mapping of *for* to the region containing *dg2* is authoritative (Osborne et al., 1997); and although some alleles (e.g. gamma irradiation-induced) might be expected to impact on neighbouring genes as well as *dg2* (there are several genes within 10 kb of *for*), there is no cyclic nucleotide phosphodiesterase within megabases of the *for* locus. Additionally, differences in cGK levels between the non-lethal alleles of *for* are either modest (Osborne et al., 1997), or undetectable (this work), yet there is still an impact on cGMP signalling. We propose that a solution is offered by the concept of feedback; in order to maintain signal integrity, relatively modest changes in cGK activity elicit relatively large changes in cG-PDE, so compensating for differences in kinase levels. PDEs undergo post-translational modification by phosphorylation, interactions with other proteins and by proteolytic cleavage (Francis et al., 2001). It is possible that small changes in cGK can profoundly affect the activity of cG-PDE in tubule. Thus the polymorphism at the *for* locus may indeed act to modulate cGMP signalling, but through an unexpected route. This concept is consistent with a previous observation that tubules in which nitric oxide synthase was overexpressed by around twofold showed only a modest increase in stimulated secretion, because cG-PDE was upregulated by nearly tenfold (Broderick et al., 2003). Accordingly, there is evidence that, in at least this tissue, cG-PDE activity can vary over quite a wide range in order to compensate for relatively modest perturbations elsewhere in the pathway.

We have thus uncovered a central role for cG-PDE in tubules of a *dg2* allele. Furthermore, we show that the capa peptides modulate cG-PDE activity as an effective mechanism of increasing cGMP content *in vivo*.

There are now obvious and exciting avenues for further study: it may be that modulation of cG-PDE may provide an interesting and general explanation for the effects of the *foraging* polymorphisms in other contexts, such as susceptibility to parasitism and neuronal activity.

We would like to thank Professor N. Pyne, University of Strathclyde for reagents for PDE assays, Professor M. Sokolowski, University of Toronto at Mississauga, for the kind gift of for^R and for^S lines, and Dr P. Rosay, University of Bordeaux, for helpful advice on locomotion assays. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC), UK and a Wellcome Trust Studentship to J.P.D.

References

Broderick, K. E., Kean, L., Dow, J. A. T., Pyne, N. J. and Davies, S. A. (2004). Ectopic expression of bovine type 5 phosphodiesterase confers a renal phenotype in *Drosophila*. *J. Biol. Chem.* **279**, 8159-8168.

Broderick, K. E., MacPherson, M. R., Regulski, M., Tully, T., Dow, J. A. T. and Davies, S. A. (2003). Interactions between epithelial nitric oxide signaling and phosphodiesterase activity in *Drosophila*. *Am. J. Physiol.* **285**, C1207-C1218.

Cabrero, P., Radford, J. C., Broderick, K. E., Veenstra, J., Spana, E., Davies, S. and Dow, J. A. T. (2002). The CRF gene of *Drosophila melanogaster* encodes a diuretic peptide that activates cAMP signalling. *J. Exp. Biol.* **205**, 3799-3807.

Davies, S.-A. (2000). Nitric oxide signalling in insects. *Insect Biochem. Mol. Biol.* **30**, 1123-1138.

Davies, S. A., Huesmann, G. R., Maddrell, S. H. P., O'Donnell, M. J., Skaer, N. J. V., Dow, J. A. T. and Tublitz, N. J. (1995). CAP_{2b}, a cardioacceleratory peptide, is present in *Drosophila* and stimulates tubule fluid secretion *via* cGMP. *Am. J. Physiol.* **269**, R1321-R1326.

Davies, S. A., Stewart, E. J., Huesmann, G. R., Skaer, N. J. V., Maddrell, S. H. P., Tublitz, N. J. and Dow, J. A. T. (1997). Neuropeptide stimulation of the nitric oxide signaling pathway in *Drosophila melanogaster* Malpighian tubules. *Am. J. Physiol.* **42**, R823-R827.

Day, J. P., Houslay, M. D. and Davies, S. A. (2003). Cloning and characterisation of a novel cGMP-specific phosphodiesterase from *Drosophila melanogaster*. *BMC Meeting Abstracts: 1st International Conference on cGMP: NO/sGC Interaction and its Therapeutic Implications* **1**, p: 0014.

de Belle, J. S., Hilliker, A. J. and Sokolowski, M. B. (1989). Genetic localization of foraging (*for*): a major gene for larval behavior in *Drosophila melanogaster*. *Genetics* **123**, 157-163.

de Belle, J. S., Sokolowski, M. B. and Hilliker, A. J. (1993). Genetic analysis of the *foraging* microregion of *Drosophila melanogaster*. *Genome* **36**, 94-101.

Dousa, T. P. (1999). Cyclic-3′,5′-nucleotide phosphodiesterase isozymes in cell biology and pathophysiology of the kidney. *Kidney Int.* **55**, 29-62.

Dow, J. A. T. (1999). The multifunctional *Drosophila melanogaster* V-ATPase is encoded by a multigene family. *J. Bioenerget. Biomembr.* **31**, 75-83.

Dow, J. A. T. and Davies, S. A. (2001). The *Drosophila melanogaster* Malpighian tubule. *Adv. Insect Physiol.* **28**, 1-83.

Dow, J. A. T. and Davies, S. A. (2003). Integrative physiology, functional genomics and epithelial function in a genetic model organism. *Physiol. Rev.* **83**, 687-729.

Dow, J. A. T., Maddrell, S. H., Davies, S. A., Skaer, N. J. and Kaiser, K. (1994b). A novel role for the nitric oxide-cGMP signaling pathway: the control of epithelial function in *Drosophila*. *Am. J. Physiol.* **266**, R1716-1719.

Dow, J. A. T., Maddrell, S. H., Gortz, A., Skaer, N. J., Brogan, S. and Kaiser, K. (1994a). The malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. *J. Exp. Biol.* **197**, 421-428.

Edwards, A. S. and Scott, J. D. (2000). A-kinase anchoring proteins: protein kinase A and beyond. *Curr. Opin. Cell Biol.* **12**, 217-221.

Foster, J. L., Higgins, G. C. and Jackson, F. R. (1996). Biochemical properties and cellular localization of the *Drosophila* DG1 cGMP-dependent protein kinase. *J. Biol. Chem.* **271**, 23322-23328.

Francis, S. H., Turko, I. V. and Corbin, J. D. (2001). Cyclic nucleotide phosphodiesterases: relating structure and function. *Prog. Nucl. Acid Res. Mol. Biol.* **65**, 1-52.

French, P. J., Bijman, J., Edixhoven, M., Vaandrager, A. B., Scholte, B. J., Lohmann, S. M., Nairn, A. C. and de Jonge, H. R. (1995). Isotype-specific activation of cystic fibrosis transmembrane conductance regulator-

chloride channels by cGMP-dependent protein kinase II. *J. Biol. Chem*. **270**, 26626-26631.

Gambaryan, S., Hausler, C., Markert, T., Pohler, D., Jarchau, T., Walter, U., Haase, W., Kurtz, A. and Lohmann, S. M. (1996). Expression of type II cGMP-dependent protein kinase in rat kidney is regulated by dehydration and correlated with renin gene expression. *J. Clin. Invest.* **98**, 662-670.

Hall, K. U., Collins, S. P., Gamm, D. M., Massa, E., DePaoli-Roach, A. A. and Uhler, M. D. (1999). Phosphorylation-dependent inhibition of protein phosphatase-1 by G-substrate. A Purkinje cell substrate of the cyclic GMP-dependent protein kinase. *J. Biol. Chem*. **274**, 3485-3495.

Kalderon, D. and Rubin, G. M. (1989). cGMP-dependent protein kinase genes in *Drosophila*. *J. Biol. Chem.* **264**, 10738-10748.

Kean, L., Cazenave, W., Costes, L., Broderick, K. E., Graham, S., Pollock, V. P., Davies, S. A., Veenstra, J. A. and Dow, J. A. T. (2002). Two nitridergic peptides are encoded by the gene capability in *Drosophila melanogaster*. *Am. J. Physiol.* **282**, R1297-R1307.

Osborne, K. A., Robichon, A., Burgess, E., Butland, S., Shaw, R. A., Coulthard, A., Pereira, H. S., Greenspan, R. J. and Sokolowski, M. B. (1997). Natural behaviour polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. *Science* **277**, 834-836.

Oster, H., Werner, C., Magnone, M. C., Mayser, H., Feil, R., Seeliger, M. W., Hofmann, F. and Albrecht, U. (2003). cGMP-dependent protein kinase II modulates *mPer1* and *mPer2* gene induction and influences phase shifts of the circadian clock. *Curr. Biol.* **13**, 725-733.

Pereira, H. S. and Sokolowski, M. B. (1993). Mutations in the larval foraging gene affect adult locomotory behavior after feeding in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **90**, 5044-5046.

Pfeifer, A., Aszodi, A., Seidler, U., Ruth, P., Hofmann, F. and Fassler, R. (1996). Intestinal secretory defects and dwarfism in mice lacking cGMP-dependent protein kinase II. *Science* **274**, 2082-2086.

Schlossmann, J., Ammendola, A., Ashman, K., Zong, X., Huber, A., Neubauer, G., Wang, G. X., Allescher, H. D., Korth, M., Wilm, M. et al. (2000). Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. *Nature* **404**, 197-201.

Sokolowski, M. B. and Hansell, K. P. (1992). The foraging locus: behavioral tests for normal muscle movement in rover and sitter *Drosophila melanogaster* larvae. *Genetica* **85**, 205-209.

Vaandrager, A. B. and de Jonge, H. R. (1996). Signalling by cGMP-dependent protein kinases. *Mol. Cell. Biochem.* **157**, 23-30.