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REVIEW

Cell signalling mechanisms for insect stress tolerance

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ABSTRACT

Insects successfully occupy most environmental niches and this success depends on surviving a broad range of environmental stressors including temperature, desiccation, xenobiotic, osmotic and infection stress. Epithelial tissues play key roles as barriers between the external and internal environments and therefore maintain homeostasis and organismal tolerance to multiple stressors. As such, the crucial role of epithelia in organismal stress tolerance cannot be underestimated. At a molecular level, multiple cell-specific signalling pathways including cyclic cAMP, cyclic cGMP and calcium modulate tissue, and hence, organismal responses to stress. Thus, epithelial cell-specific signal transduction can be usefully studied to determine the molecular mechanisms of organismal stress tolerance *in vivo*. This review will explore cell signalling modulation of stress tolerance in insects by focusing on cell signalling in a fluid transporting epithelium – the Malpighian tubule. Manipulation of specific genes and signalling pathways in only defined tubule cell types can influence the survival outcome in response to multiple environmental stressors including desiccation, immune, salt (ionic) and oxidative stress, suggesting that studies in the genetic model *Drosophila melanogaster* may reveal novel pathways required for stress tolerance.

KEY WORDS: Cyclic AMP, Cyclic GMP, Calcium, Stress, *D. melanogaster*, Malpighian tubule

Introduction

Insects are exposed to multiple environmental stressors across a variety of habitats. In particular, insects are routinely exposed to desiccation, osmotic and xenobiotic stress and so have evolved highly successful strategies to combat these. There are multiple mechanisms for stress tolerance, thus allowing insects to successfully occupy virtually all ecological niches. It is becoming apparent that the molecular mechanisms for stress resistance and/or tolerance occur in key tissues, specifically epithelia. Epithelial tissue such as salivary glands, crop, gut and Malpighian tubules are barriers between the external and internal environments and so perform crucial roles in stress sensing and response. Moreover, organismal homeostasis depends on epithelial tissue, in particular, the osmoregulatory system. This comprises the fluid-secreting Malpighian tubules (in most insect species that have these) and the fluid-absorbing hindgut, which together maintain organismal ion and water homeostasis (Dow, 2013). *Drosophila melanogaster* has been used for studies in genetics and as a model insect for more than 100 years because of the molecular genetic tools available. This species is now increasingly used for work in biomedicine as well as in fundamental and applied biology, to understand mechanisms of function from molecule, cell and tissue to organism (Bellen et al., 2010; Dow, 2012a; Schneider, 2000).

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The Malpighian tubules as stress sensors

As insect Malpighian tubules are fluid-secreting tissues, tubule ion transport pathways have been extensively mapped (Beyenbach, 2003; O'Donnell et al., 2003; Spring et al., 2009; Wieczorek et al., 2009), with tubules from *D. melanogaster* holding the distinction of being the fastest fluid-transporting epithelia known in biology (Maddrell, 2009). However, much more remains to be discovered, including neuroendocrine control of ion transport and fluid secretion (Coast, 2007). It is now also known that insect tubules perform many more functions than just osmoregulation. Tissue-specific transcriptomics of *D. melanogaster* (Chintapalli et al., 2007; Wang et al., 2004) have led the way in assigning novel functions to *D. melanogaster* tubules (Dow, 2009). Importantly, assignation of novel functions such as detoxification and xenobiotic handling, and stress sensing of oxidative, osmotic (ionic/salt) and immune challenges by transcriptomics analysis (Chintapalli et al., 2007; Dow, 2009; Wang et al., 2004), has been underpinned by functional and physiological analysis (Chahine and O'Donnell, 2011; Daborn et al., 2012; Davies et al., 2012; Naikkhwah and O'Donnell, 2011; Torrie et al., 2004; Yang et al., 2007). Thus, the tubules are mission-critical tissues for insect survival.

Drosophila melanogaster tubules emerge from the hindgut, just behind the junction with the midgut, and constitute a pair of anterior and posterior tubules (Beyenbach et al., 2010). Recent work indicates that the anterior and posterior tubules exhibit transcriptome and functional asymmetry (Chintapalli et al., 2012), suggesting further intriguing possibilities of anterior- and posterior-specific roles for each pair of tubules. Tubules consist of two major cell types, the principal and stellate cells (Dow, 2009), which allow functional separation of ion transport and cell signalling pathways (Dow and Davies, 2003) for physiological function. Principal cells contain the large ion transport complexes, e.g. the vacuolar H^+-ATPase (V-ATPase) (Allan et al., 2005; Wieczorek et al., 2009) and the Na^+/K^+ exchanger (Torrie et al., 2004). Stellate cells, by contrast, express water channels (aquaporins) (Dow et al., 1995; Kaufmann et al., 2005) and control chloride flux (Denholm et al., 2013; Dow, 2012b; O'Donnell et al., 1998). Functional analysis so far suggests that the signalling pathways which mediate stress responses occur in the principal cell; however, the role of the stellate cell cannot be excluded.

Signalling pathways in the tubule principal cell

Signalling pathways, specifically those for calcium and cyclic nucleotides, were initially investigated in tubules from large and physiologically amenable insects, e.g. locusts and crickets (Anstee et al., 1980; Morgan and Mordue, 1985; Phillips, 1982), as well as medically relevant insects, e.g. *Rhodnius prolixus* (Maddrell et al., 1971). Work on the development of *D. melanogaster* tubules as a genetic model for fluid-transporting epithelia subsequently indicated

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List of abbreviations

cAMP	3′,5′-cyclic adenosine monophosphate
capaR	capa receptor
cGK	cGMP-dependent kinase
cGMP	3′,5′-cyclic guanosine monophosphate
cG-PDE	cGMP-hydrolysing PDE
CNG	cyclic nucleotide gated (channels)
CRF	corticotropin-releasing factor
DH-31	calcitonin-like neuropeptide
DH-44	CRF-related diuretic hormone
DILP	*D. melanogaster* insulin-like peptide
DNOS	*D. melanogaster* calcium/calmodulin-sensitive nitric oxide synthase
EPAC	exchange protein directly activated by cAMP
ERK	extracellular signal-regulated kinase
GC	guanylate cyclase
IP_3K	inositol 1,4,5-trisphosphate 3-kinase
NFAT	nuclear factor of activated T-cells
NO	nitric oxide
PDE	phosphodiesterase
PGRP	peptidoglycan recognition protein
PKA	protein kinase A
rGC	receptor-guanylate cyclase
ROS	reactive oxygen species
SOD	superoxide dismutase
TRPL	transient receptor potential-like (channel)
UAS	upstream activation sequence
V-ATPase	vacuolar H^+-ATPase

signalling pathways that modulated tubule fluid secretion (Dow et al., 1994a; Dow et al., 1994b). These signalling pathways were shown to be 3′,5′-cyclic adenosine monophosphate (cAMP) and, later, 3′,5′-cyclic guanosine monophosphate (cGMP). Moreover, work in *D. melanogaster* tubules showed for the first time that the gaseous second messenger, nitric oxide (NO), modulated renal function (Dow et al., 1994a). More recent work has shown very complex regulation of cAMP, cGMP and calcium signalling in tubule principal cells (Davies, 2006; Dow and Davies, 2003) (Fig. 1), especially in relation to signalling cascades initiated by neuropeptides (Davies et al., 2013). Mosquito tubules also contain principal and stellate cells, and research into cell signalling mechanisms in mosquito tubules has revealed important insights into control of epithelial function in blood-feeding insects via both cell types (Coast et al., 2005; Kersch and Pietrantonio, 2011; Pollock et al., 2004; Radford et al., 2004; Schepel et al., 2010).

The current state of knowledge for cyclic nucleotide and calcium signalling in the tubule principal cell will be discussed in the following sections. This will provide some insight into the complexity of these signalling pathways *in vivo*; the newly discovered role(s) of some of these signalling pathways in organismal stress tolerance will then be described.

Cyclic nucleotide signalling

In *D. melanogaster* tubules, fluid secretion into the tubule lumen is energised by the V-ATPase located on the tubule principal cell apical membrane (Allan et al., 2005; Davies et al., 1996; Dow, 1999). Transepithelial fluid secretion rates in the tubule main segment are stimulated by cAMP or cGMP (Dow et al., 1994b) and the V-ATPase is also thought to be the ultimate target of cyclic nucleotide signalling in the tubule principal cell, because of increased transepithelial potential difference in intact tubules treated with either cAMP or cGMP (Bijelic and O'Donnell, 2005; Davies et al., 1995) (Fig. 1). Recent work in mosquito tubules has demonstrated that cAMP stimulates both V-ATPase activity and assembly of the holoenzyme (Tiburcy et al., 2013), which supports the findings in other insects [e.g. the blowfly (Baumann and Bauer, 2013)] and in other systems (Bond and Forgac, 2008). Although it is also possible that cyclic nucleotide kinases such as protein kinase A (PKA) may act to phosphorylate modulatory proteins for the V-ATPase, how may cyclic nucleotides increase the transepithelial potential difference across tubule principal cells? Recent evidence shows that cGMP directly increases ATP concentration in tubules. cGMP is transported into principal, but not stellate, cells via transporters (Riegel et al., 1999), and application of exogenous cGMP to intact tubules results in increased ATP concentration (Davies et al., 2013). Such increased availability of ATP substrate for the V-ATPase may ultimately increase V-ATPase activity.

cAMP signalling

Neuropeptide signalling via the corticotropin-releasing factor (CRF)-related diuretic hormone (CRF-related DH), also known as DH-44, activates cAMP signalling in tubule principal cells (Cabrero et al., 2002). DH-44 is a ligand for two receptors, DH-44 R1 and R2 (Hector et al., 2009; Johnson et al., 2005). DH-44 R2 is encoded by CG12370 (Hector et al., 2009), and is expressed in epithelial tissue but most highly in tubules in the adult fly (Chintapalli et al., 2007; Robinson et al., 2013). cAMP signalling, induced by exogenous cAMP (Riegel et al., 1998) as well as DH-44, increases fluid secretion rates by acting on the tubule principal cell (Cabrero et al., 2002; Dow et al., 1994b). Interestingly, DH-44 also increases total cAMP hydrolysing activity by cAMP phosphodiesterases (PDEs) (Cabrero et al., 2002), suggesting that targeted breakdown of cAMP is required for DH-44 action in addition to generation of cAMP.

Calcitonin-like neuropeptide, DH-31, also raises cAMP in *D. melanogaster* tubules (Coast et al., 2001), but it is possible that DH-44 and DH-31 target different downstream effectors. In tubules of the malarial mosquito *Anopheles gambiae*, DH-31, but not DH-44, increases basolateral Na^+ conductance (Coast et al., 2005).

Recent use of targeted genetically encoded optogenetic probes has allowed further unique insights into cAMP signalling in the tubule (Efetova et al., 2013). A photoactive adenylate cyclase transgene (bPAC) can be activated to rapidly and reversibly generate cAMP pulses in a cell-type-specific manner using the GAL4/UAS binary expression system (Elliott and Brand, 2008). The GAL4/UAS targeted expression system utilises transgene expression directed by the binding of yeast transcription factor GAL4 (for which there are no endogenous targets in the fly) to its upstream activation sequence (UAS), allowing expression of any transgene cloned downstream of UAS. Cell- and tissue-specific GAL4 lines ('driver' lines) are available for many cells and tissues in *D. melanogaster* (Duffy, 2002; Sözen et al., 1997; Yang et al., 1995), allowing highly specific, targeted expression of transgenes *in vivo*. In particular, the GAL4 lines for tubule cell types and regions are highly specific (Rosay et al., 1997; Sözen et al., 1997; Terhzaz et al., 2012; Terhzaz et al., 2010b). Use of the targeted photoactive adenylate cyclase transgene bPAC in either tubule principal or stellate cells showed that PKA is necessary for basal fluid secretion rates in principal cells only, but is required for stimulated fluid secretion in stellate cells. Thus, in *D. melanogaster* tubule at least, PKA does not increase the activity of the V-ATPase under stimulated conditions. This work also demonstrated a novel role for the cAMP exchange protein, EPAC (Borland et al., 2009), in stimulated fluid secretion rates. PKA and EPAC have key roles in compartmentalised cAMP signalling (Houslay, 2010), and the work with the bPAC transgene provides the first evidence for insect tubules that compartmentalised cAMP signalling is essential for both basal and stimulated fluid

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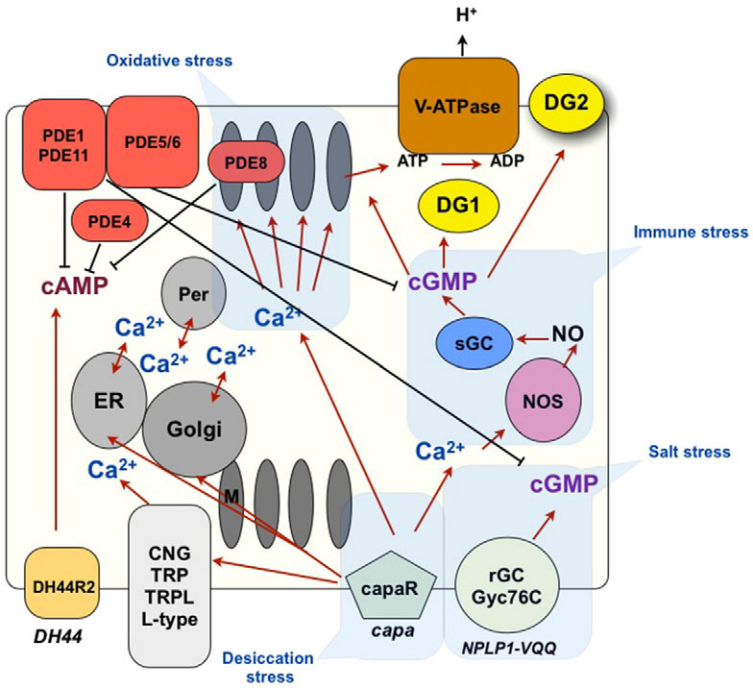

Fig. 1. Signalling pathways and components in the *Drosophila melanogaster* Malpighian tubule principal cell. The metabolically active principal cell contains the electrogenic V-type proton-motive ATPase (V-ATPase) at the apical membrane. 3′,5′-cyclic adenosine monophosphate (cAMP), 3′,5′-cyclic guanosine monophosphate (cGMP) and calcium (Ca^{2+}) signalling pathways are indicated, with neuropeptide receptors [DH-44R2 (Hector et al., 2009), capaR (Terhzaz et al., 2012), receptor guanylate cyclase Gyc76c (Overend et al., 2012)] and Ca^{2+} channels [L-type (MacPherson et al., 2001), transient receptor potential (TRP) and TRP-like (TRPL) (MacPherson et al., 2005) and cyclic nucleotide-gated (CNG) (Broderick et al., 2003)] on the basolateral membrane. Intracellular organelles, i.e. the endoplasmic reticulum (ER), Golgi body and peroxisomes (Per) are indicated in grey. Populations of mitochondria (M; in grey) are illustrated in the vicinity of the basolateral and apical membrane (Terhzaz et al., 2006). Cell signalling components that have been experimentally determined to act in the principal cells for organismal stress tolerance are indicated by light blue shading as follows: oxidative stress (Brown et al., 2013; Terhzaz et al., 2010a; Terhzaz et al., 2010b), immune stress (Davies and Dow, 2009; McGettigan et al., 2005), salt stress (Overend et al., 2012) and desiccation stress (Terhzaz et al., 2012). Abbreviations are as follows: sGC, soluble guanylate cyclase; NOS, nitric oxide synthase; DG, cGMP-dependent protein kinase; PDE, phosphodiesterase. Red arrows indicate stimulatory pathways; black headless arrows indicate inhibitory pathways.

secretion rates, and may also explain different downstream signalling by different neuropeptides utilising the same second messenger, e.g. DH-44 and DH-31.

In addition to stimulation of fluid secretion, cAMP (and cGMP) has also been shown to increase transepithelial cation transport across the main segment of the *D. melanogaster* tubules (Bijelic and O'Donnell, 2005). This suggests that multiple ion transport events can be modulated by each cyclic nucleotide.

cGMP signalling

cGMP signalling in the tubule principal cell relies on generation of the signal via either receptor or soluble guanylate cyclases (GCs) and breakdown via PDEs (Fig. 1). Several receptor GCs (rGCs) are expressed in tubules, including Gyc76c, CG33958 (also known as CG5719) and CG34357 (previously CG9783) (Davies, 2006). cGMP is also generated via NO-stimulated soluble GC (Davies et al., 1997; Dow et al., 1994a) in only tubule principal cells (Broderick et al., 2003). Neuroendocrine stimulation of principal cell cGMP occurs via the capa peptide family, which stimulate NO and the soluble GC (Davies et al., 2013; Kean et al., 2002), and also by NPLP1-4, which causes a rise in cGMP via the Gyc76c rGC (Overend et al., 2012). cGMP stimulates tubule fluid secretion and increases transepithelial potential (Bijelic and O'Donnell, 2005; Dow et al., 1994b), but also activates the cognate serine/threonine cGMP-dependent kinases (cGKs) DG1 and DG2 (Kalderon and Rubin, 1989; MacPherson et al., 2004b; Osborne et al., 1997) at micromolar concentration (MacPherson et al., 2004b). DG1 is localised to the cytosol; by contrast, DG2 is membrane localised. Using transgenic flies bearing principal cell-targeted gain-of-function dg1 and dg2 constructs, it was demonstrated that DG1 transduces a cytosolic cGMP signal, whereas DG2 transduces the cGMP signal generated at the basolateral membrane (MacPherson et al., 2004b). Thus, each kinase, although with similar EC_{50} values for cGMP (MacPherson et al., 2004b), transduces a localised cGMP signal. Moreover, dg1 is almost exclusively expressed in tubules and in hindgut (Chintapalli et al., 2007; Robinson et al., 2013) whereas dg2 is expressed throughout the fly and also regulates behaviour (Reaume and Sokolowski, 2009), so it is possible that these two cGKs have entirely different roles in tubule principal cells.

cAMP and cGMP have been shown to play a role in stellate cells (Kerr et al., 2004), although the endogenous pathways for cAMP and cGMP are not known for this tubule cell type. Recent research, however, has demonstrated that cGMP acting through DG1 (but not DG2) can inhibit transepithelial responses induced by both tyramine and *D. melanogaster* leucokinin (Ruka et al., 2013), both of which increase calcium signalling and chloride conductance (Blumenthal, 2003; Cabrero et al., 2013; O'Donnell et al., 1996; Radford et al., 2004; Terhzaz et al., 1999). Thus, a yet-unidentified inhibitory process for tyramine and *D. melanogaster* leucokinin signalling in stellate cells is cGMP/DG1-mediated.

Degradation of cGMP occurs by the cGMP-hydrolysing PDEs (cG-PDEs), whose activity can be regulated to maintain cGMP levels. For example, cG-PDE activity in tubules is depressed by *Manduca sexta* CAP2b (MacPherson et al., 2004a), a member of the capa neuropeptide family (Tublitz and Truman, 1985a). Thus, capa peptides regulate cGMP concentration in the principal cells by both generation and breakdown of cGMP.

Further regulation of cGMP signalling can occur as a result of interactions between the cGKs and the cG-PDEs. cGKs phosphorylate cG-PDEs (Francis et al., 2011), e.g. PDE5, thus modifying their activity. The *D. melanogaster* PDE5 orthologue (DmPDE5/6) is a cGMP-specific PDE (Day et al., 2005), although it is not known whether it is phosphorylated by either DG1 or DG2. However, DmPDE5/6 contains two consensus serine/threonine phosphorylation sites for cGK/PKA-KKRS and KRPS, as well as the regulatory GAF domains present in PDE5 (Davies and Day, 2007), so it is likely that DmPDE5/6 is regulated by cGK (and possibly PKA). Cross-talk between the cAMP and cGMP pathways may also occur as both DG1 and DG2 can be activated by 20 μmol l^{-1} cAMP (MacPherson et al., 2004b). Also, some of the PDEs are dual specificity enzymes, capable of hydrolysing both cAMP and cGMP (e.g. PDE1 and PDE11), so this allows for further cross-talk between cAMP and cGMP under physiological conditions.

Although the only route for degradation of cyclic nucleotides is via the PDEs (Bender and Beavo, 2006), extrusion of cyclic nucleotides in some cell types also contributes to reduction of cyclic nucleotide levels. In tubule principal cells, DmPDE5/6 is expressed at the apical membrane, and controls cGMP efflux into the lumen (Day et al., 2006), suggesting that control of PDE levels in tubule principal cells is not only due to breakdown by PDEs. The transporters for cGMP are not yet known, although White has been shown to encode an ABC transporter for cGMP in tubule principal cells (Evans et al., 2008).

Calcium signalling in tubule principal cells

Calcium (Ca^{2+}) is a ubiquitous second messenger molecule in all cell types and tissues, and in the tubule, it modulates fluid secretion rates, V-ATPase activity, and downstream signalling and ion transport events (Davies and Terhzaz, 2009).

Use of genetically encoded aequorin-based luminescent Ca^{2+} reporters specifically targeted to only tubule principal cells using the binary GAL4/UAS expression system (Brand and Perrimon, 1993) was first used to demonstrate *in vivo* Ca^{2+} signalling in defined populations of tubule cells (Kean et al., 2002; Rosay et al., 1997; Terhzaz et al., 2012). This work demonstrated that the *Manduca sexta* neuropeptide CAP2b, a member of the capa peptide family (Davies et al., 1995; Loi and Tublitz, 2004), stimulated a rise in intracellular (Ca^{2+}) ($[Ca^{2+}]_i$) in only principal cells, comprising a fast $[Ca^{2+}]_i$ spike followed by a slow $[Ca^{2+}]$ rise. The fast $[Ca^{2+}]_i$ rise was shown to occur via phospholipase Cβ and inositol 1,4,5-trisphosphate receptor (Pollock et al., 2003), and also involves Ca^{2+} release via the Golgi sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) channel (Davies and Terhzaz, 2009). CAP2b was also shown to stimulate Ca^{2+} influx through principal cell plasma membrane Ca^{2+} channels – transient receptor-like (TRPL), L-type and cyclic nucleotide-gated (CNG) channels (Fig. 1) – which contributes to the slow CAP2b-stimulated $[Ca^{2+}]_i$ rise (Broderick et al., 2003; MacPherson et al., 2001; MacPherson et al., 2005). The physiological effects of CAP2b and endogenous *D. melanogaster* capa peptides, Drome-capa-1 and -2 (Davies et al., 2013), act similarly on *D. melanogaster* principal cells, and Drome-capa-1 was subsequently shown to modulate organellar Ca^{2+} signalling in the mitochondria (Terhzaz et al., 2006), and also the Golgi- and peroxisome-localised secretory pathway Ca^{2+}/Mn^{2+}-ATPases (Southall et al., 2006).

Luminescent and fluorescent Ca^{2+} reporters (Davies and Terhzaz, 2009) targeted to mitochondria of tubule principal cells also showed that Drome-capa-1 triggered mitochondrial Ca^{2+} uptake via the mitochondrial calcium uniporter at the apical membrane, resulting in activation of these mitochondria in the vicinity of the V-ATPase (Terhzaz et al., 2006). Drome-capa-1-activated mitochondria increase ATP production, driving proton pumping of the V-ATPase complex (Terhzaz et al., 2006). However, the Drome-capa-1 receptor resides on the principal cell basolateral membrane, so Drome-capa-induced activation of the apical mitochondria demonstrates a novel spatio-temporal mode of action.

Overall, this highly complex repertoire of intracellular and plasma membrane calcium channels, ATPases and intracellular organelles (reviewed in Davies and Terhzaz, 2009) results in increased $[Ca^{2+}]_i$ by capa peptides (Kean et al., 2002). This $[Ca^{2+}]_i$ rise can trigger the activation of *Drosophila* calcium/calmodulin-sensitive nitric oxide synthase (DNOS) (Regulski and Tully, 1995), as Drome-capa-1 increases principal cell $[Ca^{2+}]_i$ from 87 to 255 nmol l^{-1}, which is close to the EC_{50} for calcium activation of DNOS. This results in the production of NO and subsequent increased cytoplasmic [cGMP] (Kean et al., 2002) due to NO-induced activity of the soluble GC (Davies et al., 1997) (Fig. 1).

Further cross-talk can occur between the cGMP and Ca^{2+} signalling pathways, as it has been demonstrated that exogenous cGMP induces $[Ca^{2+}]_i$ in tubule principal cells, which is abolished by plasma membrane Ca^{2+} channel blockers (MacPherson et al., 2001). Also, targeting a DNOS transgene to only principal cells shows potentiation of capa-peptide-induced $[Ca^{2+}]_i$ signals, potentially via Ca^{2+} influx through plasma membrane CNG channels (Broderick et al., 2003). Thus there is positive feedback regulation of Ca^{2+} signalling by NO/cGMP, to further increase $[Ca^{2+}]_i$, which then activates apical mitochondria and, subsequently, the V-ATPase. This may further explain the activation of mitochondrial ATP production by cGMP (potentially by increasing Ca^{2+} influx through CNG channels), thus, activating the V-ATPase and driving fluid secretion (Fig. 1).

cGMP signalling in stress responses

Immune stress

Tubules are immune tissues and express all components of the Imd (McGettigan et al., 2005) and Toll (Chintapalli et al., 2007; Robinson et al., 2013) innate immunity pathways. Acutely dissected tubules can bind and internalise lipopolysaccharide, a component of gram-negative bacterial coat, and can mount a significant bacterial killing response (McGettigan et al., 2005) via production of antimicrobial peptides. Antimicrobial peptides produced by the tubules are derived from both the Imd and Toll signalling pathways, and include diptericin, attacin, cecropin, metchnikowin, defensin and drosomycin (McGettigan et al., 2005; Tzou et al., 2000), which may be secreted into either the haemolymph or into the gut. Tubules sense immune challenge by expressed peptidoglycan recognition proteins (PGRPs) (Charroux et al., 2009; Kurata, 2010). For example, PGRP-LE, which binds diaminopimelic-acid-containing peptidoglycan from gram-negative bacteria, is localised to the tubule principal cells (Kaneko et al., 2006). Interestingly, a fragment of PGRP-LE consisting only the PGRP domain acts as an extracellular receptor, possibly allowing tubules to sense pathogen invasion from the haemocoel. Tubules may also express transporters that can transport peptidoglycan fragments across the membrane (Kaneko et al., 2006).

Activation of Imd-associated antimicrobial peptide gene transcription occurs via the action of the NF-kB orthologue, Relish. Relish expression is enriched in epithelia and in fat body, both in adult and larval stages (Chintapalli et al., 2007; Robinson et al., 2013). Immune challenge causes phosphorylation and endoproteolytic cleavage of Relish, resulting in a nuclear-translocated Rel homology domain, and the Iκb-like domain, which remains cytoplasmic (Stoven et al., 2003). Nuclear translocation of Relish is utilised to assess Relish activity *in vivo*, in intact tubules. Under resting conditions, Relish is mainly localised to the tubule basolateral membrane, with some localisation to the large nuclei of principal cells (Fig. 2A). Treatment of tubules with gram-negative bacterial coat peptidoglycan and subsequent activation of the Imd pathway promotes complete nuclear translocation of Relish (Fig. 2A). Nitric oxide activates the Imd pathway and antimicrobial peptide expression in tissues including the tubules (McGettigan et al., 2005). Moreover, NO-stimulated diptericin expression in the principal cells is dependent on functional soluble GC (Davies and Dow, 2009). Here, we show that cGMP regulates Relish translocation in a dose-dependent manner (Fig. 2C). Nanomolar concentrations of cGMP (1–100 nmol l^{-1}) induce Relish translocation to the nucleus. By contrast, higher (μmol l^{-1})

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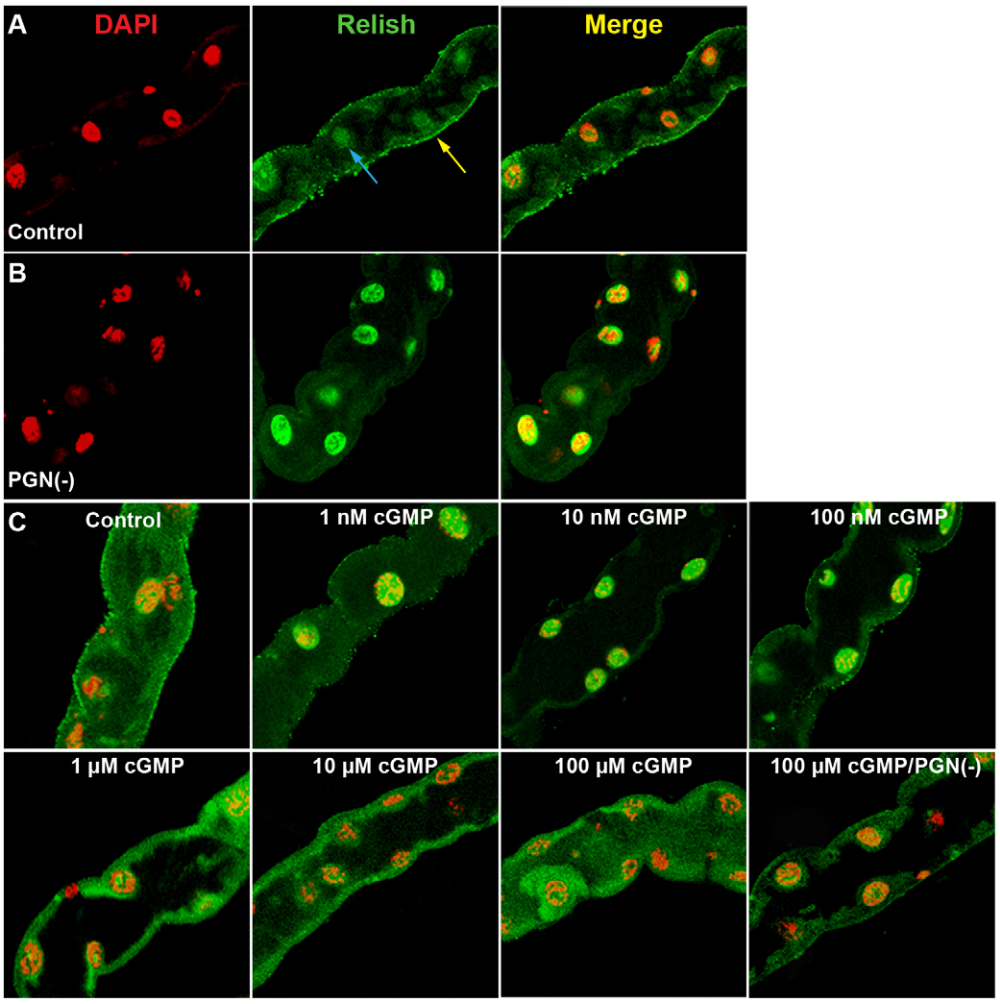

Fig. 2. cGMP-induced Relish translocation in intact tubules. (A) Confocal microscopy images of main segment of intact tubules from adult progeny of tubule principal cell driver c42 GAL4 and UAS-Relish-His6 (Hedengren et al., 1999) lines stained with DAPI (principal cell nuclei, red) and FITC (Relish, green). Strong co-localisation of DAPI and FITC signals are yellow or yellow/green. In control tubules, Relish is localised to the basolateral membrane, with weak localisation to the nucleus (arrow). Merged image of DAPI/FITC staining confirms Relish localisation at the basolateral membrane, with weak localisation to the nucleus. (B) As A, but treated with peptidoglycan (PGN), 5 µg ml^{-1} (Guntermann and Foley, 2011). Relish is completely translocated to principal cell nuclei upon immune challenge; note absence of membrane staining. The merged DAPI/FITC image confirms exclusive localisation of Relish to nuclei (yellow). (C) Concentration-dependent cGMP modulation of Relish translocation. Intact c42/UAS-Rel-His6 tubules were untreated (control, as in A), or treated with cGMP at concentrations from 1 nmol l^{-1} to 100 µmol l^{-1} (as shown) for 3 h prior to staining with DAPI and FITC. Confocal images of merged DAPI/FITC-labelled tubules are shown. Last panel: 100 µmol l^{-1} cGMP + 5 µg ml^{-1} PGN. Tubule diameter is 35 µm in all panels.

concentrations of cGMP block nuclear localization of Relish, and tubules pre-treated with 100 µmol l^{-1} cGMP prior to peptidoglycan treatment show reduced translocation of Relish to the nucleus. Thus, a saturating concentration of cGMP prevents nuclear translocation of Relish, even upon immune challenge. cGMP thus modulates Imd pathway signalling. It remains to be resolved whether the cGKs modulate Imd activity downstream of NO and cGMP (Davies et al., 2009).

Salt stress

Salt, or osmotic (ionic), stress on the whole organism is sensed and transduced by epithelial tissue, notably the tubule and gut. A microarray study on whole-fly responses to salt (NaCl) stress revealed gene changes across distinct functional gene groups, where the most significantly altered gene groups were those most highly expressed in tubules or in gut (Stergiopoulos et al., 2009). The transcription factor NFAT (nuclear factor of activated T-cells), which is enriched in tubules (Chintapalli et al., 2007; Robinson et al., 2013), also plays a role in salt tolerance (Keyser et al., 2007). Thus, the tubule is a key tissue for salt tolerance in the fly. Interestingly, salt stress induces an early immune gene transcriptional programme in epithelial tissue (Stergiopoulos et al., 2009).

More recently, we have shown that induction of salt stress in whole adult flies causes nuclear translocation of Relish in tubule principal cells (Overend et al., 2012). Salt stress also induces significantly increased expression of Imd pathway antimicrobial peptides diptericin and cecropin, and of the Toll pathway antimicrobial peptide drosomycin, in isolated tubules, thus validating the whole-fly salt stress microarray dataset.

Furthermore, the endogenous *D. melanogaster* peptide NPLP1-VQQ (NLGALKSSPVHGVQQ) (Baggerman et al., 2005) was shown to be a ligand for the tubule-enriched Gyc76c rGC, where a small but significant increase in cGMP levels was demonstrated using a peptide library screen in *D. melanogaster* S2 cells for activators of Gyc76c. *Drosophila melanogaster* rGCs have remained without identified ligands until now, and NPLP1-VQQ is the first putative ligand identified for a *Drosophila* rGC. NPLP1-VQQ also has a physiological role: it stimulates fluid secretion rates by intact tubules. NPLP1-VQQ/Gyc76c activation also results in Relish nuclear translocation and increased diptericin expression in tubule principal cells. It is possible that only a small increase in cGMP is observed upon NPLP1-VQQ stimulation of Gyc76c (Overend et al., 2012), as this is required to be within the stimulatory [cGMP] range for Relish translocation (Fig. 2). Finally, this work showed that targeted knockdown of Gyc76c in only tubule principal cells prevents cGMP production, localisation of Relish to the nucleus and induction of diptericin expression. Thus, Gyc76c and cGMP signalling modulates Relish/Imd pathway activation in tubule principal cells.

As Gyc76c/cGMP is a modulator of the Imd pathway (Overend et al., 2012), it was likely that NPLP1-VQQ/Gyc76c activation would enhance survival of immune challenge. However, targeted knockdown of Gyc76c to tubule principal cell showed that flies are

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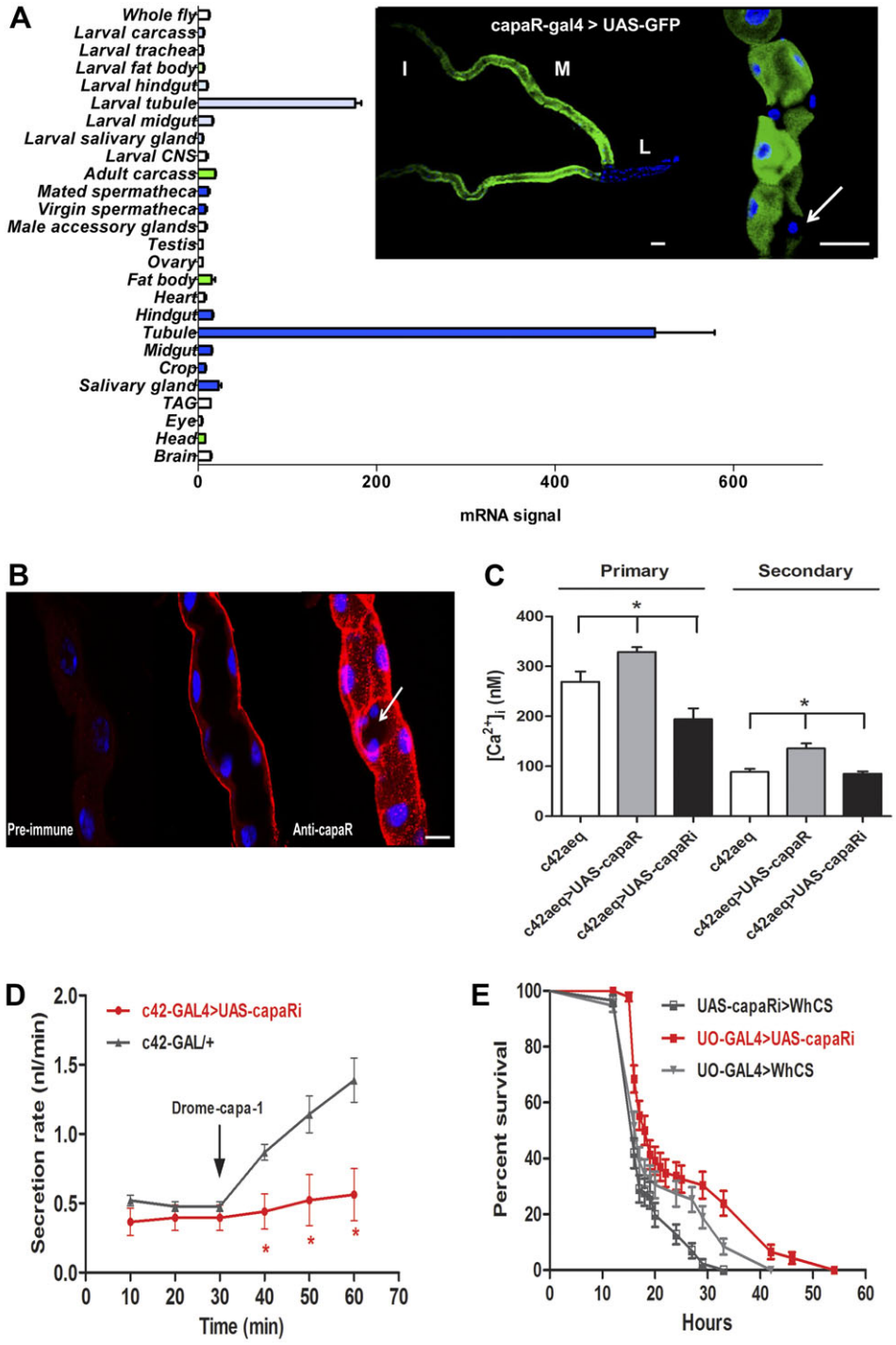

Fig. 3. See next page for legend.

not compromised for immune challenge, but rather for salt stress. cGMP has been shown to have both immune and stress-associated transcriptional targets by microarray (transcriptome) analysis of cGMP-treated tubule samples (Davies et al., 2012). Thus, cGMP signalling, and, therefore, pathway components (e.g. rGCs), modulate both immune and stress-responsive genes.

Signalling mechanisms in oxidative stress tolerance

It has been recently demonstrated that the Malpighian tubule is a major sensor for oxidative stress for the whole fly, as it is enriched for antioxidant genes (Terhzaz et al., 2010b). Production of reactive oxygen species (ROS) as a byproduct of ATP production by a very metabolically active tissue that is packed with mitochondria means that the tubule must be able to detect ROS and oxidative stress. Mitochondria in tubule principal cells are placed in either an apical or basal membrane location, and are differentially activated to produce ATP in response to a neuropeptide stimulus, i.e. Drome-capa-1 (Terhzaz et al., 2006). Mitochondria are thus critical determinants of tubule function, and therefore of organismal survival. Mitochondria are also key organelles for Ca^{2+} homeostasis

Fig. 3. CapaR is tubule-specific and modulates Ca^{2+} signalling, fluid homeostasis and desiccation tolerance. (A) Expression levels of capaR mRNA in adult and larval tissues. Mean (±s.e.m.) mRNA expression data were collated from Affymetrix tissue-specific array datasets (Chintapalli et al., 2007; Robinson et al., 2013) for adult and larval tissues as indicated. Blue shading (dark, adult; light, larvae) indicates epithelial tissues; green shading (dark, adult; light, larvae) indicates fat body or tissues containing fat body, e.g. adult head and carcass. 'mRNA signal' indicates capaR mRNA abundance. Tubules from adult progeny of the capaR promoter-driven GAL4 line, capaR-GAL4 (Terhzaz et al., 2012), crossed with UAS-GFP, showed green fluorescent protein (GFP) fluorescence in only the tubule main segment, and specifically in principal cells. Stellate cells do not show GFP fluorescence (arrow). Tubule regions are indicated by M (main segment), I (initial segment) and L (lower tubule). (B) *Drosophila* capaR is expressed in principal cells of the Malpighian tubule. Staining of wild-type adult tubules with pre-immune serum showed non-specific staining. Immunocytochemistry using anti-capaR rabbit polyclonal antibody and anti-rabbit IgG-Texas Red conjugate reveal basolateral membrane localization of capaR in tubule principal cells. Merge of z-stacks reveals exclusion of a stellate cell (arrow). Nuclei are labelled blue with DAPI. Scale bar, 30 μmol l^{-1}. (C) Manipulation of capaR affects cytosolic $[Ca^{2+}]_i$ levels in intact tubules. Tubules were dissected from calcium reporter flies, c42>UAS-apoaequorin (c42aeq) (Rosay et al., 1997) and flies in which the aequorin transgene was expressed in a capaR transgenic background – either c42aeq>UAS-capaR RNAi (capaR knockdown) or c42aeq>UAS-capaR. Resting cytoslic $[Ca^{2+}]_i$ levels were measured, after which tubules were stimulated with 10^{-7} mol l^{-1} Drome-capa-1 (Davies et al., 2013; Kean et al., 2002) to obtain stimulated cytosolic $[Ca^{2+}]_i$ readings. Primary and secondary pooled data for cytosolic $[Ca^{2+}]_i$ levels are shown as nmol l^{-1} $[Ca^{2+}]_i$ (means ± s.e.m., *N*=6, **P*<0.05, Student's *t*-test). (D) CapaR modulates fluid homeostasis. Fluid transport by *Drosophila* c42-GAL4>capaR RNAi renal tubules is significantly decreased (as determined using a Student's *t*-test, **P*<0.05) compared with the parental GAL4 line when the tubule is stimulated by application of Drome-capa-1 (10^{-7} mol l^{-1}) (Kean et al., 2002). Secretion rates are expressed as nl min^{-1} (means ± s.e.m., *N*=6). (E) Knock-down of capaR expression in principal cells enhances organismal survival to desiccation stress. Increased capaR expression in principal cells using the principal cell GAL4 driver UO-GAL4 (Terhzaz et al., 2010b) does not alter survival of desiccated flies compared with parental controls (Terhzaz et al., 2012). However, survival data from desiccation tolerance assays show that reduced capaR expression in principal cells via targeted RNAi against the capa gene (UO-GAL4>UAS-capaRi, red line) significantly increases survival of desiccated flies compared with controls (*P*<0.001 against both controls; log rank test, Mantel-Cox). Parental lines (UO-GAL4 and UAS-capaRi) were outcrossed to a CantonS wild-type allele of White (WhCS) (Green, 1959) to maintain the equivalent genetic load of each UO-GAL4 and UAS-capaRi compared with the progeny, UO-GAL4 >UAS-capaRi. Adapted from Terhzaz et al. (Terhzaz et al., 2012).

in tubules and in all other biological systems (Rizzuto et al., 2012). As mitochondria are so important for cellular function, dysregulation of mitochondrial Ca^{2+} in humans leads to disease states (Duchen et al., 2008), and *D. melanogaster* is successfully being utilised to produce new leads for diseases of oxidative stress, including neurodegeneration (Jaiswal et al., 2012).

Interestingly, work in *D. melanogaster* tubules has demonstrated that specific signalling genes expressed in only tubule principal cells can alter organismal susceptibility to oxidative stress. Modulation of principal cell inositol phosphate signalling, specifically via inositol 1,4,5 trisphosphate 3-kinase (IP_3K), using the GAL4/UAS system has been shown to increase ROS production. Precise targeting of either gain-of-function or loss-of-function (RNAi) *D. melanogaster* IP_3K-1 constructs to only tubule principal cells showed that IP_3K-1 increased H_2O_2 production, pro-apoptotic caspase-9 activity and mitochondrial membrane potential. IP_3K-1 also significantly increased mitochondrial Ca^{2+} under oxidative stress conditions, leading to apoptosis. Flies in which IP_3K-1 is overexpressed in only tubule principal cells are also significantly more susceptible to oxidative stress. Intriguingly, IP_3K-1 modulates epithelial cell apoptosis without involvement of bcl-2-type proteins.

Recently, other tubule principal cell candidate genes for oxidative stress tolerance have been discovered. Signalling by *D. melanogaster* insulin-like peptides (DILPs) has previously been successfully investigated in terms of neurobiology, nutritional status and ageing (Birse et al., 2011; Nassel, 2012; Partridge et al., 2011; Söderberg et al., 2012). There are seven DILPs, and DILP-5 has been shown to be expressed in tubule principal cells, together with the receptor for DILPs, *Drosophila* Insulin Receptor, dINR (Söderberg et al., 2011). Knockdown of DILP5 in tubule principal cells increases survival to oxidative stress induced by paraquat feeding. Interestingly, knockdown of DILP5 in larval principal cells results in increased lifespan. It is possible that DILP signalling modulates activity of the mitochondrial Mn^{2+} superoxide dismutase (SOD), as knockdown of sod2, encoding this SOD in only principal cells, reduces oxidative stress tolerance.

Other work involving mitochondrial function demonstrated that defects in mitochondrial proteins, e.g. the ADP/ATP translocase (encoded by sesB, which is notably enriched in tubule), cause reduced cytosolic and mitochondrial calcium signals, as well as reduced fluid secretion by intact tubules (Terhzaz et al., 2010a). Furthermore, sesB mutant tubules contain only 10% of the ATP levels but five times the ROS levels of control tubules, and sesB mutant flies display reduced resistance to oxidative stress. Thus, mitochondrial function in tubule principal cells is crucial for oxidative stress tolerance.

Most recently, cAMP signalling has been implicated in oxidative stress tolerance in *Drosophila*. Mammalian PDE8 is a cAMP PDE that is phosphorylated by PKA, resulting in enhanced enzyme activity (Brown et al., 2012). The PDE8A isoform is localised to mitochondria (Tsai and Beavo, 2011), and in *D. melanogaster*, DmPDE8 is the orthologue of mammalian PDE8A (Davies and Day, 2007). In a recent study, PDE8A was shown to have a novel binding partner, Raf-1, which allows modulation of downstream extracellular signal-regulated kinase (ERK) signalling via phosphorylation of ERK by Raf-1 kinase (Brown et al., 2013). DmPDE8 is abundantly expressed in epithelia, including the tubules, and PDE8 deletion mutants show reduced phospho-ERK signals. Critically, this biochemical interaction was shown to be relevant to oxidative stress tolerance in the organism, especially given the putative localisation of PDE8 in mitochondria as well as the role of the tubules in stress resistance. PDE8 deletion mutants are significantly more susceptible to oxidative stress imposed by either H_2O_2 or paraquat feeding, compared with parental controls (Brown et al., 2013). This work demonstrates for the first time in insects that cAMP and ERK signalling mediates oxidative stress tolerance.

cGMP and calcium cross-talk: desiccation stress tolerance

Capa peptides stimulate both cGMP and Ca^{2+} signalling, with several points for cross-regulation between the signalling pathways (Davies et al., 2013). Capa peptides modulate diuresis in all insects tested, and thus such complexity in downstream signalling may be necessary for maintenance of fluid homeostasis. Fluid homeostasis is critical for survival, as in many environments, desiccation is a major threat to terrestrial organisms. This is particularly relevant to insects, which have a small size and thus a large surface area to volume ratio. However, insect osmoregulatory systems may be adapted for water conservation, and insects may survive desiccation by regulating fluid secretion (excreted water loss) by the tubules, an energetically efficient strategy. *Drosophila melanogaster* has been used effectively in studies of insect desiccation tolerance: excreted water loss rates are

reduced in desiccated *D. melanogaster* (Folk and Bradley, 2003), and work with wild desiccation-resistant *Drosophila* populations showed single-feature polymorphisms in several gene groups. These included tubule ion transport genes, e.g. sodium and potassium channels/transporters and chloride transporters. Intriguingly, genes associated with Drome-capa-1 signalling pathways in tubule principal cells were also implicated, including 1,4,5-trisphosphate receptor, TRPL and DNOS (Telonis-Scott et al., 2012).

The *D. melanogaster* capa receptor, capaR, is a G-protein coupled receptor and is encoded by gene CG14575 (Iversen et al., 2002; Park et al., 2002; Terhzaz et al., 2012). CapaR has been identified in other insect species, including *A. gambiae* (Olsen et al., 2007; Pollock et al., 2004) and *R. prolixus* (for RhoprCAPA-α2) (Paluzzi et al., 2010). In the lepidopteran species *M. sexta*, the Drome-capa-1 orthologue MAS-capa-1 stimulates heart rate (Tublitz and Truman, 1985a; Tublitz and Truman, 1985b) and also hindgut contraction (Tublitz et al., 1992), suggesting that *M. sexta* capaR is expressed in heart and in hindgut. Furthermore, in *R. prolixus*, capaR is expressed in anterior midgut as well as the tubules (Paluzzi et al., 2010). This is not the case in *D. melanogaster*, where the capaR gene is almost uniquely expressed in both the adult and larval tubules (Chintapalli et al., 2007; Robinson et al., 2013; Terhzaz et al., 2012) (Fig. 3A). Furthermore, Drome-capa-1 does not modulate heart rate in *D. melanogaster* (Loi and Tublitz, 2004), nor does it affect calcium signalling in adult midgut (S.T. and S.A.D., unpublished). Thus in *D. melanogaster* (and perhaps in Diptera), capa signalling only affects the tubules, which are key tissues for fluid homeostasis.

CapaR is localised at the tubule principal cell basolateral membrane (Terhzaz et al., 2012) (Fig. 3B) and activated by both Drome-capa-1 and -2 to elevate $[Ca^{2+}]_i$ (Terhzaz et al., 2012). Tubules from transgenic principal cell-specific RNAi capaR knockdowns showed that both Drome-capa-1-stimulated $[Ca^{2+}]_i$ and fluid secretion are abolished (Terhzaz et al., 2012), (Fig. 3C,D). Thus capaR modulates Drome-capa-1-induced $[Ca^{2+}]_i$ and fluid secretion – and, therefore, fluid homeostasis – so there was a possibility that capaR modulates organismal responses to desiccation stress. The capaR RNAi *D. melanogaster* lines were assessed for desiccation tolerance using survival assays, in comparison with parental lines. The data clearly showed that significantly reduced expression of capaR in only tubule principal cells was sufficient to prolong survival to desiccation compared with control lines, suggesting that reduced Drome-capa-modulated diuresis by the tubule, and associated reduction of fluid loss, is crucial for desiccation tolerance (Fig. 3E).

Other neuropeptides, such as *D. melanogaster* Short neuropeptide F and tachykinin, also have roles in desiccation tolerance. Interestingly, downstream components of tachkykinin and insulin signalling in the tubule principal cells also modulate desiccation tolerance. Overexpression of ribosomal S6 kinase in only principal cells reduces survival under desiccation conditions, whereas targeted expression of a dominant negative S6 kinase results in increased desiccation tolerance (Söderberg et al., 2011).

Thus there are multiple, complex signalling pathways in tubule principal cells that operate for this stress response alone.

Conclusions

There is still much to be learned about the regulation of cell signalling pathways by individual signalling components in insect epithelia. Furthermore, there are multiple layers of control, including localisation and compartmentalisation (Ahmad et al., 2012; Wong and Scott, 2004), post-translational modification, and transcriptional regulation and control. For cGMP signalling, in particular, even in mammalian systems, these are recently discovered processes (Francis et al., 2011), and so further understanding of cGMP signalling in insect stress responses will have new and wide-ranging implications.

New roles in stress biology are also being discovered for Ca^{2+} signalling pathways, especially mitochondrial Ca^{2+} (Mammucari and Rizzuto, 2010). Performing such fundamental work in insects will also reveal new mechanisms in human stress signalling (Becker et al., 2010; Jaiswal et al., 2012). However, the impact of environmental stress on agriculturally friendly insects, as well as on insect pests, is very much on the current agenda worldwide, and so understanding the molecular mechanisms of insect stress tolerance will be invaluable for deciphering insect survival in response to environmental stressors.

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Competing interests

The authors declare no competing financial interests.

Author contributions

S.A.D. and J.A.T.D. conceived the study, designed the experiment(s), interpreted the findings being published, and drafted and revised the article; P.C., L.A. and S.S. designed and executed the experiment(s); S.T. and G.O. designed and executed the experiment(s) and revised the article.

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