

Review

Organellar calcium signalling mechanisms in *Drosophila* epithelial function

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Summary

Calcium signalling and calcium homeostasis are essential for life. Studies of calcium signalling thus constitute a major proportion of research in the life sciences, although the majority of these studies are based in cell lines or isolated cells. Epithelial cells and tissues are essential in the regulation of critical physiological processes, including fluid transport; and so the modulation of such processes *in vivo* by cell-specific calcium signalling is thus of interest. In this review, we describe the approaches to measuring intracellular calcium in the genetically tractable fluid-transporting tissue, the *Drosophila* Malpighian tubule by targeting cell-specific protein-based calcium reporters to defined regions, cells and intracellular compartments of the intact Malpighian tubule. We also discuss recent findings on the roles of plasma membrane and intracellular calcium channels; and on organellar stores – including mitochondria, Golgi and peroxisomes – in Malpighian tubule function.

Key words: *Drosophila*, calcium, aequorin, Golgi, mitochondria, peroxisomes.

Introduction

Calcium (Ca^{2+}) is a ubiquitous second messenger molecule in all cell types and tissues, and plays a direct role in health and disease (Berridge et al., 2000). As such, research into calcium signalling has been an intensive area of investigation for some decades, and current known mechanisms of calcium signalling in many cell types, notably neuronal, endothelial and epithelial cells, include the calcium signals associated with spatial and temporal resolution within the cell (Knot et al., 2005; Petersen et al., 2005). Although much of biology is concerned with excitable cells, epithelial cells are critically important to the maintenance of life, and the understanding of the fundamental aspects of calcium signalling in relation to epithelial tissue function is of significant interest. Calcium signalling is well understood in the pancreatic acinar cell (Petersen and Tepikin, 2008), and the discovery of the family of transient receptor protein (TRP) channels in the context of renal function has widened our understanding of the functional role of calcium handling and signalling in tissue and organismal function (Hoenderop et al., 2003; Hsu et al., 2007; Mensenkamp et al., 2006). Use of transgenic mouse models has demonstrated physiological roles for calcium and phosphate regulation of intestinal and renal function *via* the transcription factor, *klotho* (Renkema et al., 2008), as well as the importance of the TRP channels in physiological function and disease (Nilius et al., 2007).

In an organotypic context, the use of genetic model organisms is necessary, as the physiological assay of cell or tissue ‘output’ in transgenic or mutant animals allows precise analysis of gene and protein function *in vivo*. Whilst vertebrate models are clearly relevant in the biomedical arena, the use of *Drosophila melanogaster* allows greater scope for cell- and tissue-targeted genetic intervention. Also, signalling genes and proteins are structurally and functionally conserved across evolution, so the invertebrate context is not irrelevant to studies of general mechanisms of cell signalling. Furthermore, although *Drosophila* provides an excellent developmental model, it also comes with

several functional physiological phenotypes in both adult and larval stages, including that of epithelial transport. The most well-studied tissue in this regard is the *Drosophila* Malpighian tubule, a fluid-transporting organ that is critical in osmoregulation and ion homeostasis (Coast, 2007), detoxification (Torrie et al., 2004; Yang et al., 2007) and immunity of the fly (Dow and Davies, 2006; Kaneko et al., 2006).

Probing organellar calcium signals in cells, tissues and organisms

Fluorescent calcium-binding dyes, e.g. the ratiometric Fura-2 and single-wavelength fluors like fluo-3, Oregon Green and Calcium Green, have been used extensively to measure cytosolic calcium signals in vertebrate cells, with much success (Meldolesi, 2004). However, there are drawbacks to the use of fluorescent calcium-binding dyes, including the effects of pH on such compounds, cell damage induced by delivery of the fluorophore (although membrane-permeant variants of these compounds now exist so as to allow for non-invasive delivery) and non-retention of fluorophores by transporting epithelia, e.g. application of Fura-2 to *Drosophila* Malpighian tubules results in rapid extrusion of the fluorophore by organic anion transporters (J. A. T. Dow and T. Cheek, unpublished). Finally, accurate measurements of calcium in intracellular compartments are extremely limited using fluorescent dyes. So although such dyes are still very much used in calcium signalling research, other calcium-binding probes, based on calcium-binding proteins, are preferable.

Aequorin

The development of protein-based calcium probes has been such an important leap forward for the life sciences that the 2008 Nobel Prize for Chemistry was awarded for the discovery of Green Fluorescent Protein (GFP) to Osamu Shimomura, Martin Chalfie and Roger Tsien. The first protein indicator for calcium measurements was calcium-binding aequorin purified from the

jellyfish, *Aequorea victoria* (Shimomura et al., 1962; Shimomura et al., 1963). Aequorin is stable and non-toxic in cells and reversibly binds calcium ions in a linear concentration range in the presence of a chromophoric ligand, coelenterazine and molecular oxygen. Binding of calcium causes de-stabilisation of the aequorin-coelenterazine complex and results in the production of CO₂ and emission of light in the blue, UV range as luminescence, allowing quantitative measurements of calcium concentration (Fig. 1). Aequorin is then re-generated in a slow reaction upon dissociation of calcium and coelenterazine binding. Until the discovery of the aequorin gene, aequorin protein was used in cell extracts or was microinjected into cells. The discovery of the coding sequence for aequorin (Inouye et al., 1985) allowed aequorin to be expressed in cells and in tissues from different organisms, including plants (Knight et al., 1993). Following this, the first transgenic animals for apoequorin were generated, which were transgenic *Drosophila* in which cell-specific calcium measurements were made in intact tissue, the Malpighian tubule (Rosay et al., 1997). Aequorin is now also used in stable vertebrate and insect cell lines as a functional read-out for screening and high-throughput applications for proteins of biomedical interest and in drug discovery, e.g. G-protein coupled receptor and tyrosine kinases (Dupriez et al., 2002; Le Poul et al., 2002; Torfs et al., 2002).

A further development of the aequorin technology resulted in targeted aequorin to intracellular compartments (Pinton et al., 2007; Robert et al., 2000). For this purpose, aequorin constructs were modified with targeting sequences for: endoplasmic reticulum [(ER) the ER-retention signal KDEL or C_H1 domain of Igγ2b heavy chain] (Kendall et al., 1992a; Montero et al., 1995); mitochondria (subunit VIII of human cytochrome c oxidase) (Rutter et al., 1993); Golgi [transmembrane portion of sialyltransferase (ST), a resident protein of the lumen of the trans-Golgi and trans-Golgi network (TGN)] (Pinton et al., 1998); and for the nucleus (nuclear localisation signal of glucocorticoid receptor) (Brini et al., 1994). Due to the very high calcium concentration in the ER, the aequorin for the ER needs to be of lower calcium sensitivity and so is engineered with a point mutation of Asp119-Ala (Kendall et al., 1992b). All of these targeted aequorins have been used very successfully in cell lines to reveal that all cellular organelles thus examined can act as dynamic calcium stores. Most recently, a role for peroxisomes in calcium signalling has been demonstrated using both cytosolic aequorin (Southall et al., 2006) and aequorin targeted to peroxisomes (Lasorsa et al., 2008) using the yeast peroxisomal targeting sequence 1 (PTS1) (Wanders and Waterham, 2006). Some of these targeted aequorin reporters have been successfully used in an organismal context: mitochondrial and nuclear aequorin in transgenic *Drosophila* (Terhzaz et al., 2006); and cytosolic aequorin in transgenic mice (Yamano et al., 2007).

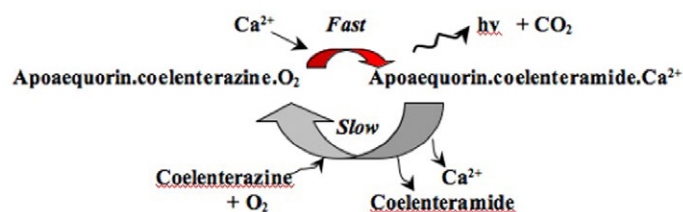


Fig. 1. Aequorin action. Reversible binding of calcium to apoequorin in the presence of coelenterazine and oxygen. Based on: <http://biophysics.bumc.bu.edu/faculty/head/index.ht>

In *A. victoria*, aequorin is associated with GFP, and green bioluminescence is emitted from GFP upon calcium binding by aequorin, due to bioluminescence resonance energy transfer (BRET) between the aequorin and GFP. Thus, expression of a GFP:aequorin fusion *via* a transgene results in increased light emission upon calcium binding compared with aequorin alone, and this has been utilised to generate a novel aequorin variant, GFP-aequorin (Baubet et al., 2000). GFP-aequorin has been successfully targeted to mitochondrial matrix, ER, synaptic vesicles and to the postsynaptic density of mammalian neurons (Rogers et al., 2005); and has now been used in transgenic *Drosophila* to demonstrate calcium transients in mushroom bodies of adult brain (Martin et al., 2007). Transgenic mice with the GFP-aequorin construct expressed in the mitochondrial matrix have also been generated (Rogers et al., 2007), which allow the analysis of calcium signals during various physiological states, including waking/sleeping. In order to improve detection of light from deep areas in mammalian tissues, the GFP-aequorin probe has also been engineered by fusing either enhanced yellow fluorescent protein (Venus) or monomeric red fluorescent protein (mRFP1) to aequorin. Light transmission through skin and thoracic cage is enhanced by using the Venus-aequorin probe whereas the emission spectrum by mRFP1-aequorin allows the detection of calcium signals in brain tissue (Curie et al., 2007).

Protein-based fluorescent calcium probes

Use of calmodulin-based fluorescent reporters as calcium probes has also been of immense value in calcium signalling research and the extremely rapid pace of development of novel probes over the last decade in the fields of cell biology, signalling, biophysics and bioengineering has meant that this area is currently very well reviewed (Chudakov et al., 2005; Mank and Griesbeck, 2008; Miyawaki et al., 2005; Rudolf et al., 2003). The majority of such protein-based fluorescent reporters involve GFP from *A. victoria*. However, fluorescent reporters are also being developed from endogenous fluorescent molecules from other organisms including Anthozoa, allowing monitoring of red fluorescence, e.g. dsRed (Verkhusha and Lukyanov, 2004).

Engineering of GFP-derivatives, e.g. cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP), fused with calmodulin and other calcium-binding proteins, i.e. M13, allows a correlation between calcium binding and changes in FRET (fluorescence resonance energy transfer) (Fig. 2) [reproduced with permission from Rudolf et al. (Rudolf et al., 2003)]. The cameleons (Truong et al., 2001; Truong et al., 2007) (Fig. 2Ba) are composed of CFP-calmodulin fused with YFP-M13 peptide. Calcium binding causes a conformation change in the calcium-binding proteins, with a subsequent change in FRET. Camgaros are based on YFP-calmodulin fusions (Baird et al., 1999; Griesbeck et al., 2001), where calcium binding leads to increased YFP fluorescence. The cameleons and camgaros have been successfully used *in vivo*, notably in transgenic flies in the monitoring of neural activity (Fiala et al., 2002; Reiff et al., 2005; Yu et al., 2003). Camgaroo2 (Griesbeck et al., 2001) has also been used successfully in hippocampal slices (Pologruto et al., 2004) and also in transgenic mice under tetracycline (TET) control (Hasan et al., 2004). Pericams based on calmodulin/M13 fusion with circularly permuted GFP derivatives [YFP or enhanced green fluorescent protein (EGFP)] are activated by calcium-binding, which leads to changes in fluorescence (Nagai et al., 2001) (Fig. 2Bc). Proteins that are circularly permuted have their natural termini joined, resulting in a circular protein, which can be reconfigured to create

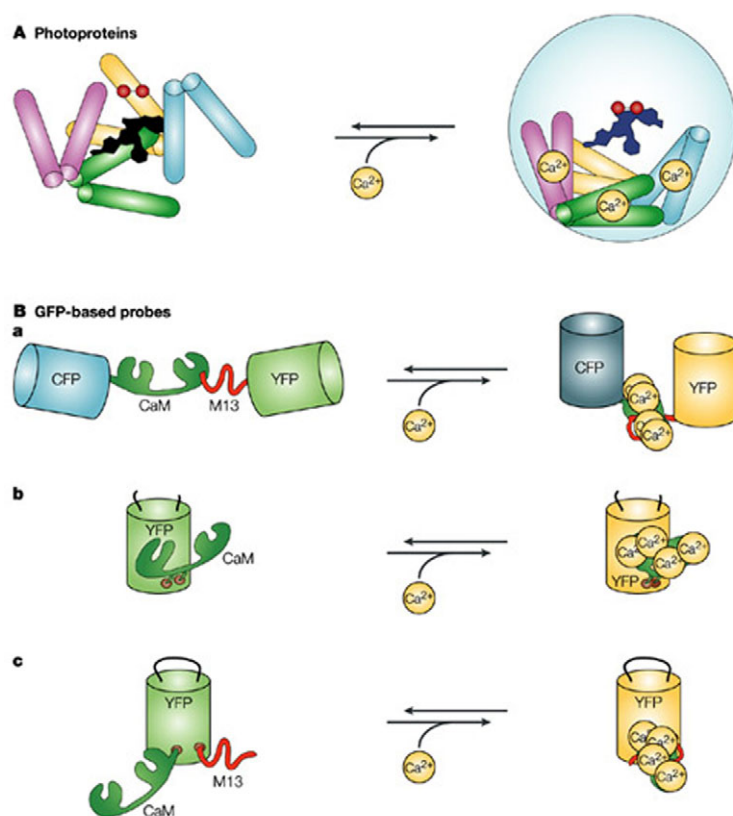


Fig. 2. Diagrammatic summary of protein-based calcium reporters. (A) As shown in Fig. 1, the apoprotein apoaequorin (rod-shaped structure) binds calcium leading to peroxidation (red circles indicate the peroxide) of the coenzyme, coelenterazine (blue), resulting in the release of blue light. (B) GFP-based probes (a) Cameleon, based on fusion of calmodulin (CaM) and the CaM-binding peptide M13 with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Binding of Ca^{2+} leads to FRET between CFP and YFP, with decreased CFP fluorescence and increased YFP fluorescence. (b) Camgarioo probe: Ca^{2+} -induced conformational change in CaM leads to an increased YFP fluorescence. (c) Pericam: the Ca^{2+} -induced interaction between CaM and M13 leads to changes in the fluorescence characteristics of circularly permuted (cp)YFP. The EGFP pericam is similar. Figure reproduced with permission from Rudolf et al. (Rudolf et al., 2003).

new C- and N-termini. The permuted protein may exhibit useful altered characteristics such as reduced substrate binding or can be used to generate fusion proteins by attaching a second polypeptide to the newly created termini. Pericams include inverse pericam (becomes dimmer in the presence of calcium), flash pericam (becomes brighter with calcium) and ratiometric pericam (exhibits calcium-dependent changes in excitation wavelength and allows quantitative measurements of calcium). In *Drosophila*, mitochondrial-targeted pericam ('Mitycam') has been used to image mitochondrial calcium in the Malpighian tubule principal cells (Terhzaz et al., 2006) whereas inverse pericam has been successfully used to image calcium changes in mice (Hasan et al., 2004). Most recently, improved pericams have been developed, which provide brighter fluorescence on calcium binding (Souslova et al., 2007).

In addition to the calmodulin/GFP probes described, newly developed calcium-binding fluorescent probes that show very fast kinetics and big fluorescent changes on calcium binding include one based on troponin C (Mank et al., 2006), which has also been used *in vivo*, in both mice and *Drosophila* (Garaschuk et al., 2007; Heim et al., 2007; Reiff et al., 2005). Interestingly, while ALL the protein-based calcium probes described here have been used in cell lines, few (as already noted) have been used in organisms. Furthermore, it has been difficult to compare the efficacy of different calcium reporters *in vivo*, as all such studies are carried out in different organisms, utilising different agonists, with different phenotypes as end-points. In a heroic study which compared the efficiencies of 10 protein-based reporters in transgenic *Drosophila* based on the fluorescence changes in larval pre-synaptic boutons (Reiff et al., 2005), it was found that flash pericam and the camgaroos did not report a change in neural activity in the larvae although Yellow Cameleon 3 (Griesbeck et

al., 2001) and pericam with EGFP (Nagai et al., 2001) showed linear changes that correlated with neural activity with a good level above background. Thus, although there are many protein-based calcium indicators now available, the real utility of these lies in their use *in vivo*, which ultimately depends on how such indicators work in the organismal, and not cell line, context.

The *Drosophila* Malpighian tubule: cell-specific calcium signalling mechanisms

The use of protein-based calcium probes allows expression in specific cells and tissues *in vivo* via targeted expression of transgenes. In *Drosophila*, the GAL4-UAS system (Brand and Perrimon, 1993), which has been further developed to allow conditional expression (McGuire et al., 2004), is the system of choice for targeted expression of transgenes *via* cell- or tissue-specific GAL4 drivers. Genes of interest are cloned downstream of the GAL4 promoter, Upstream Activating Sequence (UAS) and transgenic lines generated. The transgenes are expressed in the progeny of crosses between the GAL4 lines and the UAS lines, which can be maintained as stable lines if the transgenes have no deleterious effects on the flies. In order to investigate calcium signalling using aequorin in *Drosophila*, UAS-apoaequorin flies were generated (Rosay et al., 1997). These flies, the first transgenic animals for a calcium reporter, were used to monitor calcium signals in live intact tissue and provided the first *in vivo* measurements of cytosolic calcium concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) in *Drosophila* Malpighian tubule (Rosay et al., 1997) and brain (Rosay et al., 2001). Malpighian tubules are free-floating, single-cell thick organs connected to the hindgut (see Fig. 3F). Each tubule is $2\text{ mm} \times 35\text{ }\mu\text{m}$ and is composed of two main cell types: Type I (principal cells) and Type II (stellate cells). Other cell types also exist, including bar-shaped cells and tiny endocrine cells (Sozen et

al., 1997). Targeting of apoaquorin to different cells and regions of the tubule using different GAL4 drivers (Sozen et al., 1997) (Fig. 3A–J), reconstitution with coelenterazine, and measurement of luminescence in intact tubules showed that only principal cells in the main, fluid-transporting segment responded with increased $[Ca^{2+}]_{cyt}$ to the neuropeptide CAP_{2b} (Fig. 3K–P). CAP_{2b} is a member of the capa family of peptides, including capa-1 and capa-2 (Predel and Wegener, 2006), which all mobilise calcium, nitric oxide, the cyclic nucleotide cGMP and stimulate fluid transport in tubules with similar kinetics (Davies et al., 1995; Kean et al., 2002). This work also demonstrated that *Drosophila* leucokinin (Terhzaz et al., 1999) increased $[Ca^{2+}]_{cyt}$ in only stellate cells. Leucokinins modulate fluid transport *via* calcium signalling in many insects (Gade, 2004), including the disease vectors *Aedes aegypti* (Beyenbach, 2003), *Anopheles stephensi* and *Anopheles gambiae* (Radford et al., 2004). Therefore, peptidase-resistant leucokinins are potential lead compounds for dipterocides (Teal et al., 1999).

The cell-specific stimulation of the tubule by the capa peptides and the leucokinins is due to localisation of the respective receptors: the *Drosophila* leucokinin receptor is expressed only in tubule

stellate cells (Radford et al., 2002), and the capa receptor is localised to tubule principal cells (S.T., unpublished). The use of targeted aequorin for calcium measurements thus allowed ‘cell-identified’ approach to calcium signalling studies.

CAP_{2b} acts *via* phospholipase $C\beta$ ($PLC\beta$), inositol 1,4,5-trisphosphate (IP_3) and inositol 1,4,5-trisphosphate receptor (IP_3R) to generate $[Ca^{2+}]_{cyt}$ and increased fluid transport (Pollock et al., 2003). *Drosophila* leucokinin also acts *via* IP_3 (Radford et al., 2002) and IP_3R (Pollock et al., 2003). As IP_3R is associated with the ER (Mikoshiba, 2007), this suggests that calcium mobilisation from the ER can modulate calcium homeostasis in principal and stellate cells.

The aequorin work also demonstrated that principal and stellate cells utilise ER-associated calcium differently from each other (see Fig. 4). This was the first demonstration that different cells in the same intact tissue respond differentially to the same pharmacological compound, suggesting necessary caution in interpretation of data from whole tissue pharmacology, whether in insects or vertebrates. Fig. 4 shows that thapsigargin, which causes increased cytosolic calcium *via* an inhibition of the ER Ca^{2+} -

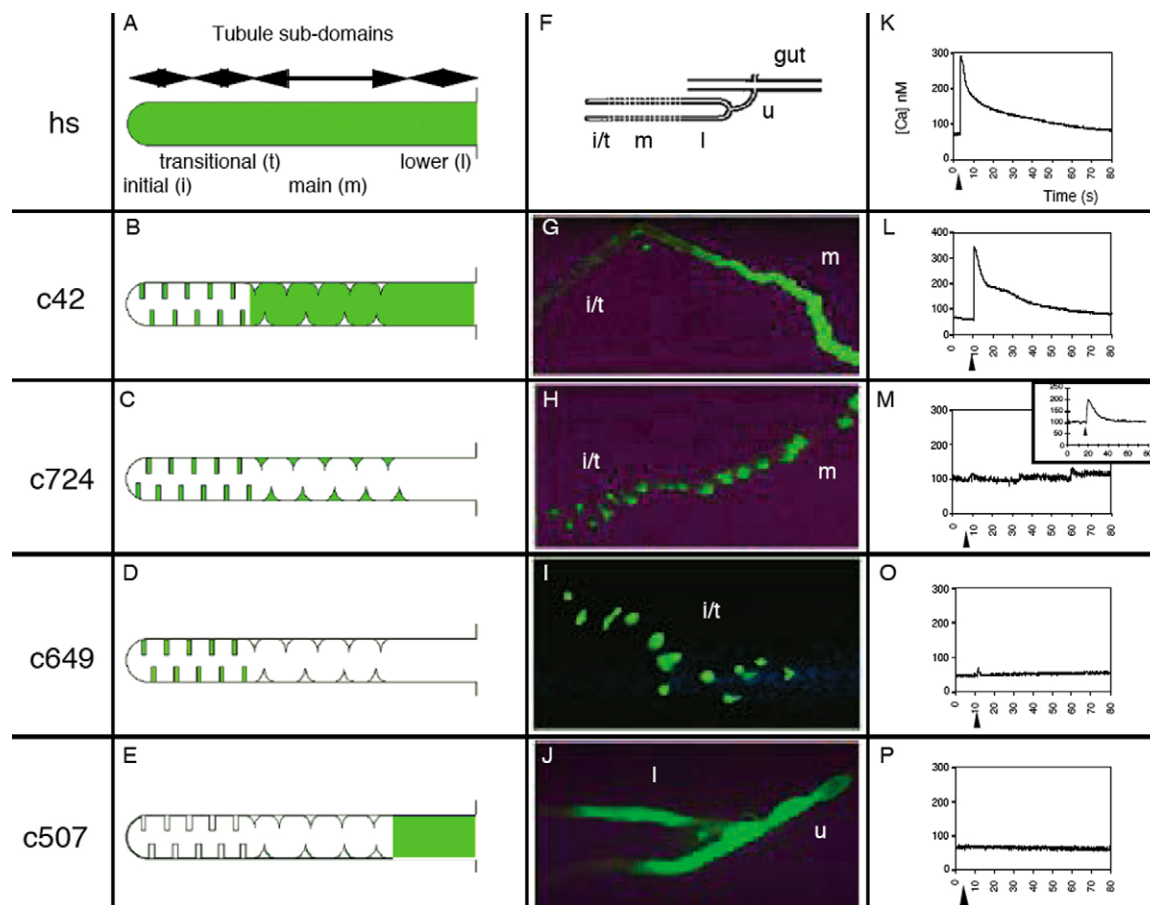


Fig. 3. Spatial resolution of calcium signals *in vivo*. (A–E) shows schematic diagrams of GAL4 expression patterns (green) in the Malpighian tubule of adult *Drosophila*. (A) Ubiquitous expression *via* heat-shock GAL4. (B) c42 GAL4 driver, which drives expression in the principal cells in main segment, bar-shaped cells of initial and transitional segments, and lower tubule. (C) c724 GAL4 driver, which marks all stellate cells in main segment, and bar-shaped cells of initial and transitional segments (Sozen et al., 1997). (D) Line 649 marks bar-shaped cells. (E) Line 507 marks the lower tubule and ureter. (F) Representation of paired anterior tubules connected to the hindgut by the ureter (u), with initial (i), transitional (t), main (m) and lower (l) segments also indicated. (G–J) GAL4-driven GFP expression, confirming expression expected as shown in panels A–E. (K–P) Typical calcium responses (in nmol Ca^{2+}) of the different GAL4/UAS-apoaquorin lines to $1\mu\text{mol } CAP_{2b}$ are shown. The inset in panel M shows the typical response of the c724/aequorin Malpighian tubule to $10^{-8}\text{ mol leucokinin}$, showing that lack of response to CAP_{2b} is not due to a compromised tubule. Reproduced from Rosay et al. (Rosay et al., 1997).

ATPase and therefore prevents uptake of calcium into the ER, does cause a rise in cytosolic calcium in both principal and stellate cells, as well as increased fluid transport (Rosay et al., 1997). However, in the absence of extracellular calcium, which unmasks the 'run-down' of $[Ca^{2+}]_{cyt}$ due to extrusion of calcium from the ER in the absence of re-uptake, only the principal cells show sensitivity to this process. Thus, the principal and stellate cells show differentiated responses to thapsigargin and may utilise different calcium stores to maintain calcium homeostasis.

Therefore, although both principal and stellate cells utilise ER calcium, it is clear that the increased IP_3 -induced $[Ca^{2+}]_{cyt}$ in principal cells cannot be due solely to ER, as there is a dependence on calcium influx from the extracellular medium; as well as the existence of non-ER, hormone-sensitive organellar calcium pools.

The involvement of plasma membrane calcium channels in cellular calcium and fluid transport

The impact of extracellular calcium on $[Ca^{2+}]_{cyt}$ and fluid transport by the Malpighian tubule is dramatic. Reduction of external calcium from 4 mmol l^{-1} to 0 mmol l^{-1} abolishes CAP_{2b} -stimulated $[Ca^{2+}]_{cyt}$ and completely prevents CAP_{2b} -stimulated fluid transport; as well as abolishing the thapsigargin response in principal cells (Rosay et al., 1997). Thus, tubule function requires extracellular calcium influx into principal cells *via* plasma membrane channels.

L-type and cyclic nucleotide gated channels

Voltage-gated calcium channels, including L-type channels (Calin-Jageman and Lee, 2008), were once considered unique to excitable cells; however, studies in vertebrates have provided evidence for functional L-type calcium channels in non-excitable cells, including renal cells (Hayashi et al., 2007). The *Drosophila* orthologues of vertebrate L-type calcium channel α subunit are Dmca1A (cacophony, previously nightblind, CG1522) (Smith et al., 1996) and Dmca1D (CG4894) (Zheng et al., 1995). Tubules express both these channel subunits and L-type calcium channel antagonists, i.e. verapamil (a phenylalkylamine) and nifedipine (a dihydropyridine) inhibit CAP_{2b} -stimulated $[Ca^{2+}]_{cyt}$ and fluid transport (MacPherson et al., 2001). Verapamil also partially abolishes the thapsigargin-stimulated $[Ca^{2+}]_{cyt}$ in principal cells, suggesting that the thapsigargin-induced increase in $[Ca^{2+}]_{cyt}$ is not necessarily ER-associated but rather is due to a phenylalkylamine-sensitive calcium channel. This supports the data in Rosay et al. (Rosay et al., 1997) and Fig. 4, which showed abolition of the thapsigargin-

induced $[Ca^{2+}]_{cyt}$ response when extracellular calcium was removed. Immunolocalisation studies indicate that α_1 subunits of the L-type channels are localised to the basolateral and apical membranes of the principal cells in tubule main segment; the spatial distribution of the α_1 subunits are associated with different affinities for the inhibitors of these calcium channels: high affinity binding of verapamil occurs at the basolateral surface whereas low affinity verapamil binding and binding of dihydropyridine occurs at the apical membrane. Interestingly, dihydropyridine also binds to the apical membrane of the initial segment in Malpighian tubule. This segment, especially in anterior tubules, is known to be a calcium store for the fly; and anterior tubules store 25–30% more calcium than posterior tubules (Dube et al., 2000b; Dube et al., 2000a); so the dihydropyridine-sensitive L-type channel may be involved in transepithelial calcium transport.

The entry of calcium is also modulated by a cyclic nucleotide-gated channel (CNG), encoded by *cng*, expressed in head and in tubules. Calcium entry into principal cells is enhanced by the cyclic nucleotide cGMP, resulting in increased $[Ca^{2+}]_{cyt}$ and fluid transport. Furthermore, both the $[Ca^{2+}]_{cyt}$ and fluid transport response induced by cGMP are verapamil- and nifedipine-sensitive, and are abolished by a reduction of calcium in the extracellular medium (MacPherson et al., 2001). Treatment of tubules with $1\text{ }\mu\text{mol}$ cGMP also causes slow rise in mitochondrial calcium concentration, with a peak of $100\text{--}150\text{ nmol l}^{-1}$ $[Ca^{2+}]_{mitochondria}$ occurring at 200 s post-stimulation (S.T., unpublished), suggesting that $[Ca^{2+}]_{mitochondria}$ accurately reflect changes in $[Ca^{2+}]_{cyt}$ due to activity of CNG channels.

Although expression of *cng* is widespread throughout the adult fly, *cng* expression is 2–4-fold increased in Malpighian tubule, midgut and hindgut compared with the whole fly (www.flyatlas.org). This suggests that CNG channels contribute significantly to epithelial tissue function.

TRP channels

The TRP ion channel family constitutes 29 vertebrate TRP members, classified into seven subfamilies (TRPC, TRPV, TRPM, TRPML, TRPP, TRPA, TRPN). TRP was originally identified in *D. melanogaster*, from screens for phototransduction mutants (Montell et al., 1985). *Drosophila* TRP is classified within the C subfamily, which also includes the closely-related TRPL (Xu et al., 1997) and TRPgamma channels (Xu et al., 2000), which can form heteromultimeric channels with TRP. TRP is activated *via* a Gq-coupled PLC mechanism in *Drosophila* photoreceptors and the TRPC, V and M subfamilies are regulated by lipid

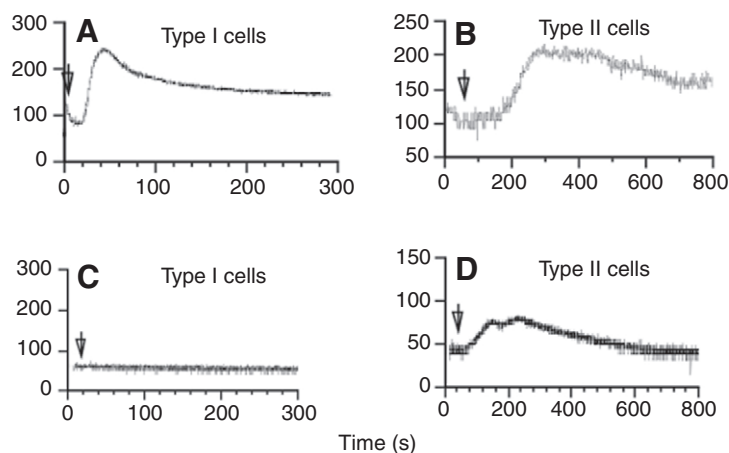


Fig. 4. Differential responses of principal and stellate cells to thapsigargin in intact Malpighian tubule. (A,B) Representative $[Ca^{2+}]_{cyt}$ traces for (A) Type I cells (c42-aeq) and (B) Type II cells (c710-aeq) in normal medium, and stimulated with 1 mmol thapsigargin (arrow). Thapsigargin increases $[Ca^{2+}]_{cyt}$ in both type I and type II cells. (C,D) Representative $[Ca^{2+}]_{cyt}$ traces for (C) Type I cells (c42-aeq) and (D) Type II cells (c710-aeq) in Ca^{2+} -free medium, and stimulated with 1 mmol thapsigargin (arrow). Thapsigargin increases $[Ca^{2+}]_{cyt}$ only in type II cells. From Rosay et al. (Rosay et al., 1997).

messengers including diacylglycerol (DAG) metabolites, polyunsaturated fatty acids (PUFAs) (Hardie, 2007), which are produced from DAG by putative DAG lipase. Recent work has identified the *Drosophila* DAG lipase, encoded by *inaE*, which regulates TRP/TRPL function in photoreceptors (Leung et al., 2008).

Whilst almost all work to date on *Drosophila* TRP channels has been confined to photoreceptors, investigation of TRP channel function in *Drosophila* Malpighian tubule provided the first evidence of TRP channel function in fluid-secreting epithelia, and also showed that the established model of TRP/TRPL interaction based on photoreceptor work, was not the sole model for TRP channel activation (MacPherson et al., 2005). Tubules express all known *Drosophila* TRPC channels, i.e. TRP, TRPL and TRPgamma, which are localised to the principal cells of the main segment. In contrast to photoreceptors, a *trp* null had no effect on CAP_{2b}-stimulated fluid transport, although CAP_{2b}-stimulated fluid transport rates were reduced in tubules from a *trpl* null, as well as a *trpl;trp* double mutant. Fluid transport rates reverted to normal on genetic rescue of the *trpl* null with a *trpl* transgene, and introduction of the *trp* null into the rescue background did not compromise the normal secretion rates.

Use of targeted aequorin to tubule principal cell using the c42 GAL driver showed a reduction of [Ca²⁺]_{cyt} in the *trpl* mutant but not in a *trp* null. Furthermore, a correlation between TRPL protein levels and CAP_{2b}-stimulated fluid transport and calcium signalling was demonstrated in the *trp* and *trpl* mutants.

Taken together, our data suggested that tubules required TRPL but not TRP for normal epithelial function – unlike photoreceptors, which are dependent on functional TRP. Furthermore, it is probable that in Malpighian tubule, TRPL-TPPL homomultimers or TRPL/TRPgamma multimers are formed, which allow normal tubule function in the *trp* null background. A novel mutant of *trp* in which the calcium pore selectivity was modified by a Asp621 mutation, thus eliminating calcium permeation *in vivo* and resulting in altered photoresponses and retinal degeneration (Liu et al., 2007), was without effect on tubule secretion rates (P. Cabrero, S.A.D. and R. C. Hardie, data not shown). Finally, a key scaffolding component of the TRPL/TRP complex, INAD (Li and Montell, 2000), is not expressed in tubules (MacPherson et al., 2005), further confirming that TRP/TRPL complexes do not occur in *Drosophila* tubules. Thus, TRPC channels, particularly TRPL, are important for epithelial function.

Analysis of gene expression levels of other TRP family members in tissues of the whole adult fly or larvae was undertaken using the *Drosophila* transcriptome database, www.flyatlas.org (Chintapalli et al., 2007) (Table 1). The data suggests that other novel TRP family members may be important for epithelial function, either in Malpighian tubule or in the gut – in addition to the roles already suggested for these TRP channels.

Intracellular calcium release channels in Malpighian tubule function

IP₃R and ryanodine receptor (RyR)

Although IP₃R is clearly associated with the ER (Mikoshiba, 2007), studies in many tissues, including epithelia, demonstrate alternative and complex arrangements for IP₃R isoforms (Bush et al., 1994). In rat kidney, the three IP₃R isoforms are expressed in different cell types, with type 1 and type 2 IP₃R expressed in the cytoplasm. However, type 3, although also cytoplasmic, is localised to the basolateral side (Monkawa et al., 1998). In rat colonic epithelium, however, IP₃R2 is localised to the nucleus and IP₃R3 in the cytoplasm at the apical region.

Attempts to localise *Drosophila* IP₃R in tubule cells have had only limited success, even though PLC-mediated calcium signalling *via* IP₃R has been established for tubule principal and stellate cells. Use of a rabbit polyclonal antibody against *Drosophila* IP₃R has demonstrated perinuclear staining of IP₃R in principal cells (Pollock, 2005), suggesting either a potentially nuclear localisation of IP₃R or association of IP₃R with ER around the nucleus.

To add to the complexity of calcium signalling *via* intracellular release calcium channels, tubules also express ryanodine receptor (RyR), an intracellular calcium release channel normally associated with the sarcoplasmic reticulum of cardiac and striated muscle (Wehrens et al., 2005). In *Drosophila*, RyR is encoded by a single gene, *RyR-44F*, which is most highly expressed in brain, thoracoabdominal ganglion, crop and hindgut in the adult fly (www.flyatlas.org). Although expression levels of the gene are not high in tubules, immunocytochemical localisation of RyR in tubules reveals staining only in principal cells, with stellate cells excluded; the staining pattern is also reticular throughout the cell (Fig. 5). Functionally, ryanodine itself, a plant alkaloid, has an inhibitory effect on neuropeptide-stimulated fluid transport by tubules: CAP_{2b}-stimulated fluid transport is sensitive to ryanodine at all concentrations between 10⁻⁸ and 10⁻³ mol whereas drosokinin-stimulated fluid transport is only affected by very high concentrations of ryanodine, especially 10⁻³ mol. The differential effect of ryanodine on capa- or drosokinin-stimulated fluid transport may reflect differences in the store mobilisation of calcium elicited by the different peptides (i.e. capa: Golgi and mitochondria, see below; and drosokinin: ER) or by the fact that ryanodine is transported into the principal cells but not into stellate cells; or by the simplest explanation that stellate cells do not have ryanodine receptors, so the inhibition of drosokinin-stimulated transport at 10⁻³ mol ryanodine is non-specific. At 10⁻⁵ mol, however, ryanodine inhibits the CAP_{2b}-induced rise in cytosolic [Ca²⁺] by ~50% and reduces the drosokinin-induced cytosolic [Ca²⁺] rise by ~30% (Pollock, 2005).

Table 1. Highest sites of expression of *Drosophila* orthologues of members of TRP sub-families

| TRP subfamily | Gene | Proposed function | Malpighian tubule mRNA signal/whole fly signal | Malpighian tubule enrichment |
|---------------|-------------------------|--------------------------------|------------------------------------------------|------------------------------|
| TRPV | <i>Nanchung, CG5842</i> | Sensory perception of sound | 12/6 | 2 |
| TRPM | <i>*CG34123/CG30078</i> | | 22/10 (42/10); 49/19 (41/19); 86/13 (25/13)* | 2(4); 2.5 (2); 6.16 (2)* |
| TRPN | <i>nompC CG11020</i> | Mechanosensory/sound sensation | 17/5 | 3 |
| TRPA | <i>trpA1 CG5751</i> | thermotaxis | Hindgut: 57/4 | Hindgut: 12 |

Data are extracted from flyatlas.org, from on tissue-specific Affymetrix microarray experiments (Chintapalli et al., 2007). TRPP (polycystin) has a *Drosophila* orthologue: *Pkd2*, which is enriched in testes and sperm. TRPML has no annotated *Drosophila* orthologue (Flybase). *Three different datasets based on three distinct Affymetrix probe sets for *CG34123*-significant enrichment for tubule is seen with all three probes. Data in brackets are for larval tubule. TRP, transient receptor protein.

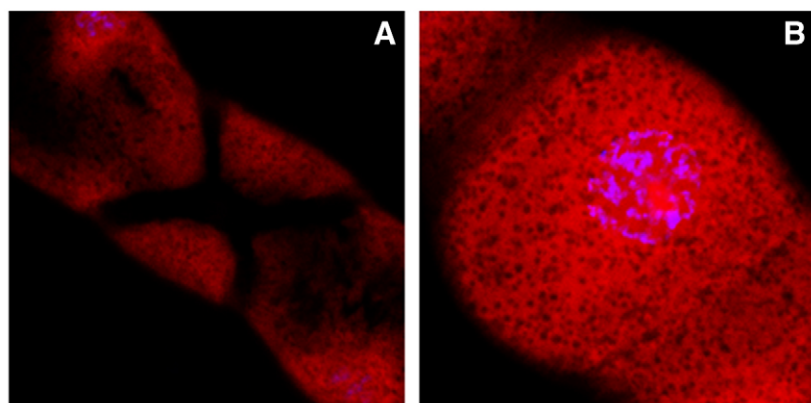


Fig. 5. Immunocytochemistry of ryanodine receptor (RyR) in Malpighian tubules. RyR expression (red staining) in principal cells in intact Malpighian tubules using a rabbit polyclonal antibody to *Drosophila* RyR verified in Pollock (Pollock, 2005). (A) Red staining is observed in only principal cells; note exclusion of star-shaped stellate cell from staining. (B) Higher magnification shows reticular pattern of RyR expression, in a principal cell containing a DAPI-stained nucleus. Data from S.T.

However, given the lack of RyR in stellate cells, this suggests that the inhibitory effects of ryanodine occur *via* the IP₃R. Furthermore, treatment of intact tubules from *itpr* mutants (Pollock et al., 2003) with ryanodine causes a reduction in the already reduced rate of CAP_{2b}-induced fluid transport associated with loss-of-function of the IP₃R. Thus, although tubules do express RyR,

the inhibitory effects of ryanodine on calcium signalling and fluid transport may be due to the impact of ryanodine on IP₃R; however, this does not rule out the possibility of an interaction between RyR and IP₃R, in principal cells at least. Although RyR is the dominant calcium release channel in striated muscle (Missiaen et al., 2000), functional interactions between RyR and IP₃R have been shown

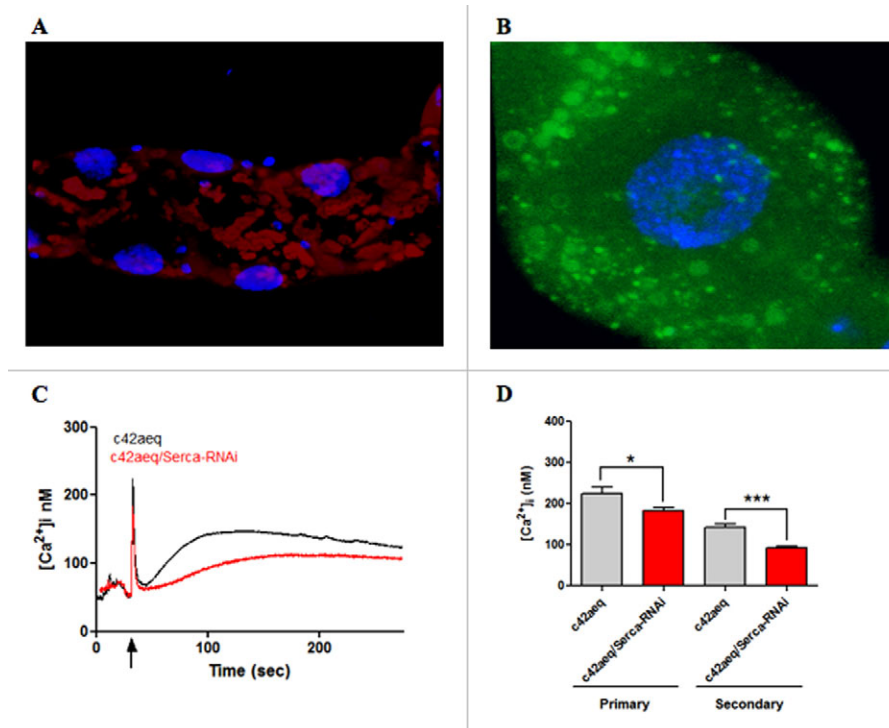


Fig. 6. *Drosophila* sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) in Malpighian tubules. (A) Immunocytochemical staining in tubule principal cells using a mouse monoclonal antibody (VE121G9) against SERCA1 ATPase from skeletal muscle SR (Knudson et al., 1989). (B) Green fluorescent protein (GFP) was C-terminally fused to the open reading frame of CG3725 and cloned into the UAS transformation vector pUAST. Transgenic UAS-CG3725 flies were generated, and crossed to the principal cell driver, c42 to target SERCA expression to principal cells. Intact tubules were dissected and imaged with confocal microscopy. (C) Transgenic lines bearing an RNAi construct against the SERCA gene were generated from plasmid constructs as follows: an inverted repeat of a 685 base pair fragment of SERCA was cloned into the P-element vector pWIZ (Lee and Carthew, 2003). Primers (5'-GGGCTCTAGAGATGGAAGACGGTCACTCG-3' and 5'-GGGCTCTAGACAGGCCAGTGCCGATGACG-3') were used to generate the SERCA region containing *AvrII* restriction sites on both ends: the PCR product was subcloned into the pWIZ vector using *AvrII* and *NheI* sites as a tail-tail inverted repeat flanking the *w* (*white*) intron. The cloning of the SERCA fragment into pWIZ was analysed by restriction analysis and verified by sequencing. Transgenic lines were generated using standard methods for P-element-mediated germline transformation (BestGene Inc, USA) with independent insertions of pWIZ-SERCA. The transgenic line was crossed with a c42/aequorin line, which allows expression of aequorin and SERCA RNAi in Malpighian tubule principal cells (see Fig. 1). Calcium levels were measured *via* luminometry in intact Malpighian tubules, which were treated with 10⁻⁷ mol l⁻¹ (final concentration) of capa-1 (Kean et al., 2002) to stimulate PLC-mediated IP₃ and calcium signalling. A typical calcium trace from such an experiment is shown. (D) Pooled data from experiments shown in C, data are nmol [Ca²⁺]_i ± s.e.m., N=8. 'Primary' refers to the fast (ms) rise in Ca²⁺ (panel C) whereas 'secondary' refers to the slower, sustained Ca²⁺ rise (panel C). Significance assessed by Student's *t*-test, where *P*<0.05 (*) or *P*<0.001 (***). Data from S.T.

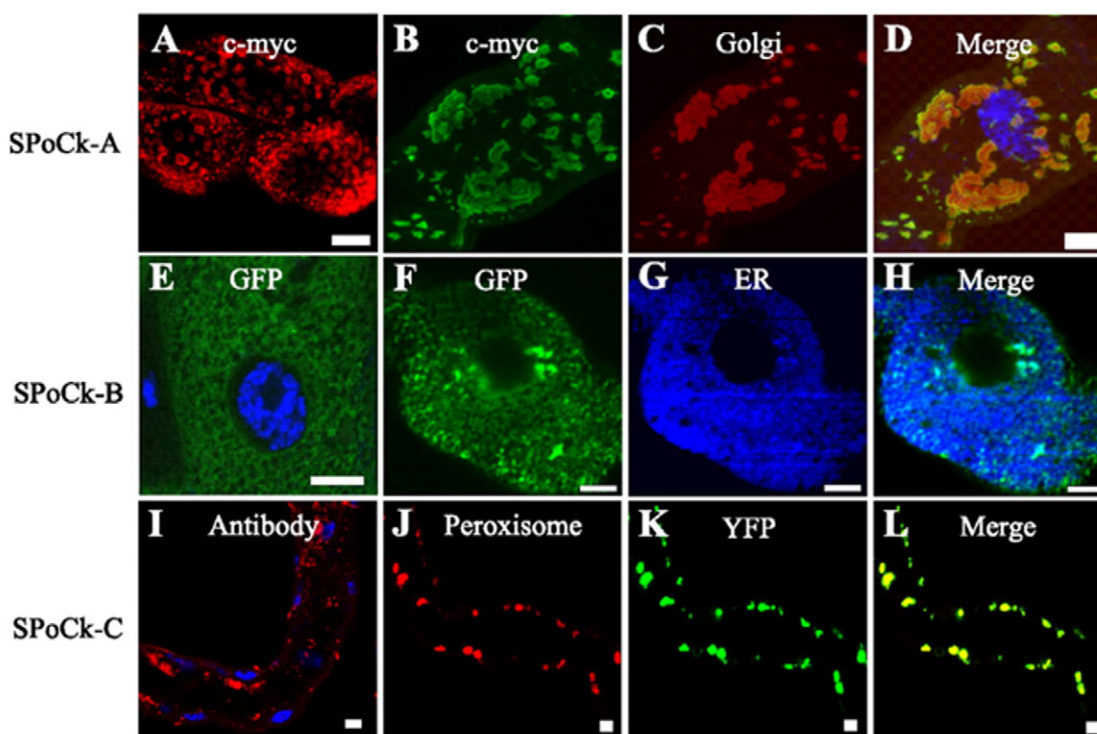


Fig. 7. Intracellular localisation of SPoCk isoforms *in vivo* in principal cells of the Malpighian tubule. (A) Golgi apparatus localisation of SPoCk A, shown by staining of c-myc tagged-SPoCk A with Texas Red secondary antibody, in principal cells of the tubule (B–D) Colocalisation (D) of SPoCk A [c-myc tagged, FITC secondary antibody, green (B)] with affinity-purified rabbit anti-SPoCk A (red; C). (E) ER-localisation of SPoCk B. The nucleus is labeled blue with 4',6-diamidino-2-phenylindole (DAPI). (F–H) Colocalization (H) of SPoCk B [green fluorescent protein (GFP)-tagged, green; F] with blue ER tracker dye (blue; G). (I) Peroxisomal localisation of SPoCk C. Endogenous SPoCk C in the tubule was detected with a SPoCk C-specific antibody; nuclei are labelled blue with DAPI. (J–L) Colocalisation (L) of SPoCk C [yellow fluorescent protein (YFP)-tagged, green; J] with catalase (red; K). All scale bars, 10 μ m. Reproduced with permission from Southall et al. (Southall et al., 2006). The specificity of all antibodies, including anti-catalase and anti-COPII antibodies was verified by Western blot analysis (Southall et al., 2006).

Chinese Hamster Ovary cells (George et al., 2003) and in pancreatic acinar cells (Gerasimenko et al., 2006).

SERCA

The *Drosophila* sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is encoded by CG3725 (*Ca-P60A*) – a complex gene containing nine exons. Studies of *Drosophila* SERCA in cell lines confirm the importance of SERCA in calcium homeostasis (Raymond-Delpuch et al., 2004) but *in vivo* studies reveal physiological roles of SERCA. Generation of conditional mutants for the SERCA gene allowed analysis of the role of store-derived calcium in neuromuscular physiology; and demonstrated that muscle excitability is dependent on SERCA-regulated, voltage-gated calcium channels and calcium-activated potassium channels (Sanyal et al., 2005). SERCA mutants have also been used to model cardiac dysfunction in the larval heart and to show that heart beat frequency is reduced in SERCA mutants (Sanyal et al., 2006).

In vertebrates, SERCA exists as three main isoforms, localised to different cell types and sub-cellular locations. SERCA1 in fast-twitch skeletal muscle and SERCA2a in cardiac muscle are located in the sarcoplasmic reticulum (SR), however, SERCA 2 and 3 are located in the ER of other cell types, although mitochondrial localisations may not be excluded (Misquitta et al., 1999). In the *Drosophila* tubules, SERCA has been localised to the Golgi apparatus, an observation reached *via* immunocytochemistry to SERCA (Fig. 6A) (similar staining is observed with Golgi-localised

secretory pathway ATPase, SPoCkA) (Fig. 7) and *via* expression of epitope-tagged SERCA in principal cells, which shows an intracellular, vesicle-like localisation (Fig. 6B). Furthermore, RNAi of the SERCA gene *in vivo* demonstrates an impact of Golgi-localised SERCA on the IP_3 -derived calcium spike and associated calcium influx in principal cells (Fig. 6C,D). Thus, IP_3 -induced calcium signalling in the principal cell is dependent on calcium storage in the Golgi and release by SERCA.

Secretory pathway calcium ATPase, SPoCk

In addition to SERCA and plasma membrane calcium ATPase (PMCA) pumps, the secretory pathway calcium ATPase (SPCA) comprises a third class of calcium pumps (Wuytack et al., 2002). SPCA pumps have an equal selectivity for transporting Mn^{2+} and Ca^{2+} , unlike the SERCA pumps, and are insensitive to potent SERCA inhibitors such as thapsigargin (Sorin et al., 1997). Work in yeast has shown that SPCAs may be involved in regulation of Mn^{2+} -mediated removal of superoxide radicals (Lapinskas et al., 1995). Expression of *C. elegans* SPCA in vertebrate cell lines has shown that SPCA can establish baseline cytosolic Ca^{2+} oscillations, without involvement of ER (Missiaen et al., 2001). Thus, SPCA may be involved in non-ER-associated calcium homeostasis.

In *Drosophila*, SPCA is encoded by CG7651 (SPoCk), and is most highly expressed in brain and in thoracoabdominal ganglion (www.Flyatlas.org). However, SPoCk encodes three transcripts, SPoCk A–C, with SPoCk A and C (but not SPoCk B) being expressed in tubules. Generation of transgenic lines for all three

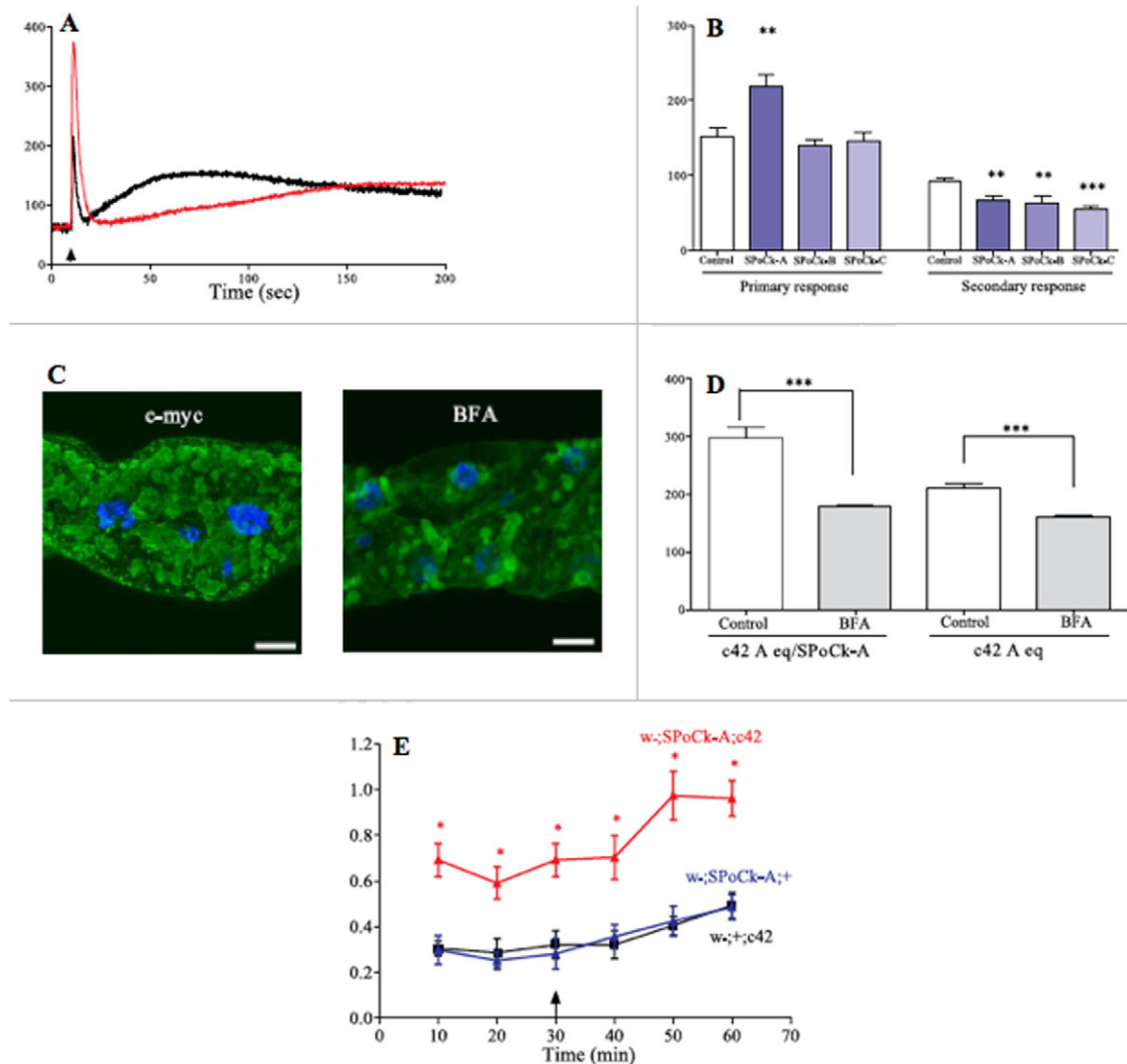


Fig. 8. SPoCk A modulates calcium signalling and fluid transport. (A) UAS-SPoCk A was crossed with a *c42/aequorin* line, which allows expression of aequorin and SPoCk A in Malpighian tubule principal cells (see Fig. 1). Calcium levels were measured *via* luminometry in intact tubules, which were treated with 10^{-7} mol (final concentration) of capa-1 (Kean et al., 2002). A typical calcium trace from such an experiment is shown, comparing SPoCk A over-expressors (*aeq/+; SPoCk A/+; c42*, red) with parental *c42/aequorin* tubules (black). Cytosolic calcium levels are expressed as nmol [Ca²⁺]cyt (*y*-axis). (B) Pooled data from experiments shown in A, including data from SPoCk B and SPoCk C over-expressors. Data are nmol [Ca²⁺] \pm s.e.m., *N*=8. 'Primary' refers to the fast (ms) rise in Ca²⁺ (panel A) whereas 'secondary' refers to the slower, sustained Ca²⁺ rise (panel A). (C) Treatment of intact *SPoCk A/+; c42* tubules with Brefeldin A (panel 'BFA') shows disruption of Golgi and loss of Golgi-associated SPoCk A (c-myc epitope-tagged, green). (D) Brefeldin A (BFA) treatment results in significantly lower capa-1-stimulated primary calcium rises in *aeq/+; SPoCk A/+; c42* compared with parental *c42/aequorin* Malpighian tubules. Data are nmol [Ca²⁺] \pm s.e.m., *N*=8. (E) Over-expression of SPoCk A in tubule principal cells (red) is sufficient to confer increased basal rates of fluid transport, as well as increased capa-1-stimulated fluid transport, compared with parental lines (black, blue). Data are expressed as mean fluid transport rates over time (nl min⁻¹), \pm s.e.m., *N*=10. Significance assessed by Student's *t*-test, where *P*<0.05 (*), *P*<0.01 (**) or *P*<0.001 (***). From Southall et al. (Southall et al., 2006).

transcripts and targeted expression of these transcripts in tubule principal cells revealed that each SPoCk isoform was differentially localised to intracellular compartments *in vivo* (Fig. 7): SPoCk A to the Golgi, SPoCk B to ER (under conditions of ectopic expression) and SPoCk C to peroxisomes. These data were confirmed by expression of each SPoCk isoform in *Drosophila* S2 cells (Southall et al., 2006) and suggested regulation of tubule function *via* novel calcium stores and SPCA.

Using *c42 aequorin* flies, SPoCk isoform expression was driven in principal cells, and cytosolic calcium levels assessed (Fig. 8A,B,D). SPoCk A, in Golgi, mediates the rapid rise in intracellular calcium induced by capa-1 and impacts on re-calcium

entry (Fig. 8A). Also, RNAi to the SPoCk gene results in reduction of the rapid rise in intracellular calcium induced by capa-1 (Southall et al., 2006). Interestingly, only SPoCk B affects IP₃-induced calcium signalling *via* the ER in *Drosophila* S2 cells but does not modulate calcium signals *in vivo* (Fig. 8B) confirming the lack of transcript B function in tubules. Disruption of the Golgi apparatus with brefeldin A (Fig. 8C) reduces capa-1 induced calcium signalling (Fig. 8D); confirming that in tubules, the Golgi is a major site of calcium release and homeostasis. Targeted expression of SPoCk A to tubule principal cells results in markedly increased capa-1 stimulated fluid transport (Fig. 8E) whereas expression of SPoCk-RNAi in principal cells abolishes capa-1 stimulated fluid

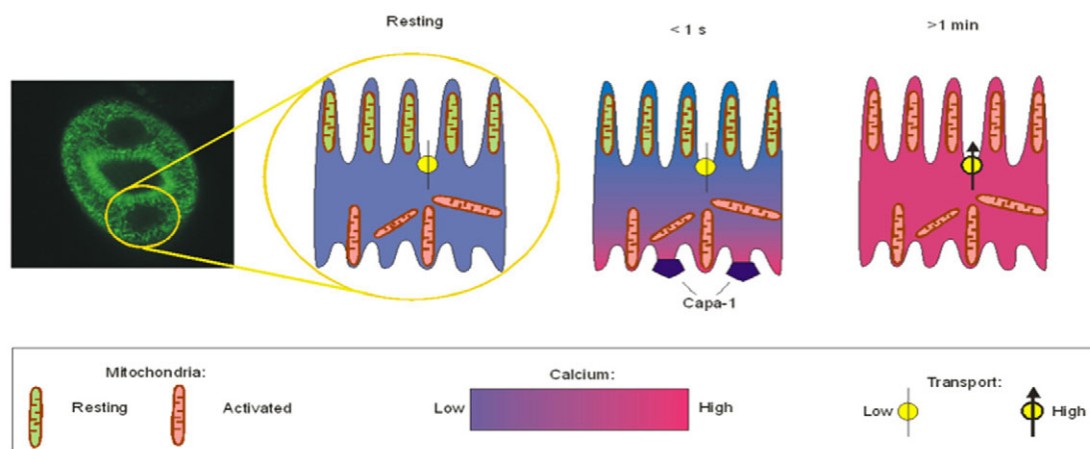


Fig. 9. Calcium and mitochondrial activity in capa-1 stimulated Malpighian tubules. Left panel: confocal section through mitycam-expressing tubule, showing packing of mitochondria into apical (luminal) microvilli). Right three panels: schematic of selective action of capa-1 on apical microvilli, under resting conditions, within a second of capa-1 stimulation, and during the sustained phase 1–10 min later. Reproduced with permission from Terhzaz et al. (Terhzaz et al., 2006).

transport (Southall et al., 2006). Thus, calcium signalling by Golgi-associated SPCA regulates fluid transport by the tubule.

Calcium stores and calcium homeostasis

The idea that only the ER can act as a dynamic calcium store is out-dated. The Golgi, nucleus, mitochondria and even peroxisomes are now known to modulate calcium homeostasis in cells, and to be critically involved in cell function. Furthermore, experimental measurements of calcium in endosomes/lysosomes, the nucleoplasm and nuclear envelope, and secretory granules are possible (Gerasimenko and Tepikin, 2005).

In tubules, although IP_3 -mediated calcium signalling *via* ER in the principal and stellate cells has been established, substantial involvement of other organelles in calcium homeostasis is now known.

Mitochondria

Mitochondria are key calcium stores, and whose activation state is coupled to calcium dynamics, and to cellular events including cell death (Rizzuto et al., 2000; Romagnoli et al., 2007; Szabadkai and Duchon, 2008). Moreover, key mitochondrial enzymes are calcium regulated, and so mitochondrial function is a calcium-sensitive process.

In tubules, the measurement of mitochondrial calcium *in vivo* has been achieved using aequorin targeted to mitochondria *via* subunit VIII of human cytochrome c oxidase (Terhzaz et al., 2006). This allowed the measurement of a slow, 100 s rise in mitochondrial calcium-dependent on uptake *via* the mitochondrial calcium uniporter – which was triggered by activation of PLC-mediated IP_3 signalling by capa-1. Furthermore, development of a transgene encoding mitochondrial-targeted pericam ('Mitycam') allowed imaging of mitochondrial calcium in individual principal cells *in vivo*, confirming the slow calcium rise measured with aequorin, deriving from populations of principal cells in several tubules. Imaging with Mitycam provided novel data on the mechanism of calcium sensing upon capa-1 challenge, and showed that apical and basolateral mitochondria in tubule principal cells respond differentially to capa-1, with the apical mitochondria being responsive to the neuropeptide. Assessment of mitochondrial activity *via* a potential-sensitive dye indicated that only apical

mitochondria 'sensed' capa-1, which also increases ATP production by the tubule. The dependence of ATP generation upon calcium increases in mitochondria was demonstrated by the reduction of capa-1-induced tubule ATP production upon inhibition of the mitochondrial calcium uniporter. Thus, a novel mode of regulation exists for capa-1-stimulated fluid transport, where a concerted action of cytosolic calcium, mitochondrial calcium and alteration in the activity profile of mitochondrial proteins (as assessed by proteomics) occurs to re-model the mitochondrial matrix (Fig. 9).

Golgi and peroxisomes

Transcript A of the *Drosophila* SPCA $\text{Ca}^{2+}/\text{Mg}^{2+}$ pump, SPoCk A, is expressed in the Golgi of tubule principal cells and significantly impacts on capa-induced calcium signalling and fluid transport (Fig. 8) (Southall et al., 2006). Thus, in tubule principal cells, the Golgi, and not ER, is the primary IP_3 -inducible calcium store.

The novel localisations of SPoCk isoforms in principal cells also provide new insights into the role of novel calcium stores and pools in epithelial function. Analysis of SPoCk C gene expression in regions of the tubule demonstrates that transcript C is expressed more highly in tubules from females compared with males. This is associated with the higher expression of SPoCk C protein in female tubules compared with males (Fig. 10A). It is possible that SPoCk C is more abundant in female tubules due to the extra demands of oogenesis for calcium, as the chorion is calcium rich (35%) (Keramaris et al., 1991). The greater abundance of SPoCk C vesicles in females may be associated with the storage excretion of calcium in spherites throughout life providing an osmotically inactive bulk pool of calcium that could be mobilised during oogenesis.

Drosophila contain a pair of anterior tubules and a pair of posterior tubules in the abdomen, connected to the hindgut but free floating in the hemocoel. Whilst the entire tubule secretes calcium, the initial and transitional segments of the anterior tubules are capable of transporting the entire animal's Ca^{2+} content in less than 2 h (Dube et al., 2000b). Although some calcium is secreted in soluble form, most is sequestered into vesicles called spherites (Wessing and Eichelberg, 1978) commonly observed in insect epithelia (Turbeck, 1974).

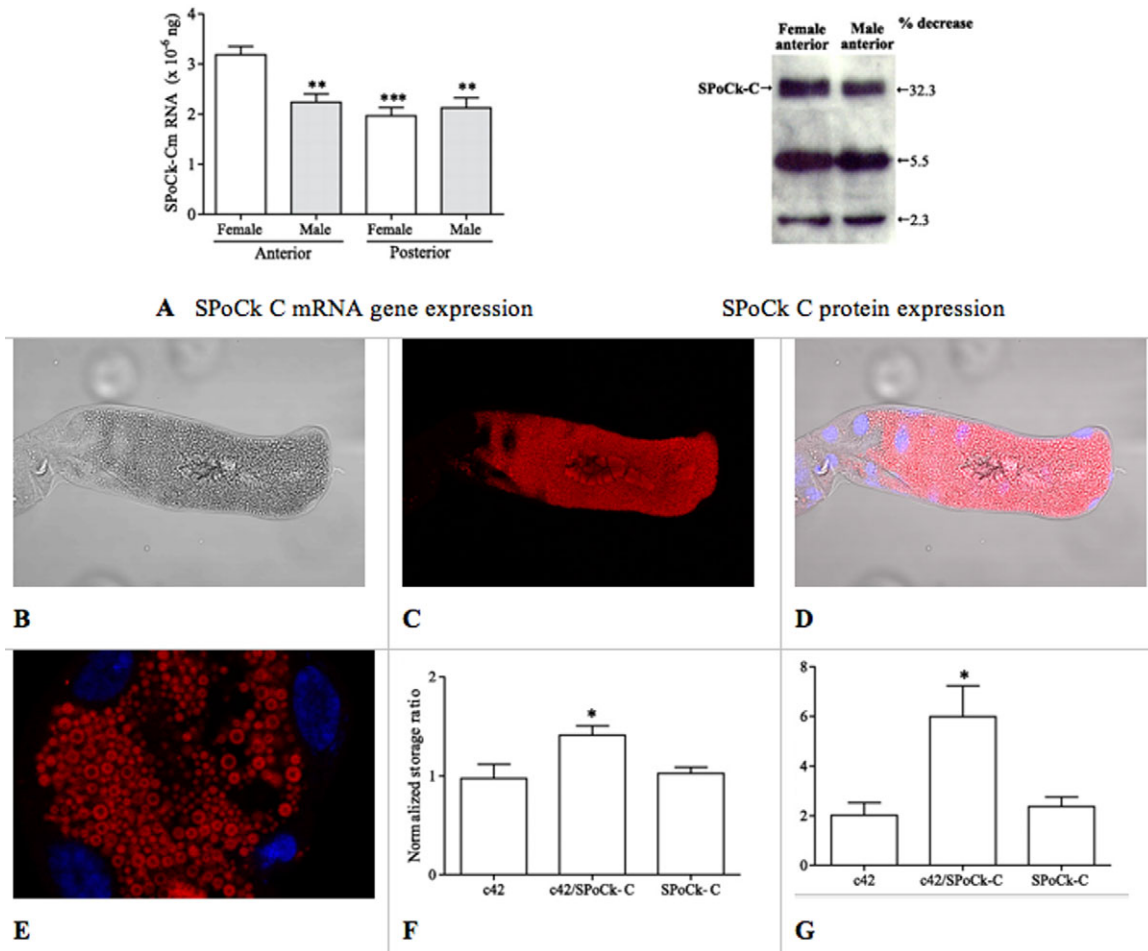


Fig. 10. Functional correlates of SPoCk C. (A) Sexually dimorphic SPoCk C expression in Malpighian tubules: mRNA expression of SPoCk C in female anterior tubules was significantly higher than in males and was increased compared with female posterior ($***P<0.0001$), male anterior ($**P=0.0015$) and male posterior tubules ($**P=0.001$). Data are shown as means \pm s.e.m., $N=4$. SPoCk C protein expression was significantly increased in female anterior tubules by ~32% [band intensity determined using ImageJ software (<http://rsb.info.nih.gov/ij/>)] compared with male anterior tubules. (B–E) Peroxisome-specific expression of SPoCk C isoform. (B) Bright-field image of initial segment of the anterior tubule; (C) confocal image of the initial segment of the anterior tubule, stained with anti-SPoCk C antibody; (D) overlay of B and C. Note that the lumen of the tubule is packed with SPoCk C-positive concretion bodies. Nuclei are stained blue with DAPI; (E) high-power view of the tubule initial segment showing the annular nature of anti-SPoCk C staining. Peroxisomal SPoCk C is associated with Ca^{2+} storage excretion. (F–G) Ca^{2+} storage (F) and transport (G) of $^{45}\text{Ca}^{2+}$ in SPoCk C-overexpressing tubules. Tubules were incubated in radioactively labeled Ca^{2+} , and the 'normalised storage ratio' was calculated as the ratio of specific activities of the tubules to bathing fluid. Over-expression of SPoCk C resulted in a significantly increased Ca^{2+} storage compared with the parental UAS-SPoCk C ($*P<0.014$) and c42 ($*P<0.033$) parental lines. Data are means \pm s.e.m., $N=4$ tubules. The 'transport ratio' was calculated as the ratio of specific activities of the secreted fluid to that of bathing fluid. Over-expression of SPoCk C resulted in a significantly increased Ca^{2+} transport compared with parental UAS-SPoCk C ($*P<0.014$) and c42 GAL4 ($*P<0.04$) lines. Data are means \pm s.e.m., $N=8$ tubules. From Southall et al. (Southall et al., 2006).

Antibodies to SPoCk C labelled the periphery of such vesicles in the lumen of the initial segment of the tubule (Fig. 10B–E). Thus, spherites are formed as concretions within specialized peroxisomes with high levels of SPoCk C in their membranes, so facilitating calcium uptake and storage (Fig. 10F,G) and then discharged into the tubule lumen. Thus, whereas SPoCk A modulates fluid transport *via* Golgi calcium, SPoCk C modulates another important property of the tubule: bulk calcium transport and sequestration. This was demonstrated by targeted over-expression of SPoCk C in principal cells, which resulted in increased calcium storage and excretion by the tubules, as determined by transport experiments using $^{45}\text{Ca}^{2+}$ (Fig. 10F,G).

In vertebrates, peroxisomes are abundant in kidney and liver and are associated with disease and stress conditions (Wanders and Waterham, 2006). Thus, deciphering the mechanisms of

peroxisomal calcium storage, transport and their impact on cellular calcium homeostasis in a genetically tractable epithelial model like the tubule may be a useful avenue of research. This may be achieved by measuring peroxisomal $[\text{Ca}^{2+}]$ using targeted aequorin or GFP-aequorin and monitoring links between the change of $[\text{Ca}^{2+}]$ in the cytoplasm and peroxisome. Furthermore, defective peroxisomal biogenesis has been shown to impact on mitochondrial ultrastructure and activity (Baumgart et al., 2001), so potential interactions between peroxisomal and mitochondrial calcium pools should be investigated.

The Golgi is a critically important organelle for calcium homeostasis (Dolman and Tepikin, 2006) and is clearly an essential calcium store in the tubule, thus modulating tubule physiology. Work in acinar cells has also shown that Golgi and mitochondria associate, and influence calcium gradients at particular zones in the

cell (Dolman et al., 2005). Given the prominence of mitochondrial calcium stores in tubule principal cells, such interactions between mitochondrial or other stores (e.g. ER), and Golgi may well occur. Thus, it will be important to make measurements of Golgi $[Ca^{2+}]$ in the intact tubule. The existence of multiple protein probes in which to survey intracellular calcium pools *in vivo*, coupled with transgenic technology, should open up several key avenues of research into cellular calcium homeostasis in defined cellular subtypes and epithelial tissue function.

Conclusions

We have described calcium signalling mechanisms in organelles of the tubule principal cell, and also the complexity of capa action that mobilises calcium signals from different intracellular organelles. The action of capa is in marked contrast to that of Drosokinin, which acts on tubule stellate cells. Although capa peptides have been shown to act *via* IP_3 and IP_3R (Pollock et al., 2003), the multiple organellar targets of capa-1 suggest that either IP_3 is generated at these different sites in the cell or, more likely, that capa-1 utilises additional routes for calcium mobilisation *via* calcium entry channels like CNG (Broderick et al., 2003; MacPherson et al., 2001) or TRPL (MacPherson et al., 2005), which promote calcium release and/or uptake into the Golgi, mitochondria or peroxisomes. Drosokinin, however, seems to act only *via* IP_3 (Pollock et al., 2003; Radford et al., 2002). Drosokinin stimulates a calcium rise in cells, which are transfected with the ER-localised SPoCk-B (which is not expressed in principal cells) but does not do so in either SPoCk A- or SPoCk C-transfected cells (Southall et al., 2006). This confirms that Drosokinin modulates an ER-derived IP_3 signal, thus increasing $[Ca^{2+}]_{cyt}$. Therefore, although both neuropeptides can act *via* IP_3 , in the tubule, Drosokinin is diagnostic of ER-derived IP_3 signalling whereas capa-1 is not, suggesting that calcium signalling mechanisms in the principal cell are necessarily more complex.

Although calcium signalling has been shown to modulate fluid transport by the Malpighian tubule, it is clear that the other important roles of the tubule in immune sensing and detoxification, may also be calcium-regulated processes. A recent study on larval fat body has demonstrated involvement of calcineurin in innate immunity (Dijkers and O'Farrell, 2007); and our unpublished work (S.T. and S.A.D.) shows involvement of principal cell calcium signalling in the innate immune response by the tubule.

The impact on calcium signalling and calcium homeostasis on tubule function is extremely significant, and affords challenges in the understanding of novel regulation of epithelial cell and tissue function. Given the importance of the tubule in detoxification, immunity and survival of the organism, there are many novel calcium-mediated events to be discovered. Targeted protein-based probes for such studies will be important tools for such studies; and will reveal dynamics of calcium signalling between distinct cellular pools.

List of abbreviations

| | |
|--------|-------------------------------------------|
| BRET | bioluminescence resonance energy transfer |
| CFP | cyan fluorescent protein |
| CNG | cyclic nucleotide-gated channel |
| DAG | diacylglycerol |
| EGFP | enhanced green fluorescent protein |
| ER | endoplasmic reticulum |
| FRET | fluorescence resonance energy transfer |
| GFP | green fluorescent protein |
| IP_3 | inositol 1,4,5-triphosphate |

| | |
|-------------|----------------------------------------------|
| IP_3R | inositol 1,4,5-triphosphate receptor |
| PLC β | phospholipase C β |
| PMCA | plasma membrane calcium ATPase |
| PTS1 | peroxisomal targeting sequence 1 |
| PUFAs | polyunsaturated fatty acids |
| RyR | ryanodine receptor |
| SERCA | sarco/endoplasmic reticulum Ca^{2+} ATPase |
| SPCA | secretory pathway calcium ATPase |
| TRP | transient receptor protein |
| YFP | yellow fluorescent protein |

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