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Cell-type specific calcium signalling in a *Drosophila* epithelium

Philippe Rosay, Shireen A. Davies, Yong Yu, M. Ali Sözen, Kim Kaiser* and Julian A. T. Dow*

Division of Molecular Genetics, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G11 6NU, UK

*Author for correspondence

SUMMARY

Calcium is a ubiquitous second messenger that plays a critical role in both excitable and non-excitable cells. Calcium mobilisation in identified cell types within an intact renal epithelium, the *Drosophila melanogaster* Malpighian tubule, was studied by GAL4-directed expression of an aequorin transgene. CAP_{2b}, a cardioactive neuropeptide that stimulates fluid secretion by a mechanism involving nitric oxide, causes a rapid, dose-dependent rise in cytosolic calcium in only a single, genetically-defined, set of 77 principal cells in the main (secretory) segment of the tubule. In the absence of external calcium, the CAP_{2b}-induced calcium response is abolished. In Ca^{2+}-free medium, the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin, elevates $[Ca^{2+}]_i$ only in the smaller stellate cells, suggesting that principal cells do not contain a thapsigargin-sensitive intracellular pool. Assays for epithelial function confirm that calcium entry is essential for CAP_{2b} to induce a physiological response in the whole organ. Furthermore, the data suggest a role for calcium signalling in the modulation of the nitric oxide signalling pathway in this epithelium. The GAL4-targeting system allows general application to studies of cell-signalling and pharmacology that does not rely on invasive or cytotoxic techniques.

Key words: Aequorin, Calcium, *Drosophila*, GAL4, Neuropeptide, CAP_{2b}, Leucokinin, DNOS

INTRODUCTION

The *Drosophila* renal (Malpighian) tubule provides a unique epithelial model for in vivo studies of membrane transport processes and intracellular signalling mechanisms using a combined approach of transgenics, molecular genetics, biochemistry and physiology. Recent developmental work (Hoch et al., 1994; Sözen et al., 1997) and classical morphology (Wessing and Eichelberg, 1978) suggest that functional sophistication is achieved by a relatively simple structure, numbering in total around 179 cells. This simple structure can further be resolved genetically into six subregions and six cell subtypes that correlate with known functional specialisations (Sözen et al., 1997). Fluid secretion by the Malpighian tubule is energised by an apical plasma membrane V-ATPase driving an amiloride-sensitive proton/alkali metal ion exchanger (Dow et al., 1997). Our current working hypothesis allocates different aspects of ion transport to different tubule regions, each of which can comprise more than one cell-type. For example, the main segment, which alone generates urine, contains two cell types, the principal (type I) and the stellate (type II) cells. Active cation transport is confined to the principal cells (Dow et al., 1997), whereas stellate cells have been proposed as the route for passive counterion and water fluxes (Dow et al., 1997; O'Donnell et al., 1996). Although such functional specialisation is not confined to *Drosophila*, we are uniquely able to mark genetically populations of cells using the P{GAL4} system (Sözen et al., 1997), thus providing a framework for further analysis.

Tissues with such cellular and regional heterogeneity can exhibit complex control pathways that may be difficult to analyse in situ with conventional techniques. Secretion by the tubule is stimulated by peptide and amine modulators (Davies et al., 1997), and by treatments that raise intracellular levels of cAMP, calcium or cGMP (Dow et al., 1994b). Fluid secretion is stimulated by the *Drosophila* neuropeptide CAP_{2b}, ELYAFPRV-amide (Huesmann et al., 1995), and leucokinin IV (Davies et al., 1995). CAP_{2b} acts to stimulate secretion via the activation of the nitric oxide (NO) signalling pathway and cGMP, but not via cAMP (Davies et al., 1995, 1997). Tubules have recently been shown to express DNOS, a Ca^{2+}/calmodulin-sensitive nitric oxide synthase (NOS), and CAP_{2b} stimulates tubule NOS activity (Davies et al., 1997). CAP_{2b} also has a cardio-stimulatory effect on insect myocardium, mediated via a change in intracellular levels of inositol 1,4,5-trisphosphate ($InsP_3$) (Tublitz, 1988). By contrast, leucokinin IV appears to act via intracellular calcium ($[Ca^{2+}]_i$) to stimulate chloride shunt conductance (O'Donnell et al., 1996), independently of cAMP/cGMP-mediated processes (Davies et al., 1995). The endoplasmic reticular Ca^{2+}-ATPase inhibitor, thapsigargin, causes an elevation in $[Ca^{2+}]_i$ levels in many cell types (Thastrup et al., 1990) and, like leucokinin, has been shown to increase both chloride shunt conductance (O'Donnell et al., 1996) and fluid secretion rates (this work) in *Drosophila* tubules. In addition, thapsigargin causes an elevation in basal cGMP levels which are not increased further on treatment with CAP_{2b} (S. A. Davies, unpublished), suggesting that CAP_{2b}-mediated NOS activation may be a Ca^{2+}-dependent process. As

the NO signalling pathway has been extensively characterised in tubules (Davies et al., 1995, 1997; Dow et al., 1994a), interactions ('cross-talk') between this and distinct signalling pathways can now be studied. Does CAP_{2b}, therefore, act via the elevation of $[Ca^{2+}]_i$ which in turn activates the NO/cGMP signalling pathway; and if so, in which cell-types do these signal transduction processes occur?

In the tubule, as in many tissues, it is difficult to study cell-specific regulation of $[Ca^{2+}]_i$ by classical techniques: small cell size precludes $[Ca^{2+}]_i$ measurement with ion-selective microelectrodes, and Ca^{2+}-sensitive fluorescent dyes are actively excreted by the cells (J. A. T. Dow, T. Cheek and S. M. Wilson, unpublished). We have thus developed a Ca^{2+} reporter expression system in *Drosophila* in order to measure cell-type-specific $[Ca^{2+}]_i$ changes in vivo in an organotypic context. Aequorin is a Ca^{2+}-sensitive luminescent protein isolated from the coelenterate *Aequorea victoria*. It is a complex of apoaequorin, a 21 kDa polypeptide, and coelenterazine, a hydrophobic luminophore. Aequorin has been previously used in several vertebrate and invertebrate contexts for monitoring $[Ca^{2+}]_i$ changes, both by micro injection of purified protein and by expression as a transgene (Brini et al., 1995). It has not until now been used for *Drosophila*, however, nor have previous experimental approaches in other organisms allowed much control over the cell-type specificity of expression. In *Drosophila*, this can be achieved with the binary GAL4/UAS_G system (Brand and Perrimon, 1993; Fischer et al., 1988). By using a variety of cell-type specific P{GAL4} lines (Sözen et al., 1997) to direct cell-specific expression of a GAL4-responsive transgene, we have targeted aequorin expression to the different cellular components of the Malpighian tubule.

In this paper we have established the basic parameters of the aequorin expression system using the tubule model: functionality and sensitivity of aequorin in *Drosophila*; cytoplasmic and cell-specific expression of aequorin. Using our aequorin expression system, we show that the neuropeptide, CAP_{2b}, rapidly elevates $[Ca^{2+}]_i$ levels in a dose-dependent manner only in principal cells and only in the tubule main segment. The CAP_{2b} response is absolutely dependent on the presence of extracellular Ca^{2+}. Thapsigargin-induced increases in principal cell $[Ca^{2+}]_i$ are similarly dependent on $[Ca^{2+}]_e$. Using the fluid secretion assay for tubule function, it has been possible to correlate cell-specific intracellular signalling mechanisms with physiological function in this tissue.

MATERIALS AND METHODS

Materials

A Calcium Calibration Buffer Kit, with Magnesium II, coelenterazine, and thapsigargin, was purchased from Molecular Probes. Thapsigargin was dissolved in ethanol before use; stocks were calculated to give less than 1% (v/v) final ethanol concentration, a level known not to inhibit tubule fluid secretion. Schneider's medium and Ca^{2+}-free Schneider's medium were obtained from GIBCO Life Technologies. All other chemicals were obtained from Sigma.

Drosophila methods

Drosophila were maintained on a 12 hours dark:12 hours light cycle on standard corn meal-yeast-agar medium at 25°C. GAL4 is a yeast transcription factor that is functional in *Drosophila*, although there are no endogenous GAL4-responsive elements in this organism. Its pattern and timing of expression are dependent upon the genomic context of the inserted P{GAL4} element (O'Kane and Gehring, 1987). GAL4 can be used to drive expression of secondary reporters linked to the GAL4-responsive promoter, UAS_G. Here, cell-specific expression of transgenes was driven by a panel of P{GAL4} lines with documented tubule-specific expression patterns (Sözen et al., 1997). The secondary reporters for GAL4 activity were a second chromosome insertion of UAS_G-*lacZ* (obtained from A. Brand); UAS_G-*aequorin* (see below) and UAS_G-*GFP* (green fluorescent protein) (p{UAS-GFP.S65T}T10, Bloomington stock). There is no detectable *lacZ* expression, GFP expression or aequorin activity in the absence of GAL4.

Construction of the aequorin reporter

The apoaequorin-coding region from cDNA clone pAQ2 (Knight et al., 1991) was cloned downstream of the GAL4-responsive promoter of the vector pUAST (Brand and Perrimon, 1993). Germ-line transformed *Drosophila* were then generated and two functionally equivalent lines, UAS_G-*aeq*30D and UAS_G-*aeq*49A, were obtained that have the transgene at the indicated cytogenetic locations (P. Rosay and K. Kaiser, unpublished data). The resultant progeny of crosses between the UAS_G-aequorin lines and the P{GAL4} lines used are designated as follows: P{GAL4} c42 ×UAS_G-aeq: c42-aeq (principal cells in main and lower segments and bar-shaped cells); P{GAL4} c710 (or c724) ×UAS_G-aeq: c710-aeq (or c724-aeq) (stellate cells in main segment and bar-shaped cells); P{GAL4} c649 ×UAS_G-aeq : c649-aeq (bar-shaped cells); P{GAL4} c 507 ×UAS_G-aeq: c507-aeq (lower tubule segment). Additionally, a P{GAL4-Hsp70.PB} ×UAS_G-aeq cross generated progeny designated hs-aeq (ubiquitous expression). GFP-expressing lines were generated in the same way using the same panel of P{GAL4} lines.

CCD imaging

Individual tubules were placed in Schneider's medium, permeabilised with buffer containing 0.1% (v/v) Triton X-100 and photographed with a CCD imager (Berthold Night Owl) linked to a Zeiss Axiophot microscope equipped with a Neofluar 20×lens (0.50 NA).

Antibody staining

Intact tubules were fixed in 4% (v/v) paraformaldehyde for 30 minutes, washed twice for 1 hour in PBS containing 1% (w/v) Sigma cold fraction V bovine serum albumin and 1% (v/v) Triton X-100 (PAT) and incubated overnight in 3% (v/v) normal goat serum containing mouse polyclonal antiserum against either aequorin (Knight et al., 1991) diluted 1:50; or rabbit polyclonal antiserum against βgalactosidase (Cappel), diluted 1:2,000, in PAT. After three washes in PAT (1 hour), tubules were subsequently incubated with the appropriate FITC-labelled secondary antibody (1:250 dilution; Vector Labs), washed twice for 1 hour in PAT and once for 5 minutes in PBS. All of the above was carried out at room temperature. Stained tubules were mounted in VectaShield (Vector). Whole-mount tubules were examined with a Molecular Dynamics Multiprobe laser scanning confocal microscope. The excitation (480 nm) and emission (530±15 nm) barrier filters used were appropriate to the fluorescein-based label of the secondary antibody. Three-dimensional reconstructions were performed using the programme 'ImageSpace 3.1' (Molecular Dynamics).

Detection of GFP expression patterns

Transgenic tubules from P{GAL4}×UAS_G-GFP lines were dissected, mounted on slides in PBS and examined with a Molecular Dynamics Multiprobe laser scanning confocal microscope, as described above.

$[Ca^{2+}]_i$ measurements

For reconstitution of intracellular aequorin, 16-30 tubules (depending on P{GAL4} expression patterns) from 4 to 14-day-old adults were dissected in Schneider's medium and placed in 100 μl of the same buffer with coelenterazine added to a final concentration of 2.5 μM

and incubated in the dark for 4-6 hours. Bioluminescence recordings were carried out using an LB9507 luminometer (Berthold Wallac). Tubule viability was consistently monitored at the end of each experiment by the final application of thapsigargin; an increase in $[Ca^{2+}]_i$ implied the integrity of $[Ca^{2+}]_i$ stores and thus of cell metabolism.

Accurate quantitation of calcium levels at any point in the experiment requires that the total available luminescence (i.e. the amount of reconstituted aequorin) is known, and that only a small fraction is consumed during the experiment (Cobbold and Rink, 1987). Accordingly, at the end of each experiment, tissues were disrupted in 350 μl lysis solution (1% (v/v) Triton X-100, 100 mM $CaCl_2$), causing discharge of the remaining aequorin and allowing estimation of the total amount of aequorin in the sample by integration of total counts. In every experiment, less than 2% of available aequorin was discharged during the $[Ca^{2+}]_i$ measurement phase. Calcium concentrations for each time point in an experiment were calculated by backward integration, using a program written in Perl, based on the method described by Button and Eidsath (1996).

Light output also depends on the aequorin transgene, the type of coelenterazine, and on the intracellular milieu (Knight et al., 1996). Accordingly, the luminescence characteristic curve was calibrated using a Calcium Calibration Buffer Kit (Molecular Probes). A calibration curve was drawn which correlates free calcium concentrations (*p*Ca) to the rate (k) of aequorin consumption (Cobbold and Rink, 1987). The equation obtained for *D. melanogaster* tubules, $p\text{Ca}=0.332588\times(-\log(k))+5.5593$, agreed closely with that established previously for the same aequorin/coelenterazine combination in plant cells (Knight et al., 1996).

Where required, the concentration of external free calcium, $[Ca^{2+}]_e$, was reduced in normal Schneider's medium by the addition of either 2 or 5 mM EDTA and the resulting $[Ca^{2+}]$ calculated, using corrections for pH and ionic strength (Maxchelator 6.5; C. Patton, Stanford University).

Fluid secretion

Malpighian tubules were isolated into 10 μl drops of Schneider's medium under liquid paraffin and fluid secretion rates measured in tubules as detailed elsewhere (Dow et al., 1994b).

Statistics

Data are presented as mean ± s.e.m. The significance of difference between data sets were analysed where appropriate using Student's *t*-test (unpaired), taking $P=0.05$ as the critical level.

RESULTS

Cell-specific cytoplasmic targetting of functional aequorin

The GAL4-responsive transgene allowed aequorin expression to be targeted to the different cellular components of the Malpighian tubule. Expression and cellular targeting of the apoaequorin polypeptide were demonstrated by immunofluorescence staining using anti-aequorin antibodies (Fig. 1A). GAL4-directed aequorin expression occurs with a pattern equivalent to that of GAL4-directed βgalactosidase or GFP expression (see Fig. 2G-J), and is distributed throughout the cytoplasm. This is advantageous compared with Ca^{2+}-sensing dyes, which are frequently sequestered into vacuolar compartments by living cells.

Functional aequorin was generated in vivo by incubating whole tubules in the presence of coelenterazine. Fig. 1B shows cell-type specificity of aequorin activity, demonstrated using a CCD imager. In P{GAL4}c42-UAS_G-aequorin (c42-aeq) tubules, luminescence is observed from principal cells in the main segment. As instantaneous luminescence is a function both of $[Ca^{2+}]_i$ and of the amount of available aequorin, the time course for conversion of apoaequorin to aequorin can be estimated by luminometry, as shown in Fig. 1C. The kinetics are similar to those of apoaequorin conversion in *Drosophila* homogenates (not shown), and of apoaequorin purified from *Aequorea* (Knight et al., 1991). Subsequent experiments employed a reconstitution time of 4-6 hours and between 16 and 30 tubules. Comparable total luminescence (see Materials and Methods) between different P{GAL4}-UAS_G-aequorin lines was achieved by adjusting the total number of transgenic tubules per sample. Exposure to coelenterazine had no impact on tubule function as assessed by the fluid secretion assay (Fig. 1D); secretion rates of coelenterazine-treated and untreated tubules are indistinguishable.

Regulation of calcium fluxes by the neuropeptide CAP_{2b} is confined to principal cells

Having established a cell-specific non-invasive technique to measure $[Ca^{2+}]_i$, we next determined if CAP_{2b}, a neuropeptide known to stimulate fluid secretion through the NO signalling pathway, increased $[Ca^{2+}]_i$. Effects of CAP_{2b} on aequorin luminescence from whole tubules were first examined in a line (hs-aeq) expressing GAL4 ubiquitously under heat-shock control (Jarman et al., 1993). As shown in Fig. 2K, 1 μM CAP_{2b} causes a rapid (<100 milliseconds) rise of $[Ca^{2+}]_i$, that remains significantly above background for 90 seconds, on CAP_{2b} stimulation. A time resolution of 100 milliseconds is the detection limit of the instrument; it may be that the actual response is even more rapid. This time course of CAP_{2b}-induced $[Ca^{2+}]_i$ elevation is more rapid than that of known CAP_{2b}-induced physiological responses: $t_{1/2}$ of CAP_{2b}-induced fluid secretion is 10 minutes while $t_{1/2}$ of the CAP_{2b}-induced electrical response is 5 minutes (Davies et al., 1995), suggesting that, consistent with its putative role as a second messenger, $[Ca^{2+}]_i$ elevation precedes CAP_{2b}-induced physiological responses of the tissue.

Cell-type specificity of the response was examined using P{GAL4} lines that independently and strongly mark the principal and stellate cell components in different segments of the tubule (Fig. 2L-P). There was no detectable CAP_{2b}-induced increase in $[Ca^{2+}]_i$ in P{GAL4} lines that selectively mark stellate and bar-shaped type II cells (c724-aeq, Fig. 2C,H,M); bar-shaped cells (c649-aeq, Fig. 2D,I,O); or the lower tubule and ureter (c507-aeq, Fig. 2E,J,P). Type II cells in line c724-aeq are nevertheless capable of responding to the diuretic peptide leucokinin IV (Fig. 2M, inset), confirming that the lack of response to CAP_{2b} is not due to a problem in viability or general responsiveness. In tubules from the P{GAL4} line c42-aeq, that marks the lower tubule and selectively marks principal cells and bar-shaped cells in the main segment (Fig. 2B,G), CAP_{2b} causes a rise in $[Ca^{2+}]_i$ (Fig. 2L) that is similar in kinetics and amplitude (87±7, basal level, to 255±54 nM, $n=8$) to that seen in the GAL4 line under heat-shock control (hs-aeq) (Fig. 2K). Taken together, the data in Fig. 2 therefore show that the $[Ca^{2+}]_i$ response to CAP_{2b} occurs only in principal cells in only the main segment of the tubule. They also implicitly show that Ca^{2+} signals do not pass between type I and type II via gap junctions.

The effect of CAP_{2b} on $[Ca^{2+}]_i$ is concentration-dependent

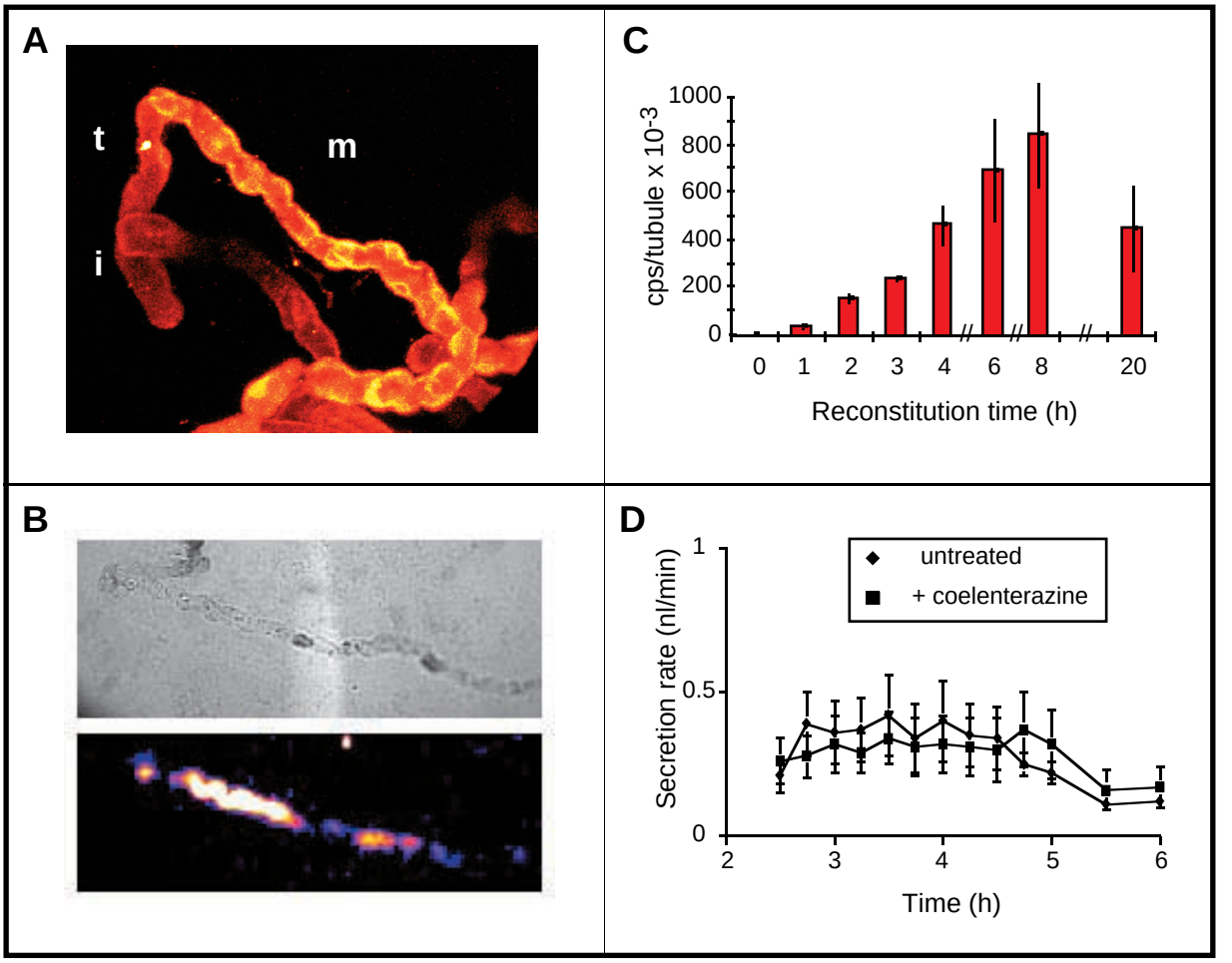

Fig. 1. Basic characterisation of the GAL4/UAS-aequorin system. (A) Cell-type specific apoaequorin expression in the main segment of the Malpighian tubule. P{GAL4} line c42 drives cytoplasmic βgalactosidase expression in principal cells of the main segment (see Fig. 2B and G). c42-aeq expression exhibits the same specificity when labelled with antiserum against aequorin. Cytosolic expression of aequorin is apparent only within the main segment. Staining of the initial and transitional segments is at background levels. m, main segment; t, transitional segment; i, initial segment. Scale is given by the tubule diameter (~35 μm). (B) Cell-type specificity of aequorin activity. c42-aeq tubules were permeabilised in a buffer containing 0.1% (v/v) Triton X-100, which increases $[Ca^{2+}]_i$ to saturation. Upper panel, bright field image of the main segment. Lower panel, luminescence signal (CCD image); exposure time 5 minutes. (C) Time course of apoaequorin conversion in whole tubules. Data were averaged for c42-aeq and hs-aeq backgrounds (n=4) since the kinetics were identical. Each bar represents the amount of calcium-dependent luminescence produced as a function of time after the addition of coelenterazine. (D) Tubule fluid secretion (Dow et al., 1994b) is unaffected by coelenterazine. Tubules were incubated in Schneider's medium with or without 2.5 μM coelenterazine and fluid secretion rate determined. Basal secretion rates are indistinguishable between the sets of tubules.

over the range 10^{-8} to 10^{-6} M (Fig. 3). Concentrations as low as 10 nM produce a detectable response. In early experiments, the $[Ca^{2+}]_i$ response detected at each concentration was found to differ only in magnitude, and not in kinetics or time course; and so subsequently, 10^{-8}-10^{-7} M was adopted as a working concentration range that reliably elicited a response well above the system noise, while using a relatively small number of tubules. The dose-response curves to CAP_{2b} obtained for hs-aeq and c42-aeq are indistinguishable (statistical analysis of all data points showed no significant difference). By contrast, the dose-response curve for c724-aeq shows that the $[Ca^{2+}]_i$ responses in stellate cells to all concentrations of CAP_{2b} are not statistically different from the mock injection.

Source of CAP_{2b}-induced rise in $[Ca^{2+}]_i$

If CAP_{2b} causes Ca^{2+} release from an internal store, perhaps via $InsP_3$, then the CAP_{2b}-stimulated rise in $[Ca^{2+}]_i$ should be blocked by prior discharge of Ca^{2+} internal pools. Tubules are known to be sensitive to the endoplasmic reticulum Ca^{2+}-ATPase blocker, thapsigargin (O'Donnell et al., 1996). Pre-treatment with 10 μM thapsigargin blocks CAP_{2b} stimulation, both of elevation of $[Ca^{2+}]_i$ (Fig. 4A) and of fluid transport (Fig. 4B). These data are summarised in Table 1: no CAP_{2b}-induced Ca^{2+} influx is observed after 15 minutes of treatment in 10 μM thapsigargin (26±5%, n=8) as compared with mock-stimulated tubules (16±3%, n=18).

These data show clearly that high concentrations of thapsigargin block the physiological effect of CAP_{2b}. However, 1 μM thapsigargin did not completely block CAP_{2b} elevation of $[Ca^{2+}]_i$ (data not shown). This suggests that thapsigargin might be acting in some other manner at higher concentrations: indeed, a direct action of thapsigargin at high concentration on

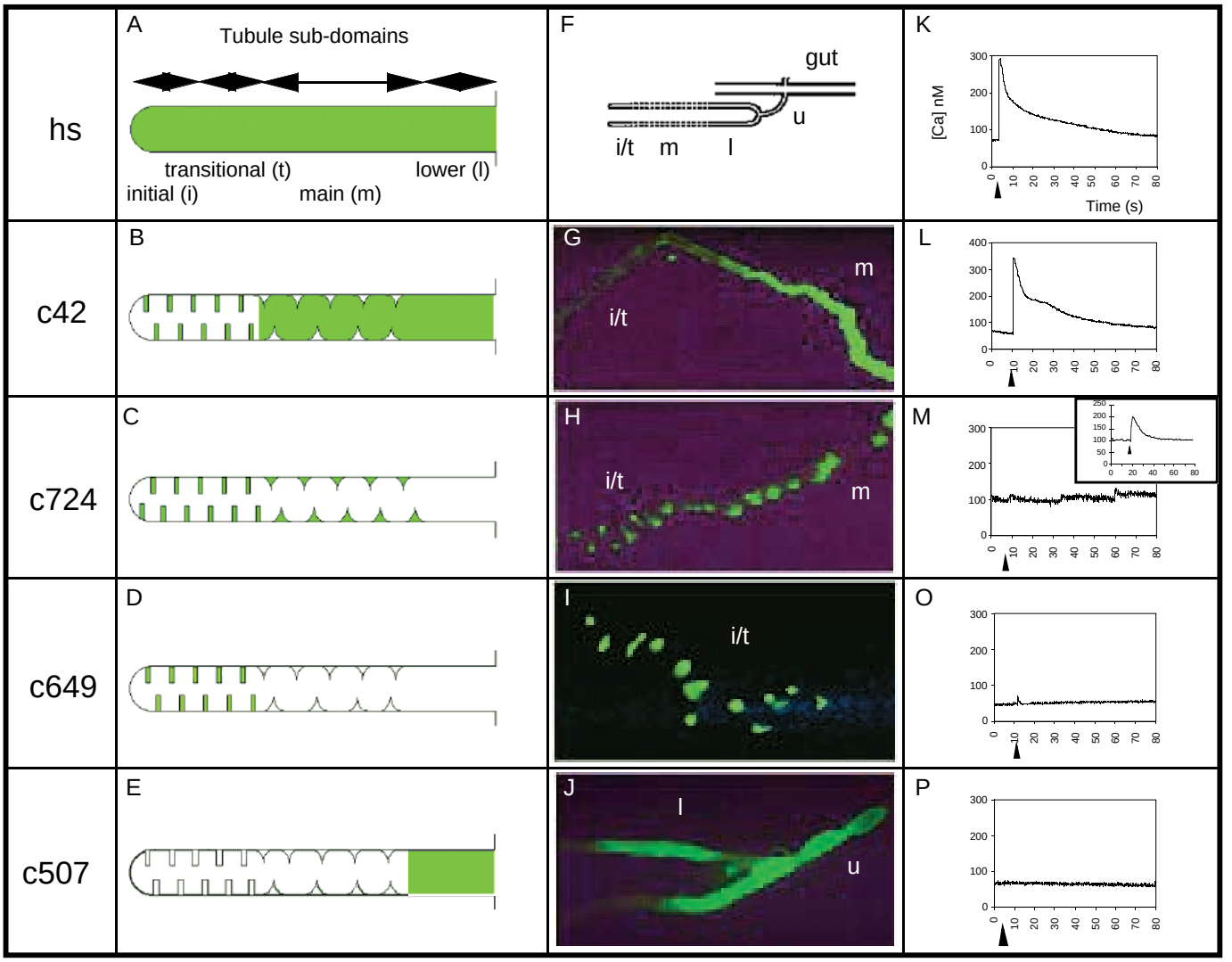

Fig. 2. Cell- and region-specific CAP_{2b}-stimulated calcium responses in the tubule. (A-E) Schematic representation of a Malpighian tubule showing domains of GAL4 expression (green) in the indicated lines: (A) P{GAL4-Hsp70.PB}, ubiquitous. (B) Line c42 expresses GAL4 in principal (but not stellate) cells of the main segment, in bar-shaped cells of the initial and transitional segments, and in the lower tubule. (C) Line c724 marks all type II cells, viz. stellate cells of main segment and bar-shaped cells of initial and transitional segments (sensu Sözen et al., 1997). (D) Line c649 marks bar-shaped cells. (E) Line c507 marks the lower tubule and the ureter. (F) Schematic representation of paired anterior tubules connected to the hindgut by the ureter (u); i, t, m, l are initial, transitional, main and lower segments respectively. (G-J) GAL4-directed GFP expression. (K-P) Typical responses to 1 μM CAP_{2b} in P{GAL4}-UAS_G-aequorin lines. Arrowheads indicate time of CAP_{2b} application. (M, inset) Response of c724-aeq to 10^{-8} M leucokinin IV. Tubule viability was in all cases verified at the end of each experiment by the application of thapsigargin at a final concentration of 1 μM (data not shown).

plasma membrane Ca^{2+} channels has been reported (Buryi et al., 1995; Shmigol et al., 1995).

Thapsigargin acts differently on principal and stellate cells

Thapsigargin alone can elicit stimulation of fluid secretion at 10 μM (Fig. 4A), and this effect can be replicated at 1 μM (Fig. 5A) or even 50 nM (data not shown). Previously, this had been interpreted as a selective $[Ca^{2+}]_i$ stimulation of the chloride shunt conductance in stellate cells (O'Donnell et al., 1996). However, it is now clear that $[Ca^{2+}]_i$ also plays a role in principal cell control. To dissect the effects of tissue application of thapsigargin, $[Ca^{2+}]_i$ was measured separately in principal and stellate cells after stimulation by 1 μM thapsigargin. This elicited an increase in $[Ca^{2+}]_i$ in both cell types (Fig. 5B-C). This implies that thapsigargin acts both to stimulate the apical V-ATPase in principal cells (the ultimate effect of CAP_{2b}) and to increase the chloride shunt conductance in stellate cells. It is the latter effect which must generate the classical electrophysiological signature of thapsigargin in this tissue, a collapse of transepithelial potential (O'Donnell et al., 1996).

To establish whether thapsigargin acts in its role in releasing intracellular calcium stores, the experiment was repeated in the absence of internal calcium. Under these conditions, any increase in calcium must be from internal stores. Thapsigargin

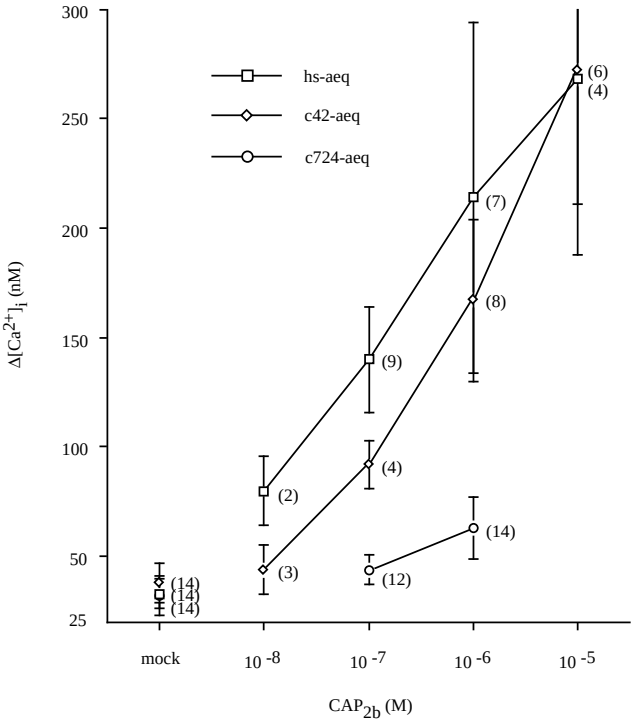

Fig. 3. Dose-response relationship of CAP_{2b} effect on $[Ca^{2+}]_i$ elevation. The number of measurements for each data-point is indicated in brackets. $\Delta[Ca^{2+}]_i$ values were determined as the difference between $[Ca^{2+}]_i$ in CAP_{2b}-stimulated and mock-injected samples. The three different P{GAL4} backgrounds gave similar basal $[Ca^{2+}]_i$ values: c42-aeq, 87±7 nM (*n*=20); hs-aeq, 76±6 nM (*n*=35); c724-aeq, 115±5 nM (*n*=50).

Table 1. Effect of external calcium and thapsigargin on the CAP_{2b}-induced $[Ca^{2+}]_i$ increase

	n	$\Delta[Ca^{2+}]_i$	Percentage	$[Ca^{2+}]_e$
Control	10	188±14	100±8	4.0
EDTA 2 mM	12	143±19	76±10	2.5
EDTA 5 mM	6	94±32	50±17	0.8
Calcium-free	8	30±8	16±4	0.0
Thapsigargin 10 μM	8	50±9	26±5	4.0

c42-aeq tubules were incubated in Schneider's medium (control), in Schneider's medium containing EDTA or thapsigargin, or in Ca^{2+}-free Schneider's. After 5 minutes of incubation (15 minutes with thapsigargin), maximal $[Ca^{2+}]_i$ levels (nM) after CAP_{2b} injection were measured. $\Delta[Ca^{2+}]_i$ was determined as in Fig. 3.

still stimulates fluid secretion in Ca^{2+}-free medium (Fig. 5D). However, under such conditions there is an increase in $[Ca^{2+}]_i$ only in stellate cells; there is no effect on principal cells (Fig. 5E-F). This shows that thapsigargin acts in a fundamentally different manner in principal and stellate cells in the same epithelium. Only the stellate cells have a detectable thapsigargin-sensitive internal pool.

Does CAP_{2b} signal to the plasma membrane via internal stores?

These data force a reappraisal of the thapsigargin blockade of CAP_{2b} action. If there is no detectable thapsigargin-sensitive pool in principal cells, high concentrations of thapsigargin might be acting to block Ca^{2+} entry, as has been documented in other tissues. In this case, CAP_{2b} might act to raise $[Ca^{2+}]_i$ by triggering Ca^{2+} entry, rather than release from internal pools. To distinguish these possibilities, c42-aeq tubules were stimulated with CAP_{2b} in solutions containing variable

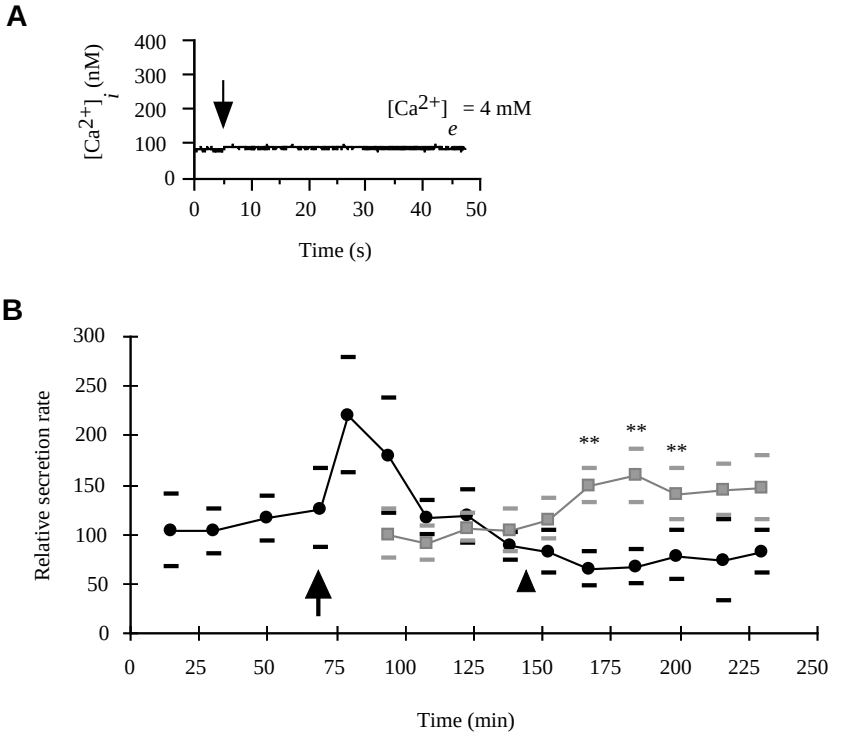

Fig. 4. CAP_{2b} action is blocked by 10 μM thapsigargin. (A) CAP_{2b} stimulation (arrow) of $[Ca^{2+}]_i$ is abolished after thapsigargin (10 μM) treatment of tubules. (B) CAP_{2b} stimulation of fluid secretion is abolished after thapsigargin (10 μM) treatment of tubules. Transgenic tubules (line c42) were bathed in Schneider's medium, treated (●) or untreated (control, ■) with thapsigargin at 10 μM (arrow) and then challenged 75 minutes later (arrowhead) with CAP_{2b} (0.1 μM final). To aid comparison between different groups, fluid secretion rates are standardised to the average of the first three readings. Asterisks denote a significant difference between treatments at a given time-point.

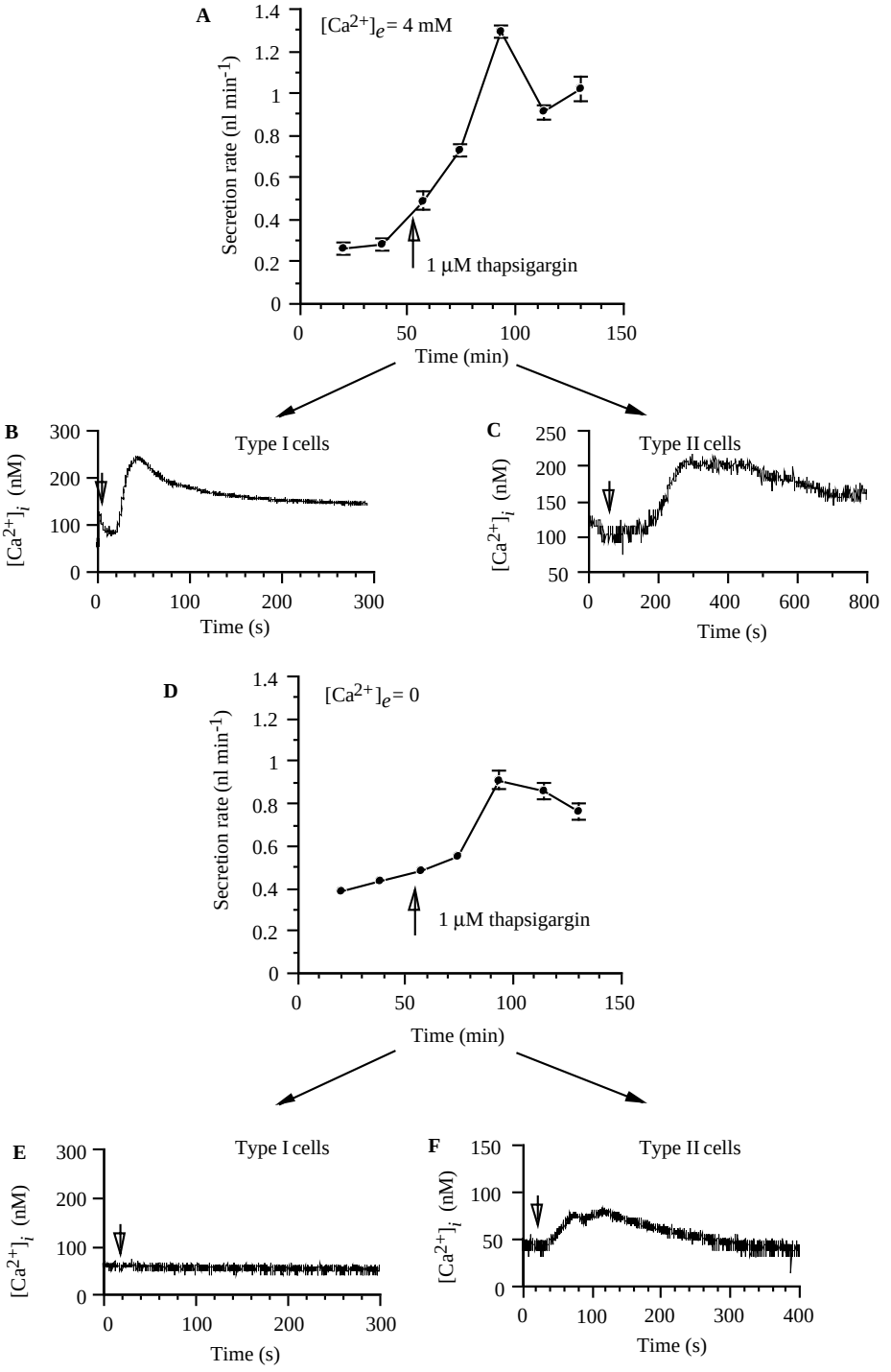

Fig. 5. Thapsigargin acts to stimulate fluid secretion through distinct cell-specific mechanisms. (A) Fluid secretion by Malpighian tubules in normal medium, and stimulated with 1 μM thapsigargin at 59 minutes. Data are presented as mean ± s.e.m. (n=3). (B-C) Representative $[Ca^{2+}]_i$ traces for (B) Type I cells (c42-aeq) and (C) Type II cells (c710-aeq) in normal medium, and stimulated with 1 μM thapsigargin (arrow). Thapsigargin increases $[Ca^{2+}]_i$ in both type I and type II cells. (D) Fluid secretion by Malpighian tubules in Ca^{2+}-free medium, and stimulated with 1 μM thapsigargin at 58 minutes. Data are presented as mean ± s.e.m. (n=6). (E-F) Representative $[Ca^{2+}]_i$ traces for (E) Type I cells (c42-aeq) and (F) Type II cells (c710-aeq) in Ca^{2+}-free medium, and stimulated with 1 μM thapsigargin (arrow). Thapsigargin increases $[Ca^{2+}]_i$ only in type II cells.

$[Ca^{2+}]_e$, and changes in principal cell $[Ca^{2+}]_i$ monitored. The magnitude of the CAP_{2b} response declines uniformly as $[Ca^{2+}]_e$ is reduced, and is abolished in Ca^{2+}-free medium (Fig. 6A-D). This correlates well with physiological data: stimulation of fluid secretion by CAP_{2b} is also dependent on the presence of external calcium (Fig. 6E). However, 0.1 mM cAMP was nonetheless able to stimulate fluid secretion of tubules in Ca^{2+}-free medium (data not shown), implying that the tubules were viable, and that cAMP signalling is not dependent on $[Ca^{2+}]_e$. Additionally, oscillatory behaviour was sometimes observed in low $[Ca^{2+}]_e$ (Fig. 6B,C): this is a characteristic of Ca^{2+}-mediated signalling systems, normally interpreted as feedback interactions between distinct Ca^{2+}-pools (Berridge, 1997).

Table 1 summarises the results of CAP_{2b}-induced $[Ca^{2+}]_i$ response in tubules treated as in Fig. 6. Pre-incubating tubules in Schneider's medium containing reduced $[Ca^{2+}]_e$ (2.5 mM compared to 4.0 mM) reduces the magnitude of the $[Ca^{2+}]_i$ response to CAP_{2b} (76±10%, n=12) compared to control tubules (100%, 4.0 mM $[Ca^{2+}]_e$). The response is reduced

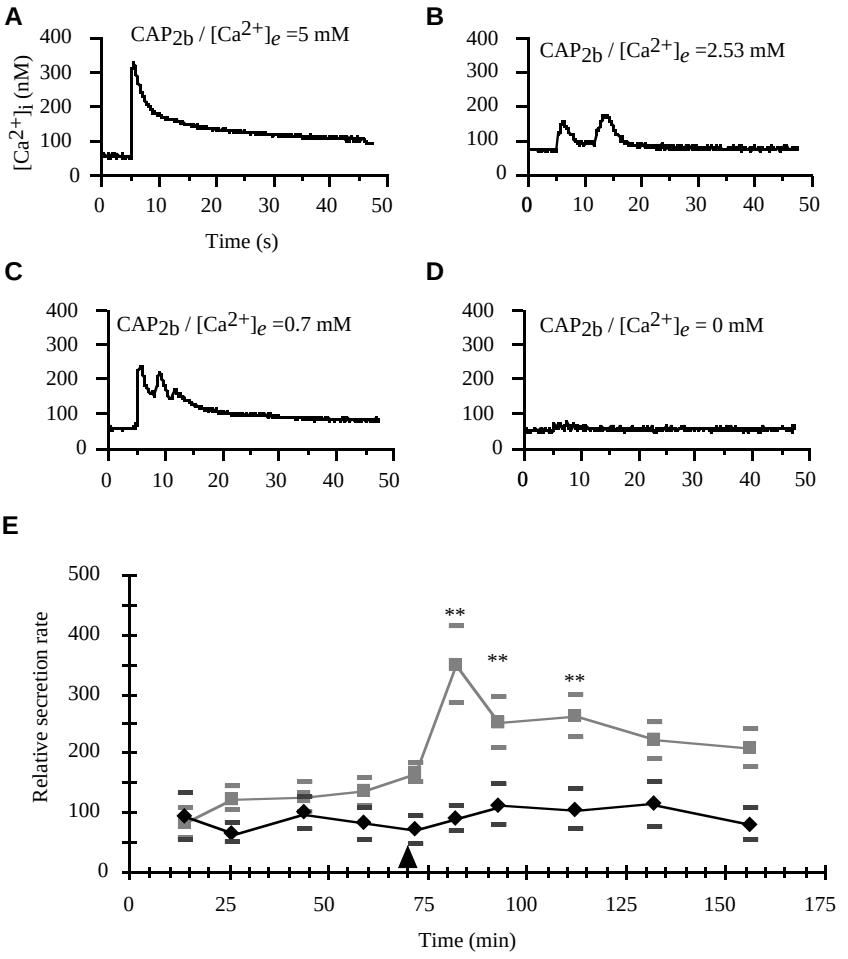

Fig. 6. CAP_{2b}-induced responses require $[Ca^{2+}]_e$. $[Ca^{2+}]_i$ response of principal cells in c42-aeq tubules to CAP_{2b} (10^{-7} M) injected at t = 5 seconds, in Schneider's medium containing (A) 4 mM Ca^{2+}; (B) 2.5 mM Ca^{2+}; (C) 0.7 mM Ca^{2+}; (D) in Ca^{2+}-free Schneider's medium. (E) Fluid secretion response of tubules to CAP_{2b} in Ca^{2+}-free medium. Transgenic tubules (line c42) were bathed in Schneider's medium and washed three times at 65 minutes by replacing the medium with Ca^{2+}-free Schneider's medium (◆). Fluid secretion rates for control, unwashed tubules are also shown (■). Tubules were challenged with CAP_{2b} (10^{-7} M) at 70 minutes (arrowhead). To aid comparison between different groups, fluid secretion rates are standardised to the average of the first three readings. Asterisks denote a significant difference between treatments at a given time-point.

further (50±17%, *n*=6) but not completely abolished, when tubules are incubated in 0.8 mM $[Ca^{2+}]_e$. By contrast, no Ca^{2+} influx is triggered by CAP_{2b} in a calcium-free environment (16.1±4.3%, *n*=8). Tubules in Ca^{2+}-free medium were also treated with 1 mM EDTA and then stimulated with CAP_{2b}. These tubules did not have a significant response to CAP_{2b} compared to a mock stimulation, nor did the response differ significantly from that seen in CAP_{2b}-stimulated tubules in nominally Ca^{2+}-free medium without EDTA.

Taken together, these data show that the CAP_{2b}-mediated Ca^{2+} response of principal cells is absolutely dependent on external calcium, and that this Ca^{2+} influx is necessary for the action of CAP_{2b} on fluid secretion.

DISCUSSION

We have utilised the GAL4/UAS_G binary expression system to investigate cell-specific calcium mobilisation events for the first time in *Drosophila*. The technique described here offers significant advantages for the analysis of calcium signalling in cellularly heterogeneous tissues. The expression of the GAL4-directed transgene, apoaequorin, is clearly restricted only to those sub-sets of cells marked in the different P{GAL4} lines used. The method is both cell-type specific and non-invasive. In the case of the main segment of the Malpighian tubule, the principal and stellate cells intercalate, and thus could not have been easily studied using classical pharmacology or electrophysiology. The aequorin expression system should therefore be of great utility in the study of the pharmacology of calcium signalling events in other tissues; for example, the brain, for which a large number of P{GAL4} lines with cell-type specific expression patterns are available (O'Dell et al., 1995; Yang et al., 1995).

CAP_{2b} causes rapid calcium mobilisation within the Malpighian tubule, a finding that could not have been predicted on the basis of previous, less direct, experimental data (O'Donnell et al., 1996). Moreover, the whole-tubule response is attributable to just the genetically defined subset of 77±0.3 (Sözen et al., 1997) principal cells in the main (fluid-generating) segment, of the tubule. The lack of stellate cell response to CAP_{2b} stimulation additionally implies that type I and type II cells of the main segment are not coupled by gap junctions to any significant extent.

CAP_{2b} must act to increase fluid secretion rates solely by an initial rapid rise of $[Ca^{2+}]_i$ in principal cells. CAP_{2b} has been shown to stimulate tubule NOS activity (Davies et al., 1997). It is probable that this rise in $[Ca^{2+}]_i$ is sufficient to trigger the activation of DNOS (Davies et al., 1997), a calcium-sensitive isoform of NOS (Regulski and Tully, 1995). The maximal CAP_{2b} concentrations employed here, 10^{-6} M, elevate principal cell calcium levels from 87±7 to 255±54 nM (line c42-aeq, Fig.

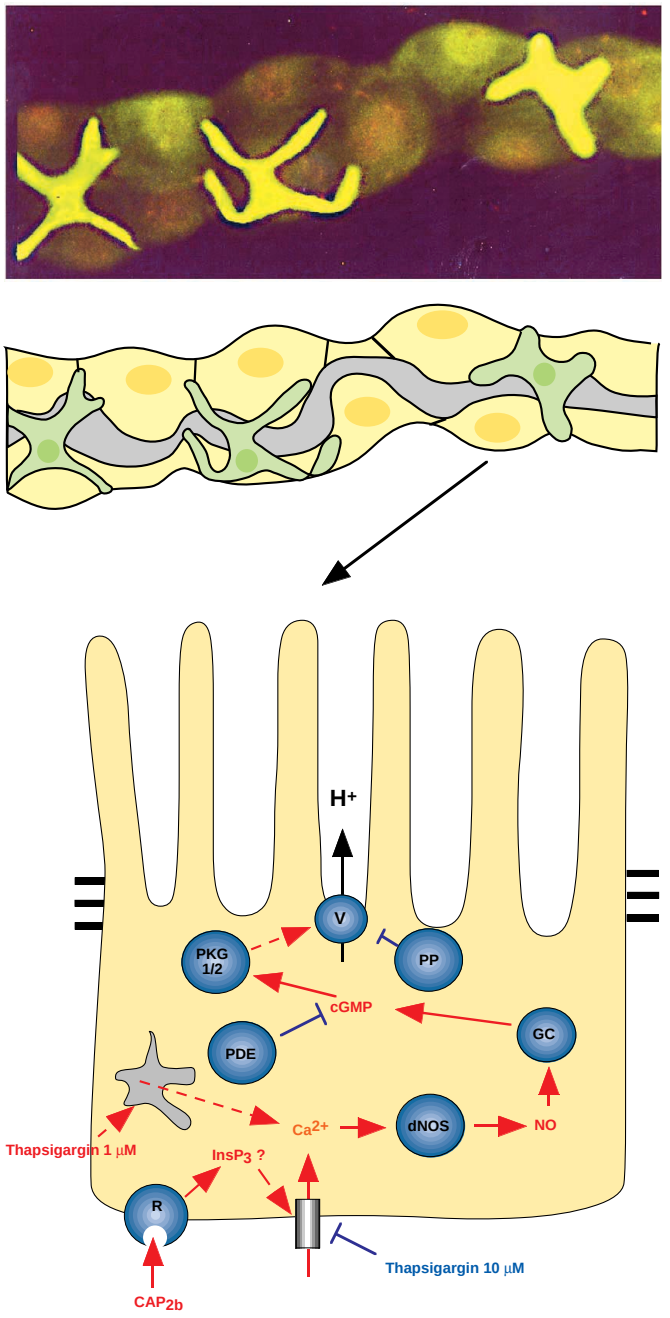

Fig. 7. Summary of current models for CAP_{2b} and calcium signalling in *D. melanogaster* Malpighian tubule principal cells. Top panel, epifluorescence view of a region from tubule main segment, showing architecture of principal and stellate cells. Stellate cells are visualised with anti-βgalactosidase and detected with an FITC-coupled second antibody: tissue is counterstained with ethidium bromide, highlighting the larger principal cell nuclei. Middle panel, abstraction of the above. Lower panel, diagrammatic representation of the CAP_{2b} signalling pathway in principal cells: see text for further details. For reasons of clarity, the cAMP signalling pathway in principal cells has been omitted. Diagram redrawn from O'Donnell et al. (1996).

3), a value close to the EC_{50} for DNOS, 300±60 nM (Müller, 1994). This implies that dNOS is responsive over the range of CAP_{2b} concentrations employed here. This may account for the observation that thapsigargin treatment results in increased basal cGMP levels which are not further increased on CAP_{2b} stimulation (S. A. Davies, unpublished). Our data thus provide strong evidence for a calcium-mediated link between CAP_{2b} and NOS/cGMP activation of fluid secretion (Fig. 7).

Interestingly, the effects of both CAP_{2b} and cGMP on both fluid secretion and on the apical V-ATPase (as determined electrophysiologically) are known to show an inverted-U shaped activation curve, with maxima around 10^{-8} M for CAP_{2b} and 10^{-4} M for cGMP (Davies et al., 1995; O'Donnell et al., 1996). This was interpreted as a feedback regulation of signalling through this pathway, probably downstream of cGMP (Davies et al., 1995; O'Donnell et al., 1996). As we show that the $[Ca^{2+}]_i$ response rises uniformly over the CAP_{2b} concentration range in question (Fig. 3), this confirms that any autoinhibitory mechanism must lie downstream of $[Ca^{2+}]_i$ signalling in this pathway (Fig. 7).

Although we show here that thapsigargin elevates $[Ca^{2+}]_i$ in both cell types of this epithelium, the nature of the response is very different. Only in stellate cells is there an identifiable thapsigargin-sensitive pool; in principal cells, external Ca^{2+} must be present for thaspisgargin to have an effect. It remains unclear how low concentrations of thapsigargin are able to elicit increases in principal cell $[Ca^{2+}]_i$ in the presence of external calcium; at this stage, we cannot exclude the possibility that thapsigargin depletes a principal cell store that is too small to detect, but which nonetheless is capable of signalling to plasma membrane calcium channels. However, the inability of low concentrations of thapsigargin to block CAP_{2b} signalling means that, whether or not a small thapsigargin-sensitive pool is present in principal cells, this is not the route by which CAP_{2b} elevates $[Ca^{2+}]_i$.

Putative mechanisms of agonist-induced calcium mobilisation in epithelia and secretory cells have been debated extensively (Shuttleworth, 1997). There are various sub-models for capacitative entry, in which mobilisation of $[Ca^{2+}]_i$ from internal stores signals to the plasma membrane to induce Ca^{2+} entry; and for non-capacitative entry, in which agonists activate plasma membrane channels directly without the intermediate involvement of internal stores. While a definitive proof in our system will require further work, the presence of a classical thapsigargin effect in calcium free medium only in stellate cells suggests that both models may be represented in the same tissue.

CAP_{2b} has been previously shown to generate $InsP_3$ in *Manduca sexta* heart (Tublitz, 1988); so it is plausible that the $[Ca^{2+}]_i$ response, induced by CAP_{2b} in *D. melanogaster* tubules, might also occur via $InsP_3$ signalling. Although receptor-stimulated $InsP_3$ generation can activate the capacitative calcium entry pathway in non-excitable cells (Putney, 1986, 1993), *D. melanogaster* tubule principal cells lack a measurable thapsigargin-sensitive pool. However, $InsP_3$ has recently been shown also to activate a class of plasma membrane $InsP_3$ receptor, and so trigger direct Ca^{2+} entry (Putney, 1997). Such a model would elegantly explain our data for Ca^{2+}-mediated signalling by CAP_{2b} in this epithelium.

The *Drosophila* Malpighian tubule is the first epithelium in which neuropeptide-activated signalling pathways can be dissected in specific cell sub-types and correlated with whole

organ function. Our ability to manipulate such a system genetically offers exciting prospects for further analysis.

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