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# *norpA* and *itpr* mutants reveal roles for phospholipase C and inositol (1,4,5)-trisphosphate receptor in *Drosophila melanogaster* renal function

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**Summary**

**Mutants of *norpA*, encoding phospholipase Cβ (PLCβ), and *itpr*, encoding inositol (1,4,5)-trisphosphate receptor ($IP_3R$), both attenuate response to diuretic peptides of *Drosophila melanogaster* renal (Malpighian) tubules. Intact tubules from *norpA* mutants severely reduced diuresis stimulated by the principal cell- and stellate cell-specific neuropeptides, $CAP_{2b}$ and *Drosophila* leucokinin (Drosokinin), respectively, suggesting a role for PLCβ in both these cell types. Measurement of $IP_3$ production in wild-type tubules and in Drosokinin-receptor-transfected S2 cells stimulated with $CAP_{2b}$ and Drosokinin, respectively, confirmed that both neuropeptides elevate $IP_3$ levels.**

**In *itpr* hypomorphs, basal $IP_3$ levels are lower, although $CAP_{2b}$-stimulated $IP_3$ levels are not significantly reduced compared with wild type. However, $CAP_{2b}$-stimulated fluid transport is significantly reduced in *itpr* alleles. Rescue of the $itpr^{90B.0}$ allele with wild-type *itpr* restores $CAP_{2b}$-stimulated fluid transport levels to wild type. Drosokinin-stimulated fluid transport is also reduced in homozygous and heteroallelic *itpr* mutants.**

**Measurements of cytosolic calcium levels in intact tubules of wild-type and *itpr* mutants using targeted expression of the calcium reporter, aequorin, show that mutations in *itpr* attenuated both $CAP_{2b}$- and Drosokinin-stimulated calcium responses. The reductions in calcium signals are associated with corresponding reductions in fluid transport rates.**

**Thus, we describe a role for *norpA* and *itpr* in renal epithelia and show that both $CAP_{2b}$ and Drosokinin are PLCβ-dependent, $IP_3$-mobilising neuropeptides in *Drosophila*. $IP_3R$ contributes to the calcium signalling cascades initiated by these peptides in both principal and stellate cells.**

Key words: $CAP_{2b}$, leucokinin, photoreception, TRP, TRPL, Drosokinin, *Drosophila*, inositol (1,4,5)-trisphosphate receptor ($IP_3R$), *itpr*, *norpA*.

## Introduction

The accepted paradigm for hormonally stimulated increases in intracellular cytosolic calcium concentration ($[Ca^{2+}]_i$) in non-excitable cells centres on G-protein-coupled activation of phospholipase Cβ (PLCβ) upon ligand–receptor binding, resulting in an increase in intracellular levels of the second messengers diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate ($IP_3$). $IP_3$ binds to its receptor on the endoplasmic reticulum (ER), $IP_3R$, which functions as a calcium-release channel. Calcium is released from intracellular stores, with associated calcium entry *via* plasma membrane calcium channels by an unknown mechanism termed ‘capacitative calcium entry’ (Berridge, 1997; Putney, 1997).

The processes of calcium signalling *in vivo* have been intensely investigated in *Drosophila* phototransduction, where novel calcium channels and associated signalling proteins have been discovered in forward genetic screens (Hardie and Raghu, 2001). Importantly, studies in *Drosophila* have subsequently revealed vertebrate and human homologues of such signalling complexes (Harteneck et al., 2000). Light-driven phototransduction has been shown to be PLC-dependent; as such, most studies of PLC function *in vivo* in *Drosophila* have utilised the photoreceptor model. Two genes encode PLCβ in *Drosophila*: *norpA* and *Plc21C*. Mutants in *norpA* severely reduce phototransduction (Pak et al., 1970). Molecular cloning of the gene, together with biochemical analyses of PLC activity in the eye of *norpA* mutants showed that *norpA* encodes a retinal-specific PLC similar to bovine brain PLC (Bloomquist et al., 1988). By contrast, *Plc21C* is more generally expressed, with a 7 kb transcript in the eye and central nervous system (CNS) and a 5.6 kb transcript in heads and bodies (Shortridge et al., 1991).

Mutagenic analysis of the $IP_3R$ has also been informative in insects. In *Drosophila*, a single gene, *itpr-83*, encodes $IP_3R$ (Hasan and Rosbash, 1992; Yoshikawa et al., 1992). *Drosophila* $IP_3R$ has most similarity to type I $IP_3R$ in vertebrates. Embryonic expression of *itpr* has been documented, and a delayed-development phenotype has been identified in mutants (Venkatesh and Hasan, 1997). In the adult, expression has been shown in photoreceptors, brain and antennae, with expression also documented in the eye, which suggests a role for $IP_3R$ in chemosensation and in visual processes. However, in spite of the documented role of PLC in the eye, recent work has shown that $IP_3$ signalling is unnecessary for phototransduction to occur (Hardie and Raghu, 1998; Venkatesh and Hasan, 1997).

While *norpA* and *itpr* have been assigned neural roles – indeed, *norpA* is generally considered to be visual system specific – it is likely that much more general roles for these genes exist but have yet to be documented due to lack of an informative physiological phenotype. The *Drosophila* Malpighian tubule is an ideal genetic model for transporting epithelia and provides a robust phenotype for the integrative physiology of cell signalling and transport genes (Dow and Davies, 2001). Previous work has established that ion transport and cell signalling process are compartmentalised into different tubule cell subtypes: the principal and stellate cells (Dow and Davies, 2001). Furthermore, direct measurements of cell-specific intracellular calcium signalling mechanisms using targeted aequorin show a direct modulation of fluid transport by agents that mobilise intracellular calcium (O'Donnell et al., 1998; Rosay et al., 1997; Terhzaz et al., 1999; MacPherson et al., 2001). The *Drosophila* neurohormones capa-1 (a member of the $CAP_{2b}$ family; Kean et al., 2002) and *Drosophila* leucokinin (Drosokinin; Terhzaz et al., 1999) have been shown to stimulate fluid transport rates, which are associated with increases in cytoplasmic calcium concentration in principal and stellate cells, respectively, in tubule main segment. The $CAP_{2b}$ response is dependent on extracellular calcium (Rosay et al., 1997); furthermore, it has been recently demonstrated that TRP/TRPL (transient receptor potential/TRP-like) and L-type calcium channels play a role in this response (MacPherson et al., 2000, 2001). However, the relative contribution of intracellular calcium stores to these neurohormone-induced responses is still unclear. Use of the ER $Ca^{2+}$-ATPase inhibitor thapsigargin in the presence of extracellular calcium results in elevation of intracellular calcium levels in both principal and stellate cells and increased fluid transport rates (Rosay et al., 1997). However, in the absence of extracellular calcium the response is abolished in principal cells but remains in stellate cells. Thus, it appears that the contribution of ER calcium stores, and thus of calcium signalling *via* $IP_3R$ to capacitative calcium entry in tubule cells, is cell-type specific. Alternatively, the putative thapsigargin-sensitive pool in principal cells is very small and is emptied too rapidly to monitor. It is thus of interest to try to dissect the different contributions of calcium signalling genes in the context of principal and stellate cell function.

In this study, we show that mutations in *norpA* reduce stimulation of fluid transport by both $CAP_{2b}$ and Drosokinin. Furthermore, $CAP_{2b}$ and Drosokinin both elevate $IP_3$ levels. Thus, PLCβ and $IP_3$ are involved in stimulated fluid transport; we have thus utilised *itpr* mutants to define the contribution of $IP_3R$ to neurohormone-mediated increases in epithelial fluid transport. Genetic blockade of $IP_3R$ function results in inhibition of neuropeptide-fluid transport rates associated with either $CAP_{2b}$ or Drosokinin. In *itpr* mutants, downregulation of fluid transport is associated with reductions in neuropeptide-stimulated intracellular calcium levels. Thus, PLCβ-mediated $IP_3$ signalling plays a functional role in calcium signalling and epithelial fluid transport in *Drosophila*, confirming that the important *norpA* and *itpr* genes are not neural specific.

## Materials and methods

### *Materials*

Coelenterazine was purchased from Molecular Probes (Leiden, The Netherlands) and dissolved in ethanol before use. Schneider's medium was obtained from GIBCO Life Technologies (Invitrogen Ltd, Paisley, UK). Neuropeptides $CAP_{2b}$ (pyroELYAFPRV-amide; Davies et al., 1995) and Drosokinin (NSVVLGKKQRFHSWG-amide; Terhzaz et al., 1999) were synthesised by Research Genetics, Inc. (Invitrogen Ltd). All other chemicals were obtained from Sigma (Pool, UK).

### Drosophila *stocks*

*Drosophila melanogaster* (Meigen) were maintained on a 12 h:12 h L:D cycle on standard cornmeal–yeast–agar medium at 25°C. Oregon R (OrR) wild-type flies used were those described previously (Dow et al., 1994). Mutant lines of *norpA* (Bloomquist et al., 1988; Pearn et al., 1996) and *itpr* (Venkatesh and Hasan, 1997) have been described previously. *itpr* lines were used as out-crossed lines to OrR to rule out the effects of balancer chromosomes on the tubule phenotype. Multiple alleles were utilised in this study in order to control for any effects of genetic background in individual lines. Choice of alleles was based on the health of the lines. Lines used were as follows: $norpA^{H52}/norpA^{H52}$ (temperature-sensitive allele); $norpA^{P24}/norpA^{P24}$ (kind gifts of R. C. Hardie, University of Cambridge); $itpr^{XR12}/+$ (X-ray inversion); $itpr^{90B.0}/+$, $itpr^{1664}/+$, $itpr^{1664}/itpr^{XR12}$, $itpr^{1664}/itpr^{90B.0}$ and $itpr^{1664}/itpr^{1664}$ (P-element insertions); $itpr^{WC361}/itpr^{UG3}$ (EMS alleles). *itpr* lines were slow-growing due to eclosion defects (Venkatesh and Hasan, 1997). Additionally, the following lines were also utilised: hsGAL4;$itpr^{90B.0}$ and UAS-*itpr* (Venkatesh et al., 2001). Temperature-sensitive (*ts*) and hsGAL4 lines were heat-shocked at 37°C for 30 min and left to recover at 23°C before experimentation.

To produce flies in which tubule calcium measurements could be made using the calcium reporter aequorin (Rosay et al., 1997; Terhzaz et al., 1999), it was necessary to generate *itpr* lines in an aequorin background under control of an

hsGAL4 promoter. These were based on an X-chromosome insertion of UAS-aeq, and an hsGAL4 construct on chromosome 2, leaving chromosome 3 free for *itpr* alleles. The following lines were generated and utilised for this study: aeq;hsGAL4;$itpr^{XR12}$/+, aeq;hsGAL4;$itpr^{90B.0}$/+, aeq;hsGAL4;$itpr^{1664}$/+, aeq;hsGAL4;$itpr^{1664}/itpr^{1664}$ and aeq;hsGAL4;$itpr^{WC361}/itpr^{UG3}$. Extremely poor viability of the $itpr^{1664}/itpr^{XR12}$ and $itpr^{1664}/itpr^{90B.0}$ heteroallelics in the aequorin background did not allow use of these lines for calcium measurements. Verification of aequorin expression was achieved at several stages of the crossing procedure by measuring total light output by dissected, intact tubules after lysis in Triton/$CaCl_2$ as described below. The presence of the appropriate *itpr* allele was verified in progeny of *aeq;itpr* flies by RT-PCR. Maintenance of the *itpr* phenotype was assessed by fluid transport assays in the presence of either $CAP_{2b}$ or Drosokinin.

### *Transport (fluid secretion) assays*

Flies were cooled on ice and then decapitated prior to isolation of whole tubules. Malpighian tubules were isolated into 10 µl drops of a 1:1 mixture of Schneider's medium and *Drosophila* saline (NaCl, 117.5 mmol $l^{-1}$; KCl, 20 mmol $l^{-1}$; $CaCl_2$, 2 mmol $l^{-1}$; $MgCl_2$, 8.5 mmol $l^{-1}$; $NaHCO_3$, 10.2 mmol $l^{-1}$; $NaH_2PO_4$, 4.3 mmol $l^{-1}$; Hepes, 15 mmol $l^{-1}$; glucose 20 mmol $l^{-1}$) under liquid paraffin, and fluid secretion rates were measured as described previously (Dow et al., 1994) under the different conditions described in the text. $CAP_{2b}$ and Drosokinin were added as solutions in assay medium at 30 min.

### *Heterologous expression of the Drosokinin receptor*

The recently characterised Drosokinin receptor (Radford et al., 2002) was used to assay Drosokinin-stimulated $IP_3$ production. In order to transfect S2 cells with Drosokinin receptor, the protocol described below was adopted.

#### *Expression constructs*

Primers were designed for the Drosokinin receptor (*CG1062*; Radford et al., 2002) to allow amplification from the start to the stop codon of the coding sequence. Forward primers were designed to include a 5′ Kozak translational initiation sequence (**G**/**A**NN<u>ATG</u>**G**). Amplification was carried out with EXPAND High Fidelity Polymerase (Roche Diagnostics Ltd, Lewes, UK) according to manufacturers instructions. Forward (GACATGGACTTAATCGAGCAGGAG) and reverse (TTAAAGTGGTTGCCACAAGGAC) primers were used to generate a fragment of 1626 bp. OrR cDNA was used as template. The PCR product was purified by gel extraction and directly cloned into the pMT-V5/His TOPO TA inducible expression vector (Invitrogen). The construct was verified by restriction enzyme digestion and sequencing to ensure that no mutations had been induced during cloning.

#### *S2 cell culture*

S2 cells were maintained in DES (*Drosophila* expression system) medium (Invitrogen) supplemented with 10% heat-inactivated foetal calf serum (FCS; Invitrogen). Cells were grown in suspension at an initial density of 2–4×$10^6$ cells $ml^{-1}$ at 23°C. S2 cells were transiently transfected at a density of 1×$10^6$ cells $ml^{-1}$ using calcium phosphate (Invitrogen), according to manufacturer's instructions. Cells were transfected with 20 µg of the Drosokinin receptor expression construct and were used 24 h post-induction of the metallothionein promoter with $Cu^{2+}$ ions.

### *Mass measurement of inositol (1,4,5)-trisphosphate ($IP_3$) levels*

$IP_3$ levels in tubules were measured by a quantitative radioligand-binding assay as described elsewhere (Palmer et al., 1989) using an $IP_3$-binding protein preparation derived from bovine adrenal gland.

#### *Tubule preparations*

Tubules (20 per sample) were dissected from wild-type and *itpr* mutants into 9 µl of Schneider's medium. Samples were stimulated with $CAP_{2b}$ ($10^{-7}$ mol $l^{-1}$) for 0 s (control), 2 s or 5 s in a final sample volume of 10 µl and performed in triplicate. Initial experiments showed that $IP_3$ levels peaked at 5 s post-stimulation (data not shown); this time was used for all subsequent experiments.

#### *S2 cell preparations*

To stimulate S2 cells, Drosokinin (Terhzaz et al., 1999) was diluted to working concentration in DES medium/FCS, then added to 5×$10^4$ cells (approximating to 5000 transfected cells) in DES medium/FCS to a final concentration of $10^{-7}$ mol $l^{-1}$ for the appropriate time. Initial experiments showed that peak $IP_3$ generation occurred at 10 s after peptide stimulation (data not shown). Cells were co-transfected with an eGFP (enhanced green fluorescent protein) control plasmid in order to measure transfection efficiency using a haemocytometer. The same transfection batch was used for all samples in any one data set; stimulations were performed in duplicate.

For both tubule and S2 cell preparations, reactions were terminated with 10% (v/v) ice-cold perchloric acid and samples were homogenised using a Polytron homogeniser on ice. Cellular debris was removed by centrifugation and the supernatants neutralised with 1.5 mol $l^{-1}$ KOH/60 mmol $l^{-1}$ Hepes in the presence of 2 µl Universal Indicator. Precipitated salts were spun down and the supernatants transferred to fresh tubes. Additions of 2500 d.p.m. [$^3$H]Ins(1,4,5)$P_3$ ([$^3$H]$IP_3$; specific activity 370–1850 GBq mmol $l^{-1}$; Amersham Biosciences UK Ltd, Little Chalfont, UK), incubation buffer and binding protein were made [final concentrations: 25 mmol $l^{-1}$ Tris-HCl (pH 9); 5 mmol $l^{-1}$ $NaHCO_3$; 1 mmol $l^{-1}$ EDTA; 1 mmol $l^{-1}$ EGTA; 0.25 mmol $l^{-1}$ dithiothreitol (DTT); 1 mg $ml^{-1}$ bovine serum albumin (Fraction V); 0.4 mg $ml^{-1}$ binding protein] to a final volume of 400 µl, and the samples were incubated on ice for 45 min prior to centrifugation at 12 000 ***g*** (4°C) for 1 min. Supernatants were removed by aspiration, the pellets dissolved in 1 ml of scintillation fluid and the radioactivity therein determined by scintillation counting. A

standard curve, using 0–40 pmol $IP_3$ per sample, was generated in parallel. Non-specific binding was determined using 100 pmol $IP_3$. Standard curves were plotted as *%B/Bo versus* pmol of unlabelled $IP_3$, where *B* is the specific binding of [$^3$H]$IP_3$ (at a given concentration of unlabelled $IP_3$), and *Bo* is the maximal specific binding of [$^3$H]$IP_3$ (at 0 pmol of unlabelled $IP_3$). A similar calculation was made using values of specific binding of [$^3$H]$IP_3$ of tissue samples and the $IP_3$ content therein determined using the standard curve.

Protein concentrations in tubule samples were assessed by Lowry assays. Three replicate samples were pooled for assay of $IP_3$ content in order to obtain measurable levels of $IP_3$. Duplicate samples were assayed for each experimental sample.

### *Measurements of $[Ca^{2+}]_i$ using an aequorin transgene under heat-shock control*

hsGAL4;aeq (Rosay et al., 1997) were used as control animals, and protocols used were essentially those previously described. For each assay, 20–40 tubules from 4–14-day-old adults were dissected in Schneider's medium 2 h after heat-shock (37°C for 30 min). Tubules were pooled in 160 µl of the same buffer and aequorin reconstituted with the cofactor coelenterazine (final concentration, 2.5 µmol l$^{-1}$). Bioluminescence recordings were made with a luminometer (LB9507; Berthold, Pforzheim, Germany); recordings were made every 0.1 s for each tube. Each tube of 20 tubules was used for a single data point: after recording $[Ca^{2+}]_i$ levels, tissues were disrupted in 350 µl lysis solution [1% (v/v) Triton X-100/100 mmol l$^{-1}$ $CaCl_2$], causing discharge of the remaining aequorin and allowing estimation of the total amount of aequorin in the sample. Calibration of the aequorin system and calculation of $[Ca^{2+}]_i$ were performed as previously described (Rosay et al., 1997). Mock injections with Schneider's medium were applied to all samples prior to treatment with neuropeptides.

## Results

### *PLCβ contributes to $CAP_{2b}$ and Drosokinin-induced fluid transport*

Two *norpA* alleles of differing severity were available for study. *norpA$^{P24}$* eyes do not express norpA protein, as assessed by western blots, and show markedly reduced PLC activity (Pearn et al., 1996); as expected, *norpA$^{P24}$* shows no electroretinogram response to any light stimulus. This allele, therefore, would be expected to display a severe phenotype if *norpA* acted in tubule function. In *norpA$^{H52}$*, however, some *norpA* transcript is detectable at the restrictive temperature, although this is severely reduced compared with wild type (Bloomquist et al., 1988). As such, any epithelial phenotype would not be expected to be as severe as that of *norpA$^{P24}$*.

Both *norpA* mutants manifest an epithelial phenotype (Fig. 1). No significant change in basal secretion rate was observed repeatedly in *norpA* mutants; however, both alleles, *norpA$^{H52}$* and *norpA$^{P24}$*, severely attenuated $CAP_{2b}$-induced

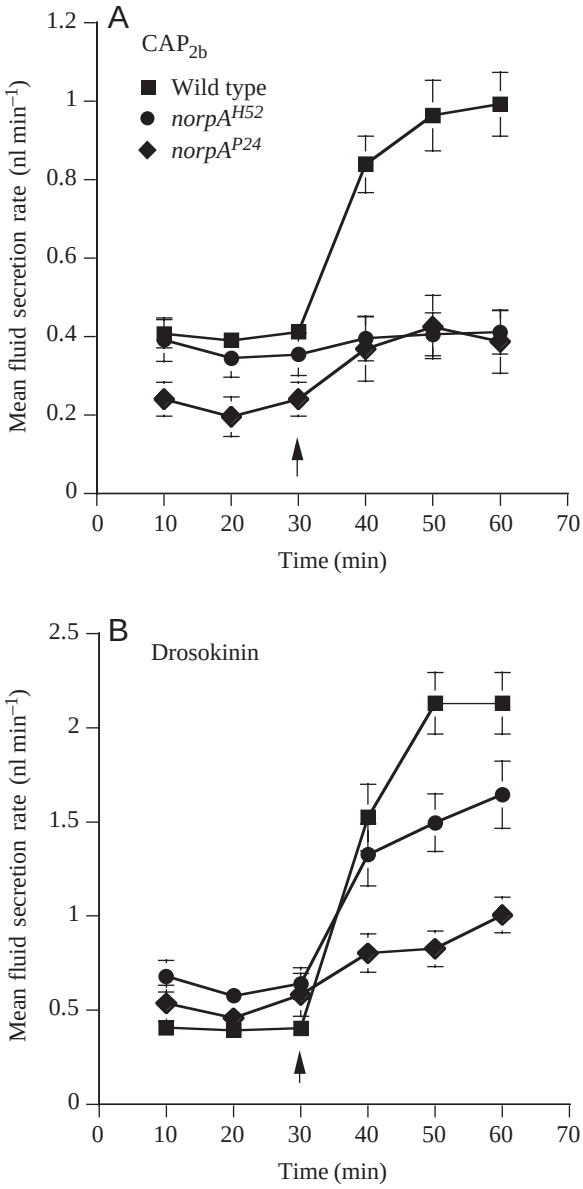


Fig. 1. An epithelial phenotype for *norpA*: phospholipase Cβ (PLCβ) is required for neuropeptide stimulation of principal and stellate cells. Fluid transport assays were performed on intact tubules from wild-type (Oregon R), *norpA$^{H52}$* and *norpA$^{P24}$* flies as described in the Materials and methods. Either (A) $10^{-7}$ mol l$^{-1}$ $CAP_{2b}$ or (B) $10^{-7}$ mol l$^{-1}$ Drosokinin were added at 30 min (arrow), and transport rates were measured for a further 30 min. Data are expressed as mean secretion rates ± S.E.M. (*N*=8).

secretion to similar extents (Fig. 1A). By contrast, for Drosokinin-stimulated fluid transport, the *norpA* alleles were distinguishable (Fig. 1B); in the eye, *norpA$^{P24}$* displays a much more severe phenotype compared with *norpA$^{H52}$*. Kinetics of the fluid secretion response in all lines were similar.

Thus, the data show that fluid transport induced by both $CAP_{2b}$ and Drosokinin requires a PLCβ-dependent signalling pathway; as such, *norpA* function is not confined to phototransduction.

Table 1. *$CAP_{2b}$ and Drosokinin stimulate $IP_3$ production*

| Ligand | Tissue | Control | Stimulation |
|---|---|---|---|
| $CAP_{2b}$ | Oregon R Malpighian tubules | 0.75±0.11 nmol μg protein$^{-1}$ | 1.26±0.10* nmol μg protein$^{-1}$ |
| Drosokinin | Drosokinin-receptor-transfected S2 cells | 1.08±0.08 pmol 5000 cells$^{-1}$ | 9.45±2.39* pmol 5000 cells$^{-1}$ |

Significant differences between inositol (1,4,5)-trisphosphate ($IP_3$) content in control and stimulated samples are denoted by * ($P$<0.05, Student's *t*-test, unpaired samples, $N$=4).

### *$CAP_{2b}$ and Drosokinin stimulate $IP_3$ production*

$CAP_{2b}$ and Drosokinin have both been shown to act through intracellular calcium; so, if their action relies on PLC (Fig. 1), they should also each act to raise $IP_3$ in tubules. Table 1 shows that $CAP_{2b}$, which acts only on principal cells, increases $IP_3$ levels in intact wild-type tubules. This, together with the data in Fig. 1, shows that $CAP_{2b}$ acts *via* a phosphoinositide (PI)-PLC-dependent mechanism.

*Aedes* leucokinins have been shown to stimulate $IP_3$ production in mosquito tubules (Cady and Hagedorn, 1999). However, as Drosokinin exerts its effects solely *via* stellate cells in *Drosophila* tubules (Terhzaz et al., 1999), and as *Drosophila* tubules each contain only 22 stellate cells (Sozen et al., 1997), Drosokinin-stimulated $IP_3$ levels could not be reliably quantified in intact tubules (data not shown). Accordingly, an *in vitro* approach was used. *Drosophila* S2 cells transfected with the Drosokinin receptor have been shown to display increased cytosolic intracellular calcium when stimulated with Drosokinin (Radford et al., 2002). Data in Table 1 show that Drosokinin increases $IP_3$ content in these cells by approximately eightfold. As the Drosokinin receptor has been localised to only stellate cells *in vivo* (Radford et al., 2002), it is probable that Drosokinin stimulates $IP_3$ production in stellate cells in intact tubules. As with $CAP_{2b}$, Drosokinin action requires activation of PI-PLC.

### *Resting and $CAP_{2b}$-stimulated levels of $IP_3$ are significantly reduced in* itpr *mutants*

Intracellular signalling is frequently regulated by feedback. If $IP_3$ signalling contributes to the biological effects of neuropeptides in tubules, lesions in the $IP_3$R might feed back to downregulate the $IP_3$ signalling molecule *in vivo*.

Measurement of basal $IP_3$ levels in wild-type and *itpr* tubules shows that disruption of $IP_3$R results in a reduction in resting $IP_3$ levels. Significant reductions in basal $IP_3$ levels are

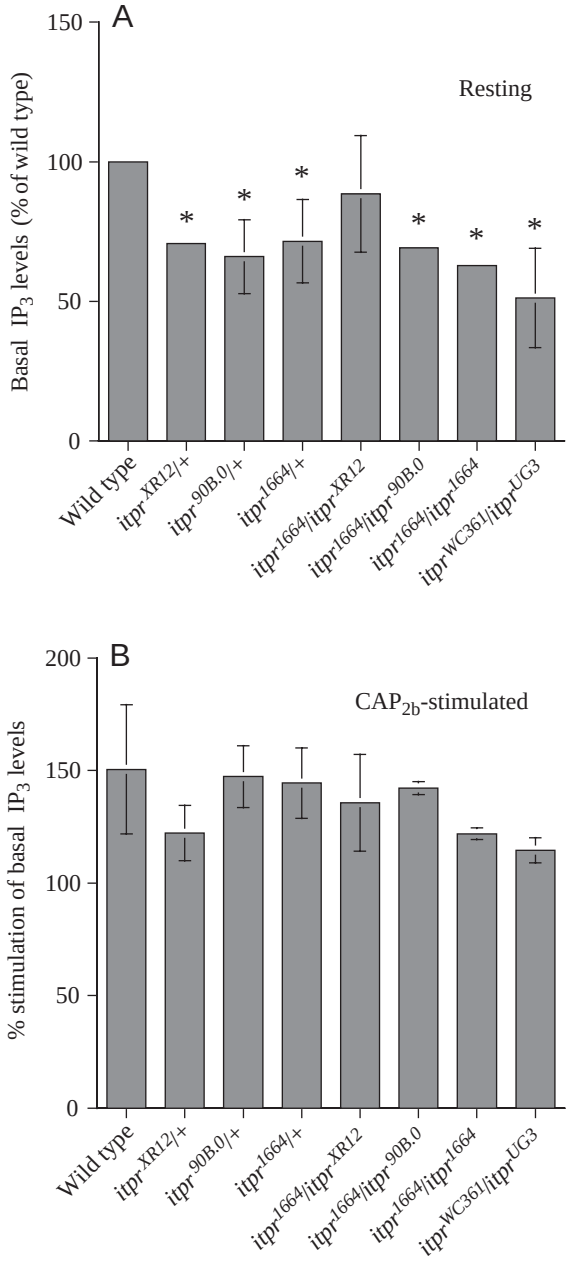


Fig. 2. Resting and $CAP_{2b}$-stimulated inositol (1,4,5)-trisphosphate ($IP_3$) levels in *itpr* mutants. (A) Resting $IP_3$ levels are shown for tubules from the following lines: Oregon R (control), $itpr^{XR12}/+$, $itpr^{90B.0}/+$, $itpr^{1664}/+$, $itpr^{1664}/itpr^{XR12}$, $itpr^{1664}/itpr^{90B.0}$, $itpr^{1664}/itpr^{1664}$ and $itpr^{WC361}/itpr^{UG3}$. In order to aid comparison between experiments, data are shown as the % difference between $IP_3$ levels in *itpr* mutants compared with wild type (100%) ± S.E.M. ($N$=4). Typical $IP_3$ content of wild-type tubules was as described in Table 1. (B) $CAP_{2b}$ stimulates $IP_3$ production in *itpr* lines. Stimulated $IP_3$ levels were measured in $CAP_{2b}$-stimulated tubules ($10^{-7}$ mol l$^{-1}$, 5 s). Data are expressed as the % increase of unstimulated $IP_3$ levels (calculated as [stimulated $IP_3$]/[resting $IP_3$]×100%; [$IP^3$] measured in pmol μg protein$^{-1}$) ± S.E.M. ($N$=3–4). Significant differences between $IP_3$ content in wild-type and *itpr* lines are denoted by * ($P$<0.05, Student's *t*-test, unpaired samples).

observed in heterozygous alleles, homozygous *itpr*$^{1664}$/*itpr*$^{1664}$ and heteroallelic lines (Fig. 2A).

$IP_3$ levels were also measured in neurohormone-stimulated intact tubules. As Drosokinin-stimulated $IP_3$ production was not measurable in intact tubules, experiments were conducted only on $CAP_{2b}$-stimulated wild-type and *itpr* tubules (Fig. 2B). $IP_3$ content is increased in all $CAP_{2b}$-stimulated *itpr* tubules, although to a lesser extent in homozygous *itpr*$^{1664}$/*itpr*$^{1664}$ and heteroallelic *itpr*$^{WC361}$/*itpr*$^{UG3}$ lines compared with wild type. However, these differences are not statistically significant. Thus, tubules from *itpr* mutants are able to generate $IP_3$ in response to $CAP_{2b}$, suggesting that PI-PLC-dependent signalling is not compromised in these lines.

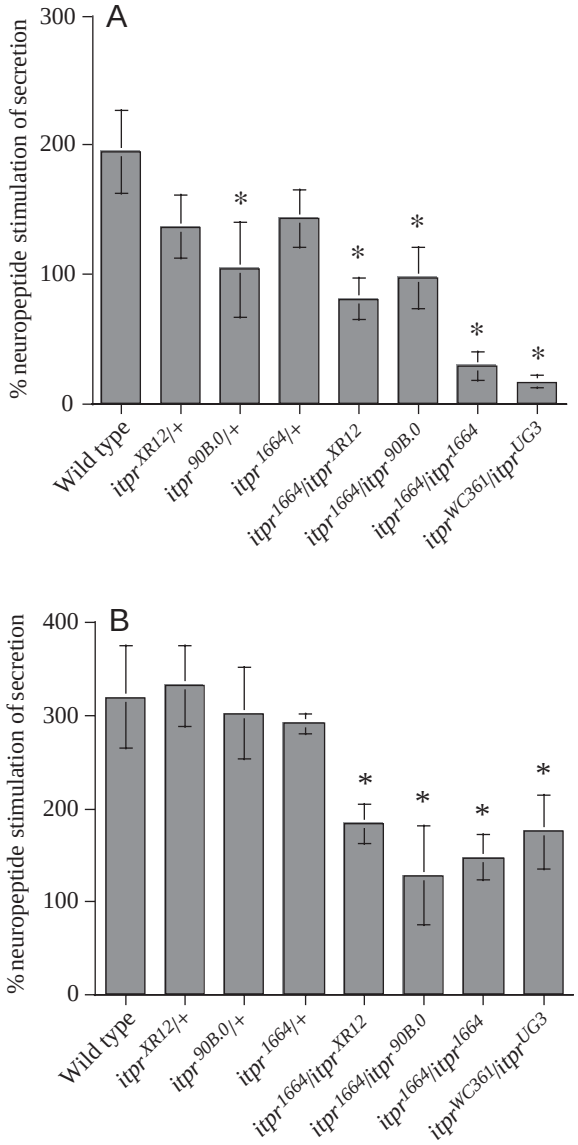


Fig. 3. $CAP_{2b}$- and Drosokinin-stimulated fluid transport are inhibited in *itpr* mutants. Fluid transport assays were performed on intact tubules as described in Fig. 1 for the following lines: Oregon R (control), *itpr*$^{XR12}$/+, *itpr*$^{90B.0}$/+, *itpr*$^{1664}$/+, *itpr*$^{1664}$/*itpr*$^{XR12}$, *itpr*$^{1664}$/*itpr*$^{90B.0}$, *itpr*$^{1664}$/*itpr*$^{1664}$ and *itpr*$^{WC361}$/*itpr*$^{UG3}$. Either (A) $10^{-7}$ mol $l^{-1}$ $CAP_{2b}$ or (B) $10^{-7}$ mol $l^{-1}$ Drosokinin were added at 30 min, and transport rates were measured for a further 30 min. No change in basal secretion rate was observed in *itpr* mutants. Furthermore, kinetics of the fluid secretion response in all lines were similar (data not shown). To aid comparison between stimulated transport rates, data are expressed as the % stimulation of secretion [(maximal stimulated rates minus the mean of three basal secretion rate readings)/(mean basal rate)×100% ± S.E.M.; $N$=15–20] upon stimulation with $CAP_{2b}$ or Drosokinin. Stimulated fluid transport rates that are significantly different from wild type are denoted by * ($P$<0.05, Student's $t$-test, unpaired samples).

*Diuresis is inhibited by disruption of* itpr

As mutations in *norpA* result in an epithelial phenotype, we investigated the possibility of uncovering such phenotypes in *itpr* mutants. We show that $CAP_{2b}$ stimulation of fluid transport is inhibited in heterozygous, homozygous and heteroallelic *itpr* alleles (Fig. 3A). Significant inhibition is observed in *itpr*$^{90B.0}$/+, *itpr*$^{1664}$/*itpr*$^{XR12}$ and *itpr*$^{1664}$/*itpr*$^{90B.0}$. However, $CAP_{2b}$-stimulated fluid transport is almost completely abolished in the homozygous *itpr*$^{1664}$/*itpr*$^{1664}$ and heteroallelic *itpr*$^{WC361}$/*itpr*$^{UG3}$ lines. It is possible that the non-correlation between the severity of the alleles (*itpr*$^{1664}$/*itpr*$^{90B.0}$ *versus* *itpr*$^{1664}$/*itpr*$^{1664}$) and $CAP_{2b}$-stimulated transport in these alleles is due to either differences in genetic background or by impact of the mutations on other interacting signalling pathways that are activated by $CAP_{2b}$.

By contrast, Drosokinin-stimulated fluid transport is not affected by either heterozygous *itpr*$^{XR12}$/+, *itpr*$^{90B.0}$/+ or *itpr*$^{1664}$/+ alleles (Fig. 3B). Significant inhibition is only observed in the heteroallelics *itpr*$^{1664}$/*itpr*$^{XR12}$, *itpr*$^{1664}$/*itpr*$^{90B.0}$ and *itpr*$^{WC361}$/*itpr*$^{UG3}$ and in the homozygous *itpr*$^{1664}$/*itpr*$^{1664}$. Furthermore, there are marked differences in the severity of inhibition between Drosokinin- and $CAP_{2b}$-stimulated fluid transport, especially in *itpr*$^{1664}$/*itpr*$^{1664}$ and *itpr*$^{WC361}$/*itpr*$^{UG3}$ alleles.

Thus, *itpr* acts in both principal and stellate cells to transduce $CAP_{2b}$ and Drosokinin diuretic signals.

*Rescue of* itpr$^{90B.0}$ *restores* $CAP_{2b}$*-stimulated fluid transport levels*

Sometimes, phenotypes ascribed to mutant loci in *Drosophila* are subsequently found to be caused by second-site mutations or other genetic accidents incidental to the original study. It is thus desirable to confirm that mutant effects are genuinely due to the locus of interest. Crosses were established between heterozygous hsGAL4;*itpr*$^{90B.0}$ and UAS-*itpr* homozygotes to allow rescue of *itpr*$^{90B.0}$. Tubules from flies with the w/UAS-*itpr*;+/hsGAL4;+/*itpr*$^{90B.0}$ genotype were used in transport assays. Control lines used were OrR and UAS-*itpr*. Non-heat-shocked w/UAS-*itpr*;+/hsGAL4;+/*itpr*$^{90B.0}$ were not used as controls, as the heat-shock promoter is 'leaky' and is transcribed at 25°C (G. Hasan, unpublished).

Fig. 4 shows that expression of *itpr* restores $CAP_{2b}$-stimulated fluid transport to levels indistinguishable from wild-type. Although disruption of *itpr* may compromise

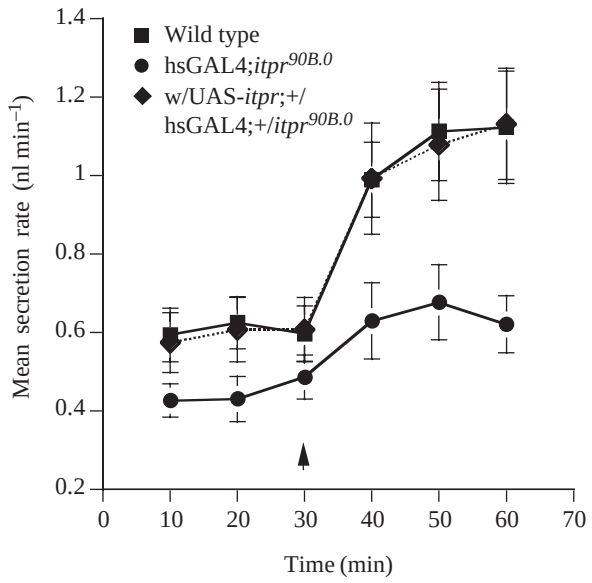


Fig. 4. *itpr* rescues the transport phenotype of the $itpr^{90B.0}$ allele. Fluid transport assays were performed on the hsGAL4;$itpr^{90B.0}$ line. The data show that $CAP_{2b}$-stimulated fluid transport is decreased in this line. Rescue of hsGAL4;$itpr^{90B.0}$ with UAS-*itpr* results in wild-type levels of stimulated fluid transport. Fluid secretion rates were measured for 30 min prior to addition of neuropeptide (arrow), after which measurements were taken for a further 30 min. Data are expressed as mean fluid secretion rates (nl $min^{-1}$) ± S.E.M. ($N$=6–10). UAS-*itpr* tubules display similar secretion rates to those of wild-type tubules (data not shown).

associated signalling pathways resulting in downregulation of $CAP_{2b}$-stimulated transport, successful rescue of $itpr^{90B.0}$/+ with UAS-*itpr* provides strong evidence that the epithelial phenotype observed in this line is associated with mutated *itpr*.

### *$CAP_{2b}$-induced calcium signalling is impaired in* itpr *mutants*

Previous work has shown that capa peptides (Kean et al., 2002) increase intracellular calcium in tubule main segment principal cells (Rosay et al., 1997) *via* plasma membrane calcium channels (MacPherson et al., 2000, 2001). However, release from intracellular stores is not measurable in this cell type (Rosay et al., 1997), thus calling into question the role of $IP_3$R-sensitive stores in principal cells. If release of calcium from $IP_3$-sensitive internal stores does occur upon $CAP_{2b}$ stimulation, changes in this response may be observed in *itpr* mutants.

Using targeted aequorin, cytosolic calcium measurements in wild-type and *itpr* tubules stimulated with $CAP_{2b}$ were performed; typical traces are shown in Fig. 5A. Fig. 5Ai shows the biphasic rise, consisting of a rapid primary peak followed by a slow secondary rise in cytosolic calcium in $CAP_{2b}$-stimulated wild-type aeq;hsGAL4 tubules. This calcium signature is also observed in capa/$CAP_{2b}$-stimulated wild-type tubules (Kean et al., 2002). The response is reduced in heterozygous *itpr* alleles, aeq;hsGAL4;$itpr^{XR12}$/+ and aeq;hsGAL4;$itpr^{90B.0}$/+ (Fig. 5B), which may result in the transport phenotype observed. Interestingly, in heterozygous $itpr^{1664}$/+, the primary response is unaffected, with only the secondary response being reduced; in this line, stimulated fluid transport is not significantly different from control (Fig. 3A). By contrast, the alleles that display the most severe transport phenotype ($itpr^{1664}/itpr^{1664}$ and $itpr^{WC361}/itpr^{UG3}$) also display an attenuated calcium response to $CAP_{2b}$ (Fig. 5Av,vi,B). Both reduction in the primary peak and loss of the secondary rise are observed in these lines. However, none of the *itpr* alleles completely abolish $CAP_{2b}$-stimulated calcium signalling. This is consistent with *itpr* being an essential gene and with viable alleles all being hypomorphs rather than nulls.

These results thus suggest that $IP_3$R-mediated calcium release from intracellular stores contributes significantly to calcium signalling, and consequent diuresis, in principal cells.

### itpr *mutants reduce Drosokinin-induced calcium signals*

We have shown previously that leucokinin (Rosay et al., 1997; O'Donnell et al., 1998) and endogenous *Drosophila* leucokinin (Terhzaz et al., 1999) elevate intracellular calcium levels in stellate cells. Furthermore, experiments in calcium-free medium show that stellate cells display emptying of intracellular calcium stores in the presence of the ER-calcium ATPase inhibitor, thapsigargin (Rosay et al., 1997). Thus, Drosokinin-stimulated calcium increases should be reduced in *itpr* mutants.

Data in Fig. 6A show typical traces of Drosokinin-stimulated increases in cytosolic calcium in wild-type and *itpr* tubules. A rapid response is observed in wild-type tubules (Fig. 6Ai; Terhzaz et al., 1999; Radford et al., 2002). This response is severely reduced in $itpr^{1664}/itpr^{1664}$ and $itpr^{WC361}/itpr^{UG3}$ flies (Fig. 6Av,vi,B). Drosokinin-stimulated fluid transport is also reduced in these lines (Fig. 3B); thus, functional $IP_3$R contributes to Drosokinin-stimulated calcium signalling and fluid transport.

## Discussion

The role of PLC *in vivo* has been most studied in *Drosophila* phototransduction. Experimental evidence suggested that *norpA* and *plc21* were expressed in the eye or CNS (Bloomquist et al., 1988; Shortridge et al., 1991); furthermore, the dependence of phototransduction on PI-PLC supported specific roles for these genes in the eye. However, RT-PCR data show that *norpA* and *plc21* are both expressed in tubules (M. R. MacPherson and V. P. Pollock, unpublished). We have shown here that *norpA* mutants display an epithelial phenotype, which is revealed upon neuropeptide stimulation. Furthermore, the severity of the epithelial response for each allele correlates with that observed in the eye and also with the levels of expression of *norpA* and biochemical activity of PLC. However, while we have established an epithelial role for *norpA*, it has been difficult to assess the contribution of *plc21*

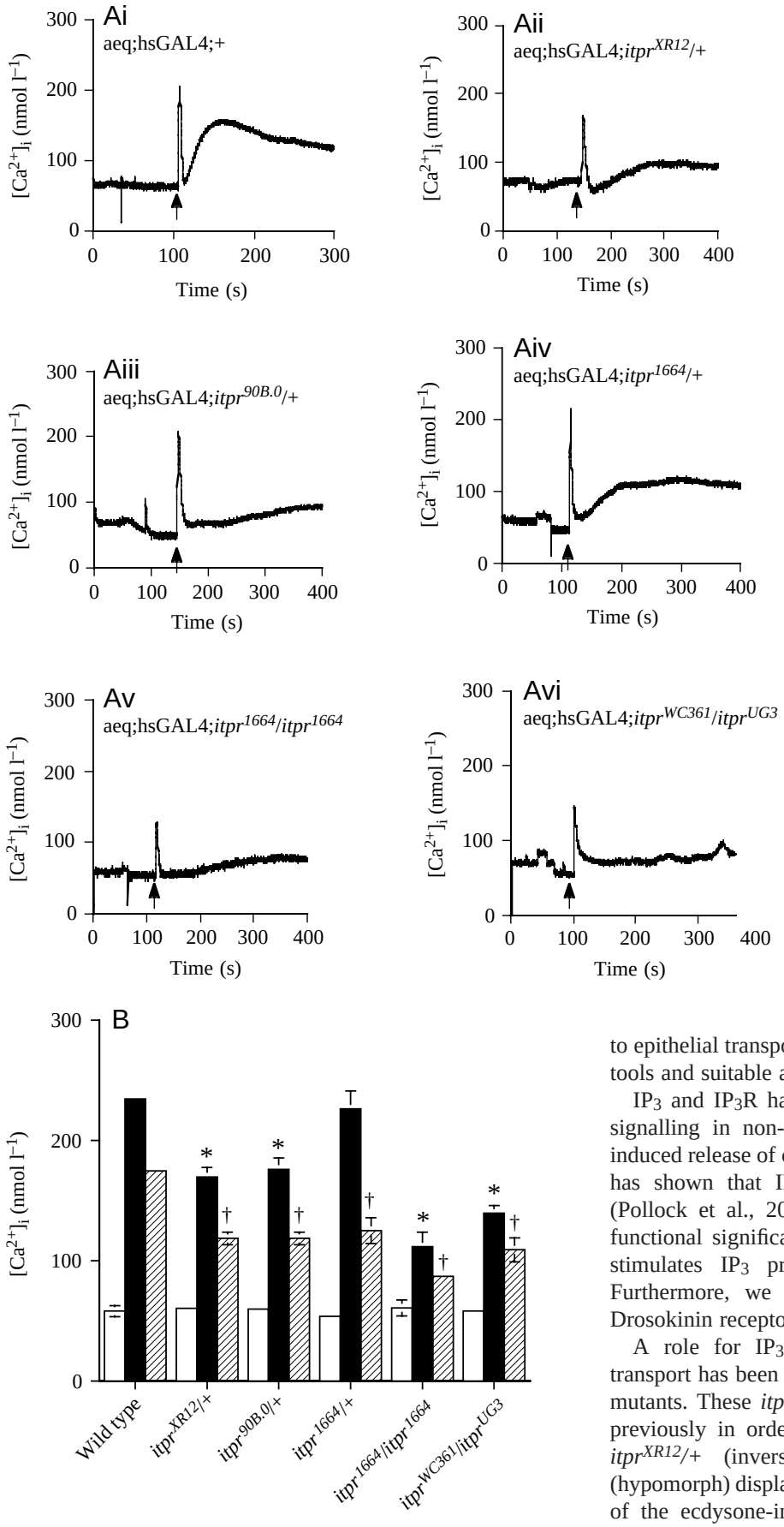


Fig. 5. $CAP_{2b}$-induced cytosolic calcium signals in *itpr* mutants. (A) Typical traces of changes in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in tubule principal cells stimulated by $10^{-7}$ mol $l^{-1}$ $CAP_{2b}$ (arrows) in the following lines: (i) aeq;hsGAL4;+ (control), (ii) aeq;hsGAL4;*itpr*$^{XR12}$/+, (iii) aeq;hsGAL4;*itpr*$^{90B.0}$/+, (iv) aeq;hsGAL4;*itpr*$^{1664}$/+, (v) aeq;hsGAL4;*itpr*$^{1664}$/*itpr*$^{1664}$ and (vi) aeq;hsGAL4;*itpr*$^{WC361}$/*itpr*$^{UG3}$. Each sample contains 20 intact tubules. While no changes in the resting $[Ca^{2+}]_i$ is seen in any of the mutants, changes in amplitude of the primary and/or secondary response can be observed in all lines (also in B). (B) Pooled results of changes in tubule $[Ca^{2+}]_i$ in *itpr* mutants in response to $10^{-7}$ mol $l^{-1}$ $CAP_{2b}$ are shown. Results are expressed as means ± S.E.M. ($N$=8) for background (open bars), $CAP_{2b}$-stimulated primary peaks (filled bars) and $CAP_{2b}$-stimulated secondary peaks (hatched bars) for the lines described in A. The measure of secondary peak is taken as the average $[Ca^{2+}]_i$ over 4 min post-stimulation with $CAP_{2b}$. $CAP_{2b}$-stimulated primary peaks that are significantly different from aeq;hsGAL4 tubules are denoted by *, and statistically significant differences in secondary peaks compared to wild type are denoted by † ($P$<0.05, Student's $t$-test, unpaired samples).

to epithelial transport and signalling due to our lack of genetic tools and suitable antibodies.

$IP_3$ and $IP_3R$ have been shown to be critical for calcium signalling in non-excitable cells *via* a mechanism of $IP_3$-induced release of calcium from internal stores. Previous work has shown that $IP_3R$ is expressed in Malpighian tubules (Pollock et al., 2000; Blumenthal, 2001). We now ascribe functional significance to this finding and show that $CAP_{2b}$ stimulates $IP_3$ production in intact *Drosophila* tubules. Furthermore, we show that Drosokinin mobilises $IP_3$ in Drosokinin receptor-transfected S2 cells.

A role for $IP_3R$ in neurohormone-stimulated epithelial transport has been demonstrated using an allelic series of *itpr* mutants. These *itpr* lines have been extensively investigated previously in order to determine the role of $IP_3R$ *in vivo*. *itpr*$^{XR12}$/+ (inversion), *itpr*$^{90B.0}$/+ (null) and *itpr*$^{1664}$/+ (hypomorph) display delayed moulting and reduced expression of the ecdysone-inducible gene *E74*. In terms of severity,

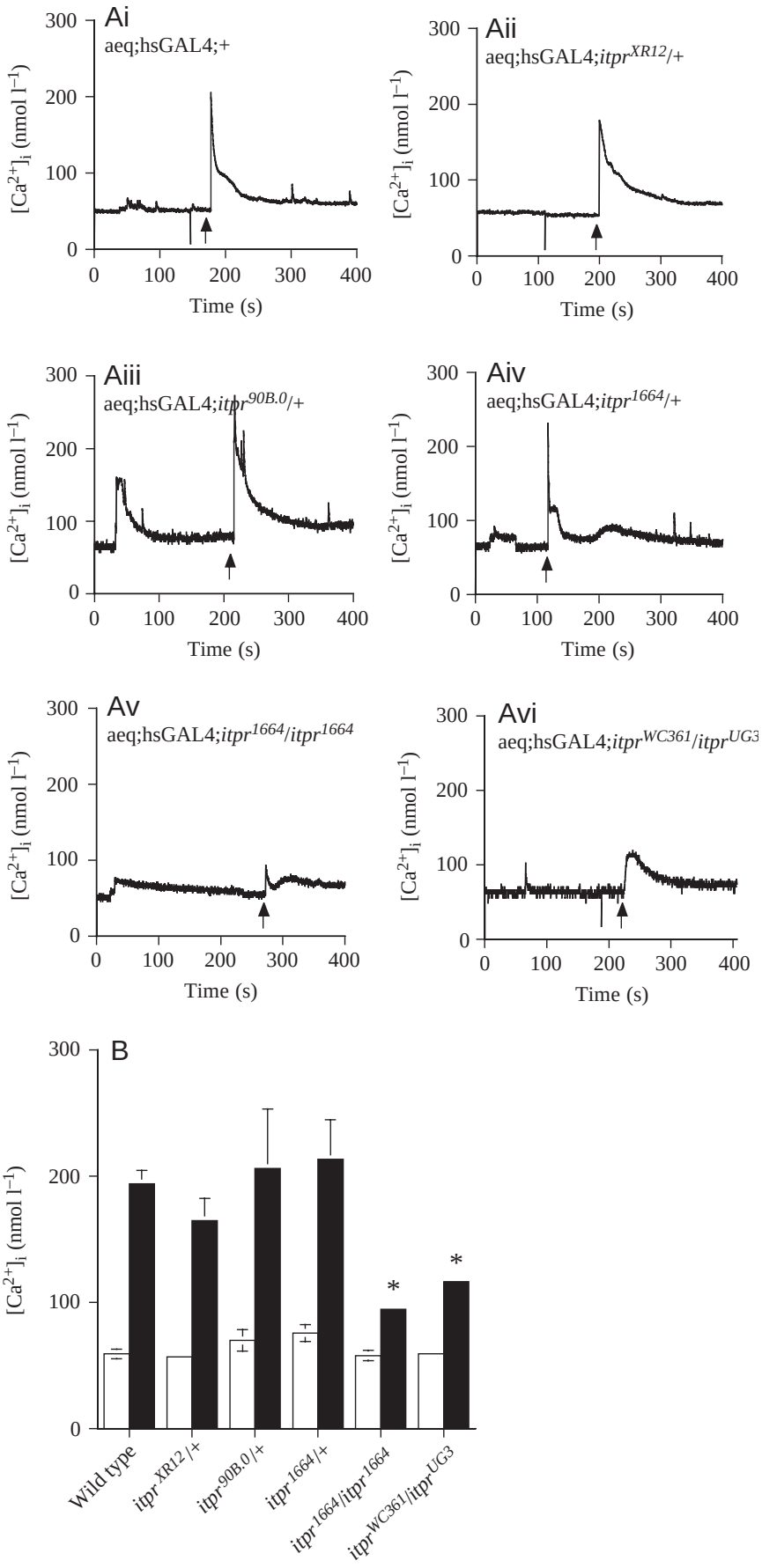


Fig. 6. Drosokinin-induced cytosolic calcium signals in *itpr* mutants. (A) Typical traces of changes in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in tubule stellate cells stimulated by $10^{-7}$ mol $l^{-1}$ Drosokinin (arrows) in the following lines: (i) aeq;hsGAL4;+ (control), (ii) aeq;hsGAL4;*itpr*$^{XR12}$/+, (iii) aeq;hsGAL4;*itpr*$^{90B.0}$/+, (iv) aeq;hsGAL4;*itpr*$^{1664}$/+, (v) aeq;hsGAL4;*itpr*$^{1664}$/*itpr*$^{1664}$ and (vi) aeq;hsGAL4;*itpr*$^{WC361}$/*itpr*$^{UG3}$. Each sample contains 20 intact tubules. While no changes in the resting $[Ca^{2+}]_i$ is seen in any of the mutants, changes in amplitude of the calcium peak can be observed in aeq;hsGAL4;*itpr*$^{1664}$/*itpr*$^{1664}$ and aeq;hsGAL4;*itpr*$^{WC361}$/*itpr*$^{UG3}$ (also in B). (B) Pooled results of changes in tubule $[Ca^{2+}]_i$ in *itpr* mutants in response to $10^{-7}$ mol $l^{-1}$ Drosokinin. Results are expressed as means ± S.E.M. ($N$=8) for background (open bars) and Drosokinin-stimulated peaks (filled bars) for the lines described in A. Drosokinin-stimulated primary peaks that are significantly different from aeq;hsGAL4;+ tubules are denoted by * ($P$<0.05, Student's $t$-test, unpaired samples).

*itpr*$^{90B.0}$ and *itpr*$^{XR12}$ are the most severe alleles (Venkatesh and Hasan, 1997). These lines, as well as *itpr*$^{WC361}$/*itpr*$^{UG3}$, have also been used in studies of olfaction, where it has been shown that, in the most severe alleles, olfactory adaptation is not maintained (Deshpande et al., 2000). $CAP_{2b}$-stimulated epithelial transport is reduced in the null mutant (*itpr*$^{90B.0}$) compared with wild-type tubules, while the most severe phenotypes are observed in *itpr*$^{1664}$ homozygotes and the heteroallelics. Rescue of the *itpr*$^{90B.0}$ transport phenotype is demonstrated with UAS-*itpr*, which strongly suggests that the tubule phenotype is associated with disruption in $IP_3R$. Interestingly, while the null mutant displays a phenotype upon $CAP_{2b}$ stimulation, only the most severe *itpr*$^{1664}$ homozygotes and the heteroallelics affect Drosokinin-stimulated secretion. Therefore, reduced $CAP_{2b}$-stimulated fluid transport in *itpr*$^{90B.0}$ may be due to principal cell-specific signalling processes associated with the null mutation.

Intriguingly, resting levels of $IP_3$ in tubules are significantly reduced in all *itpr* mutants apart from *itpr*$^{1664}$/*itpr*$^{XR12}$, suggesting a possible feedback mechanism between receptor ($IP_3R$) and second messenger ($IP_3$). However, this is not associated with an epithelial phenotype, as no difference in basal rate is observed in tubules from *itpr* lines as compared to wild-type flies.

We have previously shown that the neuropeptide $CAP_{2b}$ induces a rise in cytosolic calcium in only principal cells that is dependent on extracellular calcium; these calcium signalling events, which may mediate NO/cGMP signalling, correlate with $CAP_{2b}$-stimulated fluid transport. The $CAP_{2b}$-induced response is abolished by L-type calcium channel inhibitors and also in mutants for the plasma membrane calcium channels, TRP and TRPL (MacPherson et al., 2000, 2001). Thus, $CAP_{2b}$-induced calcium signalling occurs *via* multichannel mechanisms. We demonstrate here that $IP_3R$ plays a role in $CAP_{2b}$-induced calcium signalling (Fig. 5). Extensive work using *norpA* mutants has shown that *norpA*-encoded PLC plays a critical role in rhabdomeres. Thus, PLCβ, which is required for the cleavage of phosphatidylinositol (4,5)-bisphosphate ($PIP_2$) to $IP_3$ and diacylglycerol (DAG), is necessary for phototransduction. Interestingly, however, using the *itpr* null and $itpr^{1664}$ mutants, $IP_3$ signalling has been shown to be unnecessary to activate light-activated conductance (Raghu et al., 2000a). This response is, however, dependent on calcium entry *via* TRP/TRPL channels. DAG has been shown to activate native TRP and TRPL channels in photoreceptors and recombinant TRPL channels (Chyb et al., 1999). Furthermore, recent studies have supported the role of DAG in TRP/TRPL action: DAG kinase mutants display constitutive activation of TRP and TRPL channels (Raghu et al., 2000b), and, in vertebrate cells, TRPC3 (transient receptor potential-like channel 3) has been shown to be activated by DAG independently of $IP_3R$ (Venkatachalam et al., 2001). Thus, PLC is involved in regulation of TRP/TRPL plasma membrane calcium channels without a requirement for $IP_3$. It is possible, then, that PLC-activated DAG generation may regulate TRP/TRPL channels in tubules, which may contribute significantly to the 'multichannel' mode of action of $CAP_{2b}$. Furthermore, if DAG/TRP/TRPL signalling were compromised in some *itpr* lines, this may explain the significant impact of *itpr* alleles (for example, *itpr* null) on $CAP_{2b}$-stimulated, but not Drosokinin-stimulated, fluid transport and calcium signalling (Figs 3, 5).

Previous work has shown that leucokinin and Drosokinin increase cytosolic calcium concentration in only stellate cells in tubules, which express the Drosokinin receptor (Rosay et al., 1997; Terhzaz et al., 1999; Radford et al., 2002). This suggests that $IP_3$-mediated calcium signalling may occur in stellate cells. Activation of calcium signalling may be linked to chloride shunt conductance, which is also confined to stellate cells (O'Donnell et al., 1998). It is thus possible that Drosokinin stimulates PLCβ-dependent, $IP_3$-mediated calcium signalling in stellate cells *in vivo*, which increases chloride conductance, resulting in increased fluid transport.

Thus, Malpighian tubules, in contrast to photoreceptors, require both functional PLCβ and $IP_3R$ for neuropeptide-activated signal transduction in principal and stellate cells. The expression of *plc21* in tubules, however, and the role of DAG on plasma membrane calcium channels suggest that extremely complex mechanisms of signalling are used by tubule cells.

In summary, we have demonstrated non-neuronal, epithelial phenotypes for *norpA* (PLCβ) and *itpr* ($IP_3R$) and have correlated cell-specific signalling events for both $IP_3$ and calcium to transport phenotypes.

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