Disruption of the IP_3 receptor gene of *Drosophila* affects larval metamorphosis and ecdysone release

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Background: The inositol 1,4,5-trisphosphate (IP_3) receptor is an intracellular calcium channel that couples cell membrane receptors, via the second messenger IP_3, to calcium signal transduction pathways within many types of cells. IP_3 receptor function has been implicated in development, but the physiological processes affected by its function have yet to be elucidated. In order to identify these processes, we generated mutants in the IP_3 receptor gene (*itpr*) of *Drosophila* and studied their phenotype during development.

Results: All *itpr* mutant alleles were lethal. Lethality occurred primarily during the larval stages and was preceded by delayed moulting. Insect moulting occurs in response to the periodic release of the steroid hormone ecdysone which, in *Drosophila*, is synthesised and secreted by the ring gland. The observation of delayed moulting in the mutants, coupled with expression of the IP_3 receptor in the larval ring gland led us to examine the effect of the *itpr* alleles on ecdysone levels. On feeding ecdysone to mutant larvae, a partial rescue of the *itpr* phenotype was observed. In order to assess ecdysone levels at all larval stages, we examined transcripts of an ecdysone-inducible gene, *E74*; these transcripts were downregulated in larvae expressing each of the *itpr* alleles.

Conclusions: Our data show that disruption of the *Drosophila* IP_3 receptor gene leads to lowered levels of ecdysone. Synthesis and release of ecdysone from the ring gland is thought to occur in response to a neurosecretory peptide hormone secreted by the brain. We propose that this peptide hormone requires an IP_3 signalling pathway for ecdysone synthesis and release in *Drosophila* and other insects. This signal transduction mechanism which links neuropeptide hormones to steroid hormone secretion might be evolutionarily conserved.

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Background

Normal development of all multicellular organisms requires the function of appropriate signal transduction pathways at precise stages of differentiation. A common signalling mechanism in many types of differentiated cells is the inositol 1,4,5-trisphosphate (IP_3) signalling pathway, which involves the mobilisation of Ca^{2+} from intracellular stores through the IP_3 receptor by the action of the second messenger IP_3 [1,2]. It has been proposed that IP_3 signalling is required during key stages of development: for example, for the transient Ca^{2+} increase that occurs in both vertebrate and invertebrate eggs at fertilisation [3–5]; for normal development to proceed at the morula and blastula stages [6,7]; and for differentiation of the mesoderm [8,9]. Our interest is to define more comprehensively the nature of the developmental processes that require IP_3 signalling. In order to identify these processes, we have generated several mutant alleles of the IP_3 receptor (*itpr*) gene of *Drosophila melanogaster* and studied their developmental phenotype. Our results show that zygotic functioning of the IP_3 receptor in *Drosophila* begins postembryonically and is required for the process of larval moulting. To our knowledge, this is the first report of an IP_3 receptor mutant having a defined developmental phenotype. A previous report of IP_3-receptor-deficient mice describes adult neuronal phenotypes of epilepsy and ataxia rather than developmental defects [10].

Insect moulting is a prerequisite for metamorphosis and is controlled by the periodic release of the steroid hormone ecdysone from the prothoracic gland in response to stimulation by certain neuropeptide hormones [11–13]. A key neuropeptide hormone is prothoracicotropic hormone or PTTH, which targets the prothoracic gland (part of the ring gland in higher diptera). The PTTH–prothoracic gland relationship represents a model system that has similarities with many vertebrate steroid-hormone producing pathways [14,15]. Elucidation of the signalling events that lead to ecdysone production from the prothoracic gland in response to PTTH are therefore likely to be of general interest. In the lepidopteran *Manduca sexta*, both Ca^{2+} and cAMP appear to be required for PTTH-stimulated ecdysone release [16]. Similar experiments carried out in *Drosophila* implicate Ca^{2+} but not cAMP in ecdysone

release [17]; an involvement of IP_3 in the Ca^{2+} step has yet to be found. Here, we investigated the effects of the mutant alleles of the *itpr* gene on the transcription level of an ecdysone-inducible early gene, *E74*. Using *E74* transcripts as a measure of ecdysone levels, our data are the first to suggest that an IP_3 signalling pathway is required for the sustained release of ecdysone in *Drosophila* larvae.

Results and discussion

Disruption of the *itpr* gene

The *Drosophila* IP_3 receptor gene has been localised to band position 83A5–A9 on the right arm of chromosome 3 [18,19]. Our mutagenesis strategy utilised a *Drosophila* strain that carries a marked P-element in the 83A region. Flies from this strain were either irradiated with X-rays or mated with flies carrying a P-transposase source (see Materials and methods). Two lethal *itpr* alleles were obtained from the two experiments and these are referred to as *l(3)itpr*^{XR12} or *XR12* (X-irradiation experiment) and *l(3)itpr*$^{90B.0}$ (*90B.0*; see Supplementary material). Two other alleles were obtained by testing existing P-element lethal lines that map to the 83A region for failure to complement *XR12* and *90B.0*. These alleles are referred to as l(3)*itpr*^{B4} (*l(3)B4*) and l(3)*itpr*^{1664} (*l(3)1664*; see Supplementary material).

Molecular analysis of the *itpr* gene disruptants

In order to analyse the physical status of the *itpr* gene in the four alleles of the identified lethal complementation group, we generated a restriction map of the wild-type gene with exon–intron boundaries, using lambda clones isolated from a *Drosophila* genomic library (Figure 1a). All four alleles identified by complementation show differences from the restriction map of the wild-type *itpr* gene. These changes are summarised in Figure 1b and are derived from the data in Figure 2. Molecular analysis of the *XR12* allele (Figure 2a lanes 2, 5, 8 and 11) revealed that there was a breakpoint in the *Hin*dIII 2.5 kb fragment of the parent strain (Figure 2a, lane 4), which spans the end of the first intron and the second exon, giving rise to a smaller 2.0 kb fragment (lane 5). Additional changes in the size of the fragments obtained following digest with other restriction enzymes (e.g. *Bam*HI, lane 2; *Eco*RI, lane 8) suggest that *XR12* is an inversion of the *itpr* gene with one breakpoint between the open reading frame of the *itpr* gene and its upstream sequences. The presence of lower levels of *itpr* RNA in the heads of adult *XR12/Sb* flies supports this idea (Figure 3). As the phenotypic consequences of the other inversion breakpoint of *XR12* are unknown, however, we have not further analysed the biology of this allele.

The P[w$^+$] insert in *l(3)B4* was mapped to the 5.0 kb *Sal*I fragment which lies within the first intron of *itpr* (Figure 2a, lane 12). The hybridisation patterns of other restriction digests with *l(3)B4* DNA (Figure 2a, lanes 3,6,9) confirm this position of the P[w$^+$] insert. A correlation between the P-element insert in *l(3)B4* and the lethality of *l(3)B4* was obtained independently by generating P-element excision lines from *l(3)B4*. Southern blotting of genomic DNA from the excision lines demonstrated that reversion of lethality occurred in lines with complete excisions of the P-element (data not shown). In the strain *90B.0*, all bands corresponding to the *itpr* gene are reduced in intensity compared with

Figure 1

A restriction map of the *itpr* gene with exon–intron boundaries **(a)** and positions of molecular breakpoints in each of the *itpr* mutant alleles **(b)** derived from the data in Figure 2. The probes used in the Southern blotting analysis are also shown. Restriction enzyme sites have been denoted as B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; S, *Sal*I; and X, *Xba*I. Sites marked with an asterisk are present in the published sequence of the head *itpr* cDNA [18].

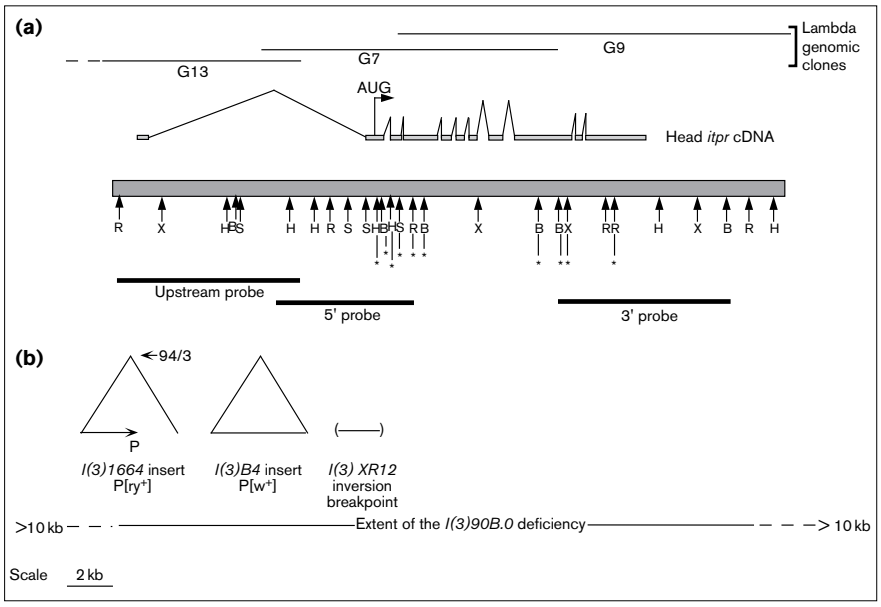
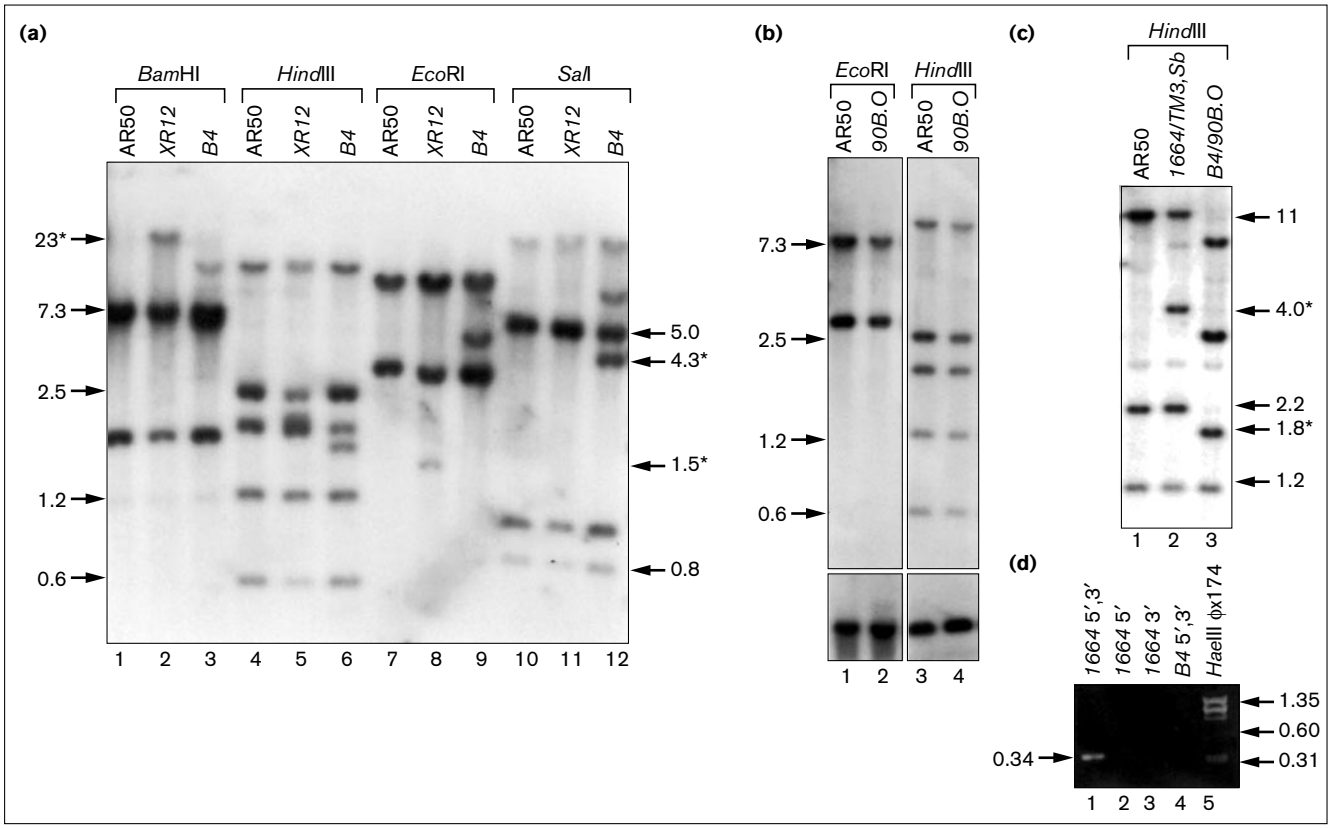


Figure 2

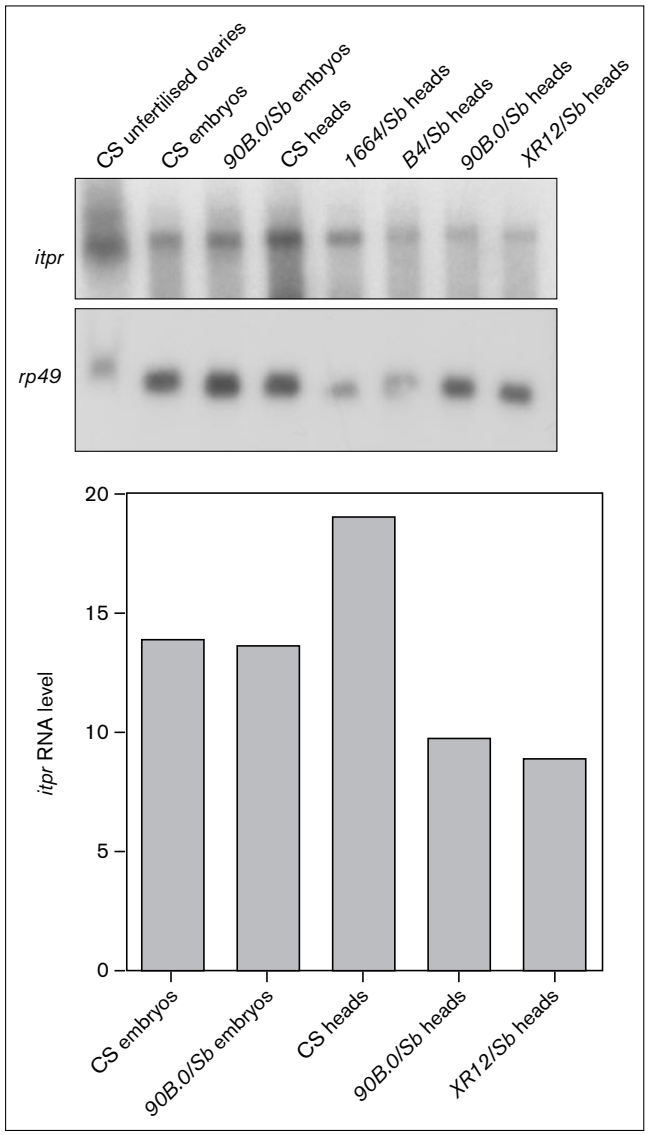


(a) Mapping of *XR12* and the P-element insert in *l(3)B4*. Genomic DNA from the three *Drosophila* strains AR50, *XR12/TM3,Sb* and *l(3)B4/TM3,Sb* was digested with restriction enzymes as indicated above each lane. The 5′ probe shown in Figure 1b was hybridised with a Southern blot of the gel. **(b,c)** Mapping of the *90B.0* deficiency. Genomic DNA from the parent *Drosophila* strain AR50 and the *90B.0/TM3,Sb* strain was digested with the restriction enzymes indicated above the lanes. A Southern blot of the gel was hybridised to the 5′ *itpr* gene probe (5′ probe in Figure 1b) in (b). In the lower panels, the same blot is shown hybridised to a probe for a gene that does not map near the *itpr* gene in order to demonstrate levels of DNA loading. Numbers on the left and right of the blot indicate band size in kb. A *Hind*III digest of genomic DNA from larvae of the genotype *l(3)B4/90B.0* is shown in lane 3 of (c). In this case, hybridisation is to the upstream probe marked in Figure 1b. **(c,d)** Mapping of the P[*ry*$^+$] insert in *l(3)1664*. Genomic DNA from AR50 (lane 1) and *l(3)1664/TM3,Sb* adults (lane 2) was digested with *Hind*III and hybridised to the upstream probe shown in Figure 1b. (d) PCR analysis of *l(3)1664/TM3,Sb* and *l(3)B4/TM3,Sb* genomic DNA using the primer(s) indicated above each lane; these P (5′) and 94-3 (3′) primers are shown in Figure 1b. *Hae*III digest of ϕX174 DNA is shown as a marker in lane 5.

the parent strain (Figure 2b, compare lanes 2 and 4 with lanes 1 and 3) indicating that the complete gene has been deleted in the *90B.0* mutant. The complete absence of the *itpr* gene in strain *90B.0* was further confirmed by analysis of DNA from larvae of the strain *l(3)B4/90B.0* (Figure 2c, lane 3); the fragments that hybridise to the upstream probe shown in Figure 1 are solely of *l(3)B4* origin. The 1.8 kb band is derived from insertion of the P[w$^+$] element in the wild-type 2.2 kb *Hind*III fragment (Figure 2c, lane 1). We have established independently that a polymorphic *Hind*III site is present within the *l(3)B4* chromosome and also within excised revertants derived from this chromosome; this site is located in the upstream region of the *itpr* gene. Consequently, the 11 kb *Hind*III band seen in DNA from wild-type *Drosophila* (Figure 2c, lane 1) is absent and replaced by two smaller bands (Figure 2c, lane 3). The breakpoints of the *90B.0* deletion do not map within the 10 kb region that flanks the termini of the *itpr* gene. By genetic complementation of *90B.0* using a neighbouring deficiency line (Df(3R)2-2) and other P-lethal lines mapped to the 82F–83A-B region, it is clear, however, that the extent of the *90B.0* deletion is small (see Supplementary material): there is no existing deficiency for either part or all of the 83A–B region.

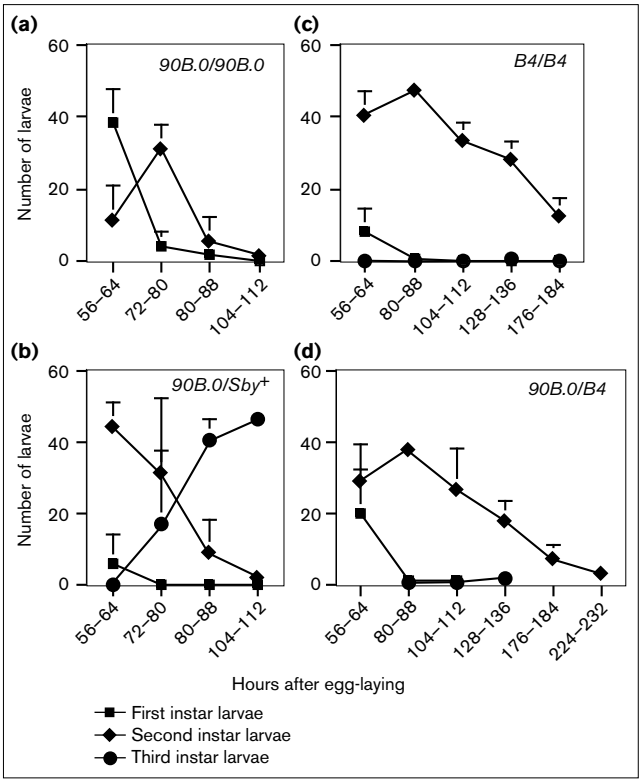
The position of the P-element in *l(3)1664* was mapped by hybridising the upstream probe to genomic DNA from *l(3)1664/Sb* flies. From the digest shown in lane 2 of Figure 2c, the *l(3)1664* insert maps to the upstream region of the *itpr* gene. The position of the insertion was further mapped by PCR analysis using a 5′ primer homologous to the ends of the P-element and a 3′ primer homologous to a

Figure 3



Qualitative and quantitative analysis of *itpr* mRNA. A northern blot of total RNA extracted from 30 pairs of mature unfertilised ovaries, polyA+ RNA extracted from 0–16 h embryos and polyA+ RNA extracted from the heads of adult flies was hybridised sequentially with an *itpr* cDNA probe and an *rp49* gene probe. RNA levels were estimated by scanning as described in Materials and methods. CS: Canton-S (wild-type) strain.

Figure 4



Progression of moulting and lethality of the alleles $itpr^{90B.0}$ and $itpr^{B4}$. The x-axis denotes hours after egg-laying and the y-axis denotes numbers of surviving larvae. Each data point is derived from the average of five vials containing fifty larvae of the appropriate genotype. The error bars represent standard deviation.

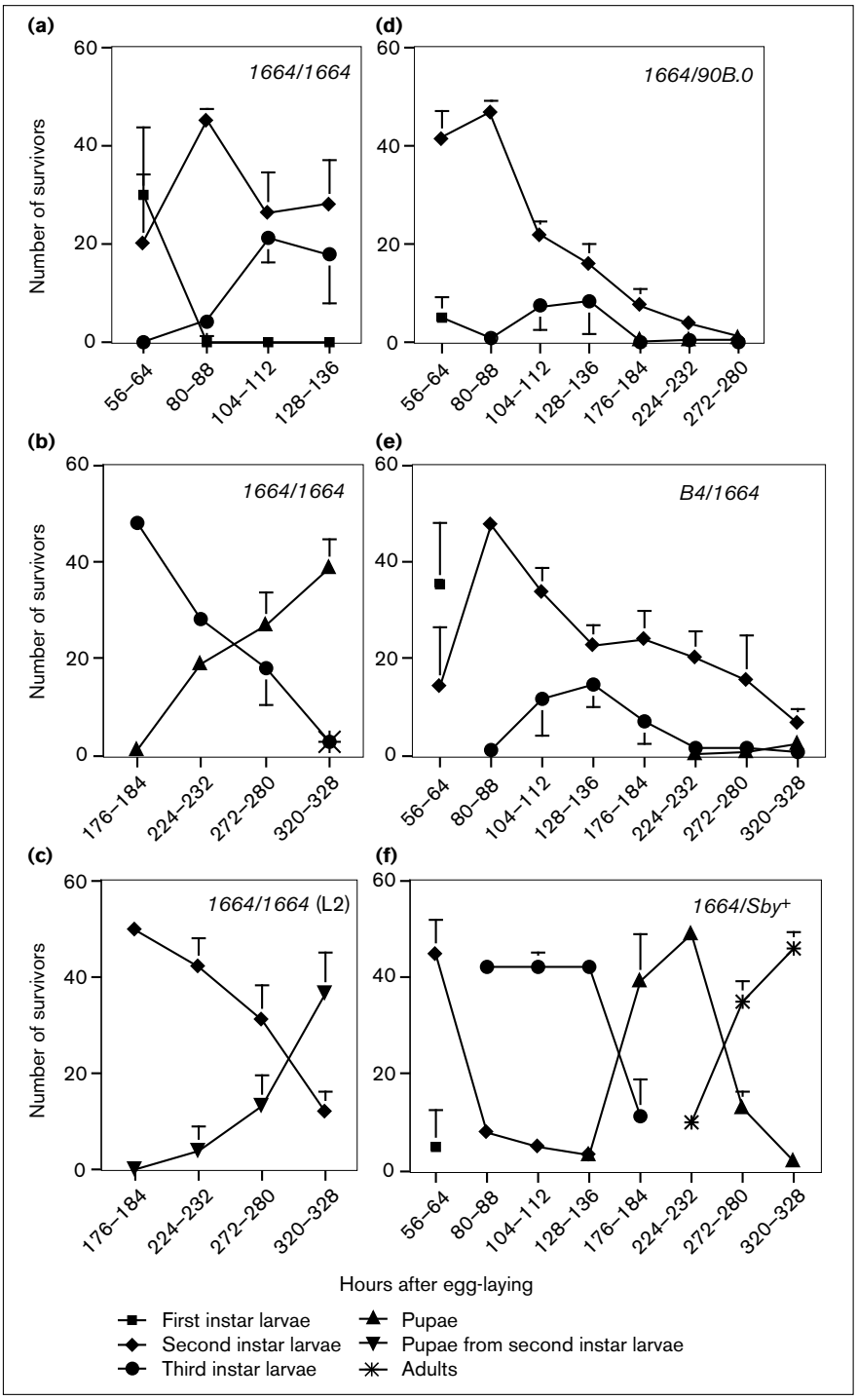
region approximately 100 bp within the first exon of the *itpr* cDNA. The size of the resulting PCR product (Figure 2d) places the P-insert approximately 200 bp upstream of the 5′ end of the *itpr* cDNA.

Temporal retardation of larval moulting in the *itpr* mutant alleles

From the molecular analysis, it is clear that *90B.0* is a null allele of the *itpr* gene. Somewhat surprisingly, we found that *90B.0* homozygous embryos develop normally and hatch as viable first instar larvae. This finding does not suggest that IP_3 receptor function is not required during embryonic development; rather, as ovaries from unfertilised females contain a substantial amount of *itpr* message (Figure 3), we assume that the maternal contribution of *itpr* is sufficient to see the embryo through to hatching and first instar larval life. A comparison of the levels of *itpr* message in embryos and heads of wild-type Canton-S (CS) and *90B.0/Sb* animals supports this idea (Figure 3). The precise stage at which zygotic expression of the *itpr* gene commences has yet to be elucidated. Lethality in *90B.0* homozygotes begins during early second instar, 72 hours after egg-laying (AEL). By 112 hours AEL, practically all *90B.0* homozygotes are dead (Figure 4a). The observation that *90B.0* homozygotes undergo the first larval moult more slowly than wild-type larvae (compare Figure 4a and 4b, 56–64 hours AEL) is significant and might be due to a hormonal imbalance that delays, but does not prevent, the first moult.

Lethality staging of the two other alleles studied, *l(3)B4* (Figure 4c) and *l(3)1664* (Figure 5), indicates that they are

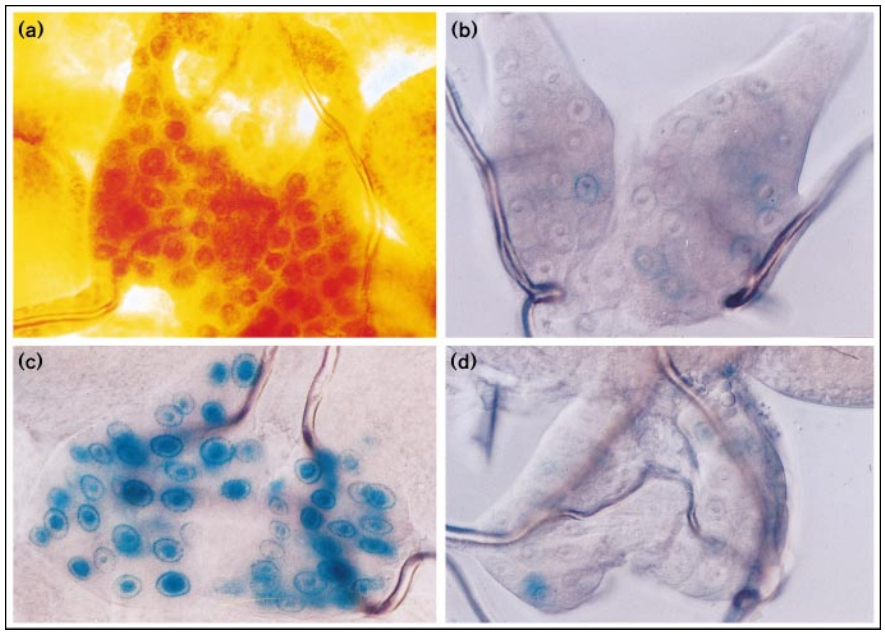
Figure 5



Moulting and lethality of the allele *itpr*^{1664} and its complementation with *itpr*$^{90B.0}$ and *itpr*$^{B4.}$ Graph axes and other notations are as in Figure 4. In **(b,c)** third and second instar larvae were separated 128 h after egg-laying and transferred to fresh vials.

both hypomorphs with phenotypes that are similar to but weaker than *90B.0*. Homozygous *l(3)B4* larvae survive longer than *90B.0* homozygotes, with most dying as late second instar larvae between 88 and 184 hours AEL (Figure 4c). Like *90B.0* homozygotes, *l(3)B4* homozygotes exhibit a delay in moulting from first to second instar, but the delay is less evident for the *l(3)B4* homozygotes (Figure 4c, 56–64 hours AEL). The third allele studied, *l(3)1664*, has a considerably weaker effect than *l(3)B4*. In our analysis of genetic complementation (see Supplementary material), *l(3)1664* is the only allele that can complement the lethality of all other alleles, but does so at a very

Figure 6



Ring glands from non-mutant (a,b) and *itpr* mutant third instar larvae (c,d). Immunohistochemical staining for IP_3 receptor expression is shown in **(a)**. Staining for β-galactosidase expression in *y/y;itpr1664/TM3,Sby*$^+$, *y/y;itpr*^{1664}*/itpr*^{1664} and *y/y;itpr*^{1664}*/itpr*$^{90B.0}$ is shown in **(b)**, **(c)** and **(d)** respectively. In all cases, photographs were taken at a magnification of 500×.

low frequency. Unlike *l(3)B4* and *90B.0*, almost 50% of *l(3)1664* homozygotes moult into third instar larvae and pupae (Figure 5a,b). Significantly, both moults are delayed compared with the *l(3)1664* heterozygotes (Figure 5a,f, 56–64 hours AEL; 80–88 hours AEL). The remaining 50% of *l(3)1664* homozygous larvae survive at the second instar stage for an extended time period and then appear to moult directly into pupae (Figure 5c). Lethality of *l(3)1664* homozygotes occurs during pupal development because very few adults emerge from the pupa: the few adults that do emerge exhibit no obvious morphological defects.

The lethality profiles of heteroallelic combinations of all three alleles (Figure 4d, 5d, 5e) further support the conclusion that the strength of the lesions caused by the three alleles is *90B.0* > *l(3)B4* > *l(3)1664*.

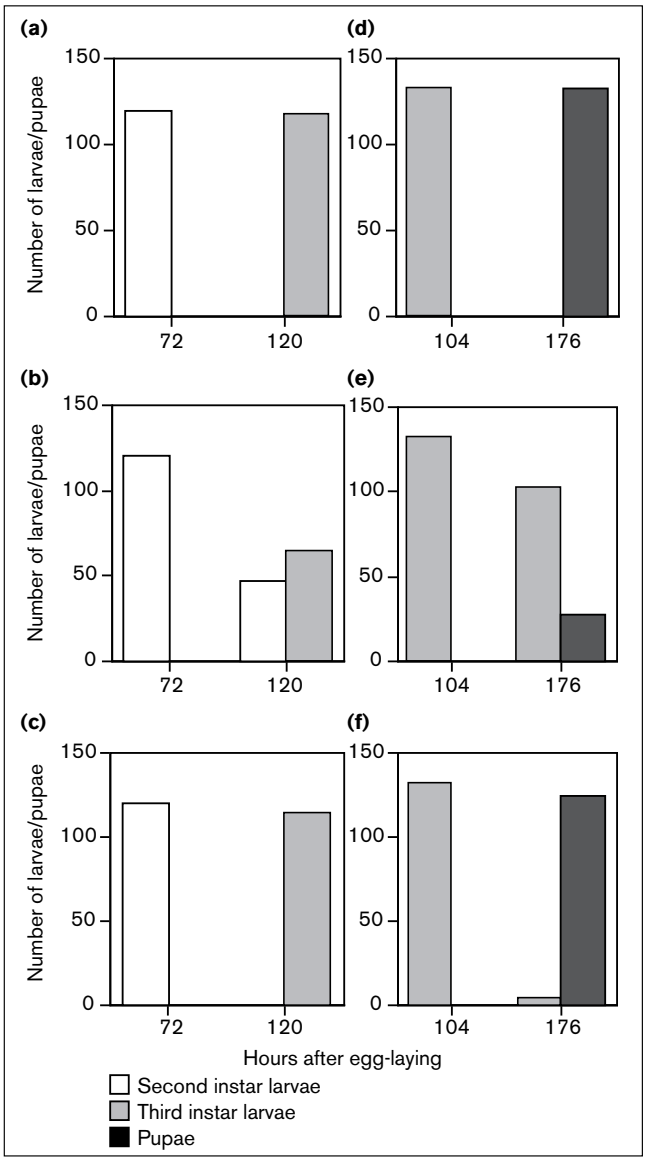
IP_3 receptor expression in larvae

To explore the cause of lethality and delay in moulting observed in the *itpr* mutant alleles, we examined all third instar larval tissues for IP_3 receptor expression. Most larval tissues such as brain, ventral nerve cord, gut and body musculature did not show any specific IP_3 expression (data not shown). Interestingly, IP_3 receptor protein was detectable in cells of the ring gland in third instar *Drosophila* larvae (Figure 6a). The ring gland is a composite endocrine gland composed of discrete regions that are analogues of the lepidopteran prothoracic gland, corpus allatum and corpus cardiacum [16]. All cells of the ring gland appear to stain equally with the anti-IP_3-receptor antibody. The only other larval tissue in which the IP_3 receptor was detected above background was the salivary gland (data not shown), which functions primarily to secrete glue proteins in late third instar larvae. This expression pattern adds to the previously described staining of myoblasts in imaginal discs of third instar larvae [9]. The presence of the IP_3 receptor protein in larval ring glands suggests that the IP_3 receptor is required either for ring gland function or for ring gland development. In order to test the latter possibility, we looked at the anatomical status of ring glands from *itpr* mutant larvae. The genotypes studied were *l(3)1664/l(3)1664* and *l(3)1664/90B.0*. In these strains, the ring gland could be visualised by staining for β-galactosidase produced by the P-insert in *l(3)1664*. In both mutant genotypes, the cellular morphology of the ring gland appeared normal (Figure 6c,d), when compared to non-mutant *1664/Sby*$^+$ heterozygotes (Figure 6b). The expression of the IP_3 receptor in the ring gland and the observation that the ring gland develops normally in both weak (*1664/1664*) and strong (*1664/90B.0*) mutant allelic combinations suggest that the IP_3 receptor is required for the normal functioning of the ring gland, possibly for signalling the release of hormone.

Exogenous ecdysone can rescue the moulting delay of *itpr*^{1664}

To test the hypothesis that levels of the steroid hormone ecdysone might be affected in *itpr* mutants, two types of experiment were performed. In the first approach, we attempted to rescue the *itpr* phenotype resulting from the weakest allele by feeding the larvae with 20-hydroxyecdysone (the active form of ecdysone in *Drosophila*).

Figure 7



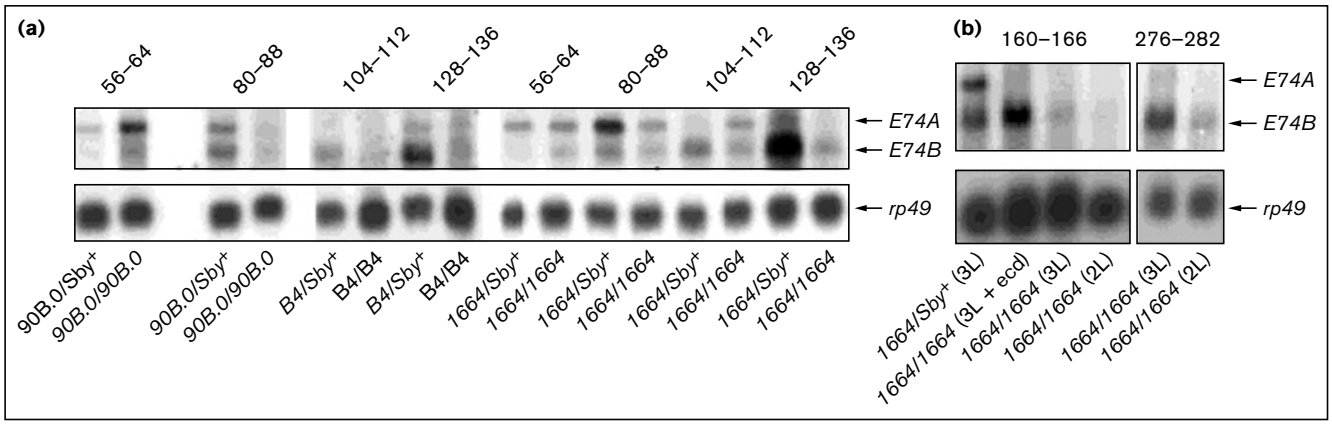
Rescue of the moulting delay in *itpr*^{1664} mutants by feeding with ecdysone. Second instar larvae, third instar larvae and pupae of the genotype *y/y;itpr*^{1664}*/TM3,Sby*$^{+}$ **(a,d)** or of the genotype *y/y;itpr*^{1664}*/itpr*^{1664} **(b,c,e,f)** were transferred to medium with ecdysone (c and f) or without ecdysone (a,b,d,e). The x-axis denotes hours after egg-laying and the y-axis denotes the number of larvae and pupae of the specified stage.

This experiment was based on a previous observation which demonstrated that the phenotype of an ecdysone-release mutant could be partially rescued by feeding ecdysone to the flies [20]. We therefore took *l(3)1664/Sby*$^{+}$ and *l(3)1664/l(3)1664* larvae at 72 hours AEL (Figure 7a–c) or 104 hours AEL (Figure 7d–f) and transferred them to medium with or without 20-hydroxyecdysone. At both larval stages, the presence of 20-hydroxyecdysone resulted in a transition of the *l(3)1664* homozygotes to the next stage of development at a comparable rate to the *l(3)1664* heterozygotes (Figure 7c,e). The absence of 20-hydroxyecdysone from the medium either prevented the transition (*1664/1664*, L2 to L3 Figure 5a and 7b), or delayed this transition (*1664/1664*, L3 to P, Figure 5b and 7e). The ecdysone-mediated rescue of the moulting delay suggests that a step upstream of ecdysone release is affected in *itpr*^{1664} mutants.

Transcription from the ecdysone-inducible *E74* gene is affected in *itpr* mutants

Ecdysone levels in larvae expressing each of the three mutant *itpr* alleles were assessed indirectly by monitoring ecdysone-inducible transcripts from the *E74* gene [21]. This gene has two ecdysone-responsive promoters that are directly activated by the binding of 20-hydroxyecdysone and the ecdysone-receptor complex. The promoter for the *E74B* transcript is activated by lower levels of ecdysone and inhibited by higher levels; the promoter for *E74A* is activated by higher levels of ecdysone [21–23]. *E74* gene transcripts thus follow an invariant order of appearance in which *E74B* always appears before *E74A* in response to a pulse of ecdysone. The relative amounts of *E74A* and *E74B* transcripts are therefore a measure of the ecdysone levels at all larval stages. Both *E74A* and *E74B* transcripts were examined at various developmental time points in flies expressing each of the three *itpr* alleles (Figure 8a). At 56–64 hours AEL, *90B.0* homozygotes had a higher ratio of *E74A* to *E74B* transcripts compared with the heterozygous controls. Taken together with the data in Figure 4a, it is apparent that a delayed but otherwise normal peak of ecdysone release occurs in *90B.0/90B.0* first instar larvae. In second instar larvae, however, both *E74A* and *E74B* transcripts were almost undetectable in the homozygotes (80–88 hours AEL). This larval stage also corresponds to the stage prior to maximum lethality (Figure 4a). Similarly in *l(3)B4* homozygous larvae, *E74A* and *E74B* transcripts were almost undetectable at the stage prior to lethality. In *l(3)1664* homozygotes, *E74A* and *E74B* RNA profiles initially followed the same pattern as in *90B.0/90B.0* (56–64 hours AEL). However, consistent with *l(3)1664* being a weaker allele, both *E74A* and *E74B* transcripts were detectable in *l(3)1664* mutants at later stages when the transcripts were no longer present in larvae expressing the two stronger alleles. The ratios of the transcripts and their actual levels of expression were abnormal, particularly at 104–112 and 128–136 hours AEL (Figure 8a). These data indicate that ecdysone levels in second instar larvae expressing each of the three alleles are reduced: in *90B.0* and *l(3)B4* mutants, the level appears to be far below that required for normal moulting to proceed; however, in *l(3)1664* mutants the ecdysone level in second instar probably varies and can almost reach the threshold of that required for moulting into third instar, thus allowing approximately 50% of them to reach third instar. In order to determine whether *E74* transcripts

Figure 8



Ecdysone-inducible transcripts of the *E74* gene at various time points of development, in heterozygous and homozygous larvae of *itpr* mutant alleles. Each lane contains approximately 10 μg of total RNA, extracted from staged larvae of the appropriate genotype. **(a)** Staged larvae from the three *itpr* allele genotypes. **(b)** Late second (2L) and late third instar (3L) larvae from *itpr^{1664}*; the effect of ecdysone (+ ecd) on E74 expression in 3L larvae is also shown. *E74A* and *E74B* transcripts were detected by hybridisation to a probe specific for the 3′ end of the *E74* gene, a region common to both *E74A* and *B* [21]. Each blot was subsequently hybridised to an *rp49* gene probe for quantification.

are indeed a good measure of ecdysone levels in *itpr* mutants, we analysed *E74* RNA in third instar larvae from *itpr^{1664}* mutants in the presence and absence of exogenous ecdysone (Figure 8b). At 160–166 hours AEL, *E74* transcripts were barely detectable in *1664/1664* homozygous larvae. In larvae of identical genotype and stage, ecdysone feeding resulted in the appearance of *E74B* transcripts. The possibility that *itpr* gene mutants affect *E74* RNA levels independently through interaction with other gene products is therefore unlikely.

It is interesting that both second and third instar homozygous *l(3)1664* larvae finally pupate after an extended period of larval life (Figure 5b,c). We examined *E74* transcripts in second and third instar *1664* homozygous larvae at the prepupal stage of 276 hours AEL (Figure 8b). The level of *E74B* RNA appeared to increase at this stage. Consistent with the observation that pupation of third instar mutant larvae is faster than second instar mutant larvae (Figure 5b,c; 224–232 and 272–280 hours AEL), the level of *E74B* is higher in third instar homozygous *1664* larvae. The observation that second instar larvae also pupate remains to be understood. Our initial classification of second and third instar larvae was on the basis of anterior spiracle morphology. In the case of *l(3)1664* homozygotes, we have also examined the morphology of teeth in the mouth hooks to corroborate the classification of the instars (data not shown). Both of these morphological criteria establish that second instar larvae are able to pupate in *l(3)1664* homozygotes. The developmental status of imaginal discs in such pupae is under investigation. In most insects, the hormonal requirements for pupation are different from those of the interlarval moults, with pupation requiring both the presence of ecdysone and the absence of juvenile hormone, JH [11,13,16], normally secreted by cells of the corpora allata. In *Drosophila*, the corpora allata is part of the ring gland and has been shown to produce a form of JH called JHB$_3$ [24]. The role of JH titres in pupation of higher dipterans, including *Drosophila*, is still a matter of speculation, however. Analysis of the aberrant pupation seen in *itpr^{1664}* could help to clarify the role of ecdysone and JH titres in *Drosophila* pupation.

Conclusions

Disruption of the *itpr* gene in *Drosophila* leads to delayed larval moulting, which appears to be caused by lowered levels of the steroid hormone ecdysone. This hormone is synthesised and secreted by the ring gland, and is required for initiating the expression of a cascade of developmental genes that regulate subsequent developmental events in the larva [25]. In the absence of this developmental sequence of events, lethality occurs. As the IP$_3$ receptor is expressed in cells of the ring gland, it seems likely that an IP$_3$ signalling pathway is required in this tissue for ecdysone synthesis and release. Although it is premature to predict other key components of the signalling pathway that require the IP$_3$ receptor, it is likely that signalling by PTTH from neurosecretory cells in the brain to the ring gland is dependent on the IP$_3$ receptor. Further investigation will be necessary to determine the stage at which IP$_3$-mediated Ca^{2+} release is required in this signal transduction pathway. Previous experiments in other organisms have suggested that although cAMP is the second messenger for the PTTH signal, Ca^{2+} is also required for this signal [16,26]. Our observation that the first larval moult can take place in the null allele *itpr$^{90B.0}$* suggests that the IP$_3$ receptor might not be essential for the initial release of ecdysone, and supports the idea that

IP_3-mediated Ca^{2+} release might be a positive modulating factor for achieving maximal levels of ecdysone. A possible mechanism for this type of modulation could be through the regulation of adenylyl cyclase activity by Ca^{2+} [27]. An advantage of generating IP_3 receptor mutants in *Drosophila* will be the ease in designing future genetic experiments to search for interacting genes. The identification of such genes should help to elucidate this well-studied but poorly understood signalling pathway.

Materials and methods

Disruption of the itpr *gene*

A homozygous viable strain AR50, which has a marked P-element in the polytene band region of 83A7–A9, was used to generate the two alleles *l(3)itpr*$^{90B.0}$ and *l(3)itpr*^{XR12}. In one experiment, the P-element was mobilised in 207 independent flies by crossing to a P-transposase source, essentially as described [28]. In another experiment, 350 males of the AR50 strain were irradiated with 4000 rads of X-rays according to standard protocols [29] and then crossed to suitably marked females. The progeny from these two experiments were screened for lethality and for the change in phenotype of the marked P-element, which in this case was eye colour. The P-transposition experiment yielded 35 independent lethal lines, which fell into 11 complementation groups. The X-irradiation experiment yielded two lethal lines from a total of 12 656 progeny screened. Genetic complementation of the lethal lines obtained by these two methods yielded only one group with alleles derived from both the P-element mobilisation experiment (*l(3)itpr*$^{90B.0}$) and the X-irradiation experiment (*l(3)itpr*^{XR12}). These two alleles were subsequently crossed to other P-lethal lines known to carry single P-elements in the 83A5-A9 region. Two more non-complementing alleles were identified this way (*l(3)itpr*^{B4} and *l(3)itpr*^{1664}.

Molecular techniques

A restriction map of the wild-type *itpr* gene was constructed by mapping sites for the restriction enzymes *Eco*RI, *Xba*I, *Hin*dIII, *Bam*HI and *Sal*I on three overlapping lambda genomic clones G7, G9 and G13. Exon–intron boundaries for the gene were obtained by comparing expected head cDNA fragment sizes with the size of fragments obtained by PCR, using both genomic DNA and genomic clones as the templates. Introns have been positioned at the mid-point of the two closest flanking primers used for the PCR. Experiments are in progress to obtain a more detailed map by direct sequencing of PCR fragments and the results from these will be published independently. The first intron, which is too large to map by PCR, was mapped using a ~300 bp 5′ cDNA fragment of the *itpr* gene as a probe on Southern blots of genomic DNA and clones. Preparation of genomic DNA from flies expressing wild-type and mutant alleles, restriction-enzyme digestion and procedures for Southern blotting were carried out according to standard protocols. PCR mapping of the P[ry$^+$] insert in *l(3)1664* was carried out with a P primer that is homologous to one end of the P-element and an *itpr* gene primer (94-3) which is derived from positions 148–168 of the 5′ untranslated region of the *itpr* head cDNA.

Northern blot analysis

PolyA$^+$ RNA was extracted from the heads of adult flies using standard procedures and approximately 3–5 μg RNA was run on a single lane of a 0.7% MOPS–formaldehyde agarose gel. The gel was transferred to a nylon membrane and the membrane hybridised to the appropriate probes sequentially. Levels of *itpr* mRNA were quantified by scanning autoradiographs and correcting for levels of RNA loading by measuring *rp49* gene transcripts. For *E74* transcript analysis, total RNA was extracted from approximately 25–50 mg of staged larvae, of each genotype, following standard RNA extraction protocols. Approximately 10 μg total RNA from each genotype and stage was used for a single experiment. As small differences in developmental times can occur after feeding with different food batches, care was taken to grow the heterozygotes and homozygotes of each allele in the same batch of food. Ecdysone feeding prior to RNA extraction was carried out as described in the section below.

Lethality staging of itpr *mutant alleles*

All mutant alleles strains are maintained as the genotype *y,w;itpr*/TM3,*Sby*$^+$. Heterozygous adults were kept for egg-laying for a period of 4–5 h. Freshly hatched first instar homozygous mutant larvae, identified by lighter pigmentation of the mouth hooks due to the *y* allele on the X chromosome, were separated from the heterozygotes, which carry a *y*$^+$ gene transposed on the third chromosome balancer TM3, and hence have normal pigmented mouth hooks. The same number of separated larvae (usually 50 per vial) were placed in vials containing *Drosophila* medium without agar and kept at 25°C. At appropriate time points, the larvae were washed and counted. Larval stages were determined by the appearance of spiracles [29].

Immunohistochemistry

Procedures for immunohistochemical staining of whole-mount tissues with the anti-IP_3 receptor affinity-purified antibody have been described in detail elsewhere [9]. Controls stained with pre-immune serum showed no specific staining (data not shown).

Staining for β-galactosidase

Hand-dissected tissues were fixed for up to 20 min in 1% glutaraldehyde in PBS. The tissues were washed twice for 5 min each in PBS and then placed in X-gal staining solution [9] overnight at 37°C.

Ecdysone feeding of larvae

A total of 120 (second instar) or 132 (third instar) staged larvae were transferred, in batches of 20 or 12 respectively, into *Drosophila* food that lacked agar. The 20-hydroxyecdysone was dissolved in double-distilled ethanol and added to the medium at a final concentration of 1mg/ml [29]. Control food vials were supplemented with only double-distilled ethanol. Larvae were washed, counted and staged as described previously.

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References

1. Berridge MJ: **Inositol triphosphate and calcium signalling.** *Nature* 1993, **361:**315-325.
2. Furuichi T, Yoshikawa S, Miyawaki A, Wada K, Maeda N, Mikoshiba K: **Primary structure and functional expression of the inositol 1,4,5-triphosphate-binding protein P400.** *Nature* 1989, **342:**32-38.
3. Galione A, McDougall A, Busa WB, Willmott N, Gillot I, Whitaker M: **Redundant mechanisms of calcium-induced calcium release underlying calcium waves during fertilization of sea urchin eggs.** *Science* 1993, **261:**348-351.
4. Buck WR, Hoffmann EE, Rakow TL, Shen SS: **Synergistic calcium release in the sea urchin egg by ryanodine and cyclic ADP ribose.** *Dev Biol* 1994, **163:**1-10.
5. Ayabe T, Kopf GS, Schultz RM: **Regulation of mouse egg activation: presence of ryanodine receptors and effects of microinjected ryanodine and cyclic ADP ribose on uninseminated and inseminated eggs.** *Development* 1995, **121:**2233-2244.
6. Reinhard E, Yokoe H, Niebling KR, Albritton NL, Kuhn MA, Meyer T: **Localized calcium signals in early zebrafish development.** *Dev Biol* 1995, **170:**50-61.
7. Stachecki JJ, Armant DR: **Transient release of calcium from inositol 1,4,5-trisphosphate-specific stores regulates mouse preimplantation development.** *Development* 1996, **122:**2485-2496.
8. Ault KT, Durmowicz G, Galione A, Harger PL, Busa WB: **Modulation of *Xenopus* embryo mesoderm-specific gene expression and dorsoanterior patterning by receptors that activate the phosphatidylinositol cycle signal transduction pathway.** *Development* 1996, **122:**2033-2041.

9. Raghu P, Hasan G: **The inositol 1,4,5-triphosphate receptor expression in *Drosophila* suggests a role for IP_3 signalling in muscle development and adult chemosensory functions.** *Dev Biol* 1995, **171:**564-577.
10. Matsumoto M, Nakagawa T, Inoue T, Nagata E, Tanaka K, Takano H, *et al.*: **Ataxia and epileptic seizures in mice lacking type 1 inositol 1,4,5-triphosphate receptor.** *Nature* 1996, **379:**168-171.
11. Gilbert LI, Combest W, Smith W, Meller V, Rountree D: **Peptides, second messengers and insect moulting.** *Bioessays* 1988, **8:**153-157.
12. Zitnan D, Sehnal F, Bryant PJ: **Neurons producing specific neuropeptides in the central nervous system of normal and pupariation-delayed *Drosophila*.** *Dev Biol* 1993, **156:**117-135.
13. Riddiford LM: **Hormones and *Drosophila* development.** In *The Development of Drosophila melanogaster*, *Vol II*. Edited by Bate M. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1994:899-939.
14. Tremblay E, Payet M-D, Gallo-Payet N: **Effects of ACTH and angiotensin II on cytosolic calcium in cultured adrenal glomerulosa cells. Role of cAMP production in the ACTH effect.** *Cell Calcium* 1991, **12:**655-673.
15. Warchol JB, Filipiak K, Ignasiak E, Nussdorfer GG, Malendowicz LK: **Oxytocin directly stimulates corticosterone secretion by dispersed rat adrenal zonae fasciculata and reticularis cells: evidence for the spreading of the oxytocin-evoked signal from responsive to unresponsive cells.** *Biomed Res* 1993, **14:**261-264.
16. Gilbert LI, Rybczynski R, Tobe SS: **Endocrine cascade in insect metamorphosis.** In *Metamorphosis*. Edited by Gilbert LI, Tata JR, Atkinson BG. San Diego: Academic Press; 1996:60-107.
17. Henrich VC: **Comparison of ecdysteroid production in *Drosophila* and *Manduca*: pharmacology and cross-species neural reactivity.** *Arch Insect Biochem Phys* 1995, **30:**239-254.
18. Hasan G, Rosbash M: ***Drosophila* homologs of two mammalian intracellular Ca^{2+}-release channels: identification and expression patterns of the inositol 1,4,5-triphosphate and the ryanodine receptor genes.** *Development* 1992, **116:**967-975.
19. Yoshikawa S, Tanimura T, Miyawaki A, Nakamura M, Yuzaki M, Furuichi T, *et al.*: **Molecular cloning and characterization of the inositol 1,4,5-triphosphate receptor in *Drosophila melanogaster*.** *J Biol Chem* 1992, **267:**16613-16619.
20. Garen A, Kauvar L, Lepesant J-A: **Roles of ecdysone in *Drosophila* development.** *Proc Natl Acad Sci USA* 1977, **74:**5099- 5103.
21. Thummel CS, Burtis KC, Hogness DS: **Spatial and temporal patterns of *E74* transcription during *Drosophila* development.** *Cell* 1990, **61:**101-111.
22. Karim FD, Thummel CS: **Ecdysone coordinates the timing and amounts of *E74A* and *E74B* transcription in *Drosophila*.** *Genes Dev* 1991, **5:**1067-1079.
23. Karim FD, Thummel CS: **Temporal coordination of regulatory gene expression by the steroid hormone ecdysone.** *EMBO J* 1992, **11:**4083-4093.
24. Richard DS, Applebaum SW, Gilbert LI: **Allatostatic regulation of juvenile hormone production in vitro by the ring gland of *Drosophila melanogaster*.** *Mol Cell Endocrinol* 1990, **68:** 153-161.
25. Thummel CS: **Flies on steroids — *Drosophila* metamorphosis and the mechanisms of steroid hormone action.** *Trends Genet* 1996, **12:**306-310.
26. Birkenbeil H: **Involvement of calcium in prothoracicotropic stimulation of ecdysone synthesis in *Galleria mellonella*.** *Arch Insect Biochem Physiol* 1996, **33:**39-52.
27. Cooper DMF, Mons N, Karpen JW: **Adenylyl cyclases and the interaction between calcium and cAMP signalling.** *Nature* 1995, **374:**421-424.
28. Tower J, Karpen GH, Craig N, Spradling AC: **Preferential transposition of *Drosophila* P elements to nearby chromosomal sites.** *Genetics* 1993, **133:**347-359.
29. Ashburner M: Drosophila*, A Laboratory Handbook*. Cold Spring Laboratory: Cold Spring Harbor Laboratory Press, 1989.

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