Interactions Between the Inositol 1,4,5-Trisphosphate and Cyclic AMP Signaling Pathways Regulate Larval Molting in Drosophila

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Manuscript received December 15, 2000
Accepted for publication February 9, 2001

ABSTRACT

Larval molting in Drosophila, as in other insects, is initiated by the coordinated release of the steroid hormone ecdysone, in response to neural signals, at precise stages during development. In this study we have analyzed, using genetic and molecular methods, the roles played by two major signaling pathways in the regulation of larval molting in Drosophila. Previous studies have shown that mutants for the inositol 1,4,5-trisphosphate receptor gene (*itpr*) are larval lethals. In addition they exhibit delays in molting that can be rescued by exogenous feeding of 20-hydroxyecdysone. Here we show that mutants for adenylate cyclase (*rut*) synergize, during larval molting, with *itpr* mutant alleles, indicating that both cAMP and $InsP_3$ signaling pathways function in this process. The two pathways act in parallel to affect molting, as judged by phenotypes obtained through expression of dominant negative and dominant active forms of protein kinase A (PKA) in tissues that normally express the $InsP_3$ receptor. Furthermore, our studies predict the existence of feedback inhibition through protein kinase A on the $InsP_3$ receptor by increased levels of 20-hydroxyecdysone.

THE two major intracellular second messengers cyclic AMP (cAMP) and Inositol 1,4,5-trisphosphate ($InsP_3$) are generally considered as modulating independent biological processes. However, several biochemical and pharmacological studies, of invertebrate and vertebrate tissues and cell lines, suggest that pathways activated by the two second messengers can interact to regulate complex cellular and physiological responses (Bygrave and Roberts 1995; Chatton *et al.* 1998; Chyb *et al.* 1999; Geneviève *et al.* 1999; Gueorguiev *et al.* 1999; Ramírez *et al.* 1999). A genetic analysis of interactions between the two pathways in the context of a whole organism has not been attempted in any system, even though such a study is likely to provide new insights into multiple levels of "signaling crosstalk." In this study we looked at interactions between cAMP and $InsP_3$ signaling mutants in *Drosophila melanogaster*. Previous work has shown that when the gene for a crucial component of $InsP_3$ signaling (the $InsP_3$ receptor) is disrupted in Drosophila, larval lethality ensues, accompanied by delays in molting (Venkatesh and Hasan 1997). Similar lethality and delayed molting phenotypes have been observed in the case of protein kinase A (PKA) mutants in Drosophila (Lane and Kalderon 1993). Expression of protein kinase A has also been demonstrated in the ring gland, which is part of the neuroendocrine axis controlling molting in Drosophila (Harvie *et al.* 1998). These observations suggest that the regulation of larval molting in Drosophila requires signaling through both cAMP and $InsP_3$ second messenger pathways. To investigate possible interactions between these pathways we have looked at combinations of $InsP_3$ receptor mutant alleles with mutants of the cAMP pathway. Mutants that decrease cAMP levels (*rut*) significantly enhanced the delayed molting phenotype of $InsP_3$ receptor mutants (*itpr*). To understand if the two pathways interact directly or function in parallel we studied the effect of ectopic expression of dominant active and dominant negative forms of PKA—a known effector of cAMP signaling. This was done both in the presence and absence of *itpr* mutant alleles and an *itpr* cDNA transgene that can rescue lethality and molting delays in $InsP_3$ receptor mutants. Our results show that $InsP_3$ signaling acts in parallel with a cAMP pathway to produce a precisely timed larval molt, and suggest that regulation of 20-hydroxyecdysone peaks during molting is through a feedback loop requiring PKA and the $InsP_3$ receptor.

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MATERIALS AND METHODS

Drosophila stocks: The *itpr* mutant alleles used in this study have been described earlier (Venkatesh and Hasan 1997). *UAS-itpr* transgenic flies were obtained by microinjection of a pUAST-*itpr* cDNA plasmid construct into Drosophila embryos and generating stable transgenic lines according to standard procedures (Spradling 1986). A total of three independent lines were obtained. The cDNA construct was made by inserting a fragment of 9.86 kb into the *Xho*I restriction enzyme site of the pUAST vector (Brand *et al.* 1994). The cDNA fragment that includes all of the embryonic cDNA (excepting a few hundred base pairs of the 3′ untranslated region) was

obtained from the plasmid pBSK-itpremb by digestion with *Xho*I (Sinha and Hasan 1999). *rutabaga* alleles rut^{178} and rut^{2080} were obtained from the laboratory of Dr. R. L. Davis. The two PKA transgenic strains *UAS-R** and *UAS-mC** were obtained from Dr. D. Kalderon.

Larval staging and molting profiles: Staging of larvae so as to obtain precise molting profiles was according to the protocol described in a previous publication with minor modifications (Venkatesh and Hasan 1997). All homozygous lethal *itpr* alleles were placed against the TM6 balancer chromosome marked with a dominant mutation *Tubby* (*Tb*), which was used for distinguishing homozygous larvae from heterozygotes. Briefly, larvae at 56–64 hr after egg laying (AEL) were transferred to fresh food vials in batches of 50. Each time point was obtained from a minimum of five such transfers. The larvae were washed into saline solution and screened for lethality and stage of development at appropriate time intervals. Larvae were scored as live if they were motile in the saline. Pupae were considered as viable unless they had turned black and did not eclose soon after. Heterozygous controls were included with each staging experiment to exclude differences due to environmental conditions. For 20-hydroxyecdysone feeding of larvae a total of 100 larvae per genotype were transferred in batches of 20 or 25 larvae per vial into standard food containing 1 mg/ml of 20-hydroxyecdysone. Staging and screening of the larvae was as described previously (Venkatesh and Hasan 1997). All staging experiments were carried out at 25° unless indicated otherwise in figure legends or text.

Conversion of $itpr^{1664}$ to $itpr^{1664GAL4}$: $itpr^{1664}$ has a $P[ry^+]$ insert in the 5′ untranslated region (UTR) of the *itpr* gene (Sinha and Hasan 1999). We generated males of the genotype *w*; $P[gal4,w^+]$/*Cyo*; $itpr^{1664}$/*TM2,* Δ*2-3, Ubx* and mated them to *w; TM3Sb/TM6Tb* females. The next generation was screened for the presence of the markers w^+ *and CyO* in the same organism. These were mated with the *w; TM3Sb/TM6Tb* strain in single pairs. Lines in which the w^+ phenotype mapped to the third chromosome were analyzed further by polymerase chain reaction (PCR) using two primer pairs in two independent reactions. In both pairs the 3′ primer used was 94-3, which maps close to the insert site in $itpr^{1664}$ (Sinha and Hasan 1999). The 5′ primer was derived from the *P*-element sequence in one case and *GAL4* sequence in the other reaction. PCR products of the appropriate size were obtained in both reactions (Venkatesh 2000). Sequencing of the PCR product obtained using the 5′ *GAL4* primer confirmed the presence of a *GAL4* insert at the same position in the 5′ UTR of the *itpr* gene as was obtained earlier for the $P[ry^+]$ insert in $itpr^{1664}$ (Sinha and Hasan 1999).

Staining for β-galactosidase: β-galactosidase staining of larval tissues was performed with minor modifications of a published protocol after dissecting the appropriate tissues from third instar larvae of the genotype *UAS-lacZ;* $itpr^{1664GAL4}$ (Raghu and Hasan 1995). The tissues were kept in staining solution overnight at room temperature and subsequently mounted in 70% glycerol. Control larval tissues of the genotypes *UAS-lacZ* and $itpr^{1664GAL4}$ were stained along with the experimental larvae. No specific staining of tissues was observed in either of these two strains.

Molecular biology: Transcript analysis for *E74* mRNAs was done as described in a previous publication (Venkatesh and Hasan 1997). For reverse transcriptase PCR (RT-PCR) analysis the appropriate larval tissues were hand dissected using RNAse-free glassware and dissection instruments, and stored on dry ice for no longer than 2 hr prior to RNA extraction. The protocol used for RT-PCR has been published recently (Raghu *et al.* 2000). Control reactions, in which no RNA was added, were carried out with every primer pair to ascertain the absence of any PCR contaminants. The reaction products were separated on 1.5–1.9% agarose gels, on the basis of the size of the predicted DNA band.

RESULTS

Adenylate cyclase and $InsP_3$ receptor mutant alleles have a negative synergistic affect on larval molting: A single gene locus, referred to as *itpr,* codes for the $InsP_3$ receptor in Drosophila (Hasan and Rosbash 1992; Yoshikawa *et al.* 1992). A homozygous null allele for the *itpr* gene ($itpr^{90B0}$) can survive up to the second instar larval stage, with a delay in first to second instar molting as the only obvious phenotype. Mutant alleles for the *rutabaga* gene, one of six adenylate cyclase genes present in the Drosophila genome, are viable and fertile (Levin *et al.* 1992). The larval molting profile of rut^{2080} homozygotes (Figure 1A), as well as a heteroallelic combination of rut^{2080}/rut^{178} (Figure 1E), appears normal when compared to the wild-type strain Canton-S (CS; data not shown). In contrast, larvae homozygous for both rut^{2080} and $itpr^{1664}$ are highly abnormal as judged by their inability to molt from second to third instar stage and their lethality as second instar larvae (Figure 1C). This phenotype is considerably stronger than either the molting delays or lethality shown by $itpr^{1664}$ homozygotes, which can molt to third instar (Figure 1B) and eventually die primarily as pupae, although a few adult survivors do emerge (Venkatesh and Hasan 1997). We also examined individual larvae of the genotypes rut^{2080}/rut^{2080}; $itpr^{1664}/itpr^{1664}$, $itpr^{1664}/itpr^{1664}$, and rut^{2080}/rut^{2080} at 80–88 hr AEL to ascertain their phenotype (Figure 2, G–I). In all cases the larvae looked normal but, depending on the genotype, were at varying stages of larval development as evidenced from their tracheal endings and mouth hooks. Specifically it was observed that the presence of rut^{2080} in the background of a weak *itpr* allele ($itpr^{1664}$) had a negative synergistic affect on larval molting. Several of these double mutant larvae had tracheal endings characteristic of the first instar stage (Figure 2G).

To assess the level at which adenylate cyclase interacts with the $InsP_3$ receptor we looked at the effect of rut^{2080} homozygotes on an *itpr* null allele—$itpr^{90B0}$. Presence of rut^{2080} homozygotes in the background of $itpr^{90B0}$ enhanced the delayed molting phenotype of the *itpr* null allele and prevented them from progressing to second instar (Figure 1D). The phenotype of these double mutant larvae at 80–88 hr was further analyzed after mounting and observing under a compound microscope (Figure 2, A, B, D, and E). Interestingly, some of the larvae appeared unable to molt from first and second instar, even though they had developed second instar spiracles and mouth hooks (Figure 2, B and E). $itpr^{90B0}$ homozygotes on their own exist as second instar at 80–88 hr (Figure 1D, solid diamond; Figure 2, C and F). Enhancement of the *itpr* null phenotype by a *rut* mutant allele demonstrates that *rut* functions either downstream or

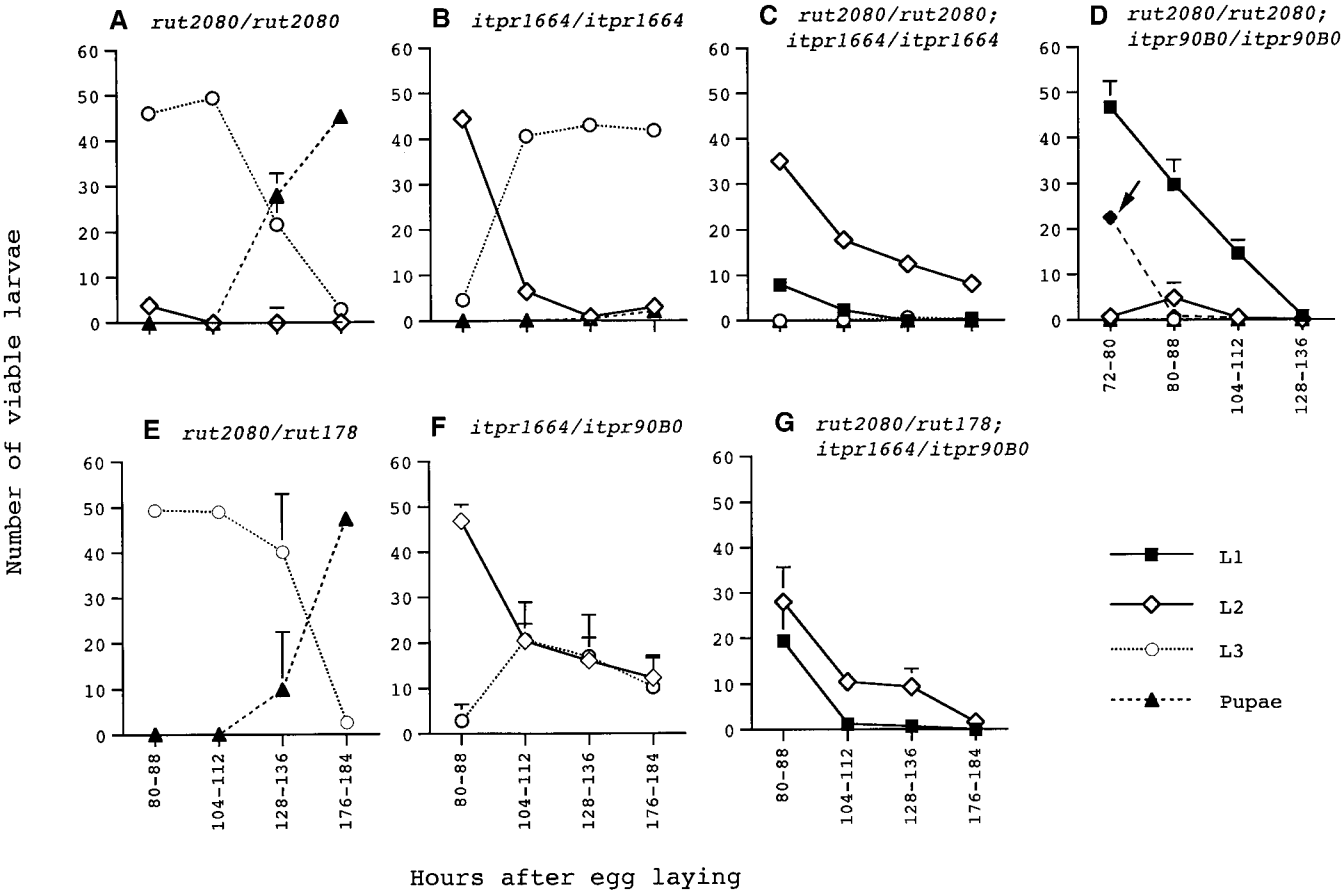

FIGURE 1.—*rutabaga* mutant alleles enhance lethality and molting delays in *itpr* mutant larvae. Symbols for each developmental stage are shown on the right. L1, first instar larvae; L2, second instar larvae; L3, third instar larvae; P, pupae. The solid diamond in D indicates second instar larvae of the genotype $itpr^{90B0}/itpr^{90B0}$ (VENKATESH and HASAN 1997). Each data point is derived from the average of five vials containing 50 larvae of the appropriate genotype. The error bars denote standard deviation. *x*-axes for A–C are as marked in E–G.

in parallel with the $InsP_3$ receptor during larval molting. To rule out the possibility of either allele-specific effects or an effect of the genetic background we also looked at molting in a heteroallelic mutant combination of *rut* and *itpr*, and found a similar enhancement of the delayed-molting phenotype (Figure 1, F and G).

Generation of an *itpr-GAL4* line and *Itpr* gene expression in larval tissues: From the synergistic interaction seen between *rut* and *itpr* alleles it would appear that both cAMP and $InsP_3$ signaling pathways regulate Drosophila larval molting. However, since enhanced lethality could arise as a consequence of general physiological defects in the organism, it was important to determine if the interaction between the two pathways occurs in a cell- or tissue-specific manner. For this purpose we used the UAS-*GAL4* system (BRAND *et al.* 1994). An *itpr-GAL4* line, referred to as $itpr^{1664GAL4}$ or *1664GAL4* for short, was generated by genetic conversion from $itpr^{1664}$ (VENKATESH and HASAN 1997), using a recently published genetic method (SEPP and AULD 1999). Presence of the *GAL4* insert in the *itpr* upstream region was confirmed by molecular analyses (see MATERIALS AND METHODS). The expression pattern of *1664GAL4* in larval tissues is shown in Figure 3. It is expressed in several neurons of the ventral ganglion and larval brain, in addition to the ring gland, fat body, and salivary glands (Figure 3, A–C). All of these tissues, except the salivary gland, are considered necessary for the process of larval molting. Drosophila prothoracicotrophic hormone (PTTH) activity is found in extracts of the brain and ventral ganglion (HENRICH *et al.* 1987, 1999; KIM *et al.* 1997). PTTH acts on the ring gland to initiate synthesis and secretion of the prohormone 3-dehydroecdysone, which is finally converted to 20-hydroxyecdysone in target tissues such as the larval fat body (GILBERT *et al.* 1996). Within the ring gland, cells that constitute the corpus allatum do not stain in the *1664GAL4* strain (Figure 3B). These cells are the source of juvenile hormone (JH), which is a modulator of ecdysteroid action (GILBERT *et al.* 1996). No expression occurs in larval imaginal discs (Figure 3D).

Immunohistochemical staining with an antiserum to the Drosophila $InsP_3$ receptor had earlier established the presence of the $InsP_3$ receptor in the larval ring

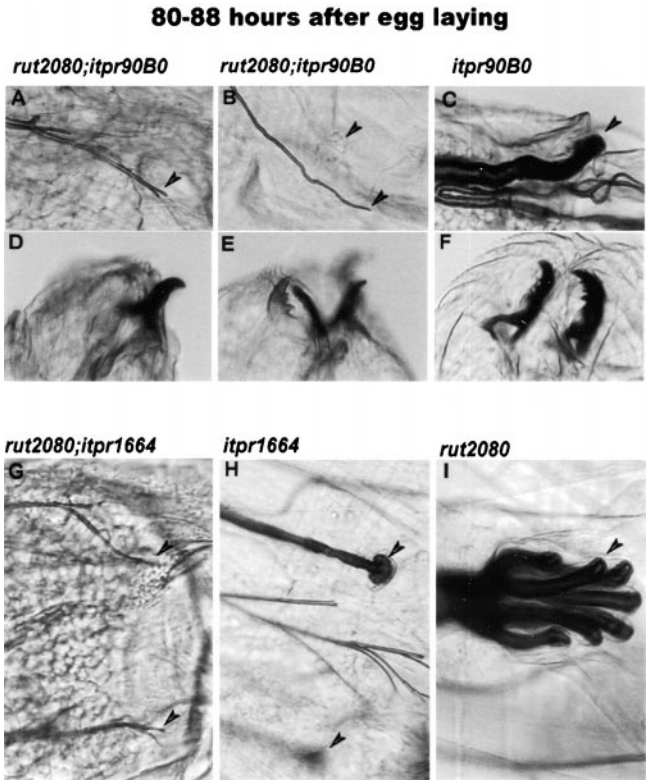

Figure 2.—Phenotypes associated with delayed molting observed in *rut-itpr* mutant strains at 80–88 hr after egg laying. Genotypes are denoted at each top. (A) Anterior tracheal end, characteristic of first instar larvae. (B) The top arrowhead indicates a second instar spiracle in the presence of a characteristic first instar tracheal end. The spiracle is not aerated and hence appears transparent. (C) An aerated trachea and second instar spiracle. (D) A first instar mouth hook characterized by the absence of teeth. (E) Both first and second instar mouth hooks are visible in the *rut-itpr* double mutant strain. (F) Second instar larval mouth hooks with four to five teeth. In G–I the arrowheads indicate first instar tracheal ends, second instar spiracles, and a third instar spiracle, respectively. A majority of wild-type larvae have spiracles similar to the one shown in I at this stage of development.

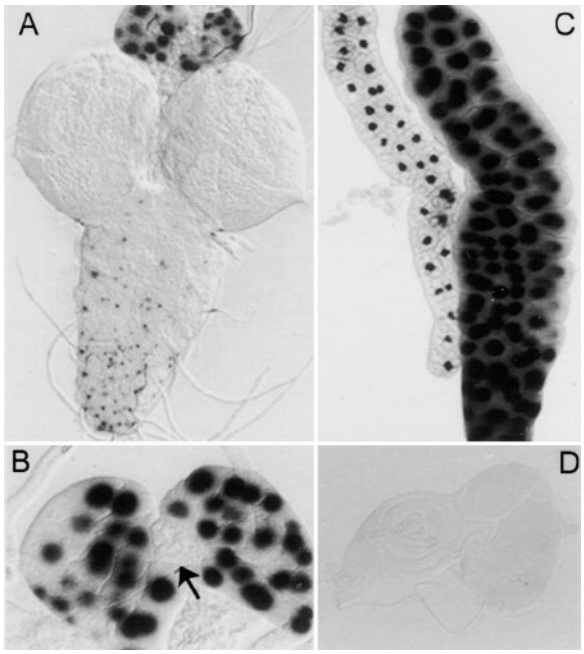

Figure 3.—β-Galactosidase staining of third instar larval tissues obtained using the *itpr*^{1664GAL4} strain. (A) A dissected larval brain with attached ring gland. (B) Ring gland at higher magnification. The arrow indicates unstained cells of the corpus allatum. (C) Salivary glands with a portion of fat body attached. (D) Eye-antennal imaginal disc.

gland (Venkatesh and Hasan 1997). Expression of *1664GAL4* in organs other than the ring gland appears to be in a subset of tissues that normally express the $InsP_3R$ in larvae (Raghu and Hasan 1995; our unpublished results). We reconfirmed the presence of $InsP_3R$ expression in larval tissues that express *1664GAL4* by RT-PCR. As shown in Figure 4, RNA for the $InsP_3R$ is detectable in larval brains including ring glands (lane 1), larval brains alone (lane 2), and in the fat body (lane 3). With primers that span the cDNA region from 3675 to 4235 bp, RNA obtained from larval brains gave two closely spaced PCR products. After cloning and sequencing, these PCR products were found to be identical to the two previously identified $InsP_3$ RNA splice forms isolated from adult heads (Figure 4, lane 4; Yoshikawa *et al.* 1992) and embryos (Figure 4, lane 5; Sinha and Hasan 1999), with a difference of 27 additional base pairs in the slower migrating form (data not shown). Thus all larval tissues that express *GAL4* in the *1664 GAL4* line also normally express the $InsP_3$ receptor, with the additional complexity that two isoforms exist in larval brain cells.

Molting delays caused by tissue-specific expression of a dominant-negative protein kinase A transgene: The *1664GAL4* line was used to drive expression of a dominant negative PKA transgene, referred to as *UAS-R**. PKA is a direct downstream effector of cAMP and the dominant negative contruct used in this study has been used successfully to study developmental and physiological phenotypes in Drosophila larvae (Li *et al.* 1995; Kiger *et al.* 1999). The molting profile of larvae of the genotype *UAS-R*/+; 1664GAL4/+* is shown in Figure 5A. They exhibit marked delays in molting, particularly at the transition time points of 80–88 hr (L2 to L3 transition), 128–136 hr (L3 to pupal transition), and 248–256 hr (pupal to adult transition), as compared with controls carrying only the *UAS-R** transgene (Figure 5B). No significant lethality was observed, presumably due to the tissue specificity and low level of GAL4 expression in the *1664GAL4* strain. Similar molting delays were obtained in larvae of the genotype *heat-shock gal4/UAS-R** when they were grown at 29°. At higher temperatures, significant levels of lethality were observed (data not shown). Next we asked the question as to whether the delayed molting observed with the *UAS-R** transgene was enhanced in an *itpr* mutant back-

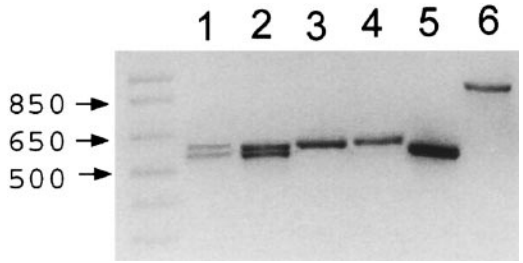

FIGURE 4.—$InsP_3$ receptor transcripts in larval tissues. Products were obtained by reverse-transcription and PCR (RT-PCR) of RNA isolated from (1) larval brains plus ring glands; (2) larval brains; (3) larval fat body; and (4) adult heads, using primers specific for the cDNA region 3673–4235. The RT-PCR reactions were separated on a 1.9% agarose gel. Positions of marker bands in base pairs are indicated on the left. The same primer pair was used for amplifying DNA products from an embryo cDNA clone (lane 5) and genomic DNA (lane 6).

ground, as might be expected from the *rut-itpr* interaction. In combination with a weak allele (Figure 5C; *1664GAL4/itpr*^{1664}), a longer delay in molting was observed at the L3 to pupal transition (152–160 hr). With a stronger allele (Figure 5E; *1664GAL4/itpr*^{90B0}) enhancement of the delay was not obvious. Significantly, expression of the *UAS-R** transgene did enhance lethality in both *itpr* allelic combinations, as is evident on comparing Figure 5, C and D, and E and F. It is possible that increased lethality of third instar larvae in *UAS-R*/+; 1664GAL4/itpr*^{90B0} organisms masks the delayed molting phenotype of this strain.

The allelic strength of *1664GAL4* appears to be weaker than its parent line *itpr*^{1664}. This is evident from comparing their respective molting profiles in combination with *itpr*^{90B0}, a null allele (Figures 1F and 5F). Since molecular analysis of *1664GAL4* indicates that the position of the *P* insert in both lines is identical (VENKATESH 2000), this change is probably due to the smaller size of the *P[w*$^+$*, GAL4]* insert as compared with the *P[ry*$^+$*]* insert in *itpr*^{1664}.

Delayed molting in larvae expressing dominant negative PKA can be rescued by exogenous 20-hydroxyecdysone: The observation that *UAS-R** expression driven by an *itpr-GAL4* line (*i.e.*, *1664GAL4*) can delay molting supports the idea that both $InsP_3$ and cAMP signaling are required in the same tissue and cell type, possibly the ring gland. This idea is further strengthened by our next observation that feeding 20-hydroxyecdysone to *UAS-R*/+;1664GAL4/+* larvae rescues the transition delay from second to third larval instar (Figure 6A). In addition, transcripts of a 20-hydroxyecdysone-inducible gene, *E74*, were altered and reduced (Figure 6B, lanes 1, 4, and 7) at specific larval stages as compared to control larvae (Figure 6B, lanes 2, 3, 5, 6, and 8). The level of *E74* gene transcripts can be used as an indirect measure of 20-hydroxyecdysone levels in larvae, since this gene has two ecdysone-inducible promoters that are directly activated by the binding of 20-hydroxyecdysone

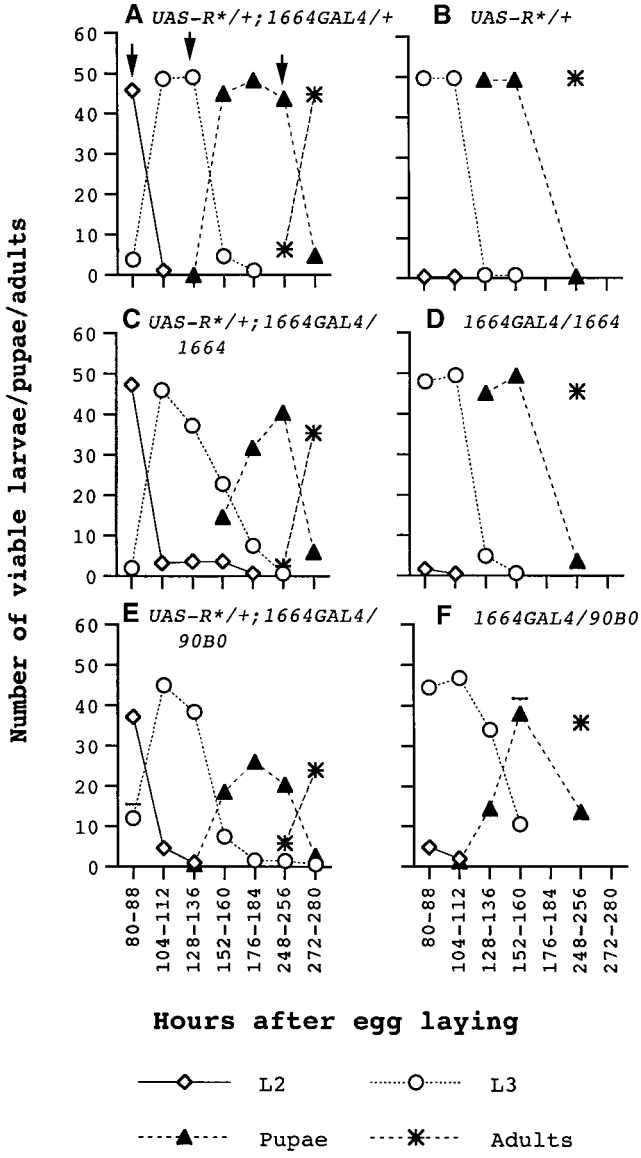

FIGURE 5.—Molting profiles of larvae expressing a dominant negative protein kinase A transgene (*UAS-R**). Results from expressing the transgene under the control of *1664GAL4*, in different genetic backgrounds, are presented on the left (A, C, and E). Arrowheads indicate time points where maximum differences are observed from larvae of the control genotypes shown in B, D, and F. *itpr*^{1664GAL4}, *itpr*^{1664}, and *itpr*^{90B0} are depicted as *1664GAL4*, *1664*, and *90B0*.

and the ecdysone-receptor complex (KARIM and THUMMEL 1991). The promoter for the *E74B* transcript is activated by lower levels of 20-hydroxyecdysone and inhibited by higher levels; the promoter for *E74A* is activated by higher levels of 20-hydroxyecdysone. *E74* gene transcripts thus follow an invariant order of appearance in which *E74B* always appears before *E74A* in response to a pulse of 20-hydroxyecdysone (THUMMEL *et al.* 1990; KARIM and THUMMEL 1991, 1992). Lower levels of *E74* transcripts in *UAS-R*; 1664GAL4* larvae, taken together with the data in Figure 5A, strongly suggest that 20-

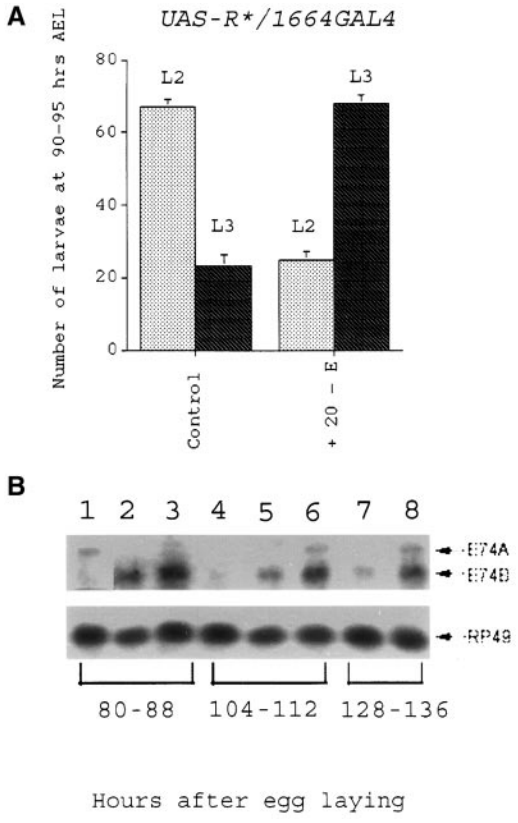

FIGURE 6.—Dependence of molting delays in *UAS-R*; 1664GAL4* larvae on 20-hydroxyecdysone (20-E). (A) 20-hydroxyecdysone feeding of early second instar larvae of the genotype *UAS-R*/+; 1664GAL4/+* rescues their delay in molting. Control larvae of the same genotype were transferred into medium containing ethanol instead of 20-hydroxyecdysone and screened in parallel. Ethanol was the solvent used for making up the 20-hydroxyecdysone solution. (B) A Northern blot with RNA isolated from staged larvae of the genotype *UAS-R*/+; 1664GAL4/+* (lanes 1, 4, and 7), *UAS-R*/+*, *+/+* (lanes 2 and 5), and *+/+; 1664GAL/+* (lanes 3, 6, and 8). The blot was hybridized sequentially to an *E74* gene probe (top; KARIM and THUMMEL 1991) and an *RP49* gene probe (bottom).

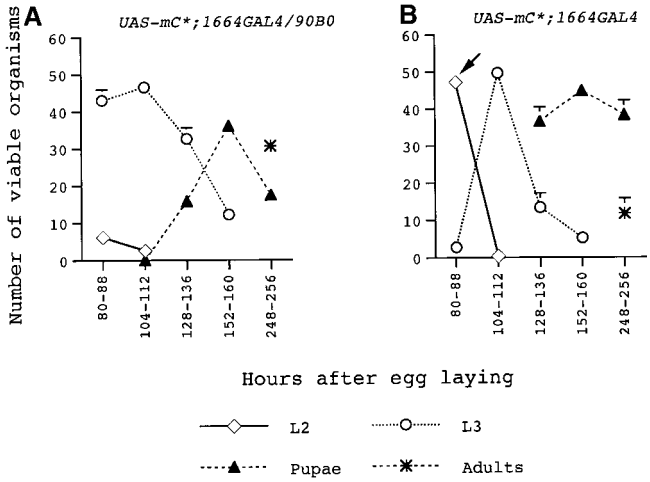

FIGURE 7.—A dominant active transgene for PKA (*UAS-mC**) does not rescue molting delays in *itpr* mutants. Molting profile of *UAS-mC*/+; 1664GAL4/90B0* larvae (A) and *UAS-mC*/+; 1664GAL4/+* larvae (B) is shown. The arrowhead indicates a delay in the L2 to L3 transition.

hydroxyecdysone peaks are either delayed or lowered at all analyzed stages of molting. Thus, both *itpr* mutant alleles and *UAS-R*/+;1664GAL4/+* larvae exhibit molting delays with similar physiological characteristics.

Molting phenotypes of larvae expressing a dominant active form of PKA: Results obtained so far suggest that signaling through adenylate cyclase (*rut*) and PKA as well as the $InsP_3$ receptor is required for correct timing of larval molts. Both pathways appear to regulate 20-hydroxyecdysone levels as demonstrated by the fact that feeding of 20-hydroxyecdysone can rescue disruption of either pathway. However, these data do not say if the two pathways interact directly or function in parallel. To test these two alternatives a dominant active form of PKA (*UAS-mC**) was expressed under the control of *1664GAL4*. If cAMP signals are directly downstream of $InsP_3$ signaling, we predicted that expression of the *UAS-mC** transgene should rescue molting delays in *itpr* mutant larvae. *UAS-mC** has been tested and found to work in Drosophila larval tissues (THE *et al.* 1997). On expressing *UAS-mC** with *1664GAL4* in the background of one copy of $itpr^{90B0}$, no change was observed in the molting pattern as compared with control larvae of the genotype *1664GAL4/*$itpr^{90B0}$ (compare Figures 7A and 5F). This result indicates that cAMP signaling is not directly downstream of the $InsP_3$ receptor. The *UAS-mC** transgene is active as evinced by the molting profile of larvae of the genotype *UAS-mC*/+;1664GAL4/+* (Figure 7B). Surprisingly, these larvae also have a delay in molting, which is most apparent at the L2 to L3 transition at 80–88 hr AEL (Figure 7B).

The observation that expression of both dominant negative and dominant active forms of PKA can delay larval molting suggests a dual role for PKA during this process, possibly at different temporal stages. To investigate this possibility we fed *UAS-mC*/+;1664GAL4* larvae with 20-hydroxyecdysone. As shown in Figure 8A the L2 to L3 molting delay caused by *UAS-mC** is not rescued by feeding of 20-hydroxyecdysone. Delay in molting caused by ectopic expression of *UAS-mC** thus appears to be at a signaling step different from that affected by *UAS-R**. This was confirmed further by expressing an *itpr* cDNA transgene with *UAS-mC** and *UAS-R** (Figure 8, C and D). While the *itpr* cDNA can rescue the L2 to L3 delay caused by *UAS-mC** it had no significant affect on the delay caused by *UAS-R*. These experiments were carried out at 29° to enhance *GAL4* activity. Similar results were also obtained at 25° (data not shown).

The *itpr* cDNA transgene used in these experiments is derived from an embryonic cDNA for the Drosophila $InsP_3$ receptor (SINHA and HASAN 1999). Its functioning in larvae was determined by its ability to rescue lethality

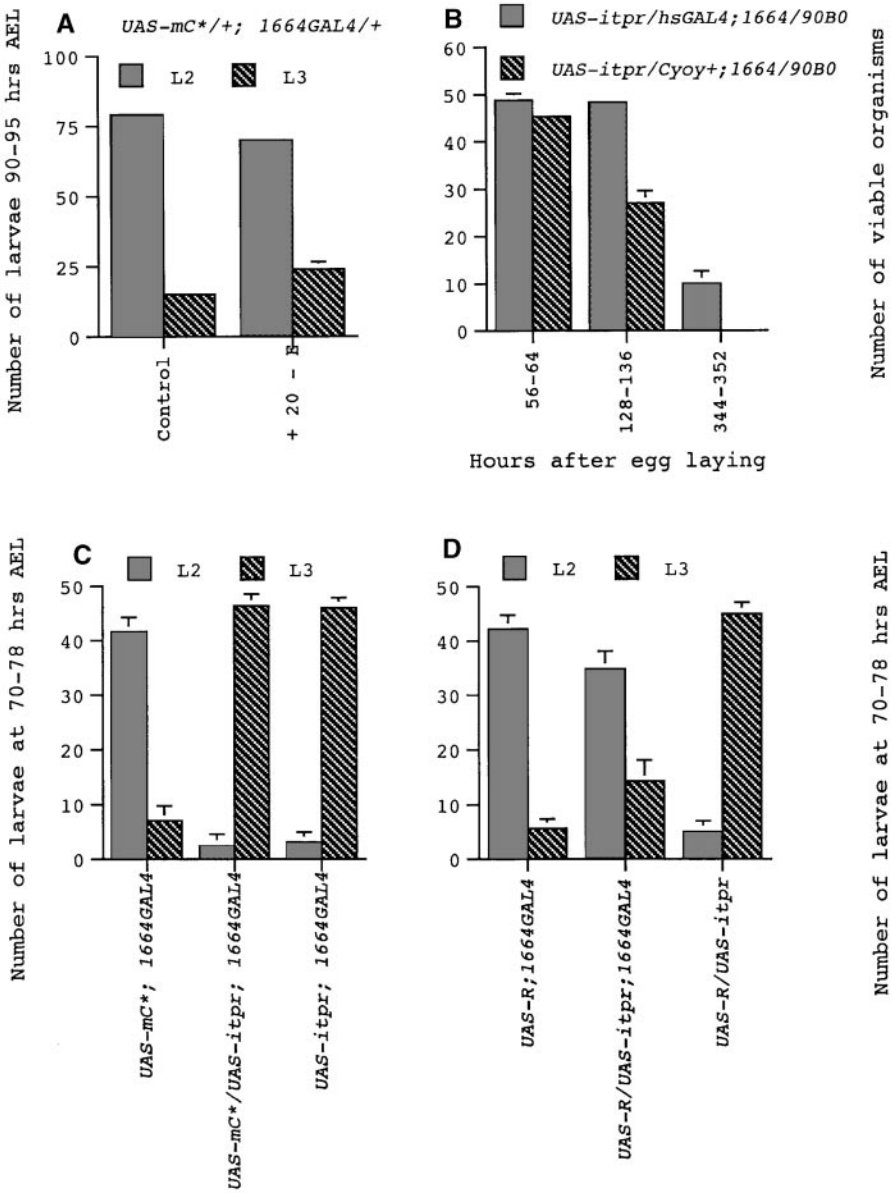

FIGURE 8.—Delay in molting in larvae expressing dominant active PKA can be rescued by overexpression of the *itpr* gene but not by 20-hydroxyecdysone. (A) Larvae of the genotype *UAS-mC*/+;1664GAL4/+* were fed with 20-hydroxyecdysone (20-E) as described in MATERIALS AND METHODS and Figure 5. The numbers of second and third instar larvae (L2 and L3) are not significantly different between larvae fed on food containing 20-hydroxyecdysone and control food. (B) Expression of the *UAS-itpr* cDNA transgene under control of a *hsp70-GAL4* construct (*hsGAL4*) at 25° rescues lethality in $itpr^{1664}/itpr^{90B0}$ (*1664/90B0*) larvae completely (128–136 hr). A partial rescue of lethality is seen in adults (344–352 hr). All genotypes have the markers *y, w* on the first chromosome. Larvae with the *hsGAL4* transgene were distinguished from control larvae, carrying a *Cyoy+* balancer, by the absence of darkly pigmented mouth hooks. (C and D) Numbers of second instar (L2) and third instar (L3) larvae in vials with the indicated genotypes. The larvae were counted at 70–78 hr after egg laying since the experiment was done at 29°. This time point corresponds to the 90- to 95-hr developmental time point at 25°. Each data point is derived from the average of five vials containing 50 larvae of the appropriate genotype. The error bars denote standard deviation.

in an *itpr* mutant lethal combination ($itpr^{1664}/itpr^{90B0}$). The *UAS-itpr* transgene completely rescued both molting delays (data not shown) and lethality in $itpr^{1664}/itpr^{90B0}$ larvae up to 128–136 hr after egg laying (Figure 8B). At later stages, a partial rescue of lethality was observed where 10–15 adult survivors were obtained in strains with the transgene as compared with no adult survivors in the control (Figure 8B). Partial rescue of lethality at later stages is not surprising since the *itpr* cDNA used is the embryonic splice form and from Figure 4 it is clear that both the adult head and embryo splice forms are expressed in larval brains. Second, the levels of expression of the transgene may not be sufficient for complete rescue since it was driven by a *heat shock-GAL4* promoter at 25°.

These two key observations, *viz.*, that the delay caused by *UAS-mC** in L2 to L3 molting is not rescued by 20-hydroxyecdysone but is rescued by *UAS-itpr*, suggested a negative feedback loop after 20-hydroxyecdysone release, which requires both PKA and the $InsP_3$ receptor (see DISCUSSION).

DISCUSSION

Ca^{2+} and cAMP in insect molting: An understanding of the signaling pathways that control insect molting has come primarily from pharmacological and biochemical studies on lepidopterans with similar studies extending to Drosophila (GIRGENRATH and SMITH 1996; GU *et al.* 1996; SONG and GILBERT 1996; BIRKENBEIL 1998; HENRICH *et al.* 1999). These studies have shown that neural factors, which include the PTTH, stimulate the

prothoracic gland (a part of the ring gland in higher Dipterans including Drosophila) to synthesize and secrete ecdysone, which is subsequently converted to its active form of 20-hydroxyecdysone in other tissues. Biochemical and molecular analysis of PTTH isolated from lepidopterans and Drosophila has shown that the peptide hormone is quite different in the two classes of insects, indicating that signaling downstream of PTTH in the prothoracic gland may also differ (Henrich *et al.* 1987; Kim *et al.* 1997). In fact, while extracellular calcium is required for secretion of ecdysone in both systems, cAMP has been demonstrated to be a second messenger only in lepidopterans (Smith *et al.* 1996; Henrich *et al.* 1999). Molecular identification of other key players, such as the PTTH receptor and the channel for entry of extracellular calcium, is still awaited. The first indication that insect larval molting is regulated by $InsP_3$ signaling came from analysis of Drosophila mutants for the $InsP_3$ receptor gene (Venkatesh and Hasan 1997). Data presented here now implicate, in addition, the cAMP pathway in control of larval molting in Drosophila. Since exogenous 20-hydroxyecdysone can rescue the molting delays caused by disruption of either pathway, it is likely that both pathways control 20-hydroxyecdysone levels during molting. Due to technical difficulties associated with measuring 20-hydroxyecdysone levels in Drosophila larvae, we have not been able to carry out these measurements directly. Instead, transcript levels of an ecdysone-inducible gene, *E74,* were used as an indirect measure of 20-hydroxyecdysone levels. Conceivably, $InsP_3$ and cAMP could also function in regulating *E74* transcription, although there is no published evidence to support this possibility. An alternate mechanism by which $InsP_3$ and cAMP could regulate larval molting is through controlling levels of juvenile hormone. We think this unlikely for two reasons. First, molting delays caused by disruption of either the $InsP_3$ or cAMP pathway occur at all transition points from second larval to third larval instar, third larval instar to pupal, and pupal to adult stages. In contrast, JH action is thought to be primarily at the second to third larval instar transition for maintaining a larval to larval molt. It is downregulated at the third larval instar to pupal transition and is absent from pupal to adult transition (Riddiford 1993). Second, *1664GAL4* does not detectably express in cells of the corpus allatum, which is the region of the ring gland that synthesizes JH (Figure 3B).

$InsP_3$ signaling during synthesis and secretion of steroid hormones: Interestingly, steroid secretion by the adrenal fasciculata-reticularis cells of mammalian adrenal glands in response to adrenocorticotrophic hormone occurs through the cAMP pathway, while $InsP_3$-mediated Ca^{2+} release is required for the steroidogenic action of Angiotensin II on adrenal glomerulosa cells (Won and Orth 1995; Burnay *et al.* 1998). An increase in cytosolic Ca^{2+} levels is thought to affect multiple steps in mammalian steroid biosynthesis, including one crucial step that requires the transfer of endogenous cholesterol from the outer to the inner mitochondrial membrane (Cherradi *et al.* 1996, 1997; Cherradi and Capponi 1998). Our data presented here support a similar model in which 20-hydroxyecdysone levels are regulated through activation of both $InsP_3$ and cAMP signals. The presence of multiple genes encoding adenylate cyclases allows *rut* mutant alleles to proceed through molting normally. Presumably, however, activity of the alternate adenylate cyclase(s) is dependent on $InsP_3$ receptor function since removal of the *itpr* gene in *rut* mutant backgrounds leads to phenotypes that are synergistic. Activation of the two second messenger pathways probably occurs in the ring gland via PTTH and other as yet unidentified neural factors. Alternate explanations whereby $InsP_3$ and/or cAMP signaling are required for PTTH release from neurons or during conversion of 20-hydroxyecdysone precursors to 20-hydroxyecdysone cannot be ruled out at this stage. In either event the two pathways act in parallel to maintain 20-hydroxyecdysone levels perhaps via nonoverlapping downstream targets.

Proposed existence of a negative feedback loop requiring PKA and the $InsP_3$ receptor during Drosophila larval molting: Since ecdysone secretion occurs as tightly regulated peaks, preceding each molt, inherent in the system should be a mechanism that inhibits ecdysone secretion once the peak level has been reached (Riddiford 1993; Gilbert *et al.* 1996). On the basis of data from the *UAS-mC** transgene in Figure 8, we suggest that increased levels of 20-hydroxyecdysone in the hemolymph initiate a negative feedback loop that requires PKA activation and inhibition of the $InsP_3$ receptor. Thus the activated PKA phenotype is not rescued by increased levels of 20-hydroxyecdysone, but is rescued by increased levels of the *itpr* transgene. Interestingly, the effect of the *UAS-mC** transgene on molting is also lost when *itpr* gene levels are reduced as in larvae of the genotype *UAS-mC*/+; 1664GAL4/itpr^{90B0}* (Figure 7A). This observation supports our idea that the *itpr* gene is downstream of the *UAS-mC** effect, and in addition suggests that the negative feedback is highly sensitive to levels of the *itpr* gene. While our results demonstrate interactions between the two signaling pathways, the molecular basis of these interactions is unknown as yet. Since mammalian $InsP_3$ receptors can be directly phosphorylated by PKA (Wojcikiewicz and Lou 1998; Haug *et al.* 1999) the possibility exists that a similar mechanism might operate in the negative feedback step predicted from our results in Figure 8. However, both predicted isoforms of the Drosophila $InsP_3$ receptor, which are present in larval tissues and derive from two known splice variant forms of the *itpr* cDNA (Yoshikawa *et al.* 1992; Sinha and Hasan 1999), lack putative PKA phosphorylation sites as determined by Prosite analysis (Hofmann *et al.* 1999). It is possible that a low-abundance isoform of the $InsP_3$ receptor exists in specific

larval cells that may be directly regulated by PKA. Additionally, there are almost certainly other unidentified players in this system that our study has not revealed as yet. It should be possible to identify some or all of these factors using suitable genetic interaction screens in the future.

The authors thank Pavan Kadandale and Rajnikanth Vangala for testing the PKA transgenes. This work was supported by a core grant from National Centre for Biological Sciences and Tata Institute of Fundamental Research and a Wellcome Collaborative Initiative Grant to G.H.

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Communicating editor: T. Schüpbach