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Follicle Cell Calmodulin in *Blattella germanica:* Transcript Accumulation during Vitellogenesis Is Regulated by Juvenile Hormone

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There is abundant calmodulin (CaM) in the oocytes and eggs of *B. germanica*. Whether oocytes accumulate CaM for immediate use or use at a later stage in their development is still unknown. We show that isolated follicle cells accumulate more CaM transcripts per unit RNA than any other control tissue. CaM transcript increases exponentially 4800-fold in follicles during the 96-hr vitellogenic period in the absence of cell division. This includes a 32-fold increase in total follicle RNA during the period and an 150-fold increase in relative titer of the CaM transcript. In comparison, levels of actin transcripts increase exponentially 1200-fold during the same developmental period. On the other hand fat body tissue shows little relative increase of CaM transcripts despite a 4-fold increase in total RNA over the 4-day developmental period. Both the CaM and actin transcripts are more highly concentrated in the Day 4 follicle cell layer, being found in 84- and 33-fold greater titer, respectively, than in fat body RNA. Deprivation of juvenile hormone (JH), by head ligation, not only causes atresia of the follicles, but also reduces accumulated CaM transcripts. Reestablishing JH titer by injection allows a selected population of follicles to develop to full size and also reinstates CaM transcript levels above that of unligated controls within 24 hr.

INTRODUCTION

Calcium metabolism is an important part of oocyte development. Sufficient hemolymph titer of Ca^{2+} is necessary for vitellogenin to bind to its oocyte membrane receptor and for its subsequent internalization (König *et al.*, 1988). In this instance, Ca^{2+} acts directly from the outside on the receptor (König *et al.*, 1988) and machinery for uptake. On the other hand, calcium within cells often does not act in its free ionic form but requires the presence of a binding protein as a mediator (Carafoli, 1987). Calmodulin (CaM) is a ubiquitous and multifunctional Ca^{2+}-dependent regulatory protein that binds to as many as 30 different target enzymes (Cheung, 1980; Means *et al.*, 1991). It is known that there is an abundance of CaM in germ cells (Klee and Vanaman, 1982). Further, during oogenesis CaM levels in the maturing oocyte undergo dramatic changes in titer that are characteristic of regulated proteins, suggesting a crucial role for CaM in oogenesis (Cicirelli and Smith, 1986; Zhang and Kunkel, 1992a) or later embryogenesis. It has been hypothesized that CaM is a key player in the physiology of the oocyte and follicle cells during vitellogenesis (Zhang, 1992).

In order to understand the role of CaM in the larger context of oogenesis our laboratory has undertaken a systematic study of the developmental changes and their causative factors in the ovariole of *B. germanica.* The panoistic ovariole of *B. germanica* is anatomically and developmentally simple. Only the terminal follicle undergoes vitellogenesis in each vitellogenic cycle. A follicle consists of primarily two cell types, the oocyte and a surrounding follicle cell epithelial layer. No cell divisions occur in the 6-day vitellogenic cycle but the binucleate follicle cell layer is growing in DNA content by endopolyploidy (Zhang and Kunkel, 1992b). Extensive changes occur in the structure of the terminal follicle cells during each vitellogenic cycle. The morphogenesis of follicle cells in the terminal follicle is accompanied by a dramatic increase in CaM in the enclosed oocyte (Zhang and Kunkel, 1994). Subsequent to ovulation and fertilization, CaM returns to low levels only after early embryogenesis, at the start of the embryonic growth phase accompanied by yolk utilization (Zhang and Kunkel, 1992a). These correlated events during vitellogenesis and embryogenesis, including the fluctuation in CaM titres, suggest a relationship that deserves investigation.

The synthetic rate of CaM by the follicle cell layer was determined to be 13-fold greater than that of oocytes and by extrapolation could produce all of the oocyte CaM present at ovulation (Zhang and Kunkel, 1994). However, the full significance of that result is in doubt for two reasons. First, measured CaM titers might underestimate actual levels due to sequestration of CaM by CaM binding proteins in vitellogenic follicles (Burgess *et al.*, 1984; Cicirelli and Smith, 1986; Floyd *et al.*, 1986; Zimmer

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and Van Eldik, 1989). Second, in studying the rate of CaM biosynthesis by the follicle cells and oocytes, the endogenous pool size of methionine had not been measured for either tissue. Hence, reported synthetic rates were minimal estimates for each tissue. Considering the volume difference between oocytes and follicle cells, it is still possible that the oocyte could produce all of its own CaM. In this study we examine follicular CaM transcripts during the vitellogenic growth phase. We show that CaM mRNA levels are exceptionally high in the follicle cells relative to the oocyte and other tissues and that this transcript level is regulated by juvenile hormone (JH).

MATERIALS AND METHODS

Animals

Cohorts of first parturition females of the German cockroach, *B. germanica*, were synchronously cultured at 30°C (Kunkel, 1973). Vitellogenic cycles were initiated by providing food at 30°C. *B. germanica* last instar male nymphs were isolated and allowed to metamorphose, providing a source of virgin males. A whole-animal paradigm for studying the effects of JH on ovarian function is based on the approximate 6-day cycle of terminal oocyte growth initiated by feeding. Starvation starting at 48 hr allows ovarian growth to continue and approach ovulation. Head ligation at 24 hr results in reduction of endogenous JH with recession in size of the terminal oocytes.

Tissue Preparation

Rapid dissection of ovaries from *B. germanica* females was carried out in chilled ISS (Anderson and Kunkel, 1990). The majority of the fat body remains associated with the abdominal integument of the carcass in the above dissection. The abdominal segments are separated from the carcass and a dorsal midline cut exposes the fat body tissue attached to the abdominal pleura. The fat body is gathered with forceps and severed from the body wall. Tissue obtained by this method is not devoid of associated tracheoles. The trilobed testes are obtained from the adult male abdomen and cleaned of associated fat body.

Injections

Earlier studies on JH-III-mediated vitellogenesis in *B. germanica* have shown that the injection of JH in an olive oil carrier is the most direct and reproducible method of hormone delivery *in vivo* when measuring both growth of the terminal oocyte (Kunkel, 1973) and induction of vitellogenin synthesis by the fat body (Kunkel, 1981). Treatment groups were maintained separately throughout experimentation to avoid transfer of hormone by animal contact.

Nucleic Acid Extractions

Total RNA was extracted from whole follicles dissected from ovaries, fat body, and testes by the protocol of Chomczynski and Sacchi (1986) as modified by Promega Inc. (1993). To produce follicle ghosts, whole follicles were punctured and their yolk extruded according to the method of Sato and Yamashita (1991). The resulting follicle ghosts were the source of follicle cell total RNA. RNA concentration was estimated spectrophotometrically using ultraviolet absorbance at 260 nm. One o.d. unit represents 40 μg of RNA. Total genomic DNA was isolated from the testes of male animals by the protocol of Sambrook *et al.* (1989).

Plasmids and Probes

The DNA fragment used in the hybridizations is a 500-bp *Nco*I–*Cla*I fragment of the plasmid pJFM 34, a gift of Dr. Kathryn Beckingham (Maune *et al.*, 1988). The plasmid contains the *Drosophila melanogaster* CaM-coding *Eco*RI fragments derived from a cDNA clone, cCD1 (Smith *et al.*, 1987; Maune *et al.*, 1988). The plasmid was digested and electrophoretically separated on a 1% agarose gel, and the 500-bp fragment was electroeluted and purified. The purified fragment was labeled using the random primer method with nucleotide mixtures containing digoxigenin uridine triphosphate (Genius systems, Boehringer–Mannheim). A digoxigenin-labeled actin RNA probe was used as a parallel control (Boehringer–Mannheim).

Hybridizations and Densitometry

The specificity of the CaM probe was determined by Northern analysis of total RNA derived from *D. melanogaster* pupae. The probe indicated the presence of a 1.7-kb and 1.9-kb transcript for CaM as reported by Smith *et al.* (1987). Genomic Southern blots (Sambrook *et al.*, 1989), suggested the presence of a single CaM gene in the *B. germanica* genome. Northern blots were performed with 15 μg of total RNA denatured in formaldehyde and separated in a 1.5% agarose gel. RNA was transferred to Magnagraph nylon transfer membranes (Micron Separations Inc.), and hybridized overnight in 5× SSC, 50% formamide, 0.02% SDS, 0.1% *N*-lauroylsarcosine, 2% blocking reagent for nucleic acid hybridization (Boehringer–Mannheim), 20 m*M* sodium maleate, pH 7.5, at 30°C. Washes were performed at a stringency calculated for a 10% mismatched hybrid (Boehringer–Mannheim). Blots were reacted with an alkaline phosphatase-conjugated antidigoxigenin antibody, and the color reaction

TABLE 1
CHANGES IN TOTAL RNA LEVELS IN FOLLICLES, FAT BODY, AND TESTES

Group	Analysis day	RNA (μg) Follicle (±SE)	Fat body (±SE)	Testis (±SE)
d0	0	0.06 (±0.00)	6.24 (±0.10)	2.13 (±0.13)
d1	1	0.17 (±0.01)	9.24 (±0.24)	2.46 (±0.16)
d2	2	0.39 (±0.02)	13.56 (±0.22)	2.92 (±0.11)
d3	3	0.67 (±0.04)	17.27 (±0.33)	3.21 (±0.18)
d4	4	1.90 (±0.11)	26.76 (±0.71)	3.49 (±0.14)
Starved	4	0.42 (±0.04)	—	—
Ligated	4	0.02 (±0.00)	—	—
Injected				
−JH	4	0.05 (±0.02)	—	—
+JH,				
responder	4	3.00 (±0.16)	—	—
atretic	4	0.02 (±0.00)	—	—

Note. Total RNA was extracted and optical density measurements at 260 nm were used to arrive at microgram amounts. Changes in RNA levels during a 4-day period postfeeding is expressed in micrograms per follicle for the whole follicle (1 follicle out of approximately 40 per animal), micrograms per animal for the fat body tissue, and micrograms per testis for testes. d0, d1, d2, d3, and d4, days postfeeding; Starved, starvation started at d2; Ligated, controls deprived of endogenous juvenile hormone by head ligation at d2; Injected, head-ligated groups injected at d3 with olive oil either −JH (without juvenile hormone) or +JH (with juvenile hormone). In the +JH group, follicles were separated into atretic and responder subgroups. Values represent averages from six collections of pooled tissues with 30 animals per collection.

was quantified by densitometry. Known amounts of total RNA were applied to the nylon membrane and the range of reactivity of both the probe and the total RNA preparation were monitored by densitometry. Quantitative assay samples were guided by a pilot assay. Experiments were repeated at least three times. Results were generally consistent between experiments but differences in the relative timing and spacing of observations and the dynamic nature of the process prevented pooling of the results to compute standard errors. All replicated experiments showed the same trends and the most complete data sets are illustrated. In preliminary experiments relative levels of CaM transcripts and actin measured by densitometry of dot blot assays were comparable to relative levels obtained by densitometry of parallel Northern blots; we therefore adopted the more rapid dot blot assays. To facilitate comparison of results obtained in the different experiments which we describe in this paper, we represent the results in the form of fat body units. The fat body unit was chosen due to its minimal developmental change with respect to CaM transcripts. We define one fat body unit as the amount of CaM transcript in 0.6 μg of total RNA of the fat body tissue at Day 4 measured by the densitometry of the color reaction described earlier. One fat body unit yields approximately 1500 color units in a typical densitometry assay, and multiples (up to threefold) of the RNA applied to the dot blot result in a similar multiple of color yield.

RESULTS

Total RNA in Growing Follicles

Changes in the amount of total RNA present in ovarian terminal follicles, fat body, and testis were measured during the 96-hr vitellogenic period following feeding (Table 1). Total RNA in female fat body increased from 6.24 μg per animal at 0 day postfeeding to 26.76 μg at Day 4, a 4-fold increase. Total RNA content per follicle increased from 0.06 μg at Day 0 to 1.9 μg per follicle, a 32-fold increase, over this developmental period. During the same 4-day period total RNA from testis increased marginally from 2.1 μg to 3.5 μg per testis.

Appearance of CaM and Actin Transcripts in Whole Follicles

Accumulation of CaM and actin transcripts was measured by densitometry. Due to the highly conserved nature of the CaM gene in a variety of species (Munjaal *et al.*, 1981), we were able to use the *D. melanogaster* cDNA probe for CaM. The CaM cDNA probe identified a 1.9-kb transcript through the entire 96-hr vitellogenic period and a 1.6-kb transcript at 72 and 96 hr in the whole-follicle total RNA (Fig. 1). Densitometry of dot blots indicates a 150-fold increase in CaM transcript levels relative to total RNA over the 96-hr period (Fig. 2). The actin gene probe identified two specific transcripts, 1.1 kb and 1.2 kb, respectively (not shown). Actin transcript levels in the ovariole increased 35-fold during the vitellogenic period, relative to total RNA (Fig. 3). Both CaM and ac-

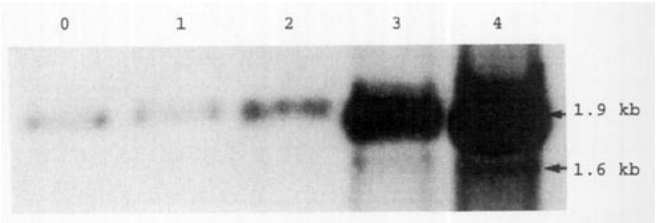

FIG. 1. Northern blot of CaM transcript accumulation during vitellogenesis in *B. germanica*, identified with a *Drosophila* CaM cDNA probe. Total RNA (15 μg) was loaded in each lane. Lanes 0, 1, 2, 3, and 4 represent total RNA collected at Days 0, 1, 2, 3, and 4 postfeeding, respectively. The size of the transcript was determined by loading 10 μg of RNA molecular weight marker from BRL and staining the gel with ethidium bromide. Arrows indicate a 1.9-kb transcript that appears early in development and a 1.6-kb transcript that appears at Day 3 in vitellogenesis.

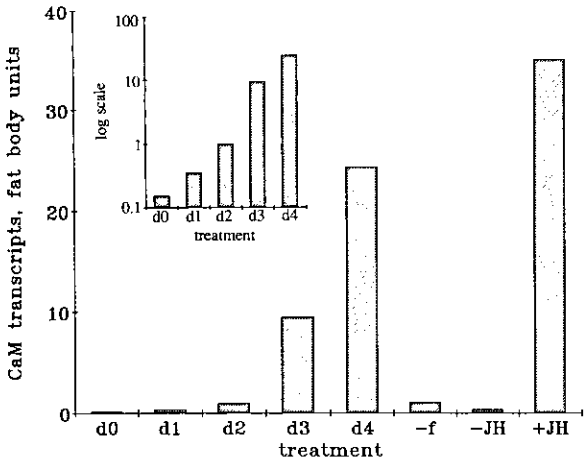

FIG. 2. Histogram of CaM transcript in follicles during development and JH regulation of accumulation. One fat body unit equals 1500 color units measured in the colorimetric assay described under Materials and Methods. d0, d1, d2, d3, and d4 represent Days 0, 1, 2, 3, and 4 postfeeding, respectively. −f, −JH, and +JH represent treatment groups of starving animals, head-ligated animals, and JH-injected animals, respectively. Olive oil control group is not shown. 0.60 μg of total RNA was used per dot. The log transform inset shows a 10-fold increase in the CaM transcript titer prior to the intense vitellogenic period and a log-linear increase over the entire period.

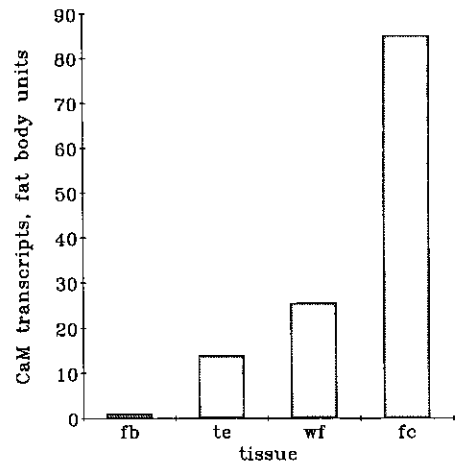

FIG. 4. Histogram of dot blot assay comparing CaM transcript levels in different tissues during follicle development. One fat body unit equals 1500 color units measured in the colorimetric assay described under Materials and Methods fb, fat body; te, testis; wf, whole follicle; fc, follicle cell. Each bar represents fat body units calibrated as color-developed for 0.6 μg of total Day 4 fat body RNA. Different concentrations of total RNA from other tissues were applied to the membrane to accomodate the range of sensitivity of the assay and fat body units were multiplied by the dilution factor of each tissue relative to fat body RNA.

tin transcript titers increased at approximately linear exponential rates as indicated in the log scale insets to Figs. 2 and 3. The slope of a regression of $\log_2$ transforms of follicle total RNA, CaM, and actin transcripts vs days of the vitellogenic cycle gives a 2.3-, 3.8-, and 2.5-fold increase in total RNA, CaM, and actin transcripts per day. The increases in the titers of CaM and actin transcripts must be multiplied by the above-mentioned 32-fold increase in total RNA to obtain the net transcript increase of 4800- and 1200-fold for CaM and actin transcripts, respectively, over the 4 days. In preliminary experiments CaM transcript continued to accumulate to higher levels through Day 5 but uncertainty in exactly when chorion deposition starts in a group of animals influenced us to narrow our observations to Days 0 to 4.

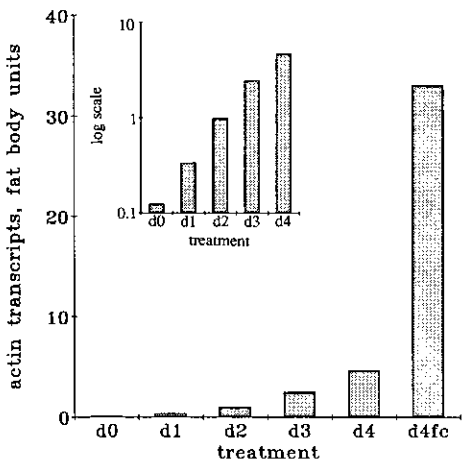

FIG. 3. Histogram of levels of actin transcripts in whole follicles and follicle cells at 96 hr during vitellogenesis in *B. germanica*, identified with a actin-specific probe. One fat body unit equals approximately 700 color units measured in the colorimetric assay described under Materials and Methods. Multiple concentrations of total RNA were used as internal controls to cover the range of sensitivity in the assay. Actin levels increase 35-fold during the 4-day vitellogenic period. A log transform inset shows a log-linear increase over the entire period.

Source of Follicular CaM Transcripts

To determine more about the source of the abundant transcripts, we prepared follicle cell total RNA from Day 4 follicle ghosts. For controls we quantified the titres of CaM transcripts in whole follicles and in fat body RNA of similar vitellogenic females. Testicular tissue RNA of virgin males was used as an additional control, since the resting level of CaM in that tissue is very high. Among the different tissue preparations used, whole-follicle total RNA contains as much as 25 times more CaM transcripts as a proportion of its total RNA than the fat body tissue at Day 4 and approximately twice that of testicular tissue (Fig. 4). Follicle cell total RNA contains 84 times higher CaM transcript titer at Day 4 postfeeding compared to that in fat body total RNA.

In spite of the fact that the total RNA derived from the follicle cell layer is equal to one-sixth of the total

RNA derived from whole follicles, the titer of CaM transcripts in the follicle cell total RNA was 3^+-fold higher than the latter. Calculating the exact amount of excess of CaM transcripts in follicle cells compared to the oocyte is difficult due to competing losses in extraction from the two tissues. However, based on the differences in the measured amounts of total RNA and the titers of CaM transcripts in the total RNA, we estimate that 75% of the CaM titer in the whole follicle is derived from the follicle cell layer. Total RNA from yolk of punctured follicles which we measured (with losses) to be two-thirds of the whole-follicle RNA showed a low titer of CaM transcripts. This observation suggests that (1) the oocyte itself makes a small contribution to the high titers of CaM transcripts observed in the whole follicle and (2) follicle cell CaM transcripts are more likely translated in the follicle cells than transported to the oocytes. In our measurement of the amounts of total RNA from yolk, we estimated the expected yield from the difference between the amounts of total RNA in whole follicles and the follicle cell layer. However, the actual yield of total RNA from yolk was at most 70% of the estimated expected yield. We could not eliminate losses in the total RNA from yolk during the yolk expulsion and collection procedure. It is thus not yet possible to calculate a complete balance sheet of CaM transcript titers in our study.

Actin transcripts are similarly found in disproportionate amounts in the follicle cell layer compared to that in the whole follicle (Fig. 3); however, they are found in a smaller multiple (33-fold) of the fat body level of actin transcript.

JH Regulation of Follicle Growth and CaM Transcript Accumulation

We examined the effect of JH on follicle growth by measuring the total RNA levels accumulated during the vitellogenic period and follicle cell CaM transcription using the JH bioassay paradigm described previously (Kunkel, 1973). Animals were separated into different treatment groups at Day 2 postfeeding. One subgroup was starved and the other deprived of JH and food by head ligation. Out of the JH-deprived animals, one set of animals received an injection of JH 24 hr after the ligation while the other set received injection vehicle only. Total RNA extractions were carried out at Day 4 postfeeding; i.e., injected JH had 24 hr to have an effect (Table 1). In head-ligated animals 50% of the follicles became atretic and total RNA levels in the other, apparently functional, follicles was 0.02 μg per follicle at Day 4. The (−JH) olive oil controls showed 0.05 μg of total RNA per follicle at Day 4 in the surviving follicles. The obviously atretic follicles of the −JH controls did not yield any measurable RNA (not tabulated). The +JH treatment group had the most dramatic effect on total RNA content. Total RNA content increased to 3.0 μg in the +JH responding follicles but only to 0.02 μg per follicle in the +JH atretic follicles. Our data is consistent with the positive gonadotrophic effect of JH in the responding follicles of *B. germanica* (Kunkel, 1973).

CaM transcript levels showed a dramatic response to JH injection (Fig. 2). In animals starved from Day 2 examined at Day 4, CaM transcript levels were comparable to normal animals at Day 2. Head-ligated (−JH) animals at Day 4 had further reduced CaM transcript levels compared to those in either fed or starved controls. The (−JH) olive oil control group had comparable CaM transcript levels to those in Day 2 fed controls (not shown). Of greatest significance, injection of JH 1 day after ligation stimulated CaM transcript in the responding follicles to levels above fed control animals at Day 4. This suggests that JH regulates accumulation of CaM gene transcripts in the cockroach follicle.

DISCUSSION

There is a 4800-fold increase in CaM transcripts during the 4-day vitellogenic period in *B. germanica* ovarian follicles. This increase in CaM transcripts is achieved by a 3.8-fold daily increase in the titer of CaM transcripts in addition to a 2.3-fold daily increase in total RNA. Actin transcript titer increases at the slower rate of 2.5-fold per day, which results in a lower total titer increase over the 4-day period. These increases occur in the absence of cell division since neither the oocyte nor the follicle cells divide during this time period. In addition our measurements suggest that most, if not all, of the follicular CaM transcripts originate from the follicle cell layer.

We have shown that systemically injected JH increases the total RNA content of the ovarian follicles dramatically and it has a specific effect on the level of CaM transcripts in the follicle cells of *B. germanica* females. The extent to which this effect has a direct effect on the follicle and on the CaM gene itself is yet to be determined. The role of JH in insect vitellogenesis has been the subject of study by many investigators (Wyatt, 1990). The best-studied aspect of JH action is the stimulation of vitellogenin synthesis by the fat body (Chen *et al.*, 1979; Bradfield, 1993; Engelmann, 1987; Wyatt, 1988). This stimulation of vitellogenin synthesis by JH is mediated by a specific transcriptional activation mechanism (Braun and Wyatt, 1992). JH is clearly necessary for the growth of cockroach follicles (Bell, 1969; Kunkel, 1973). An earlier assertion of a follicle cell level effect of JH was reported by Bell (1969). However, the nature and directness of the JH effect on follicle cells is still unknown.

During the morphogenesis of follicles in *B. germanica* during vitellogenesis, a regional rounding of the follicle cells and a transition in cell types was observed (Zhang and Kunkel, 1992b). This phenomenon, patency, has been well studied in *Rhodnius prolixus* (Abu-Hakima and Davey, 1977; Telfer *et al.*, 1982). In the *Rhodnius* follicle cell, JH mediates the phosphorylation of a 100-kDa polypeptide via a pathway that involves protein kinase C and the JH-sensitive Na^+/K^+-ATPase (Sevala and Davey, 1993), a direct effect that does not seem to involve activation of gene transcription. In *B. germanica* changes in follicle size respond to systemically injected JH (Kunkel, 1973). The associated changes in follicle cell shape mentioned earlier may well be direct effects as observed in *Rhodnius*. However, the accumulation of CaM transcripts that we report here might represent a class of JH ovarian effects at the transcriptional level and particularly in the follicle cells. The proportion of follicle CaM transcripts in the follicle cell layer is high (~75%). Depletion of endogenous JH by head ligation reduces CaM transcripts in follicles to low levels. Repair of hemolymph JH levels by injection of exogenous hormone restores CaM transcript levels.

This is the first report on JH regulation of accumulation of CaM gene transcripts. The gene for CaM in *B. germanica* is likely under transcriptional control in follicle cells during vitellogenesis. Increase in general protein synthesis in follicles and fat body has also been observed in the presence of JH in other systems (Wyatt, 1988). However, the latter is a cumulative response of the fat body tissue with a delay of 24 hr (Wyatt, 1990). The cockroach follicles respond rapidly to JH, restoring CaM transcript levels to above controls within 24 hr of injection. The accumulation of CaM transcript appeared to be regulated by JH, for the withdrawal of the hormone is followed by a steep fall in transcript levels, as observed in other systems (Jones *et al.*, 1988). The diverse roles played by CaM and its ubiquitous presence in all tissues makes it an attractive target for studying JH modulation in various JH-sensitive tissues.

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