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IN VITRO GROWTH OF CORPORA ALLATA FROM *DIPLOPTERA PUNCTATA*

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SUMMARY

An in vitro organ culture system was established to support growth of corpora allata from the cockroach *Diploptera punctata*. During a 1-wk incubation in L-15B medium supplemented with 10% fetal bovine serum (FBS) and 10% cockroach hemolymph, adult male corpora allata exhibited a cycle of de novo DNA synthesis followed by cell division. The number of S-phase cells and metaphase cells per corpus allatum were counted from whole-mount monolayers after labeling in vitro with 5′-bromo-2′-deoxyuridine and exposure to colchicine, respectively. While both FBS and cockroach hemolymph were essential for proliferation of allatal cells, the growth-promoting effect of insect hemolymph was not species-specific and adult female hemolymph was more potent than hemolymph from adult males. Furthermore, DNA synthesis of corpus allatum cells was stimulated in vitro by 20-hydroxyecdysone. This sensitive assay system will be of immense utility in the search for allatal growth factors.

Key words: corpora allata; juvenile hormone; cockroach; insect organ culture; DNA synthesis; *Diploptera punctata*.

INTRODUCTION

Ecdysteroids and juvenile hormones (JHs) regulate growth, differentiation, metamorphosis, and reproduction in insects. Production and release of these hormones is in turn regulated by brain neurohormones (Gilbert et al., 1980; Riddiford, 1985; Sehnal, 1985). Recent research has characterized both allatotropic and allatostatic neurohormones that regulate the synthesis of JH by the corpora allata (CA) (Kataoka et al., 1989; Woodhead et al., 1989; Donly et al., 1993). Yet, surprisingly little is known about extracellular signals that control CA development.

In most exopterygotes, such as cockroaches, endocrine cells retain the capacity to divide during postembryonic development (reviewed by Cassier, 1990). The CA exhibit sequential changes in cell proliferation and cellular organization in conjunction with JH synthesis. For instance, in *Diploptera punctata*, a viviparous cockroach, the number of CA cells increases before each larval molt, during the entire last stadium and soon after the imaginal molt (Szibbo and Tobe, 1981; Chiang et al., 1993; Johnson et al., 1993). This pattern of stage-specific cell proliferation suggests that as yet unknown regulatory factors control CA growth.

Since the original successes by Grace, in vitro cell and organ culture systems have been used routinely to define hormone action in the control of insect development (Grace, 1962; Marks, 1980; Mitsuhashi, 1982; Riddiford, 1985; Porcheron, 1991). Although incubation conditions for maintenance of CA activity have been established (Roller and Dahm, 1970; Feyereisen, 1985; Gadot et al., 1993), a culture system that supports growth of the gland has not been available. In this paper, we show in vitro growth of CA from adult male *D. punctata*. In the adult male, CA cells normally experience a single mitotic wave 2–3 d after ecdysis and subsequently remain quiescent (unpublished data). Here we show that a second mitotic cycle can be induced in vitro.

MATERIALS AND METHODS

Cockroaches. The *D. punctata* and *Periplaneta americana* colonies were maintained at 27 ± 1° C under a 12-h light:12-h dark photoperiodic regime and was supplied with pelleted Purina rat chow (number 5012) and water ad libitum. Adult males were collected from the colony within 24 h after ecdysis and reared in groups of 2–10.

Organ culture. Corpora allata were dissected from 4-d-old adult males, cleaned from adjacent tissues, and separated in sterile cockroach saline solution (Chiang et al., 1993). Individual CA were incubated in 10 µl L-15B medium (Munderloh and Kurtti, 1989), supplemented with various amounts of tryptose phosphate broth (TPB), fetal bovine serum (FBS) and insect hemolymph, as indicated. All incubations were carried out in 96-well culture plates sealed with parafilm membrane and maintained in a humidified chamber at 27 ± 1° C.

Preparation of insect hemolymph. Hemolymph from *D. punctata* or *P. americana* adults was diluted with a known volume of L-15B medium on ice, and stored at −70° C until needed. To prepare culture media, the stored hemolymph solution was diluted with L-15B to the desired concentration. The media were subsequently centrifuged (10 000 rpm for 20 min) and then sterilized by filtration (pore size 0.2 µm).

Mitotic index. Individual CA were incubated for 4 h in 10 µl medium containing 2 ng colchicine. The gland was desheathed with collagenase and spread into a whole-mount monolayer under a coverslip as described earlier (Chiang et al., 1989). Total number of arrested metaphase cells per corpus allatum was counted directly under a Nikon Optiphot microscope.

DNA synthesis. Individual CA were cultured in 10 µl L-15B supplemented with 5′-bromo-2′-deoxyuridine (BrdU) for 2 h in pulse labeling or for 6 d in continuous labeling. To prepare a single corpus allatum into a monolayer of cells without using a coverslip, the BrdU-labeled CA was digested with 0.1% collagenase in a hypotonic solution composed of equal parts saline and distilled water. After 10 min at 27° C, the CA was transferred into a 2 µl drop

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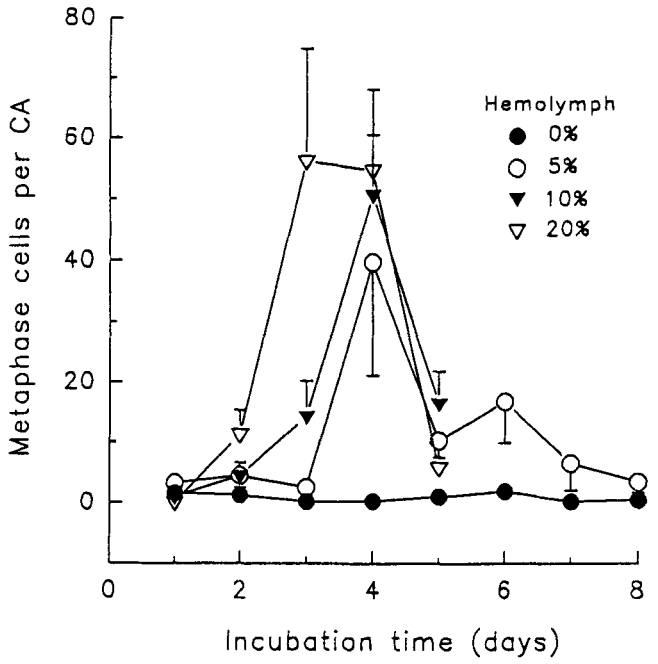

FIG. 1. The effects of hemolymph on mitotic activity of the CA. Glands from 4-d-old adult male *D. punctata* were incubated in L-15B medium containing 10% FBS, 3% TPB, and various amounts of hemolymph derived from Day 4 adult male *D. punctata*. Metaphase cells were counted from whole-mount CA monolayers 4 h after adding colchicine into the media. Each point represents the mean ± SEM of four to eight measurements.

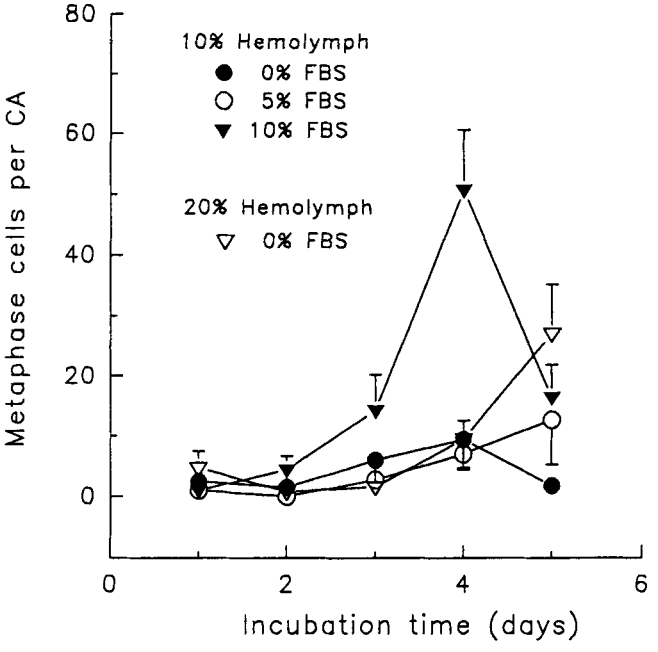

FIG. 2. The effects of FBS on mitotic activity of the CA. CA were incubated in L-15B with 3% TPB and 10% hemolymph from Day 4 adult male *D. punctata* containing various amounts of FBS, or in L-15B containing 20% hemolymph without FBS. Metaphase cells per CA were counted from whole-mount monolayers 4 h after adding colchicine into the media. Data for 10% FBS are the same as in Fig. 1. Each point represents the mean ± SEM of four to eight measurements.

of 0.1 *M* citric acid solution. Fifteen min later, as CA cells became swollen and loosely attached to each other, the CA was spread into a monolayer of cells by slowly removing the incubation solution under a dissecting microscope. The specimen was fixed and dried quickly by passing it through a flame three times. After washing the specimen three times in phosphate-buffered saline (PBS), BrdU-labeled cells were visualized by immunodetection according to the RPN 20 protocol (Amersham Corporation, Arlington Heights, IL). The number of BrdU-labeled cells per corpus allatum indicated by black DAB precipitate was counted directly under the microscope.

RESULTS

Effects of insect hemolymph. Corpora allata from 4-d-old adult males exhibited a mitotic wave in L-15B medium supplemented with 10% FBS, 3% TPB, and various amounts of Day 4 male hemolymph (Fig. 1). During the 8 d in vitro, mitotic activity was higher in media containing 10% or 20% hemolymph than 5% hemolymph, and the onset of the mitotic wave was directly related to hemolymph concentration. In the absence of hemolymph, mitotic activity was nearly undetectable during this period.

Effects of FBS. The mitotic wave was significantly delayed and attenuated as the concentration of FBS was reduced, and few CA cells divided in medium containing only 3% TPB, 10% *D. punctata* hemolymph, and no FBS (Fig. 2). The effect of FBS could not be duplicated by doubling the amount of hemolymph in the medium. Thus, it is clear that to support the proliferation of corpus allatum cells in vitro, both hemolymph and FBS are required.

Periplaneta hemolymph and the effects of TPB. When *D. punctata* hemolymph was replaced in the medium by hemolymph derived from 4-d-old adult male *P. americana*, the CA exhibited a similar pattern

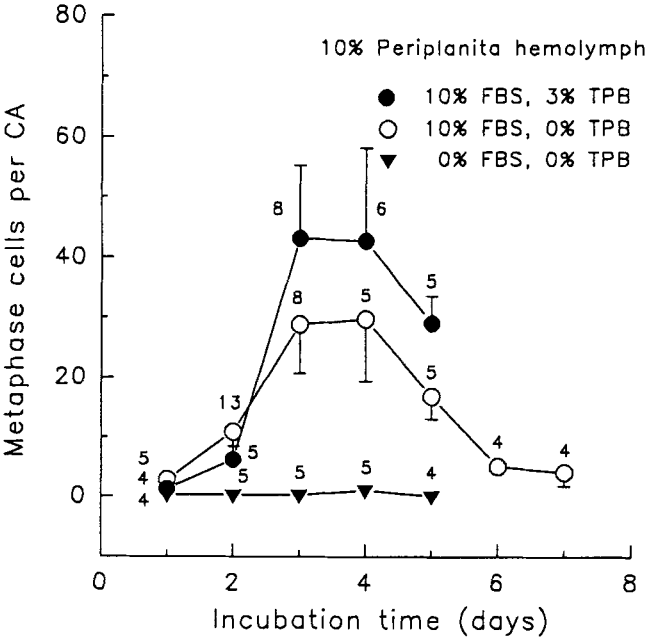

FIG. 3. The effects of FBS and TPB on mitotic activity of the CA in medium containing *P. americana* hemolymph. CA were incubated in L-15B medium containing 10% hemolymph derived from Day 4 adult male *P. americana*, with or without FBS or TPB. Metaphase cells per CA were counted from whole-mount monolayers 4 h after adding colchicine into the media. Each point is the mean ± SEM of the number of measurements indicated.

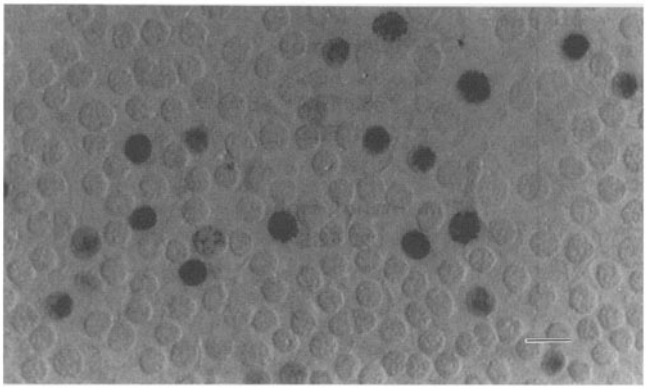

FIG. 4. A portion of a whole-mount monolayer of CA cells showing BrdU-labeled nuclei (*black dots*). This method allows counting of all cells within a single CA that have passed through S-phase during the period of exposure to BrdU. *Bar* = 10 μm.

of mitotic activity during a 5-d incubation period (Figs. 1,3). Using the same hemolymph, removal of TPB alone resulted in a similar pattern with slightly lower peak mitotic rates (Fig. 3). Removal of both FBS and TPB abolished mitosis completely in the L-15B medium containing 10% *P. americana* hemolymph.

In vitro DNA synthesis. In subsequent experiments, CA were incubated in suboptimal culture conditions in L-15B medium containing 10% FBS and 10% *P. americana* hemolymph without TPB. This condition allowed us to examine both stimulatory and inhibitory effects of various factors on the proliferation of CA cells. The pattern of DNA synthesis in the CA during 8 d of incubation in vitro was monitored by BrdU immunodetection. On each day, 3 ng/μl BrdU was added to the medium 2 h before the CA were processed for immunodetection. The number of S-phase cells during the 2-h exposure to BrdU was counted from whole-mount monolayers (Fig. 4). Fig. 5 shows the daily pattern of DNA synthesis in the CA resulting from pulse labeling with BrdU for 2 h. The patterns of DNA synthesis and mitotic activity peaked on Days 3 and 4, respectively (Figs. 3,5).

Continuous BrdU labeling. The total number of CA cells passing through S-phase was determined by continuous exposure to BrdU for 6 d in the suboptimal medium. High concentrations of BrdU (30 ng/μl and 300 ng/μl) were toxic to CA cells, resulting in low numbers of labeled cells, whereas 0.003 ng BrdU/μl was too dilute for sufficient incorporation into nuclei during DNA synthesis (Fig. 6). While 3 ng BrdU/μl was used for most studies on mammalian cells and for our pulse labeling, we used 0.3 ng/μl for continuous labeling to minimize the possibility of cytotoxic effects. At this concentration, de novo synthesis of DNA was evident after the first day of continuous incubation in vitro (Fig. 7).

Effects of female hemolymph and 20-hydroxyecdysone. Continuous BrdU labeling in vitro provides a very sensitive method to monitor slow developmental responses to changes in the incubation medium. Using this assay system, we found that DNA synthesis of CA cells was stimulated by factors from female hemolymph and by 20-hydroxyecdysone (Fig. 8). The number of BrdU-labeled cells in medium containing female hemolymph was about threefold greater than in the contralateral glands, which were incubated for 6 d in medium containing male hemolymph (Fig. 8). The stimulatory effect of female hemolymph on DNA synthesis of allatal cells could be mimicked by

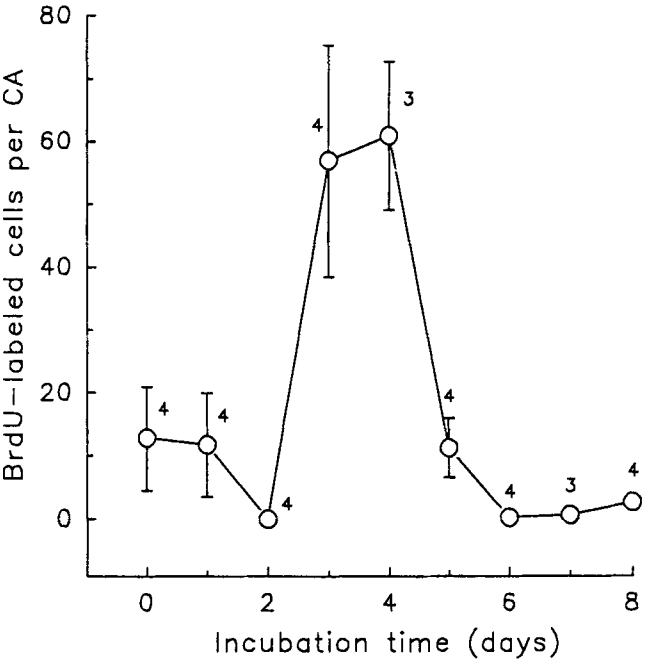

FIG. 5. The pattern of DNA synthesis of CA cells in organ culture. CA were incubated in L-15B medium containing 10% hemolymph and 10% FBS without TPB. Hemolymph was derived from 4-d-old adult male *P. americana*. The CA were exposed to BrdU (3 ng/μl) for 2 h before immunodetection and BrdU-labeled cells were counted from whole-mount monolayers. Each point is the mean ± SEM of the number of measurements indicated.

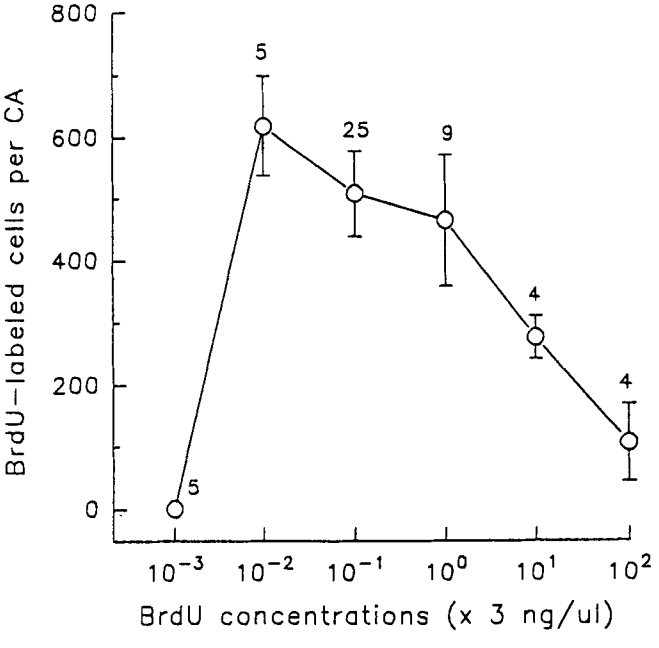

FIG. 6. Dose-response to determine an effective dosage of BrdU for detection of DNA synthesis in CA cells. Conditions of the culture medium were the same as those described in Fig. 4, except that various amounts of BrdU were present in the incubation medium for 6 d. Each point is the mean ± SEM of the number of measurements indicated.

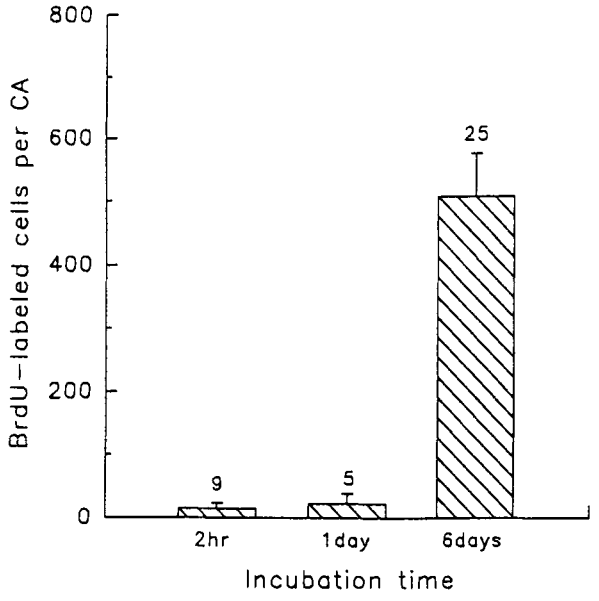

FIG. 7. De novo DNA synthesis in CA culture *in vitro*. Conditions of the culture medium were the same as those described in Fig. 4, except that less BrdU (0.3 ng/μl) was used in continuous labeling. CA were removed from the culture medium 2 h, 1 d, or 6 d later and the number of BrdU-labeled cells was counted. Data for 6 d are the same as in Fig. 6. *Vertical bars* represent SEM and the number of measurements is shown.

adding 2 μ*M* 20-hydroxyecdysone into the medium containing male hemolymph.

DISCUSSION

Our results show that corpora allata of adult male *D. punctata* can grow in L-15B medium supplemented with FBS and cockroach hemolymph. In vitro culture of CA, in which the differentiated function of the glands is maintained, has been used extensively to quantify in vitro rates of JH synthesis and to investigate hormone biosynthetic pathway (Roller and Dahm, 1970; Judy et al., 1973a, 1973b; Schooley et al., 1973; Muller et al., 1974; Pratt and Tobe, 1974; Gadot et al., 1993). However, attempts to grow CA in vitro have failed (Marks, 1970, 1976; Seshan and Levi-Montalcini, 1971), primarily because basic studies on regulation of CA development are lacking. It has been suggested that extracellular factors might regulate JH synthesis by effecting slow developmental changes in either CA cell number or in organization of cellular machinery (Tobe and Stay, 1985; Chiang and Schal, 1991, 1994; Chiang et al., 1991a, 1991b, 1991c). While the latter event is clearly evident in ultrastructural studies of *D. punctata* female CA (Johnson et al., 1985, 1993), and from direct morphometric studies (Chiang et al., 1989), the regulation of cell proliferation has received little attention, probably because a long-term in vitro system that supports CA growth has not been available. In the present paper, we show DNA synthesis followed closely by cell division in isolated CA in vitro. Although the relationship between cell proliferation and JH synthesis was not studied, this system provides a sensitive tool to assay various regulators of CA development and their effect on JH synthesis.

Continuous BrdU labeling, followed by immunodetection from whole-mount CA cell monolayers, allowed us to monitor the total number of cells that passed through S-phase during 6 d of in vitro incubation. This technique bypasses concerns that potential daily rhythms in DNA synthesis might be missed by pulse labeling. Compared with paraffin sectioning, which is time consuming and only provides estimated indices of cell proliferation, our new method of preparing an organ into a monolayer of cells takes far less time (½ h) and the nuclei retain their immunogenicity. We have successfully used this technique to monitor DNA synthesis in other insect tissues (unpublished observation), and we are currently using this sensitive assay system to search for allatal growth regulators.

Because both cockroach hemolymph and FBS were essential for proliferation of CA cells, it appears that they contain the necessary growth factors for CA cell proliferation. In fact, most primary insect cell culture cannot grow without FBS (Mitsuhashi and Goodwin, 1989). Although results from many other reports of in vitro studies suggest the existence of mitogenic factors in insect hemolymph, such factors have not been identified in invertebrates (Ferkovich and Oberlander, 1991). Our data indicate that at least some allatal growth stimulators in hemolymph are not species-specific because CA cells proliferate equally in media with hemolymph derived from either *D. punctata* or *P. americana* (Figs. 1,3). However, female hemolymph appears to be a more potent stimulator of cell proliferation (Fig. 8). One possible mitogenic factor in the hemolymph is 20-hydroxyecdysone. This hormone might be found at higher concentrations in female hemolymph than male hemolymph because ecdysteroids of ovarian origin exist only in adult females (Stay et al., 1980). In fact,

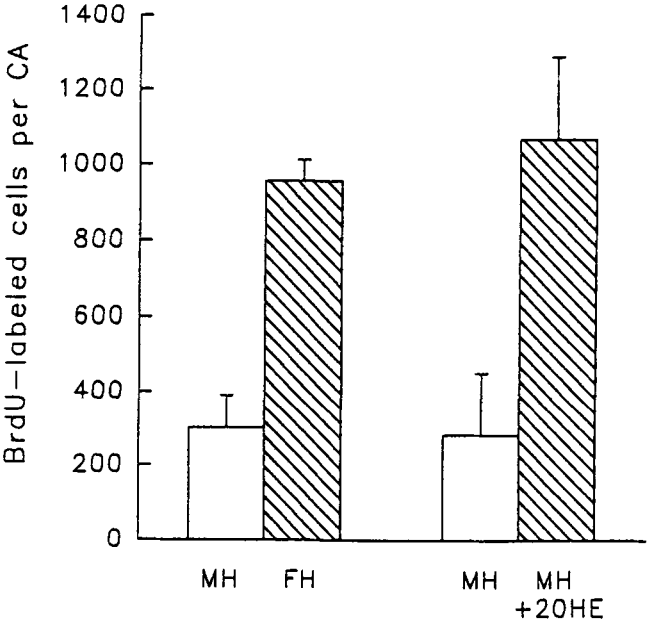

FIG. 8. Stimulation of DNA synthesis by female hemolymph or 20-hydroxyecdysone. Individual CA were cultured in 10 μl L-15B medium containing 10% FBS, 10% insect hemolymph, 0.3% TPB, and 0.3 ng/μl BrdU. Hemolymph was derived from 4-d-old *P. americana* adult males (MH) or females (FH). To determine the effects of hemolymph, the two members of each CA pair were separated and randomly assigned to the two treatment groups (n = 5). Likewise, to assay the effects of 20-hydroxyecdysone, the two separated CA were randomly assigned for incubation in medium containing male hemolymph and 0.1% ethanol with (+20HE) or without 2 μ*M* 20-hydroxyecdysone (n = 3). After a 6-d incubation, BrdU-labeled cells were visualized by immunodetection and counted. *Vertical bars* represent SEM.

supplementing male hemolymph with 2 μM 20-hydroexecdysone produced as much mitotic activity as female hemolymph (Fig. 8). Furthermore, nymphal development of CA shows a stage-specific cell proliferation coincident with the timing of an elevated level of hemolymph ecdysteroids (Chiang et al., 1993).

In vitro growth of the corpora allata suggests that brain neural factors, transmitted through nerves, are not required for the proliferation of CA cells. In fact, our in vitro and in vivo studies have demonstrated a neural inhibition on CA cell proliferation in both adult females and males (unpublished observations). The CA of adult male *D. punctata* undergo a cycle of DNA synthesis and cell division 2–3 d after the imaginal molt; cells then remain quiescent at least through Day 12, the last day that we investigated. Transection of the connecting nerves between the CA and the brain in 4-d-old males stimulates a second CA cell proliferation cycle in situ (unpublished), suggesting that mitosis is restrained in innervated CA, while the hemolymph environment contains mitogenic factors of unknown origin. Because hemolymph is included in our present in vitro assay system, we cannot exclude regulators of nervous origin that operate humorally.

Acknowledgments

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