A novel neuropeptide–endocrine interaction controlling ecdysteroid production in ixodid ticks

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SUMMARY

Ixodid (hard) ticks are blood-feeding arthropods that require a blood meal to complete each stage of development. However, the hormonal events coordinating aspects of feeding and development are only poorly understood. We have delineated a new neuropeptide–endocrine interaction in the adult tick, *Amblyomma hebraeum*, that stimulates the synthesis of the moulting hormones, the ecdysteroids. In adult female ticks, ecdysteroid synthesis could be demonstrated in integumental tissue incubated *in vitro* with a synganglial (central nervous system) extract, but not in its absence. Stimulation by the synganglial extract is both time- and dose-dependent, but is completely abolished by trypsin treatment, suggesting that the activity is due to a peptide/protein. Integumental tissue ecdysteroidogenesis is also stimulated by elevation of the cAMP concentration using forskolin and 3-isobutyl-1-methyl-xanthine, or by 8-bromo-cAMP. This suggests the involvement of at least a cAMP second messenger system in the neuropeptide–ecdysteroidogenesis axis, without precluding a role for other second messengers as well. Despite involving a quite different steroidogenic tissue, the foregoing system has some parallels with the known prothoracicotropic hormone (neuropeptide)–prothoracic gland endocrine axis of insects.

1. INTRODUCTION

Moulting and reproduction in arthropods are elicited by the polyhydroxylated steroidal moulting hormones, the ecdysteroids. According to the classical scheme of hormonal control of insect post-embryonic development, the prothoracic glands synthesize ecdysteroids (Grieneisen *et al*. 1993; Rees 1995) under the stimulus of a prothoracicotropic hormone (Bollenbacher & Granger 1985; Watson *et al*. 1989). Ecdysone, the primary ecdysteroid secreted from the prothoracic glands of insects (with the exception of Lepidoptera, which produce 3-dehydroecdysone as well; Warren *et al*. 1988), is subsequently converted into the major active hormone, 20-hydroxyecdysone, in certain peripheral tissues (Rees 1995). 20-hydroxyecdysone then initiates a cascade of events that directs moulting in immature stages (Steele & Vafopoulou 1989). Ecdysteroids are also involved in regulation of aspects of reproduction (Hoffmann & Lagueux 1985).

This model is somewhat oversimplified as there is evidence for production of ecdysteroids from sources other than the prothoracic glands (reviewed in Rees 1985; Delbecque *et al*. 1990). For example, in the immature stages of some Lepidoptera (Gersch & Eibisch 1977; Sakurai *et al*. 1991), Orthodoptera (Gersch & Eibisch 1977), Diptera (Studinger & Willig 1975; Dai & Gilbert 1991) and Coleoptera (Delbecque *et al*. 1986) the prothoracic glands degenerate before the pupal/adult ecdysteroid peak. In immature stages, abdominal and/or thoracic tissues, including the epidermis and oenocytes, have been implicated in ecdysteroid synthesis. Similarly, in adult stages of most insects, where the prothoracic glands are absent, there is evidence that the ovary, epidermis and possibly oenocytes are sources of ecdysteroids (Hagedorn *et al*. 1975; Delbecque *et al*. 1990).

Ticks (Acarina, Ixodidae), like other arthropods, progress through a series of developmental stages from embryo to adult that are dependent on environmental and hormonal cues. Although the two classes of ticks, ixodidae ('hard' ticks) and argasidae ('soft' ticks), show fundamental differences in their developmental and reproductive strategies (reviewed in Sonenshine 1991), it is likely that similar hormones to those found in insects would have similar functions in ticks, and indirect evidence suggests that this is the case (Oliver & Dotson 1993). One class of hormone, the ecdysteroids, has been unequivocally identified in ticks (Delbecque *et al*. 1978). Furthermore, appreciable evidence indicates that a major route of ecdysteroid inactivation in ticks involves fatty acyl esterification (Connat *et al*. 1984; Crosby *et al*. 1986). However, fundamental questions regarding ecdysteroid synthesis and its regulation still remain unanswered. Although it is evident that in ixodid ticks feeding and mating status are important physiological factors influencing ecdysteroid synthesis (Kaufman & Lomas 1997), there is still uncertainty as to the identity of the synthetic tissues and the factors that directly stimulate steroid synthesis. Previous reports based on indirect evidence have suggested that either the fat body (Ellis &

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Obenchain 1984) or the lateral segmental organs (part of the central nervous system; Binnington 1981; Sonenshine *et al*. 1985) may be capable of ecdysteroidogenesis. More recently Zhu *et al*. (1991 *a*) demonstrated that the epidermal tissue was capable of synthesizing ecdysteroids in immature stages of argasid ticks. However, the site of ecdysteroid synthesis in adult ixodid and argasid ticks has remained elusive.

We have now identified the integument as a tissue capable of synthesizing ecdysteroids *in vitro* in the adult ixodid tick, *Amblyomma hebraeum*, and, more importantly, demonstrated the occurrence of a protein/peptide factor from the tick synganglion (central nervous system) that can stimulate ecdysteroidogenesis in integumental tissue. Furthermore, evidence is presented that elevated cAMP, or a homologue of cAMP, mimics the effects of the synganglion factor in stimulating integumental ecdysteroidogenesis.

2. MATERIALS AND METHODS

(*a*) *Animals*

Adult *Amblyomma hebraeum* (Koch) were reared in the laboratory on rabbits according to the method of Kaufman & Phillips (1973). Engorged females (1 g and larger) were allowed to detach spontaneously (generally 8–10 d after attachment). After detachment, ticks were maintained at 26 °C and > 90% humidity.

(*b*) *Materials*

Tissue culture medium 199 (TCM199, Gibco BRL) was modified by the addition of 2.10 g l^{-1} 3-[N-morpholino]-propanesulphonic acid (MOPS; Sigma), 2.09 g l^{-1} NaCl, 25 mg l^{-1} ampicillin (Sigma) and the pH adjusted to 7.3 with NaOH. Borate buffer consisted of 0.1 M H_3BO_3 (BDH), 0.25 M $Na_2B_4O_7$ (BDH), 82 mM NaCl and the pH adjusted to 8.4 with HCl. Trypsin (EC 3.4.21.4), carboxyl esterase (EC 3.1.1.1, derived from porcine liver), aryl sulphatase from *Helix pomatia* (*Helix* hydrolase), 8-bromoadenosine 3′:5′-cyclic monophosphate (8-bromo-cAMP), forskolin, 1,9-dideoxyforskolin, 3-isobutyl-1-methyl-xanthine (IBMX) and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Company. All solvents used were of HPLC grade (BDH) or freshly redistilled.

(*c*) *Tissue dissection and incubation*

Ticks were glued (cyanoacrylate compound) to the bottom of a Petri dish and placed on ice for 20 min. They were then flooded in ice-cold TCM199, an incision made along the dorsal-lateral margin, and the dorsal integument removed and transferred to fresh ice-cold TCM199. The synganglion was excised and transferred to ice-cold TCM199 by first severing the midgut just anterior to the retrocerebral complex and all connecting nerves. Ovarian tissue was removed, transferred into fresh TCM199 and kept on ice until use. Fat body tissue was specifically taken from the margins of the integument just anterior to the spiracles and transferred into ice-cold TCM199. In this location, fat body tissue could easily be removed in 'sheets' of a relatively constant area. The ventral integument was then cleaned of remaining fat body and muscle tissue and washed several times in fresh TCM199. Dorsal and ventral integumental surfaces were rinsed three times in fresh TCM199, then covered with TCM199 and maintained on ice until the time of incubation. The integument comprises the epidermis and several cuticular layers, which are impossible to separate under our conditions.

Synganglia (four organs/100 µl TCM199), ovary and integumental tissue (one tissue/400 µl TCM199) and fat body (an area from one tick/400 µl TCM199) were cultured individually for 0 h (control) or 24 h (treatment) at 26 °C and the incubation terminated with the addition of cold methanol (1 ml for all tissues except the integument where 5 ml was used). For assay of tropic activities, five synganglial tissues were homogenized in a 1.5 ml microfuge tube using a loose-fitting, Teflon-coated plunger in 400 µl TCM199, centrifuged at 10000 *g* for 5 min (4 °C), and the supernatant (= extract) incubated with freshly prepared tissues (see §3 for tissue equivalents/incubation) for 0 h (control) or 24 h (treatment) at 26 °C.

(*d*) *Extraction of ecdysteroids from tissue cultures*

Synganglion, fat body, salivary gland or ovary cultures were transferred to a 5 ml ground-glass hand homogenizer, made to 70% (v/v) methanol:water and homogenized. Integumental cultures were transferred to a 20 ml vial, made to 70% (v/v) methanol:water and homogenized using a mechanical tissue grinder (Polytron PT 20 OD). The homogenates were centrifuged for 10 min (approximately 900 *g*), the supernatants collected and the pellets re-extracted three more times in methanol (1 ml per wash, or 5 ml for integumental tissue). Respective extracts were combined, evaporated to dryness under N_2, resuspended in 1 ml borate buffer containing 200 Units carboxyl esterase and incubated at 37 °C for 48 h to hydrolyse any ecdysteroid fatty acyl esters. The mixture was then adjusted to 10% (v/v) methanol, loaded onto a reversed-phase C_{18} Sep-pak (Waters Associates), and eluted with 10% (v/v) methanol:water (5 ml, elutes proteins and salts), 70% (v/v) methanol:water (6 ml, elutes polar ecdysteroid conjugates and free ecdysteroids), and methanol (6 ml, elutes ecdysteroid fatty acyl esters). The 70% methanol:water fraction was evaporated to dryness under N_2, reconstituted in borate buffer and the ecdysteroid content determined by radioimmunoassay.

(*e*) *Radioimmunoassay* (*RIA*)

All RIAs used ecdysone as standard with bound and unbound [23,24,-^{3}H(N)] ecdysone (NEN; 2.0 Tbq $mmol^{-1}$) being separated by ammonium sulphate precipitation (Mercer *et al*. 1987). Routinely, H-22 antiserum was used, which shows greatest specificity towards the ecdysteroid nucleus (Warren & Gilbert 1986). For ecdysteroid identification, the H-22 antiserum was complemented with DHS-1.13.5 antiserum, which shows greatest specificity for the ecdysteroid side chain (Soumoff *et al*. 1981). Ecdysone standards and samples for each RIA were assayed in duplicate.

(*f*) *High-performance liquid chromatographic analysis* (*HPLC*)

HPLC separation of ecdysteroids was carried out on the following systems: a reversed-phase Nova-Pak C_{18} cartridge (100 × 8 mm, particle size 4 µm; Waters Associates) employing either a linear gradient over 45 min of 35–100% (v/v) methanol in water followed by isocratic methanol at 1 ml min^{-1} (system 1) or a linear gradient over 20 min of 16–25% (v/v) acetonitrile:water (0.1% trifluoroacetic acid) followed by isocratic acetonitrile:water (1:3, v/v; 0.1% trifluoroacetic acid) at 1 ml min^{-1} (system 2); a Hypersil

APS-2 adsorption column (150 × 4.6 mm, particle size 3 μm; Shandon) eluted isocratically with methanol:1,2-dichloroethane (3:47, v/v) at 1 ml min^{-1} (system 3); a Lichrosorb diol column (25.0 cm × 4.6 mm, particle size 5 μm; Phase Sep) eluted isocratically with methanol:1,2-dichloroethane (1:24, v/v) at 2 ml min^{-1} (system 4). UV absorption was monitored at 254 nm (Model 441 absorption detector; Waters Associates).

(*g*) *High-performance liquid chromatography–mass spectrometry*

Synganglion-stimulated integumental tissue incubations were pooled, extracted as above, the extract evaporated to dryness under vacuum and fractionated by silicic acid column chromatography. Silicic acid columns were prepared using Silica gel 60 (Merck; sample to silicic acid ratio of 1:100) equilibrated in chloroform. The ecdysteroid extract was reconstituted in methanol, absorbed onto Celite, evaporated to dryness, and the residue applied to the silicic acid column. The column was sequentially eluted with chloroform, methanol:chloroform (3:7, v/v), methanol :chloroform (4:1, v/v) and methanol; the volume of each fraction was 10x weight of silicic acid. The methanol :chloroform (3:7, v/v) fraction (containing free and any apolar conjugated ecdysteroids) was evaporated to dryness, reconstituted in 1.0 ml borate buffer, and the apolar conjugates hydrolysed as above. After hydrolysis, the free ecdysteroids were subjected to a four-step purification method. First, the ecdysteroid sample was subjected to Sep-pak purification (see above). The 70% methanol:water fraction (v/v; containing free ecdysteroids) was evaporated to dryness, reconstituted in methanol:1,2-dichloroethane (3:50, v/v), and separated by adsorption HPLC (system 3). HPLC column eluent corresponding to the region from ecdysone to 20-hydroxyecdysone inclusive was collected. This fraction was next evaporated to dryness, reconstituted in methanol:1,2-dichloroethane (1:25, v/v) and separated by adsorption HPLC using a Lichrosorb diol column (system 4). Fractions with retention times corresponding to ecdysone and 20-hydroxyecdysone were collected, combined and subjected to reversed-phase HPLC/mass spectrometry with selected ion monitoring (HPLC/MS-SIM) using a Waters 600MS HPLC system (solvent system 2) linked to a VG Quattro triple-quadropole mass spectrometer via an atmospheric pressure chemical ionization interface operating in positive ion mode (APCI; corona discharge = 3.5 kV, probe temperature 200 °C, source temperature 120 °C, nitrogen nebulizer gas). Ions were selectively monitored at m/*z* 429, 447 for ecdysone and m/*z* 445, 463 for 20-hydroxyecdysone.

(*h*) *Peptide digestion*

Synganglial extracts were incubated for 18 h in the presence (treatment) or absence (control) of 100 Units trypsin at 37 °C. As a second control, trypsin alone was incubated in TCM199 at 37 °C for 18 h to assess autodigestion. After 18 h, the trypsin-digested (treatment) and non-digested (control) synganglial extracts, as well as the autodigested trypsin control were incubated with integumental tissues as described above, to assess stimulatory activity.

(*i*) *Second messengers*

Stock solutions of forskolin (1 mg ml^{-1}), 1,2-dideoxyforskolin (1 mg ml^{-1}) and IBMX (10 mg ml^{-1}) were prepared in redistilled ethanol. A stock solution of 8-bromo-cAMP (2 mg ml^{-1}) was prepared in distilled water. For incubations examining the effects of forskolin (100 μM forskolin), forskolin + IBMX (100 μM or 10 μM forskolin + 50 μM IBMX), or 1,9-dideoxyforskolin (100 μM 1,9-dideoxyforskolin + 50 μM IBMX), appropriate volumes of relevant stock solutions were combined, evaporated to dryness under a stream of N_2, reconstituted in 5 μl DMSO plus 50 μl redistilled ethanol and diluted to 5 ml with TCM199. For incubations with 8-bromo-cAMP (250 μM 8-bromo-cAMP), appropriate volumes of stock solution were added directly to 5 ml TCM199. Integumental tissues were subsequently cultured with 400 μl of the appropriate second messenger effectors as described above.

(*j*) *Statistics*

All data are expressed as mean ± s.e. (*n*). Statistical significance was determined by one way analysis of variance (ANOVA) or Student's *t*-test using Statview™ 4.01 (Abacus Concepts Inc., Berkeley, California, USA) on an Apple Macintosh computer.

3. RESULTS

(*a*) *Identification of an ecdysteroidogenic tissue in ticks*

The total immunoreactive ecdysteroid content of cultures was determined by extracting all ecdysteroids from the tissues (including both free and conjugated ecdysteroids), followed by enzymic hydrolysis of the conjugated ecdysteroids to their free forms. Initially, tissues were subjected to both esterase (hydrolyses apolar esters to free ecdysteroids) and *Helix* hydrolase (hydrolyses polar conjugates to free ecdysteroids) hydrolysis. The results indicated that the contribution of polar conjugates was minimal, whereas apolar conjugates represented 48–57% (two experiments) of the total ecdysteroids (data not shown).

Specific tick tissues were incubated for 24 h, either on their own or with synganglial extract. It has been previously determined that the ecdysteroid content of *A. hebraeum* tissues, such as haemolymph or ovary, remains low during the first 2–3 d after detachment but increases significantly thereafter (Kaufman 1991). For this reason, tissues cultured individually were removed from ticks and incubated on day 4 post-detachment, i.e. just at the time when the ecdysteroid titre is naturally increasing. For coculture incubations, tissues were taken from ticks at the point just prior to the natural increase in ecdysteroid titre (day 2 post-detachment from the host). Synganglial, integumental, ovary or fat body tissues taken from engorged ticks on day 4 post-detachment showed no significant increase in total ecdysteroid content when maintained in culture for 24 h (figure 1). The endogenous content of ecdysteroids in integumental tissues was appreciably greater than in the other tissues. Cocultures of synganglial-integumental tissues (five synganglial equivalents/integumental tissue) from ticks on day 2 post-detachment, however, did reveal a significant increase in total RIA-positive material over a 24 h incubation (5590 ± 329 ($n = 8$; 0 h) vs 13216 ± 679 ($n = 9$; 24 h) pg ecdysone equivalents/culture; $p <$

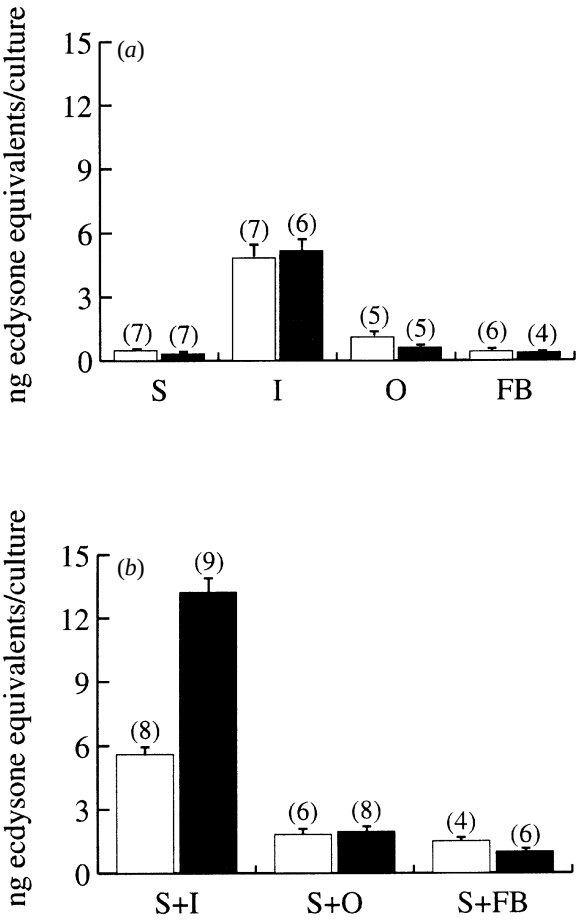

Figure 1. Steroidogenic activity of tick tissues assessed by changes in total immunoreactive material (tissue + medium; pg ecdysone equivalents/culture) for tissues maintained in culture (*a*) individually, or (*b*) in combination for 0 h (white bars) or 24 h (grey bars). S, synganglia; I, integument; O, ovary; FB, fat body. Cultures contained five synganglia, one integument, one ovary, or an area of fat body from one tick. For each coculture, five tissue equivalents of synganglial extract (SE) was added. Data presented as mean ± s.e. (sample size).

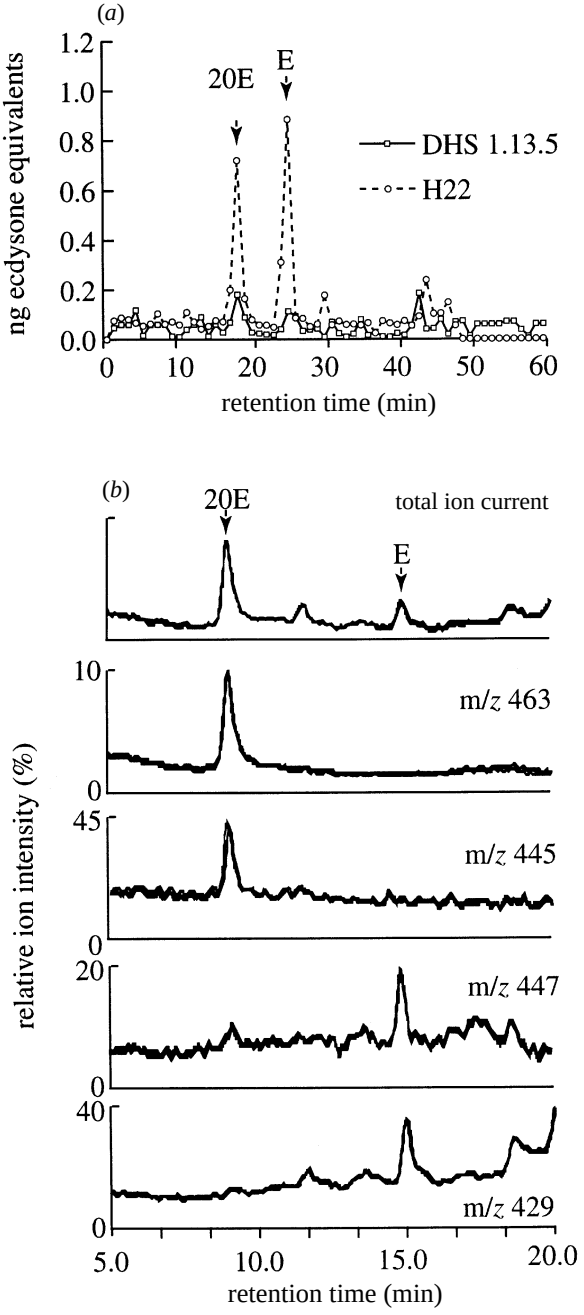

Figure 2. Identification of ecdysone and 20-hydroxyecdysone in stimulated integumental cultures. Ecdysteroids from stimulated integumental cultures were extracted as outlined in §2 and subjected to: (*a*) HPLC–RIA analysis employing a C_{18} reversed-phase column (system 1). Effluent was collected as 1-min fractions and the ecdysteroid content analysed by RIA using H-22 (open squares) and DHS-1.13.5 (open circles) antisera; different extracts were used for the analyses using the two antisera. (*b*) HPLC/MS analysis employing a C_{18} reversed-phase column (system 2) with selected ion monitoring at m/*z* 447 and 429 (characteristic of ecdysone) and m/*z* 463 and 445 (characteristic of 20-hydroxyecdsyone). Positions of elution of authentic ecdysone and 20-hydroxyecdysone are indicated by arrows.

0.001); this corresponded to an overall increase of 2.3-fold in immunoreactive material as compared to the 0 h controls (figure 1).

In preliminary experiments using either unstimulated integumental tissues or synganglial-stimulated integumental tissues, separate extraction of tissue and medium showed that essentially all the ecdysteroids were in the integumental tissue. Cocultures of synganglial-ovary or synganglial-fat body tissues did not show a similar increase in ecdysteroid content (figure 1).

(*b*) *Identification of ecdysone and 20-hydroxyecdysone in cultures of stimulated integumental tissues*

To identify the ecdysteroids in extracts of synganglial-stimulated integumental tissue cultures, they were subjected to HPLC–RIA analysis using a combination of the H-22 antiserum and the DHS-1.13.5 antiserum. Using these antisera, immunoreactive peaks were identified on both adsorption-HPLC (data not shown) and reversed phase-HPLC (system 1, R_t 18 min and 25 min fractions) that had similar retention times to authentic 20-hydroxyecdysone and ecdysone standards (figure 2*a*). Later-

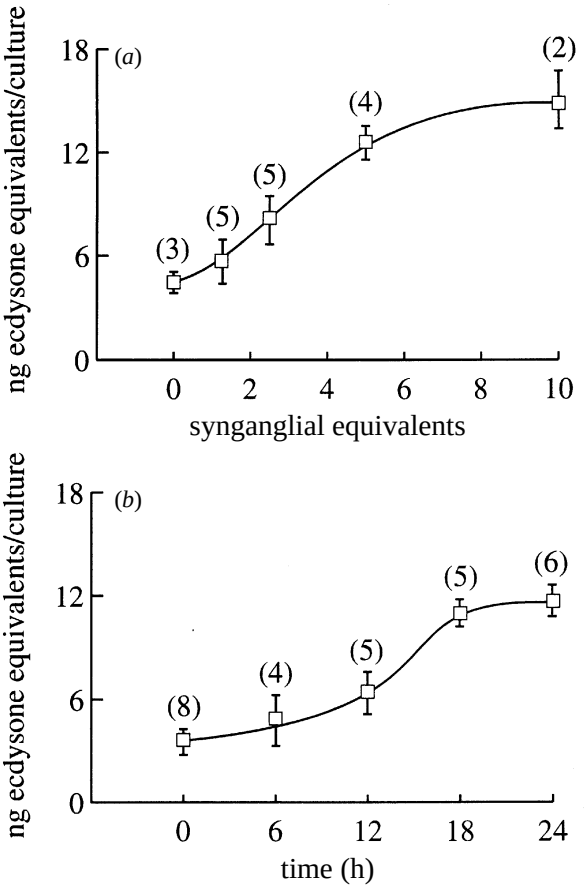

Figure 3. Dose- and time-relationship of stimulation of ecdysteroid synthesis in integumental tissue by synganglial extracts. Integumental tissues were incubated for (*a*) 24 h in the presence of varying amounts of synganglial extracts, or (*b*) in the presence of synganglial extracts (five equivalents/culture) for varying lengths of time. Data are expressed as total immunoreactive material (pg ecdysone equivalents/culture) and presented as mean ± s.e. (sample size).

eluting, unidentified immunoreactive material was also observed. The identity of ecdysone and 20-hydroxyecdysone was corroborated by pooling several synganglial-stimulated integumental tissue cultures and subjecting the extract to HPLC/MS. Selected ion monitoring of ions specific for 20-hydroxyecdysone (m/*z* 463 and 445) and ecdysone (m/*z* 447 and 429) showed prominent peaks (R_t 9.07 and 14.83 min) at positions corresponding to those of authentic 20-hydroxyecdysone and ecdysone standards, respectively (figure 2*b*).

(*c*) *Dose–response and time-course relationship of stimulation of ecdysteroid synthesis by synganglial extracts*

Incubation of integumental tissues with an increasing number of synganglial equivalents showed an increasing stimulatory effect (figure 3*a*). Integumental tissues incubated with our lowest dose of 1.25 synganglial equivalents produced only a slight stimulatory effect. The increase in total integumental ecdysteroid production became significant only after the synganglial extract dose was increased to 2.5 synganglial equivalents (1.8-fold greater than that of controls lacking synganglial extract; $p < 0.001$), whereas ten synganglial equivalents (the maximum dose tested) produced an increase of 3.3 times that of the controls.

Using a constant dose of five synganglial equivalents per integumental culture, the incubation time of each culture was varied. Under our conditions, the total ecdysteroid content of stimulated integumental tissues reached a maximum by 18 h (figure 3*b*). Increasing the length of culture time from 18 h to 24 h did not produce a significant increase in overall ecdysteroid content.

(*d*) *Biochemical nature of the synganglial stimulatory factor*

Trypsin-digested synganglial extracts were unable to stimulate integumental tissues when cultured for 24 h (2646 ± 973 pg ecdysone equivalents/culture; $n = 4$) as compared to synganglial extracts that were not pre-incubated with trypsin (6405 ± 607 pg ecdysone equivalents/culture, $n = 5$). The ecdysteroid content of the former cultures was equal to that of non-stimulated controls (2867 ± 316 pg ecdysone equivalents/culture; $n = 4$). This suggests that synganglial stimulatory activity is due to a peptide or protein.

(*e*) *Involvement of cAMP*

In insect prothoracic glands, ecdysteroidogenesis is stimulated by a peptide hormone through the second messenger, cAMP (Smith *et al.* 1984). Given the peptide/protein nature of the tick synganglial factor, the effects of enhancing the endogenous cAMP concentration or of a cAMP analogue to mimic the activity of synganglial extracts was examined. For this, the effects of forskolin (adenylate cyclase activator), 1,9-dideoxyforskolin (inactive analogue of forskolin), IBMX (phosphodiesterase inhibitor) and 8-bromo-cAMP (cAMP analogue) were examined. Integumental tissues cultured in the presence of 100 μM forskolin showed no significant increase in total ecdysteroid content as compared to the 24 h controls (figure 4). A forskolin-stimulated increase in total ecdysteroid content of integumental cultures was observed, however, when the incubation medium was supplemented with 50 μM IBMX. Such an increase was not observed with IBMX alone. Total ecdysteroid content of integumental cultures increased significantly from 4723 ± 258 pg ecdysone equivalents (24 h controls) to 11047 ± 727 pg ecdysone equivalents ($n = 8$; $p < 0.001$) in the presence of 100 μM forskolin and 50 μM IBMX (figure 4). The stimulatory effect of forskolin was dose-dependent as a ten-fold decrease in forskolin concentration, in the presence of 50 μM IBMX, showed a marked decrease in total ecdysteroid content of integumental cultures (11047 ± 727 pg ecdysone equivalents (100 μM forskolin) vs 7797 ± 466 (10 μM forskolin; $n = 5$); $p = 0.008$). Culturing of integumental tissues with the inactive forskolin analogue, 1,9-dideoxyforskolin, and 50 μM IBMX did not cause stimulation of ecdysteroid synthesis in integumental tissues and the ecdysteroid content of such cultures was

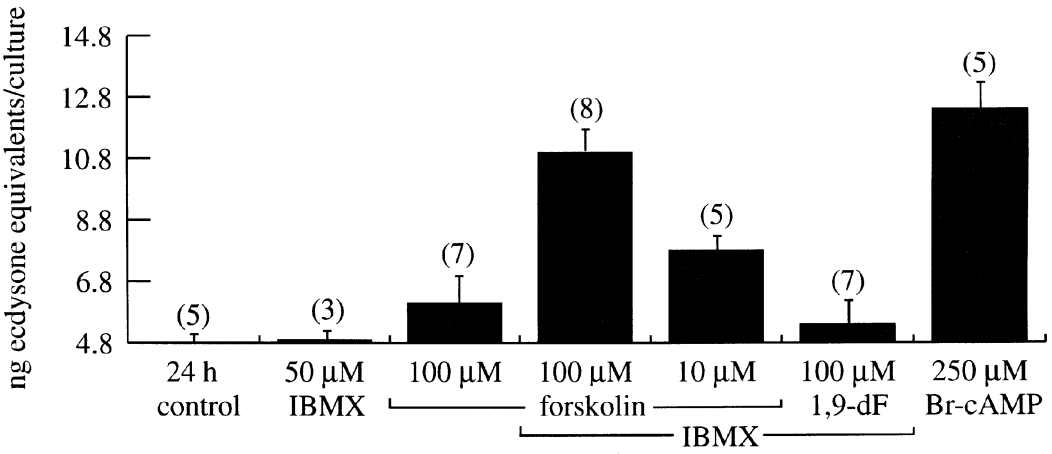

Figure 4. Involvement of cAMP in stimulating ecdysteroid steroid synthesis by integumental tissue cultures. To examine the effects of elevating the cAMP concentration, integumental tissues were cultured with either 100 μM forskolin, 100 μM forskolin + 50 μM IBMX, 10 μM forskolin + 50 μM IBMX, or 100 μM 1,9-dideoxyforskolin (1,9-dF) + 50 μM IBMX. To examine the effects of a cAMP analogue, integumental tissues were cultured with 250 μM 8-bromo-cAMP (Br-cAMP). Data are expressed as total immunoreactive material (pg ecdysone equivalents/culture) and presented as mean ± s.e. (sample size).

equivalent to 24 h control cultures. The cAMP mimic, 8-bromo-cAMP, was also able to stimulate ecdysteroid synthesis by the integumental tissue. Total ecdysteroid content of integumental tissues cultured in the presence of 250 μM 8-bromo-cAMP increased significantly from 4753 ± 258 pg ecdysone equivalents (24 h controls) to 11921 ± 659 pg ecdysone equivalents ($n = 5$; $p < 0.001$; figure 4).

4. DISCUSSION

The involvement of ecdysteroids in regulating various aspects of development and reproduction in ticks has been established (Sonenshine 1991; Oliver & Dotson 1993). The source of this hormone in immature argasid (and likely ixodid) ticks has been identified as epidermal tissue (Zhu *et al.* 1991*a*). However, no tissue has been demonstrated as a source of ecdysteroids in the adult stage. We now report that the integumental tissue is capable of ecdysteroid synthesis in adult females of *A. hebraeum*, and more importantly, demonstrate the existence of a peptide factor(s) from the synganglion that stimulates ecdysteroid synthesis in the integumental tissue.

Whereas ecdysteroid synthesis was not observed upon incubation by itself of any of the tissues examined, our results clearly indicate that the immunoreactive ecdysteroid content of integumental tissue increases after culturing in the presence of a synganglial extract (figure 1). This approach for demonstration of ecdysteroid synthesis in integumental tissues relies on a measurable increase in immunoreactive ecdysteroids after culturing for 24 h. A potential complication is the fact that ixodid ticks are known to contain inactive, acylated ecdysteroids, which are in themselves poorly immunoreactive, but can liberate free ecdysteroids upon hydrolysis with esterase. Such hydrolysis of these acyl esters during the culturing period would result in an increase in immunoreactive material after incubation, but would not represent *de novo* ecdysteroid synthesis. Both unstimulated and synganglial-stimulated integumental tissues are known to contain free as well as acylated ecdysteroids. However, hydrolysis of all extracts prior to RIA ensured that the observed increase in immunoreactive ecdysteroids in synganglial-stimulated integumental cultures was due to synthesis rather than release of free ecdysteroids from endogenous acyl esters. The possibility that the observed ecdysteroid synthesis is due to any contaminating fat body tissue associated with the integument can be eliminated as such synthesis was not observed in fat body (figure 1), in agreement with other observations (Zhu *et al.* 1991*a*). However, fat body tissue contains a potent ecdysone 20-monooxygenase, responsible for hydroxylation of ecdysone into the more active 20-hydroxyecdysone (Zhu *et al.* 1991*a*). As the H-22 antiserum used in our radioimmunoassays shows seven-fold less sensitivity towards 20-hydroxyecdysone compared to ecdysone (unpublished results), contribution of any contaminating fat body tissue would likely be limited to conversion of ecdysone into 20-hydroxyecdysone and potentially result in a decrease in the total measured immunoreactive material.

Demonstration of ecdysteroid synthesis in synganglion-stimulated integumental tissues is consistent with the study of Zhu *et al.* (1991*a*), who showed that the epidermal tissue of argasid nymphs of *Ornithodoros parkeri* is capable of *de novo* ecdysteroid synthesis. It may not be surprising that the integumental tissue of ticks can synthesize ecdysteroids. Ticks lack a tissue that is directly analogous to the prothoracic glands in immature insects, but epidermal tissue (a component of the integument) and prothoracic glands share a common ectodermal origin, and it has been demonstrated that the epidermal tissues of a variety of insects can act as a source of ecdysteroid in the absence of prothoracic glands (Rees 1985; Redfern 1989; Delbecque *et al.* 1990). In *O. parkeri* nymphs, ecdysone was the sole product of epidermal incubations, whereas in the presence of fat body, effective 20-hydroxylation occurred as well (Zhu

et al. 1991 *a*). The demonstration of both ecdysone and 20-hydroxyecdysone in integumental tissue incubations in the current work is probably due to the presence of 20-hydroxyecdysone acyl esters in the tissue before culturing, as extraction and esterase hydrolysis of ecdysteroids from non-cultured integument showed approximately equal proportions of 20-hydroxyecdysone and ecdysone.

The presence of synganglial factors(s) appears to be a requirement for steroid synthesis in the integument, as incubation of integumental tissue from ticks at a stage of increasing ecdysteroid titre (4 d post-removal) in the absence of synganglial extracts did not show an increase in immunoreactive ecdysteroids (figure 1). The addition of synganglial extracts to cultures of integumental tissue from animals 2 d post-removal caused a 2.8-fold increase in integumental ecdysteroid content. This rate of integumental ecdysteroid synthesis is comparable to the increase in ecdysteroid titre observed *in vivo*, over 24 h at a slightly later stage of development and could account for formation of the steroid. Furthermore, the conditions *in vitro* are suboptimal, with the ecdysteroid being formed from endogenous integumental precursors. Our preliminary observation that ecdysteroid produced by the integument was not released to a significant extent may not necessarily reflect the true situation *in vivo*, and it is possible that the medium may not be optimal in this respect. In the case of insect prothoracic glands or crustacean Y-organs, incubated *in vitro*, synthesized ecdysteroids appear to be largely released (Redfern 1989).

The stimulatory activity of synganglial extracts showed a dose-dependency with 1.25 synganglial equivalents causing a slight increase in total integumental ecdysteroid content (figure 3). Although this dose seems comparatively high, it could be due to insensitivity in our current *in vitro* bioassay, rather than a reflection of the true dose necessary to stimulate the integumental tissue *in vivo*. In particular, the total volume of haemolymph at this stage of development is in the range of 150–200 µl/tick (Kaufman *et al*. 1980), whereas the volume of our current bioassay incubation is 400 µl, thus constituting a dilution of 2–2.7-fold compared to the *in vivo* situation.

Stimulation of integumental ecdysteroidogenesis by synganglion extract shows a time dependence (figure 3). The occurrence of an appreciable lag period is not entirely surprising considering that the *in vivo* haemolymph ecdysteroid titre in adult *A. hebraeum* exhibits a gradual increase over several days (Lomas 1992), unlike insects where changes in ecdysteroid titres are generally more rapid (Steele & Vafopoulou 1989).

It is apparent that the activity of the synganglial steroidogenic stimulatory activity is due to a peptide/protein component(s) because all activity is destroyed by incubation of the synganglial extract with trypsin before incubation with integumental tissue. No neuropeptide has, hitherto, been identified in ticks. Release of peptide hormones from the synganglion should occur through specific neurohaemal centres and based on paraldehyde fuchsin staining studies, several neurohaemal centres have been identified in ticks (Binnington & Obenchain 1982). Furthermore, Zhu *et al*. (1991 *b*) identified three such paraldehyde fuchsin-positive centres that could also be labelled with an antibovine insulin antibody. This is significant because bombyxin, the neuropeptide responsible for stimulating ecdysteroid synthesis in the silkmoth, *Bombyx mori*, has a high amino acid sequence identity with insulin (Iwami *et al*. 1989).

The demonstration that the adenyl cyclase activator forskolin (but not its inactive analogue, 1,9-dideoxyforskolin) and 8-bromo-cAMP (cAMP analogue) can mimic the effects of the synganglial factor(s) in stimulation of ecdysteroid synthesis suggests that at least the cAMP second messenger system is involved in the action of the factor. However, it does not preclude the involvement of other second messenger systems as well. In insect endocrine systems, brain neurosecretory tropic peptides stimulate ecdysteroid synthesis via Ca^{2+}-dependent (Smith *et al*. 1996) or Ca^{2+}-independent (Hayes *et al*. 1995) elevation of cAMP. Furthermore, in crustaceans there is evidence suggesting the involvement of both cAMP and cGMP in moult-inhibiting hormone-inhibition of Y-organ ecdysteroidogenesis (Sedlmeier & Fenrich 1993; Saidi *et al*. 1994).

The combined results indicate the occurrence of a neuropeptide–second messenger–ecdysteroidogenesis endocrine axis in adult ticks, encompassing synganglial and integumental tissues. Presumably, *in vivo*, ecdysteroids are released from integumental tissue into the haemolymph and exert a hormonal effect in relevant target tissues. Characterization of the tropic neuropeptide will open up novel immunological approaches for control of tick vectors of disease.

We thank Rachael Prentice for skilled technical assistance, Mark C. Prescott for mass spectral analyses, Professors L. I. Gilbert and J. D. O'Connor for generous gifts of antisera, and The Wellcome Trust for financial support.

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Received 8 November 1996; *accepted 2 December 1996*