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0014-4754/91/010075-03$1.50 + 0.20/0

The insect gut: A new source of ecdysiotropic peptides

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Received 6 March 1990; accepted 17 May 1990

Summary. Proctodaea of European corn borer (*Ostrinia nubilalis*) and gypsy moth (*Lymantria dispar*) last instars (larvae) contain prothoracicotropic factors that stimulate the prothoracic glands (PGs) of the gypsy moth to produce both ecdysone and 3-dehydroecdysone (precursors to the insect molting hormone) in a dose-dependent manner. In a separate in vivo assay, injections of proctodaeal extracts into gypsy moth larvae that were head-ligated before the release of the molt-stimulating brain hormone, PTTH, resulted in a pupal molt.
Key words. PTTH; ecdysteroids; ketoreductase; proctodaeum; prothoracic glands.

Prothoracicotropic hormones (PTTHs) are important ecdysiotropic neurohormones that stimulate the insect prothoracic glands (PGs) to produce an ecdysteroid which is a precursor to the molting hormone, 20-hydroxyecdysone[1,2]. The only site of synthesis reported for PTTH is a pair of neurosecretory cells located in either the lateral or median protocerebrum of the brain[3–5]. In response to endogenous and/or exogenous cues, PTTH is released from its neurohemal organ[6] into the hemolymph and transported to the PGs. While it was previously thought that ecdysone was the only ecdysteroid secreted by the PGs[7,8], it is now known that PGs of the tobacco hornworm, *Manduca sexta*[9] and of the gypsy moth, *Lymantria dispar*[10], produce 3-dehydroecdysone, a precursor, which is then converted to ecdysone by a 3β-forming-3-ketoecdysteroid reductase (ketoreductase), and later by peripheral tissues to the physiologically active molting hormone, 20-hydroxyecdysone. Since Beck and his colleagues[11] reported the existence of a factor (proctodone) in the hindgut which stimulated the onset of development in diapausing ('hibernating') European corn borer (*Ostrinia nubilalis*) larvae, it was decided to examine larval proctodaeal extracts for the presence of ecdysiotropic factors. We now report that in addition to the brain, large amounts of ecdysiotropic factor(s) are present in the hindgut of *O. nubilalis*, and in the hindgut of *L. dispar*; and that these factors stimulate the production of both ecdysone and 3-dehydroecdysone by the PGs of *L. dispar*.

Proctodaea minus recta (pylorus and anterior intestine)[12] were dissected from laboratory-reared *O. nubilalis*[13] and *L. dispar* prepupae (last stage larvae in the process of, or having completed gut purge)[14], and placed in Grace's medium (GIBCO, Grand Island, NY) for 20–60 min prior to being frozen at -20 °C. When needed, proctodaea were thawed and homogenized in ice-cold Grace's medium. To remove tissue debris and large proteins, homogenates were boiled for 2.5 min, centrifuged at 16,000 × g and 4 °C (3 min), and the supernatants removed and stored on ice. A triple incubation in vitro PG assay, modified from previously described in vitro assays[15–19], was utilized to measure the ecdysiotropic activity in these extracts (fig. 1).

Results and discussion

The antibody (provided by W. E. Bollenbacher, University of North Carolina, Chapel Hill, and characterized by us [unpublished results]) used here for the RIA, detects ecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone, and 20,26-dihydroxyecdysone, but not 3-dehydroecdysone. Since the only RIA-detectable ecdysteroid produced by *L. dispar* PGs (with our antibody) is ecdysone[18], detectable ecdysteroid in 'G' or 'Gc' (fig. 1) was due to the production of ecdysone, while the detectable ecdysteroid in 'K' or 'Kc' (fig. 1) was due to the production of both ecdysone and 3-dehydroecdysone, the latter having been converted to ecdysone by the added ketoreductase.

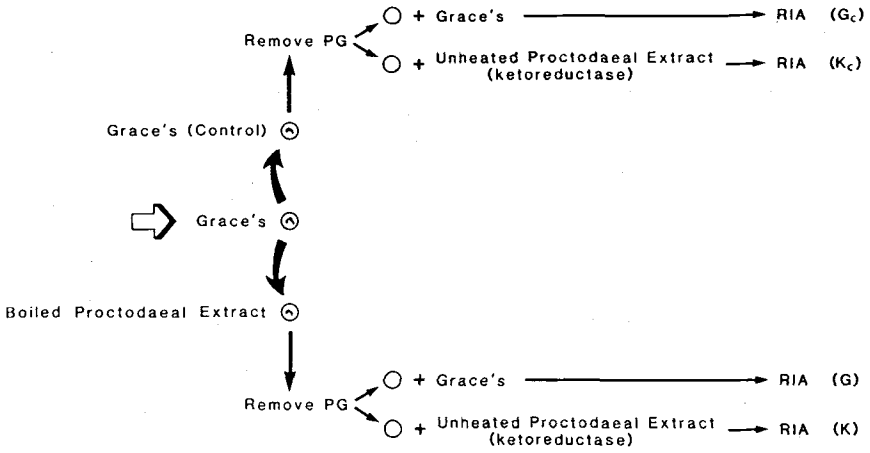

Figure 1. PGs of day-5 last stage larvae of *L. dispar*, were pre-incubated in a 25-µl drop of Grace's medium for 2 h. After this time, ecdysteroid production dropped to low levels[18]. Glands were then rinsed in Grace's medium and transferred to 25 µl of fresh Grace's medium (controls) or to 25 µl of Grace's medium containing the desired concentration of boiled proctodaeal extract. After an additional 2 h of incubation, the PGs were removed and the incubation medium was divided into 2 drops containing 11µl of fluid each. To one drop, we added 12 µl of Grace's medium; to the other, we added 12 µl (0.25 equivalents) of a crude preparation of freshly extracted unboiled proctodaeal tissue, a good source of the enzyme(s), ecdysteroid ketoreductase[21]. After a 3rd 2-h incubation, 15-µl aliquots were transferred to 6 × 50-mm tubes, and the enzymatic reaction was stopped with 30 µl of absolute methanol. Ecdysteroid titers were measured by radioimmunoassay (RIA) (30). 'Gc' and 'Kc' were controls that received Grace's medium and ketoreductase respectively for the third (last) 2-h incubation. 'G' and 'K' were experimental samples that received Grace's medium and ketoreductase respectively for the last incubation.

Therefore, to determine the titer of 3-dehydroecdysone synthesized by the PGs, total ecdysteroid in 'G' was subtracted from that in 'K'. Results were expressed (fig. 2) as an activation ratio, A_r, i.e., [G]/[Gc] for ecdysone and [K-G]/[Kc-Gc] for 3-dehydroecdysone. Typical control values were 15 to 40 pg ecdysone for 'Gc' and 75 to 150 pg ecdysone for 'Kc'. For both boiled and unboiled proctodaeal extracts, ecdysteroid content as measured by RIA was negligible.

Proctodaeal extracts from *O. nubilalis* stimulated production of both ecdysone and 3-dehydroecdysone in a dose-dependent manner (fig. 2) with maximum stimulation occurring at 0.125 organ equivalents. The A_rs reported here are approximately 5–10 × higher than those reported by Bollenbacher et al.[15] for *M. sexta*. This difference can be explained by our use of a 2-h preincubation which allowed ecdysteroid production by PGs to drop to low levels prior to the addition of the boiled proctodaeal extracts. When stimulated, *L. dispar* PGs released 3–5 times more 3-dehydroecdysone than ecdysone, but A_rs were similar because the controls for ecdysone were proportionally lower than those for 3-dehydroecdysone production. Preparations of *L. dispar* proctodaea also showed considerable ecdysiotropic activity. In contrast, extracts prepared from testes, salivary glands, mandibular glands, malpighian tubules, subesophageal ganglia and fat body produced little ($A_r < 3$) or no stimulatory activity (data not shown). The protein concentration of these extracts ranged from 0.5–10 µg while that of active proctodaeal extracts was in the range of 0.3 – 6 µg. For *O. nubilalis*, reverse-phase HPLC-RIA[20] of pooled aliquots of incubation fluid after either treatment 'G' or 'K' revealed the presence of a peak that had the same retention time as ecdysone, the peak after treatment 'K' being larger than that after treatment 'G' (fig. 3A). For 'K' very small amounts of a polar ecdysteroid and an ecdysteroid having the same retention time as 20-hydroxyecdysone also were observed. To verify that 'G' did indeed contain considerable 3-dehydroecdysone, pooled samples of 'G' were subjected to HPLC. When fractions were dried, incubated with ketoreductase (unheated proctodaeal extracts) and then assayed by RIA, detectable ecdysteroid was observed only for those fractions (47–53) that had the same retention time as 3-dehydroecdysone (fig. 3B). Additional HPLC revealed that more than 95% of this RIA-detectable ecdysteroid had the same retention time as ecdysone.

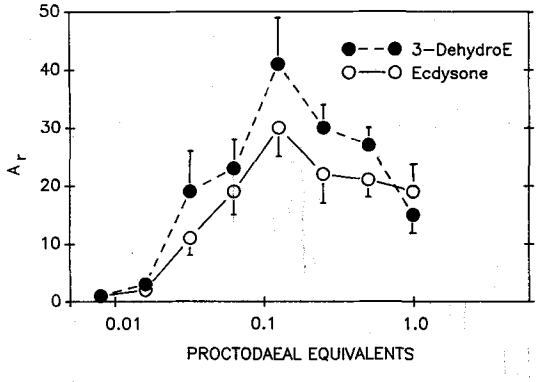

Figure 2. PG production of ecdysteroid upon stimulation by *O. nubilalis* proctodaeal extracts. A_r = [picograms (pg) of RIA-detectable ecdysteroid produced by experimental glands] / [picograms (pg) of ecdysteroid produced by their respective controls], [G] / [Gc] and [K-G] / [Kc-Gc] for ecdysone and 3-dehydroecdysone respectively (fig. 1). Each point is the mean of at least 6 separate determinations. Error bars represent the standard error of the mean.

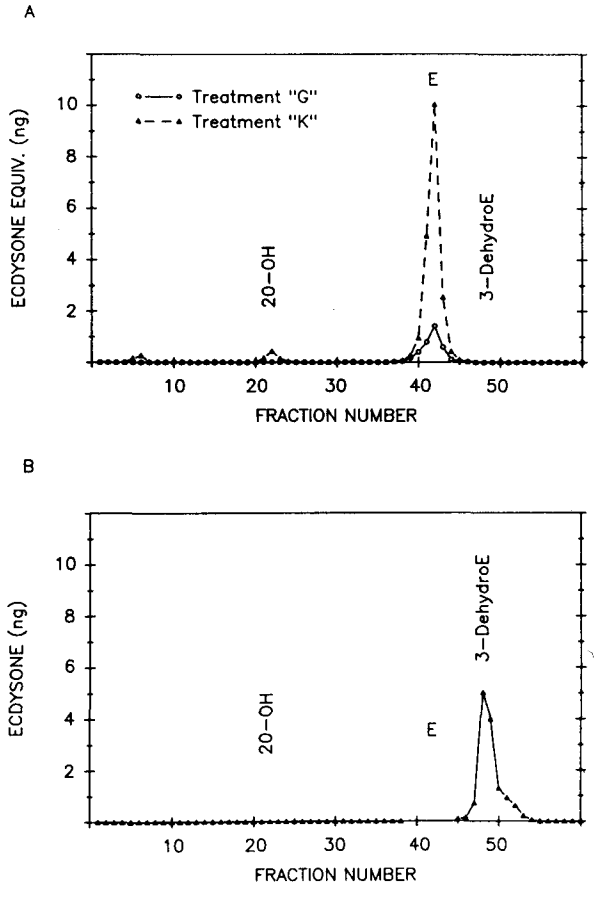

Figure 3. *A* HPLC-RIA of incubation medium from 6 *L. dispar* PGs stimulated with boiled proctodaeal extract of *O. nubilalis* and then incubated with either Grace's medium 'G', or unheated proctodaeal extract, 'K', containing an active ketoreductase as described in fig. 1. Extracts were fractionated on a reverse-phase Supelco LC-18DB column with 41% aqueous methanol as the eluting solvent. The flow rate was 2 ml · min^{-1} and fractions of 0.60 ml were collected. Fraction numbers for 20-hydroxyecdysone (20-OH), ecdysone (E), and 3-dehydroecdysone (3-DehydroE) are shown. The graph represents results from a single experiment, but duplicate runs were almost identical. *B* HPLC fractions (from treatment 'G') that were converted to RIA-detectable ecdysteroid after treatment with ketoreductase. Pooled aliquots of incubation fluid were extracted and processed for HPLC. All fractions except those having the same retention time as ecdysone were dried and then incubated with 0.25 organ equivalents of unheated proctodaeal extract (ketoreductase) for 2 h. The reaction was stopped with absolute methanol; tubes were dried and subjected to RIA. Only fractions having the same retention time as 3-dehydroecdysone were converted by the ketoreductase in the unheated proctodaeal extract to RIA-detectable ecdysteroid.

When it was originally reported that *M. sexta* PGs produced 3-dehydroecdysone, the authors showed that hemolymph contained considerable ketoreductase activity and hypothesized that the PGs also possessed some enzyme activity[9]. In earlier experiments, we did not detect any ketoreductase activity in extracts of *L. dispar* PG's[21]. However, by adding aliquots of whole homogenates of PG glands to 3-dehydroecdysone, incubating for 2 h, and analyzing the methanol-extracted incubation mixture by HPLC-RIA, we have confirmed that these glands contain ketoreductase that converts 3-dehydroecdysone to ecdysone. Thus, the ecdysone detected after incubating *L. dispar* PG glands with heated proctodaeal extract is probably the result of the conversion of the gland's product, 3-dehydroecdysone to ecdysone by the PGs themselves.

Exposure of proctodaeal extracts from *Ostrinia* to pronase destroyed 80 and 92% of the prothoracicotropic activity promoting ecdysone and 3-dehydroecdysone production, respectively, attesting to the proteinaceous nature of the gut ecdysiotropin(s). Since this factor(s) is stable to boiling and to treatment with acetonitrile-TFA and methanol-TFA (unpublished results), it is likely that it is a peptide or small protein. After centrifugation through Amicon filters (Amicon, Division of W. R. Grace and Co., Danvers, MA), activity was detected in the 3–10, 10–30, and greater than 30 KD range. Therefore, the proctodaea may contain more than one peptidic factor with prothoracicotropic activity.

To determine the effect of exposure time to the proctodaeal ecdysiotropin on PG activation, *Lymantria* PGs were incubated in Grace's medium for 2 h and then exposed to boiled proctodaeal extracts from *Ostrinia* for 2, 5, 10, 15 or 30 min prior to being transferred to fresh drops of Grace's medium. Measurements of ecdysone and 3-dehydroecdysone production after an additional 2 h of incubation revealed that a 2-min exposure to the boiled proctodaeal extract was sufficient to induce ecdysteroid production by PGs, and that 10 – 15-min treatments caused maximum stimulation. Similar results were

Percentage of head-ligated day 7 *Lymantria* 5th instars that molted to pupae after injection with various tissue extracts

Insect	Tissue	Concentration (organ equivalents)	Percent molting***
European corn borer	*OW Proctodaeum	0.5	60
		0.25	60
		0.125	60
		0.063	30
		0.032	50
		0.016	40
		0.005	25
		0.001	0
	OW Brain	2.0	70
		1.0	50
		0.5	50
		0.25	80
		0.125	70
		0.063	50
		0.032	10
		0.01	10
	****OW Midgut	0.5 – 0.06	0
	OW Testes	1.0 – 0.125	0
	OW Salivary glands	0.5 – 0.063	0
	**OB Fat body	1 segment equiv.	0
Gypsy moth	OW Proctodaeum	0.002	67
		0.0004	44
		0.00008	33

*OW, last instars that had completed gut purge. **OB, last instars that had left the medium, but had not completed gut purge. ***10, 20 larvae were used for each determination; larvae were injected on the 5th day post-ligation with 10 µl of boiled extract. ****1 midgut equivalent = the portion of the midgut in 2 abdominal segments (only segments 1–6 were utilized).

reported for the stimulation of *Manduca* PGs by brain extract[22, 23].

By means of an in vivo assay[24] in which larvae of *L. dispar* were ligated at the neck before the release of PTTH, injected with boiled extracts of proctodaea, and then scored for pupal molt, we confirmed that boiled proctodaeal extracts from both *Ostrinia* and *Lymantria* did possess ecdysiotropic activity as did similarly prepared extracts of *O. nubilalis* brains (table). Treatment with other tissue extracts (testes, salivary glands, midgut and fat body) gave negative results.

Our finding that proctodaea possess prothoracicotropic peptides is the first report of the existence of ecdysiotropic peptides outside of the brain and associated neurohemal organs. At present, the origin and function of these proctodaeal peptides are unknown. Since other researchers have reported the presence of endocrine centers in insect midgut[25–27], and since a multi-site location of regulatory peptides is well documented in the vertebrates[28], it is possible to speculate that the gut peptides reported here may be involved in the regulation of ecdysteroid production, and thus, secondarily, in the control of lepidopteran molting and metamorphosis. However, as in the vertebrates, gut peptides may have a different function in gut than in the central nervous system[29]. As mentioned previously, there are reports of a proctodaeal hormone, proctodone, that plays a role in stimulating diapause larvae of *O. nubilalis* to pupate[11]. However, this hormone purportedly stimulates the brain to release PTTH. The proctodaeal factor(s) reported in the present study is an ecdysiotropin that can act directly on the prothoracic glands of *L. dispar*. Questions regarding its structure and actual regulatory roles must now be addressed.

Acknowledgments. We wish to thank M. A. Canter, A. A. Khalidi and C. A. Masler for technical assistance, and D. L. Dahlman, H. H. Hagedorn, R. S. Hakim, M. J. Loeb and C. A. Shephard for their critical reading of the manuscript. This work was supported, in part, by a USDA Competitive Grant, No. 86-CRCR-1-2111 (E.P.M., T.J.K.), and by the Ministry of Home Affairs, Government of India, Postdoctoral Research Assistantship, No. BC-11015/5/84 (B.S.T.). Mention of a commercial or proprietory product in this paper does not constitute an endorsement by the USDA.

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