The Inhibitory Control of Prothoracic Gland Activity by the Neurosecretory Neurones in a Moth, *Mamestra brassicae*

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**ABSTRACT**—The change in both the ecdysteroid titers in the haemolymph and the amount of ecdysteroids released from the prothoracic gland (PTG) *in vitro* during larval development were determined in *Mamestra brassicae*. These showed a clear inverse relationship to the efferent electrical activity in the PTG nerves, even in the detailed changes in each day during development. An exception was the final day of the last instar. This result suggests that, as well as the activating effect of prothoracotrophic hormone (PTTH), the PTG innervating neurosecretory neurones are possibly involved in the delicate inhibitory adjustment of the amount of ecdysteroids in the haemolymph. Here, the simultaneous determinations of the amount of ecdysteroids released from PTG and of the electrical activity of nerves by using the isolated preparation *in vitro*, provides direct evidence of the inhibitory effect of PTG innervating nerve on the PTG.

**INTRODUCTION**

Various types of PTG innervating neurones were described in the previous paper [1]; both histological and electrophysiological studies showed that they are neurosecretory. The electrical activities of one of PTG innervating nerves shows the remarkable changes during the course of development, its firing frequency varying inversely with the ecdysteroid titers in the haemolymph as examined by Agui and Hiruma [2]. These results and those from surgical experiments [3] have led to the suggestion that the function of this PTG innervating nerve is to inhibit the PTG.

To analyse accurately this inverse relationship between the electrical activity of the PTG innervating nerve and ecdysteroid titer in the haemolymph, it is important to reexamine the haemolymph ecdysteroid titers by using the same animals from our strain and reared under the same condition as was used for the measurement of the electrical activity. Also, it is better to use isolated preparations for determination of both the released ecdysteroids from PTG and the electrical activity of PTG innervating nerves. Therefore, the time change of the ability to synthesize ecdysteroid in the isolated PTG *in vitro* was determined. In this paper, the simultaneous determinations of the amount of released ecdysteroid from PTG and of the electrical activity of the PTG innervating neurones were made to obtain direct evidence for the nervous inhibition of the PTG activity.

**MATERIALS AND METHODS**

Larvae of *Mamestra brassicae* were reared on artificial diet [4] at $23^\circ \pm 1^\circ C$ under a long day photoperiod (16L, 8D). The time course of development of the last instar larva was the same as described in the previous paper [1]. The first day after ecdysis was designated day 0. Female larvae were used throughout this experiment.

Ecdysteroid titers in the haemolymph were determined by radioimmunoassay (RIA). Haemolymph collection was made four times in each day of larval development at 0:00, 6:00, 12:00 and 18:00, the middle of dark period being taken arbitrarily as 24:00 (0:00). Ten $\mu l$ haemolymph was taken from each larvae, by making a small cut.

Accepted November 14, 1988
Received October 24, 1988
on a proleg, and introduced into 400 μl methanol. After mixing vigorously, this sample was cen-
trifuged at 3000 g for 20 min, methanol in the sup-
natant was evaporated at 40°C, and the sample was
stored frozen at -20°C. Before starting the RIA
procedures, the dried sample was dissolved in 100
μl borate buffer (pH 8.4). If necessary this solu-
tion was diluted to one tenth. Forty μl of this
solution were used as the biological sample in
RIA.

The amount of ecdysteroids released from PTG
in vitro was also determined by RIA. PTGs were
extirpated from the larvae of various stages of last
instar. The isolated PTGs were kept immersed in
100 μl Grace’s medium for about 1 hr. The ne-
cessity of the immersion of PTG in the culture
medium will be described later. The PTGs were
then transferred and incubated usually for 2 hr in
100 μl new Grace’s medium dropped on each
compartment of an acrylic culture well (Falcon
3047, Becton Dickson & Co.). The culture wells
were placed in a container which was filled with a
gas mixture of O₂ 95% and CO₂ 5%, saturated
with water vapor and kept at 23° ± 1°C. After the
incubation, 40 μl of the culture medium was col-
cected and used as the biological sample in RIA.
The culture method for the isolate ganglion-PTG
preparation used for studying the ecdysteroid
synthesis under the influence of PTG innervating
nerves will be described later in the corresponding
part of results.

RIA for ecdysteroids was performed according
to Borst and O’Conner [5] using [23, 24-3H] ec-
dysone (ca. 70 Ci/mmol, New England Nuclear).
The antiserum of ecdysterone (20-hydroxy-
ecdysone, Rhoto Pharmaceutical, Osaka) was pre-
pared in collaboration with Prof. E. Ohnishi,
Nagoya University, Prof. N. Ikekawa, Tokyo In-
tstitute of Technology and Dr. K. Ozawa, Megro
Institute (Osaka). Practical steps of RIA pro-
cedures were based on and modified from Bollen-
bacher et al. [6] which presented the systematic
descriptions for the study of the synthetic ability
of ecdysteroids by PTG in vitro. Antiserum was
diluted to 1/3200 with 10% rabbit serum dissolved
in the borate buffer so that about 50% of the
radioactivity of labelled ecdysone was bound in the
absence of unlabelled ecdysone. The labelled
ecdysone (ca. 8 nCi) in 80 μl buffer and 50 μl
antiserum solution was added to each of a pair of
40 biological samples described above. Duplicated
assays were performed for each sample. The
mixture was stored at 4°C for 12–16 hr. After
precipitation using ammonium sulfate, the precipi-
tate was dissolved in 50 μl distilled water and 400
μl scintillation solution (ACS II, Amersham) was
added. Radioactivity was determined using Pack-
ard scintillation counter (model 240 CL/CLD).

The standard curve for ecdysone (Sigma) was
log-linear between 10 and 200 pg and that for
20-hydroxyecdysone (Rhoto Pharmaceutical)
between 30 and 1000 pg. Therefore, in this exper-
iment, the binding curve for ecdysone was exclu-
sively used for both determinations of the haemolymph ecdysteroid and the secreted ec dys-
eroid from the PTG in vitro. All data were
expressed as ecdysone equivalent, because the
determinations in this experiment by RIA were
based on the cross reactivity of the prepared
antiserum with ecdysone, 20-hydroxy-ecdysone
and other ecdysteroids.

RESULTS

Haemolymph ecdysteroid titers

Figure 1 shows the change in haemolymph
ecdysteroid titer during development of the last
instar larva. To show clearly the small change
in ecdysteroid titer, the values from day 1 to day 7
were plotted separately with five fold magnifica-
tion in the vertical scale. There are small peaks at
each light period of day 3-6 which show gradual
increase in magnitude with the progress of the
development. It is difficult to assess directly which
peak of haemolymph ecdysteroids is the stimulant
for the appearance of transparent epidermis or for
wondering behaviour to the ground. The highest
peak prior to the pupal ecdysis was observable at
18:00 of day 8. Quantitative comparison between
ecdysteroid titers in the haemolymph and the
electrical activities of PTG innervating nerves dur-
ing development will be given at the later section
of results.
Inhibition of PTG Activity by Nerves

Fig. 1. Ecdysteroid titers in the haemolymph of *Mamestra brassicae* during the development of last instar larva. Each point is the mean (±SEM) of ten measurements. The following developmental stages are indicated by arrows: LE, larval ecdysis; TE, appearance of transparent ventral epidermis; WG, wandering to the ground; PE, pupal ecdysis. Light and dark periods in each day are shown at the lower part of the figure. See text for details of the representations of ecdysteroid titer from day 1 to day 7.

*Released ecdysteroids from PTG in vitro*

Time course of ecdysteroid synthesis by PTG in vitro was determined to find a convenient incubation condition for the study of the change in ability of the ecdysteroid synthesis during development. PTGs were extirpated at 9:00–11:00 from day 8 last instar larva and then incubated in Grace’s medium. The ecdysteroid titer determinations in Figure 1 suggest that the isolated PTG from the larvae of this stage would be sufficiently potent to produce the ecdysteroids under the influence of

Fig. 2. Time course of the ability to synthesize ecdysteroid after incubation of PTG in the Grace’s medium. Each point is the mean (±SEM) of ten measurements.
the prothoracicotropic hormone (PTTH) in the haemolymph.

The amount of ecdysteroid released from PTG into the external medium every hour after the start of incubation was determined by RIA as shown in Figure 2. The ecdysteroid synthesis shown in this figure is limited to low values in the first 1 hr and elevated significantly in the succeeding 2 hr. The rate of ecdysteroid synthesis shows the marked decrease at 4 to 6 hr after the incubation, probably due to washing out the PTTH by the repeated changes of the external medium. The exact reasons for the limited synthesis in the first hour after incubation were difficult to find here physiologically.

In consideration of the time course of ecdysteroid synthesis shown in Figure 2, the following procedure was chosen for the determination of the change in ability of ecdysteroid synthesis during development. After incubation in the culture medium for about 1 hr, the PTGs were transferred and incubated in new Grace's medium for 2 hr, the external medium being used as a samples for RIA determination. In this series, PTG isolations were made at 9:00–11:00 from the larva of each development stage, and consequently incubated for 2 hr from 10:00–12:00 to 12:00–14:00.

The results in Figure 3 shows that a measurable amount of ecdysteroid synthesis is observable at day 3 and the values are increased by day 5 where the transparent epidermis was visible. The amounts of ecdysteroid synthesis remained fairly stable for 3 days from day 5 to day 7 increased significantly at day 8.

Comparison of ecdysteroid synthesis and electrical activity of nerves to PTG

It is important to compare three results; the ecdysteroid titers in the haemolymph (Fig. 1); ecdysteroid synthesis by PTG in vitro (Fig. 3); and electrical activities of the PTG innervating nerve (Fig. 8 in the previous paper [1]) during the larval development. These results are superimposed in Figure 4, where only data in Figure 1 obtained at 12:00 are plotted for better comparison. There are clear parallels between the haemolymph ecdysteroids and the ecdysteroids synthesized in vitro, except that the rate of increase of the haemolymph ecdysteroids from day 7 to day 8 is about thirty times but only about two times in the latter case. This will be discussed below. The critical comparison of the absolute

![Graph](image)

**Fig. 3.** Ecdysteroid synthesis by PTG in vitro during the development of last instar larva. Each point is the mean (± SEM) of ten measurements. Refer to Fig. 1 for explanation of the developmental stages shown at the upper part of the figure.
values determined in both cases is difficult because the amount of haemolymph ecdysteroids cannot be compared directly with the values obtained using PTG preparation in vitro. In this connection, Warren et al. [7] published critical studies on the PTG activity of *Manduca sexta* with an important discussion of the results obtained from the RIA of released ecdysteroids in the culture medium from the isolated PTG.

Careful comparison in Figure 4 of the ability to synthesize ecdysteroid in vitro and the electrical activities of PTG innervating nerves in the isolated preparation indicate a clear inverse relationship even in the precise changes in each day during the development, day 9 being an exception. Detailed descriptions of the change of electrical activities each day have been given in the previous paper [1].

**Inhibitory action of nerves to PTG on the ecdysteroid synthesis by PTG**

The inverse relationship between electrical activity in nerve N4 to PTG and ecdysteroid synthesis by the PTG, as was demonstrated in the previous section of this paper as well as in the previous paper [1], suggest that the activity of the nerve N4 is inhibitory to the ecdysteroid synthesis or release at PTG. This suggestion can be tested directly by simultaneous determination of released ecdysteroid from PTG and of electrical activities of nerves in the same preparation in vitro.

The preparations were isolated as the ganglionic chain from brain to mesothoracic ganglion connected with PTG by the nerve N4. In the first, the nerve N4 in the isolated preparation was placed on the oil gap as shown in Figure 1A of the previous paper [1] and the ganglion and the PTG were immersed separately in the two Grace’s medium pools of the gap chamber. These oil-gap chambers were placed in a gas mixture as described above.

However, unexpected troubles occurred for two reasons. At first, after several min immersion of PTG in Grace’s medium, there was enhanced afferent activity in the nerve N4 followed by the disappearance of the efferent activity generated at the prothoracic ganglion and conducted to PTG. Analysis of this phenomena will be made in the following paper. This problem was solved by replacing the physiological saline described in the
previous paper [1] with a K-rich saline with 70 mM KCl. With this medium, the efferent activity continued fairly well during at least half the period of PTG incubation in the Grace’s medium.

Secondly, the rate of ecdysteroid synthesis by PTG in the oil-gap chamber was drastically reduced. This was due to chemical and/or physical disturbance by the paraffin oil on the synthetic activity of PTG and also to the difficulty of supplying oxygen to PTG in the incubation pool. This problem was reduced by using white vaseline instead of paraffin oil. Although the rate of ecdysteroid synthesis was reduced to about 1/5 in comparison with the case of the usual organ culture described above, the values obtained in the same experimental condition were fairly uniform.

The preparations were obtained from day 5 last instar larva. After the isolated preparations were arranged on the gap chamber in the improved manner and kept for 1 hr, the external medium was replaced by the new Grace’s medium and the samples for RIA were collected after 2 hr incubation. Nervous activity was monitored during incubation period. For the control determinations, contralateral PTGs with the connection of the nerve N4, severed just in front of prothoracic ganglion, were isolated from the same specimen used for the test above, and placed in the similar manner on the individual gap chambers. The efferent electrical activity was not routinely recorded from the nerve of this preparation.

The results are shown in Figure 5 where the rate of inhibition is expressed as a ratio divided by the value obtained from the control experiment. For comparison, ratios between right and left of a single pair of PTGs isolated from the connection with ganglia are also plotted. Thirty animals were used for each comparison; to help the immediate comparison in the figure the degree of inhibition was expressed by the number of animals showing the values which would fit the corresponding ranges of inhibition ratio. This figure shows that the action of the nerve N4 is inhibitory to the ecdysteroid synthesis at PTG. Although the small number of the preparations showed no inhibitory effect, this probably occurred by the injurious

![Fig. 5. Inhibitory effects of PTG innervating nerves on the rate of ecdysteroid synthesis by PTH. The rate of inhibition obtained by comparing the abilities of ecdysteroid synthesis in preparations both with and without PTH innervating nerves. The results obtained by the above determinations are plotted as white circles and those obtained by comparing the synthetic activities in both right and left PTGs of a single specimen as black circles. See the text for the explanation of the inhibition ration.](image-url)
effects on nerves or nerve junctions with PTG in the case of setting the isolated preparation on the gap chamber.

DISCUSSION

Based on the results suggested from the electrophysiological determinations in the first paper [1] of this series, the present work using RIA techniques was intended to establish the real function of the nerves to PTG with respect to the PTG activity. Direct determinations of the amounts of synthesized ecdysteroid under the influence of nerve N4 to the PTG clearly showed the inhibitory function of the PTG nerves on the PTG. This activity is assumed to be neurosecretory as described in the previous paper [1]. However, it is not yet clear whether the neurosecretory substance released into the PTG inhibits the synthetic or releasing process at PTG.

The high afferent electrical activity observed in nerve N4 shortly after the immersion of the isolated nervous system in Grace’s medium and the following reduction in efferent activity is similar to changes already noticed during measurements of efferent activity during development. Here it was found that the firing frequency was greatly influenced by the chemical composition of the external medium [1]. Although the details of these phenomena will be described in the following paper, the results described above indicate that the main feedback machinery for monitoring the chemical condition of haemolymph must be contained in the ordinary isolated preparation of the nervous system.

The electrical activity of nerve N4 was found to show the remarkable changes with the progress of the larval development exactly related to the change in ecdysteroid synthetic or releasing activity at PTG. This result suggests that, as well as the activation effect of PTTH, the neurosecretory neurones, which directly innervate PTG, are involved in the precise adjustment of the amount of haemolymph ecdysteroids. These may regulate the appearance of the various important events of the development, like the wandering behaviour as has been analyzed in the lepidopterous larvae by Gilbert et al. [8], Dominick and Truman [9], Fujishita et al. [10]. The exception observed at the final day of larval development to pupa probably means that the drastic decrement of the haemolymph ecdysteroids is solely governed by the chemical inactivation of ecdysteroids in the haemolymph. Similarly, the elevation to the extremely high level of haemolymph ecdysteroid titers just before pupal ecdysis (Fig. 1) may not be the consequence of regulation by the PTG innervating nerves.

ACKNOWLEDGMENTS

We wish to thank Dr. M. Fujishita, Nagoya University, for her technical support on radioimmunoassay. We also wish to thank Dr. J. Kien, University of Regensburg, for critical reading of the manuscript.

REFERENCES

