Insect Prothoracicotropic Hormone: Evidence for Two Molecular Forms

Abstract. *In an insect, the tobacco hornworm* Manduca sexta, *the cerebral neuropeptide prothoracicotropic hormone (PTTH), the primary effector of postembryonic development, exists as two molecular forms. These two PTTH's elicit characteristic in vitro dose responses of activation of prothoracic glands from different developmental stages, an indication that during development the glands change in their sensitivity to the neurohormones. Both PTTH's are active in a specific in situ bioassay. Since they may be released in situ at stage-specific times to evoke distinctly different developmental responses, the PTTH neuroendocrine axis appears to be an effective system for determining the functions of molecular forms of a neurohormone in the regulation of growth and development.*

The first evidence for the existence of a neurohormone was first obtained in a study of the gypsy moth *Lymantria dispar*, showing that molting and metamorphosis required the presence of a "brain hormone" (*1*), termed the prothoracicotropic hormone (PTTH) (*2*). This hormone activates the prothoracic glands (PG) to synthesize and secrete ecdysone, which is hydroxylated to 20-hydroxyecdysone, the ecdysteroid that elicits molting in insects. The chemistry of PTTH has been investigated for many years, but its chemical structure has remained elusive (*3*). Although its protein nature was established, its apparent molecular size ranged from 4.4 to 30 kD. Studies on the PTTH of the silkmoth (*Bombyx mori*) indicate the presence of a single 4.4-kD form (*3, 4*), but similar studies of the PTTH of *Manduca sexta* (tobacco hornworm) have suggested that PTTH may exist in more than one molecular form (*5, 6*).

A limiting factor in earlier studies of PTTH was the bioassay (*3*). However, we have developed an in vitro PG assay for the neurohormone (*7*), which monitors the direct activation of the PG by PTTH. Activation is expressed as an increase in the rate of ecdysone synthesis by the gland (*8*), and this increase occurs in a characteristic dose-dependent manner. Using this assay, which can also be used to quantify units of PTTH activity (*9*), we now provide evidence that two PTTH's are present in the *Manduca* brain.

To identify the PTTH's in the brain of day 1 pupae of *Manduca*, we established an extraction protocol for direct assessment of PTTH activity with the in vitro assay. Critical to the protocol was the interfacing of the extraction method, as well as all of the purification procedures, with the in vitro assay. The methods used were therefore dictated by the in vitro conditions required for PG activation by PTTH. Of several extraction methods tested, homogenization in a mixture of distilled water and Grace's culture medium (2:1, by volume, 4°C), subsequent heat treatment (100°C for 2 minutes), and centrifugation yielded the greatest amount of PTTH activity and interfaced most easily with the assay. Heat treatment resulted in a tenfold purification of PTTH with no loss in activity.

To determine the molecular size of *Manduca* PTTH, an extract of ~ 2500 brains from day 1 pupae (*10*) was fractionated by gel filtration chromatography; the fractions were assayed with both larval and pupal PG (Fig. 1). The profile of PTTH activity generated with day 1 pupal PG revealed a major peak at ~ 29 kD and a possible minor peak at 6 to 7 kD. Analysis of the fractions with day 3 larval PG, which appear to be more sensitive to PTTH than pupal PG (*11*), confirmed the presence of the large molecular size moiety but also clearly showed an additional, smaller 7-kD moiety. This smaller compound was apparently the minor peak of activity detected with the pupal PG. These data indicated the presence of two PTTH-like factors in the pupal brain which differentially activate the PG from different developmental stages. Comparable results have been obtained with extracts of day 3 larval brains, indicating that these factors are present in both larval and pupal brains. These two moieties have been termed big and small PTTH (*12*) and they appear to differ substantially from the 4.4-kD *Bombyx* PTTH (*13*).

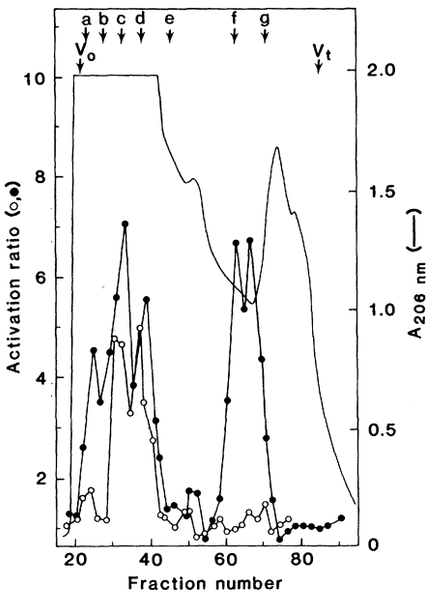

Fig. 1. Determination of the molecular weight of the PTTH present in day 1 pupal brains of *Manduca sexta*. Gel filtration was performed with a Bio-Rad P-100 column (1.6 by 90 cm) and a phosphate buffer (0.05*M* NaH_2PO_4, *p*H 6.8). The PTTH activity was quantified with the in vitro PG assay. Samples of 2.0-ml fractions were assayed with day 0 pupal (○) and with day 3 last-instar larval prothoracic glands (●) at dilutions in Grace's medium of 1:9 and 1:15 (by volume), respectively. The A_r for each fraction is the mean of three to five separate determinations. V_o and V_t denote the exclusion and inclusion volumes of the column. The molecular standards used were bovine serum albumin (*a*), ovalbumin (*b*), carbonic anhydrase (*c*), myoglobin (*d*), cytochrome (*e*), aprotinin (*f*), and the B chain of insulin (*g*).

Since these factors appeared to be neurohormones, they would most likely be peptides, as was shown by their sensitivity to proteolytic hydrolysis. When known amounts of each moiety were treated with either protease or trypsin (*14*) the activity of each factor decreased by 95 percent, as measured by in vitro assay, indicating that the PTTH's were proteins.

A distinguishing property of PG activation in vitro by the PTTH in a pupal brain extract is that the dose-response curve (*7*) is characterized by a narrow dynamic range of neurohormone concentration, that is, the number of units (*9*) needed to raise a gland from its unactivated state (where $A_r = 1$) to a maximally activated state (A_{max}). Thus the response curve obtained with the big and small PTTH activities separated by gel filtration could show (i) that they were indeed PTTH's and possibly (ii) that there could be different responses of the larval and pupal PG to each hormone. Dose-response profiles of the activation of the larval and pupal PG (Fig. 2B) by big PTTH revealed that these PG's responded to big PTTH in the same manner, which was similar to their responses to PTTH in a brain extract: (i) the narrow dynamic range of big PTTH eliciting a gland response was comparable (0.03 to 0.12 unit for larval PG, 0.04 to 0.14 unit for pupal PG); (ii) the values of A_{max} for the glands (5 for larval and 3.3 for pupal) were not significantly different ($P > 0.18$); and (iii) the ED_{50}'s (the dose eliciting half-maximal activation) were the same (0.075 unit for larval and pupal PG), indicating that the big PTTH was an equally potent activator of these glands.

By contrast, small PTTH had a different effect on the larval and pupal PG (Fig. 2A). Even though a narrow dynamic range of neurohormone-activated PG from both stages and approximately the same A_{max} was reached (larval PG 4.5, pupal PG 3.5) (see legend to Fig. 2), the larval glands were considerably more sensitive (about 20 times) to small PTTH (ED_{50} = 0.15 unit) than were the pupal glands (ED_{50} = about 3 units). These findings explain the gel filtration results, where small PTTH was not clearly and reproducibly detected when assayed on pupal PG. They also suggest that PG sensitivity to the PTTH's changes during postembryonic development, a situation that may be of physiological significance. If true, there may be stage-specific physiological roles for the small and big PTTH's.

An additional characteristic of the larval PG response to small PTTH was a decrease in the A_r to less than half the A_{max} at high concentrations of the hormone. This inhibitory effect was not observed in the activation of larval glands by high concentrations of big PTTH. These differing profiles of response of larval and pupal PG to the PTTH's indicate that these moieties may be acting on the PG via different receptors (mechanisms).

In addition to the different molecular sizes and gland sensitivities of the two PTTH's, the isoelectric points (*p*I) of big and small PTTH provided further evidence for two prothoracicotropins in the *Manduca* brain. By analytical acrylamide gel isoelectric focusing (IEF) (*15*), the *p*I's for the big and small PTTH's were 5.4 and 5.2, respectively. Minor activity at a second *p*I was found for each factor, 5.1 for big PTTH and 3.7 for small PTTH. The generation of a dose response of activation of larval and pupal PG with each of the four activities indicated that they were PTTH's. The dose responses obtained with the big and small activities were essentially identical to those obtained for the respective fractions after gel filtration (Fig. 2, A and B). These different effects of the small PTTH, particularly the reduced ability of this PTTH to activate pupal PG, were again observed, clearly distinguishing it from big PTTH. The existence of more than one *p*I for big and small PTTH may reflect posttranslational processing of each neurohormone, for example, amidation, glycosylation, and phosphorylation.

To prove that these PTTH's functioned as neurohormones in situ and were not artifacts of the in vitro assay, their biological activity was tested in the *Manduca* larval bioassay for PTTH (*16*). An extract of day 1 pupal brains injected into a test population of larvae at a dose of 1 unit of PTTH per animal causes about 50 to 70 percent of the animals to undergo a larval molt. Injections of 1 unit of big PTTH elicited molting in 67 percent of the test animals, a percentage approximating the response to 1 unit of PTTH of the whole brain extract. This response to big PTTH was dose-dependent and was essentially the same as the dose response of larval molting obtained with a pupal brain extract. By contrast, the best response obtained with small PTTH (10 units) was 20 percent. While the bioassay response to small PTTH was dose-dependent, the slope of the response was much less than that for big PTTH, an indication that small PTTH was much less potent in this assay than big PTTH. Although the bioassay was less sensitive to small PTTH, the results were sufficiently reproducible to conclude that this moiety functions as a PTTH in situ. That small PTTH is less able to elicit a response may be due to the fact that the assay measures only a molting response. It is possible that the true biological activity of small PTTH is not assessed with this particular bioassay since the PG's are differentially sensitive to small PTTH during development. Nevertheless, the data indicate that both big and small PTTH function in situ as prothoracicotropins.

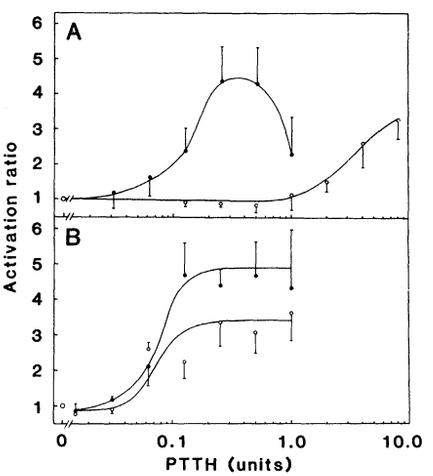

Fig. 2. Dose responses of larval (●) and pupal (○) prothoracic gland activation by small (A) and big (B) PTTH. For this study the same stock solutions of big and small PTTH at the units denoted (*9*) were used to compare the response kinetics of these PG to the neurohormones. Because of the paucity of small PTTH available for the dose-response analysis with pupal glands, a complete response curve was not generated. Thus the comparison of larval and pupal PG responsiveness to this PTTH is based upon the pupal PG response reaching an extrapolated A_{max} of about 3.5, a value consistent with that obtained with big PTTH on these glands. Because a dose of 3 units was needed to achieve an A_{50}, the physiological significance of this response needs to be investigated more thoroughly. The standard in vitro PG assay was used (*8*), and each data point is the mean ± standard error of the mean of five to nine separate determinations.

Final proof that these peptides are neurohormones will require their isolation from the hemolymph at times during development when PTTH release occurs. Preliminary studies of the biological and chemical properties of the PTTH's released in situ indicate that small PTTH is released early in the last larval instar. Activation of the PG at this time results in a subtle increase in the hemolymph ecdysteroid titer, which in turn elicits pupal commitment and wandering (*17*), processes not directly involved in, nor responsible for, molting. By contrast, the release of big PTTH during the fourth larval instar is temporally related to the dramatic increase in the hemolymph ecdysteroid titer which elicits larval molting. These results thus explain the different in situ bioassay activities of big and small PTTH. If corroborated, they present the intriguing possibility that these two prothoracicotropins have distinct roles in regulating the postembryonic development of this insect. Since the PTTH-PG axis of *Manduca* can be easily manipulated both in vitro and in situ, this system appears well suited for answering basic questions about the functions of different molecular forms of neurohormones.

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8. In the in vitro assay for PTTH, one PG of a pair in an animal serves as the experimental gland and is incubated in medium containing a portion of test sample while the contralateral control gland is incubated in medium alone. The assay is performed in Grace's medium, and the amount of medium varies depending on experimental design. It is never less than 20 percent by volume to ensure that the gland is capable of being activated maximally by PTTH. Activation is expressed as an activation ratio (A_r), which is the ecdysone synthesized and released by the experimental gland in 2 hours divided by that released by the control gland. Ecdysone synthesis is quantified by an ecdysone radioimmunoassay (RIA) in which a sample (0.01 ml) of the incubation medium (0.025 ml) is assayed directly. A macro RIA with an ecdysone standard concentration range of 0.25 to 32 ng was used (*11*).
9. The in vitro PG assay was used to quantify units of PTTH activity. A standard PTTH unit is the amount of neurohormone present in a day 1 pupal brain of *Manduca*. The value of the standard PTTH unit was determined from the dose of day 1 pupal brain extract (ED_{50}) needed to half-maximally activate the PG (A_{50}) in a dose-response curve of PG activation in vitro. For day 1 pupal brains, a mean ED_{50} of 0.06 brain equivalents elicited the A_{50}; thus with one brain, 16.7 (the reciprocal of 0.06) in vitro PG assays can be run at the A_{50}. One PTTH unit is therefore the amount of neurohormone that can generate 16.7 in vitro assays at the A_{50}. The PTTH activity in one PTTH unit represents the combined activity of big and small PTTH. Because of the different relative amounts of these two neurohormones in the brain, the different efficiencies with which they are purified, and the stage-specific differences in the PG response they elicit, the PTTH unit had to be redefined for big and small PTTH once they were separated. The definition of these units retained as its basis the ED_{50} value derived from a dose-response curve of PG activation in vitro with each neurohormone. Thus one unit of big PTTH is the amount of this hormone that can generate 16.7 in vitro assays at the A_{50} when either day 0 pupal or day 3 larval PG are used. Similarly, one unit of small PTTH is the amount of this hormone that can generate 16.7 in vitro assays at the A_{50} when day 3 larval PG are used. Thus the ED_{50} in units of big and small PTTH in the in vitro assay is 0.06 as it is with a standard PTTH unit. To quantify PTTH units in a sample requires the generation of a dose-response curve of PG activation; from this curve the number of in vitro assays that can be run with the sample at the A_{50} is determined. This number is divided by 16.7 to yield total PTTH units.
10. After heat treatment and the quantification of PTTH content, the brain extract was subjected to ultrafiltration and diafiltration with distilled water (Amicon YM-2). The retentate, free of small molecules (< 1 kD), was assayed for PTTH to ensure minimum loss of activity and then lyophilized and stored at −20°C.
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14. Big and small PTTH (12 to 14 units from same stocks as in Fig. 2) were incubated individually with 1.45 units of protease (Sigma) and 4.12 units of trypsin (Boehringer Mannheim) in tris buffer (0.046*M*, *p*H 8.1) for 4 hours at 25°C. The loss of PTTH activity was due to proteolytic hydrolysis since heat-denatured or inhibitor-inactivated enzyme had no effect on the ability of nonhydrolyzed PTTH to activate the PG.
15. Analytical isoelectric focusing was performed (LKB Instruments, application note 250). Fractions were eluted with 0.2 ml of phosphate-buffered saline (0.01*M* NaH_2PO_4, 0.15*M* NaCl, *p*H 7.2), mixed with Grace's medium 4:1 (by volume), respectively; the *p*H was adjusted to 6.8 and PTTH activity was assayed. The *p*I for big PTTH was determined on a 4 to 6.5 *p*H gradient and the *p*I for small PTTH was determined on 4 to 6.5 (*p*I 5.4) and 3.5 to 9.5 (*p*I 3.7) *p*H gradients. Ampholines were not removed before assay since at *p*H 6.8 they did not have an adverse effect on the ability of the PG to be activated by PTTH.
16. D. Gibbs and L. M. Riddiford, *J. Exp. Biol.* **66**, 255 (1977).
17. L. M. Riddiford, *Nature (London)* **256**, 115 (1976). Pupal commitment: early in the last larval instar a small increase in the hemolymph ecdysteroid titer occurs in response to PTTH activation of the PG, and this increase in the absence of juvenile hormone covertly commits the larval tissues to become pupal. The expression of this commitment occurs later in the instar in response to a second, dramatic increase in the ecdysteroid titer.
18. H. Ishizaki reported (at the Second International CNRS Symposium on the Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones in Strasbourg, France, September 1983) that adult heads of *Bombyx mori* contain a 22-kD peptide having PTTH activity in *Bombyx*. This is in addition to the 4.4-kD moiety which is not active in the *Bombyx* assay but is active in a *Philosamia cynthia ricini* assay.
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Reinitiation of Growth in Senescent Mouse Mammary Epithelium in Response to Cholera Toxin

Abstract. *Several lines of mouse mammary tissue that had been serially transplanted until mitotic senescence was reached were exposed in vivo to plastic implants that slowly released cholera toxin. Gland tissue surrounding the implants displayed new end buds, indicating reinitiation of growth and morphogenesis. The ability of cholera toxin, which elevates intracellular adenosine 3',5'-monophosphate, to temporarily reverse the senescent phenotype suggests that this mitotic dysfunction results not from generalized cellular deterioration but from specific changes in cell regulation.*

Normal cells consistently display a limited capacity for proliferation when serially transferred in culture (*1, 2*) or when passaged in young, isogeneic hosts in vivo (*3*). An example of this phenomenon, often interpreted as an expression of cellular aging, is found in mouse mammary epithelium. Young mammary ducts exhibit vigorous growth when transplanted into gland-free mammary fat pads, but undergo a progressive reduction in growth rate with serial transplantation (*4*), even though the transplanted tissue maintains its normal stromal associations (*5*) and is propagated in a hormonal and nutritional environment that is optimal for continuous proliferation and morphogenesis. This loss of replicative potential by normal cells appears irreversible, and immortal transplant lines can be obtained only by transformation into preneoplastic (hyperplastic) or neoplastic cell types (*6*).

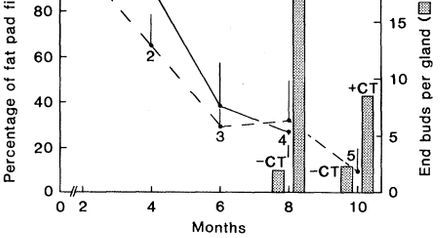

Fig. 1. Growth of mammary ducts in two of the tissue lines used for experiments represented in Fig. 2 and Table 1 (solid line, experiment 1; dashed line, experiment 3). Each transplant line was initiated by tissue taken from a 3-month-old BALB/c donor. End-bud number, a measure of growth rate, is indicated by bars at generations 4 and 5 for experiments 1 and 3, respectively. Vertical lines are 95 percent confidence intervals. *CT*, cholera toxin.

Agents that raise the intracellular concentration of adenosine 3',5'-monophosphate (cyclic AMP), such as cholera toxin (*7*), are mitogenic in vitro for a variety of epithelial cells (*8–11*); mammary cells are stimulated to increase DNA synthesis and to divide in both monolayer (*12*) and collagen gel cultures (*13*). In vivo, increased cyclic AMP in mammary tissue is correlated with pregnancy (*14*) and with several breast carcinomas (*15*). In normal mammary ductal tissue cyclic AMP is a positive growth regulator, as shown by administration of cholera toxin and other cyclic AMP–active agents to local areas of mammary glands in ovariectomized mice (*16*). Small plastic implants (*17, 18*) were used to gradually release the agents. Cholera toxin stimulated the appearance of new end buds, which grew rapidly and participated in normal ductal morphogenesis. In young intact animals, cyclic AMP–active agents did not increase the elongation rate of mammary ducts, which were already growing at the maximum rate.

We undertook experiments to determine whether serially aged, growth-retarded mammary duct cells retain the ability to respond to cyclic AMP–active agents. Each of three mammary transplant lines was initiated by transplanting fragments of mammary ducts from a 3-