A dual role of 20–hydroxyecdysone in the control of protein synthesis related to DNA puff activity in the anterior region of *Bradysia hygida* (Diptera, Sciaridae) salivary gland

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Abstract

During the last 30 h of the larval stage, the salivary glands of *Bradysia hygida* show the amplification of some genes, resulting in the formation of two successive groups of DNA puffs, which direct the synthesis of two different sets of polypeptides. Incubation of anterior (S1) salivary gland regions, at age E7, beginning of first group of DNA puffs activity, in culture medium for 2 to 10 h results in a decrease in the synthesis of the polypeptides characteristic of this period. However, during subsequent incubation (from E7 to E7+12 h–24 h), when the second group of DNA puffs is active, S1 regions were able to synthesize some polypeptides characteristic of this period. The role of 20–OH ecdysone was studied, in vitro and in vivo, during these two periods of protein synthesis in S1 regions. The presence of the hormone was shown to be necessary to maintain, in vitro, the synthesis of the first set of polypeptides and was strongly inhibitory, in vitro and in vivo, to the synthesis of the second set of polypeptides. Thus, it is likely that the activity of the two distinct groups of DNA puffs is under opposite 20–OH–ecdysone control mechanisms. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: 20–OH–ecdysone; Protein synthesis; In vitro and in vivo; DNA puffs; Salivary glands; Sciaridae

1. Introduction

DNA puffing is characteristic of the normal development of salivary gland polytene chromosomes of Sciaridae larvae and corresponds to the amplification of protein-coding genes (Breuer and Pavan, 1955; Glover et al., 1982; Paçó-Larson et al., 1992; Fontes et al. 1992, 1999; Wu et al., 1993; Laicine et al., 1984; Almeida, 1997). Similar amplification occurs with chorion genes, in the follicular ovary cells of *Drosophila* (Spradling and Mahowald, 1980). The amplification process associated with DNA puffing is triggered by the molt hormone of arthropods — 20–hydroxyecdysone (20–Ecd) (for review see Lara et al., 1991). The consecutive expression of two different sets of amplified genes, present in two groups of DNA puffs, is observed in *Bradysia hygida* salivary gland cells during a short period of about 30 h, by both morphological and biochemical techniques (Sauaia et al., 1971; Laicine et al., 1984; Paçó-Larson et al., 1992; Fontes et al. 1992, 1999; Almeida, 1997). This makes those gland cells a promising model for in vivo and in vitro study of gene activity and its control.

The fourth larval instar of *Bradysia hygida* lasts for about 10 days. During almost nine days the anterior (S1) regions of the salivary glands produce the same set of polypeptides, defining the first period of protein synthesis. The remaining 30 h are marked by striking changes in the pattern of protein synthesis. Two new and successive sets of polypeptides are synthesized, characterising the second and third periods of protein synthesis (Laicine et al., 1984). Indirect and direct evidences indicates that the activity of the amplified genes present in the DNA puffs of first (puffs C7, C5 and C4) and second groups (puffs A14, B3d, C6 and X4) is responsible for the production of the main polypeptides characteristic of second and third periods of protein synthesis. The evidences are: (a) A very tight temporal correlation between the expansion of the two groups of DNA puffs and the last two programs of protein synthesis. The production
of the main polypeptides exclusive to each of the last two periods is strongly inhibited when the DNA puff development is suppressed by treatments, like DNA synthesis inhibitors or by the incorporation of 5-bromodeoxiuridine, which do not affect either the activity of RNA puffs nor the synthesis of polypeptides not exclusive of the second and third periods (Sauaia et al., 1971; Laicine et al., 1984); (b) it was shown by the expression of fusion protein in bacteria and the production of the amplified gene \( BhB10–I \), located in DNA puff B10, which opens in a period of time intermediary between the two groups of puffs, codes for a secretory polypeptide of 23 kDa which synthesis occurs at the same time the puff opens (Fontes et al., 1999).

In this work, the control of Bradysia hygida salivary gland protein synthesis was studied in the late fourth larval instar. In vivo and in vitro experiments showed that 20–Ecd is necessary and inhibitory during the second and third periods of protein synthesis, respectively.

2. Material and methods

2.1. Animals

Bradysia hygida is maintained in the laboratory (Sauaia and Alves, 1968), has four larval instars and at 20°C its life cycle lasts for about 36 days. The 4th larval instar begins on the 12th day after hatching and lasts for about 10 days. The eyespots arise on the 6th day of the 4th larval instar and serve as age markers (Gabrusewicz-Garcia, 1964).

Late 4th instar larvae have paired salivary glands approximately 10 mm long. The glands are composed of two rows of cells with three morphologically distinct regions: S1 (anterior), S2 (granulous) and S3 (posterior). About 190 cells form each gland, and around 40 of them are in the S1 region. Only S1 regions from female larvae were used in this study.

The eyespot pattern E3 (8th day of 4th larval instar) coincides with the beginning of the gene amplification process which results in the formation of all DNA puff anlaje. Eighteen hours later (eyespot pattern E5), the 1st group of DNA puffs begins its expansion (DNA puffs C7, C5 and C4). After 2 h, in the so-called pattern E7, these three puffs are partially open. About 7 h later all of them have already receded. The expansion of the 2nd group of DNA puffs starts about 12 h after eyespot pattern E7, and a few hours before the pupal molt (that occurs about 26 h after E7) these puffs are already closed, with the exception of puff B3d. The puff B10 is the only active DNA puff between the periods of expansion of 1st and 2nd groups of DNA puffs, and its maximum size is attained at E7+8 h (Laicine et al., 1984).

2.2. In vitro incubation of S1 regions

The salivary glands were dissected in a drop of saline solution (120 mM NaCl, 1.8 mM CaCl₂, 6.7 mM KCl and 2 mM NaHCO₃) under a stereomicroscope. The S1 regions were separated and transferred to Cannon’s modified medium (Ringborg and Rydlander, 1971) either with (complete medium) or without (incomplete medium) leucine (Leu). The incubations were carried out in the well of a micro culture slide, at 20°C. A very thin layer of silicon grease was always applied on the surface of the well, in order to keep the medium as a drop. To avoid evaporation, grease was applied around the well and another slide was set up on top of the micro culture slide.

Solutions of 20–Ecd (Sigma Chemical Co., St. Louis, MO, USA) containing different concentrations of the hormone were prepared from a 400 mM stock solution in 5% ethanol. Before use, the 20–Ecd solutions were heated at 60°C for 20 minutes in order to solubilize the hormone.

2.2.1. Experimental groups

Larvae at the age E7 were dissected, the S1 regions were separated and incubated in complete medium (5 μl/pair of regions) for increasing periods of time, from 2 to 24 hours. In some experiments, 0.5 μM–10 mM of 20–Ecd was added to the complete medium. For periods longer than 16 h, the medium was changed once at the middle of the period. Following this treatment, the gland regions were incubated in the presence of radioactive precursor.

2.2.2. Control groups

Larvae at the age E7 were collected and maintained for the same periods of time used for the S1 region incubations. After each period of time, the larvae were dissected, the S1 regions were separated and incubated in presence of the radioactive precursor.

2.3. In vitro radioactive precursor incorporation

The S1 regions from experimental or control groups were placed in incomplete medium (5 μl/pair) supplemented with 1 μl/pair of [³H]–Leu solution (159 Ci/mmol, 1 μCi/μl, Amersham Life Science Ltd, Buckinghamshire, England) and incubated at 20°C for 1 h. After that the gland regions were fixed in 70% ethanol for 30 minutes and processed for SDS–PAGE and fluorography.

In all experiments there were 12 gland regions in each sample. During the period studied, different samples of S1 regions presented a very similar amount of total pro-
tein, as shown by previous measurements and estimated by visual examination of stained electrophoretic gels. The radioactivity was measured by liquid scintillation counting and showed to be quite reproducible.

2.4. Injection of solutions into the larvae

2.4.1. Second period of protein synthesis (first group of DNA puffs in activity)

Two groups of E7 larvae, were injected (Sauaia et al., 1971) with 1 µl of either a 10 µM 20–Ecd solution (dissolved in 5% ethanol) or with 5% ethanol. An additional group of larvae did not receive any injection. After 4 h, all the larvae were injected with 1 µl of [3H]–Leu solution, and 1 h later the S1 regions were removed, fixed and processed for SDS–PAGE and fluorography.

2.4.2. Third period of protein synthesis (second group of DNA puffs in activity)

Three groups of E7 larvae were selected and two of them were injected 10 h later with the hormone or ethanol or not injected. Six hours later, all the larvae were injected with 1 µl [3H]–Leu. After 1 h all the larvae were dissected, the salivary glands were fixed and the S1 regions were separated and processed for SDS–PAGE and fluorography.

2.5. SDS–PAGE and fluorography

After fixation, the S1 regions were solubilized in sample buffer (Laemmli, 1970), 10 µl/pair, at 95°C for 5 min. Polypeptides were separated on 10% SDS–polyacrylamide slab gels, according to Laemmli (1970). The Coomassie-stained gels were processed for fluorography with 20% 2,5–Diphenyloxazole (PPO) in glacial acetic acid (Laskey and Mills, 1975). The gels were dried and exposed to a prefetched X–Omat AR–5 Kodak film at −70°C.

3. Results

3.1. Newly synthesized polypeptides in salivary gland S1 regions maintained in vivo and in vitro

When we compare the profiles of protein synthesis of S1 regions of salivary glands from larvae at different ages after E7 (Fig. 1A and B, left lanes in each pair) with those from S1 regions of the same ages but maintained continuously in complete Cannon’s medium (right lanes), we note that for 2 h the synthesis is almost unchanged in relation to the control (Fig. 1A, lanes 2). From that time on, a gradual decrease in the synthesis of the main polypeptides was observed in the experimental samples, practically stopping at E7+8 h (Fig. 1A, lanes 8). In the control samples synthesis was maintained high until E7+6 h, decreasing up to E7+10 h. The polypeptide of about 23 kDa (SP23) began to be detected in the control and experimental groups at E7+4 h. In the control, its synthesis reached a maximum at E7+10 h and by E7+14 h (Fig. 1B, lanes 14) it was almost undetectable. In the experimental samples the amount of SP23, seemed similar to the control until E7+8 h, but was very reduced by E7+10 h. From this age to E7+12 h, the synthesis of all main polypeptides characteristic of second period, except SP23, stopped in the control. At E7+12 h started in vivo the third period of protein synthesis, with its new characteristic polypeptides (Fig. 1B). Surprisingly, a similar pattern of synthesis, with some bands missing, was observed in S1 regions maintained in vitro until for 18 hours (E7+18 h).

3.2. In vitro the second and third periods of protein synthesis respond differentially to 20–Ecd

In order to verify whether the presence of 20–Ecd could prevent the drastic fall in the synthesis of the main polypeptides characteristic of the second period of synthesis, the effect of a range of 20–Ecd concentrations was observed in S1 regions maintained in vitro. Since at age E7+8 h, protein synthesis is still significant in the in vivo control, while in vitro the synthesis is very low, this time point was chosen in the experiments.

All hormone concentrations (5 µM to 1 mM) used were able to partially prevent the in vitro decrease in synthesis of some of the major polypeptides characteristic of this period (Fig. 2A), whereas ethanol and medium alone had no effect (Fig. 2A, lanes Vte and Vt, respectively). The lowest concentration of 20–Ecd still effective was 0.1 µM (Fig. 2B).

The results, described in Section 3.1, showed that the synthesis of some third period polypeptides do not demand external factors for activation and maintenance, whereas others were not synthesized in vitro. The hormonal control of the synthesis of these polypeptides was examined in another series of experiments. S1 regions were incubated with or without 1 µM 20–Ecd from age E7 until E7+16 h (Fig. 3). Up to 4 h of incubation, the positive effect of 20–Ecd on protein synthesis during the second period was clear. From E7+6 h to E7+10 h, all samples, including the in vivo controls, showed very low levels of incorporation. The exception was the polypeptide SP23, whose synthesis seemed normal. At E7+12 h the in vivo control started to show some new polypeptides characteristic of the third period of synthesis, such as P50, P47 and P40. The ethanol and 20–Ecd treated S1 regions presented very low levels of synthesis. At E7+14 h and E7+16 h the synthesis became very strong in the in vivo controls. In the samples of ethanol treated S1, some of the main polypeptides synthesized at this time could be detected. An unexpected observation was the inhibition by 20–Ecd of the synthesis of the polypep-
Fig. 1. Newly-synthesized polypeptides during in vitro incubation of salivary gland S1 regions. S1 regions isolated from larvae at age E7 were incubated in medium for different periods of time (indicated in hours, below the lanes), left for 1 h in medium with [1H]-Leu, followed by SDS-PAGE and fluorography of their polypeptides. The pair of lanes for each time point corresponds to in vivo control (left lanes) and experimental groups (the right lanes). The molecular mass references are indicated in kDa at the left side in A and B. SP23 the product of gene BhB10–1 is indicated. The fluorogram in A refers to the 2nd period of protein synthesis and fluorogram in B refers to the 3rd period of protein synthesis.

3.3. In vivo, 20–Ecd also exerts a strong inhibitory effect on the third period of protein synthesis

Fig. 4 (lanes 1, 2 and 3) shows that the injection of 5% ethanol or 10 μM 20–Ecd into larvae at the age E7 does not affect (4 hours later) the second period of protein synthesis. In contrast, a very strong inhibition of protein synthesis was observed at E7+16 h, in S1 regions from larvae injected with the hormone at E7+10 h (lane 6). The injection of ethanol into larvae at this age also had some inhibitory effect on protein synthesis (lane 5) in relation to the control (lane 4).

4. Discussion

The present results provide new information towards the understanding of the processes involved in the control of gene activity by 20–Ecd in the salivary gland of Bradysia hygida. Our data show that the different groups of genes, whose activity gives rise to two successive periods of protein synthesis that occur during the end of 4th larval instar, reacted in different ways after the S1 regions were transferred from the larvae to a culture medium.

The cells of S1 regions, when explanted from larvae at the age E7, about 2 h after the beginning of the 2nd period of protein synthesis, do not maintain the synthesis of the main polypeptides characteristic of this period. This means that some essential factor(s) is (are) missing in the culture medium or that incubation conditions were harmful to the cells. Otherwise, the cells of S1 regions, incubated for prolonged times, were able to induce and sustain in vitro, for several hours, the synthesis of some third period polypeptides. This result showed that transcription and translation machinery are functional and hence that the incubation conditions were not harmful.

Probably, the inhibitory effect of the incubation on the second period of protein synthesis occurred at the transcription level of specific genes. In a preliminary experiment, we have observed by Northern blotting a rapid decrease in mRNA content corresponding to two amplified genes, BhC4–1 and BhB10–1 (data not shown). However it is also possible that an increase in the rate of mRNA degradation led to that result.

The presence of factors like insect hormones
(Buszczak and Segraves, 1998), probably is necessary to maintain the 2nd period of protein synthesis. For instance, 20–Ecd is known to trigger the process of gene amplification and gene activation in the salivary glands of Sciaridae (for review see Lara et al., 1991). In Brachysia hygida the injection of the hormone into larvae at the age E1 induces, 24 h later, the appearance of the E7 eyespot pattern and the expansion of the 1st group of DNA puffs. Both characteristics do not normally appear in control larvae until 72 h after E1 (Fontes and Paço-Larson, 1988). The induction of the 1st group of DNA puffs by the injection of 20–Ecd is accompanied by the onset of the 2nd period of protein synthesis (Carvalho and Almeida, 1993). On the other hand, when S1 regions from larvae at the age E1 were incubated in presence of 20–Ecd for up to 28 hours no induction of the 2nd period of protein synthesis was observed (results not shown).

The present results suggest that those genes characteristic of the 3rd period of protein synthesis, which were activated in the explanted S1 regions, are under the control of factor(s) produced by the cells themselves. Considering that the synthesis of major polypeptides of the third period depends on the activity of the second group of DNA puffs, the control of some of the amplified genes does not seem to fit in the model proposed by Lara et al. (1991). According to this model, the presence of 20–Ecd would be necessary during the whole period of gene amplification and puff expansion. The remainder of the 3rd period genes, which were not hormonally activated, probably are under the control of other factor(s). These factors could be the products of some of the genes characteristic of the second period of synthesis that were, under the experimental conditions, switched off.

20–Ecd works through an intracellular protein receptor complex (Yao et al. 1992, 1993; Thomas et al., 1993), one of the components is the ecdysone receptor (EcR), from the superfamily of nuclear receptors (Koelle et al., 1991), that are transcription factors. The EcR forms heterodimers with another receptor from the same superfamily, the product of the gene ultraspiracle (usp) (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990). The binding of EcR to consensus sequences in the promoter of target genes, the ecdysone response elements (EcRE) and its consequent functional activity depends on the presence of USP. Therefore, in this discussion we interpret the hormone effects on polypeptide synthesis as changes in the transcription of the amplified genes.

In this work we show that 20–Ecd is necessary and sufficient to maintain in vitro, at levels lower than in the controls, the synthesis of polypeptides characteristic of
Fig. 3. Effect of 20–Ecd on in vitro polypeptide synthesis in salivary gland S1 regions incubated for increasing periods of time. The time of incubation (hours) after the age E7 is represented below the lanes. For each time, the left lane is the in vivo control, the middle lane is a sample incubated in presence of 0.5% ethanol and the right lane is the 1 μM 20–Ecd treated S1 region. Three polypeptides characteristic of the third period of synthesis are indicated at the right side of the figure and the molecular mass references, in kDa, at the left side. Other details as in the legend of Fig. 1.

the 2nd period. On the other hand, it was strongly inhibitory for those 3rd period genes that were activated in vitro in the absence of the hormone.

At concentrations of 20–Ecd lower than 0.1 μM, the response to the hormone was not uniform during the 2nd period of protein synthesis. This suggests that the promoters of different genes may demand different concentrations of the hormone–receptor complex to be activated. In fact, data obtained in Trichosia (Amabis et al., 1977; Amabis and Amabis, 1984) demonstrated that low concentrations of 20–Ecd were not able to induce all the DNA puffs in all larvae. However, in our experiments, when higher concentrations of 20–Ecd were used, no increase in protein synthesis was observed. This could indicate that lower than normal amounts of EcR and/or USP were present in the cells. However, it is not possible to discard the possibility that other insect hormones, not present in our experiments, are responsible for the results reported here. According to Yao et al. (1992), in cotransfection assays in Drosophila, 20–Ecd seems to be the only external ligand necessary for EcR–USP dimerization. However, recent results from in vitro experiments have shown that juvenile hormone can bind to USP (Jones and Sharp, 1997). This could have a modulating role on the heterodimer activity.

There are other factors that seem to modulate the activation of genes by hormones (see for review Orti et al., 1992; Truss and Beato, 1993). The receptors of steroid hormones are highly phosphorylated proteins (Kuiper and Brinkman, 1994; Weigel, 1996; Rauch et al., 1998) and perhaps our in vitro system does not present ideal conditions for phosphorylation.

When larvae were injected with 20–Ecd at the beginning of 2nd or 3rd period of protein synthesis, the two sets of genes characteristic of these periods responded in the same manner they did when glands were incubated with the hormone.

Supported by the present results we propose that, in vivo, the role of 20–Ecd in the control of the activity of both groups of amplified genes is as follows: during cocoon spinning, some hours after E7, the high 20–Ecd and EcR concentrations, start to decrease, leading with other factors to the turning off of the amplified genes and regression of the respective DNA puffs. As a consequence, the synthesis of the main polypeptides gradually decreases, as a result of mRNA degradation, ending at E7+11 h.

At the beginning of the 3rd period of protein synthesis, age E7+12 h, the hormone must decrease at a level low enough to permit the activation of the genes characteristic of this period. It is important to note that only the decrease in the 20–Ecd concentration is not sufficient to result in the activation of genes from the 3rd period, since the in vitro incubation of S1 regions in the absence of hormone did not lead to that. Without hormone the onset of polypeptide synthesis, characteristic of the 3rd period, occurred at almost the same time as in the in vivo control.
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