IMMUNOAUTOGRAPHIC STUDY OF THE SYNTHESIS OF AN ECDYSTEROID AMPLIFIED PROTEIN IN A *DROSOPHILA* CELL LINE AND A CLONE IN VITRO

Denise CADE-TREYER and Nicole MUNSCH*

Laboratoire de Zoologie, Université Paris VI, 7 quai St Bernard, 75 230 Paris Cedex 05 and **Institut de Recherches Scientifiques sur le Cancer, Boite Postale no. 8, 94 800 Villejuif, France*

Received 2 May 1980

1. Introduction

Drosophila melanogaster cell lines maintained in vitro for several years [1] have retained their original diploid character. They are reliable material for the study of hormone action at the cellular level, with the advantage of an hormonal virgin background over in vivo or in vitro explanted tissue experiments.

Ecdysteroids, the molting hormones controlling insect development, have been shown to produce in vitro on *Drosophila* cell lines, morphological neuromimetic changes, agglutination of cells [2–4] and inhibition of cell proliferation [5–8].

After 20-hydroxyecdysone (β-ecdysone) treatment of a clone selected from the Kc line, a protein was shown to be induced after 3 days of steroid action, and was separated by electrophoresis on a non-denaturing gel [9]. Specific immune sera were elicited in rabbits injected with the protein gel band appearing after β-ecdysone stimulation [10].

To study if the steroid effect was a protein induction or an amplification and if it was an early or a late effect of the hormone, we studied its time-course of synthesis, using an immunoautoradiographic method quantified through densitometric scanning.

2. Materials and methods

2.1. Drosophila *cells and ecdysteroid treatment*

The diploid (XX haplo IV) drosophila Kc cell line [1] was grown in monolayer in Falcon flasks, in D_{22} medium [11] supplemented with 10% fetal bovine serum, at 23°C. The diploid clone 5284 derived from Kc line, has the same caryotype and was grown in the same medium. Cells were seeded at 6×10^6 cells/ml. 20-Hydroxy-ecdysone (Rhoto, Osaka) was added in the medium at final conc. 10^{-7} M, either 1 h or 3 days after subculture of the cells. This concentration was chosen as it was shown to give a maximal effect on acetylcholinesterase [3,4] or β-galactosidase [12] induction in Kc cell line.

2.2. *Protein labelling of the cells*

Cells incubated in the presence or in the absence of 20-hydroxyecdysone, were labelled for 1 h with L-[U-^{14}C]leucine 330 mCi/mmol. (Amersham Great Britain) at 0.5 μCi/ml of a cellular suspension agitated gently by shaking, in D_{51} medium lacking leucine.

2.3. *Cell culture extracts*

After labelling, cells were washed thrice in cold Shaw's glutamate–glycine–phosphate buffer (pH 7.0), 360 mOsm. They were then homogenized in 10 mM Tris–HCl (pH 7.1) by sonication at 0°C with an MSE sonicator at low power for 15 s periods with 30 s intervals. The homogenate was centrifuged at 20 000 × *g* 20 min and the supernatant used for immunodiffusion. Protein concentration was measured by the method of Lowry.

2.4. *Immunoautoradiography*

The radiolabelled 20 000 × *g* supernatant was tested by immunodiffusion in 1% agarose veronal (pH 8.2) $I = 0.025$. It was precipitated with an antiserum directed against a protein shown to be specifically induced in a Kc clone, after 3 days of treatment with 10^{-7} M β-ecdysone, as compared to the control. These antisera were produced in rabbits with the protein band cut out of a 5% acrylamide gel [10]. Quantification of the protein labelling was performed by densitometric scanning of the labelled immuno-

precipitation lines of the autoradiograms obtained on Kodirex films, with a Joyce-Loebl double beam MK III microdensitometer. Integration of the peaks was performed by weighing after their reproduction on tracing paper.

3. Results

Among the antisera raised in rabbits against the protein band, shown to be quantitatively increased after electrophoresis in 5% acrylamide gel of soluble extracts of cells of a clone derived from the Kc line and treated for 3 days with 10^{-7} M β-ecdysone [10], we selected one which was monospecific. Fig.1 shows that only one precipitation line is obtained if this antiserum is allowed to diffuse against an extract of cells treated with β-ecdysone for 2 or 3 days. The control cells, not treated with the hormone are not precipitated by the antiserum, neither are the cells treated for 5 h or 16 h with the hormone.

Therefore the question arose: is the protein induced by the steroid due to a de novo synthesis or to a protein amplification? To solve this problem we sensitized the technique of Ouchterlony by employing radiolabelled protein extracts of cells treated by the steroid or of the control cells, and by adding a 3-days cold steroid treated cell extract as a carrier. We lowered thus 50 times the threshold of detection of the specific protein. The initial 1–5 μg specific protein detected in a 100 μg cellular protein extract, fell to 20–100 ng for 100 μg cellular proteins. So, by immunoprecipitation, we could follow the increase in the rate of synthesis of a single protein species, when *Drosophila* cells in vitro were treated with 10^{-7} M β-ecdysone with varying lapses of time, after 1 h exposure to labelled amino acids.

Fig.2A,B and its corresponding densitometric

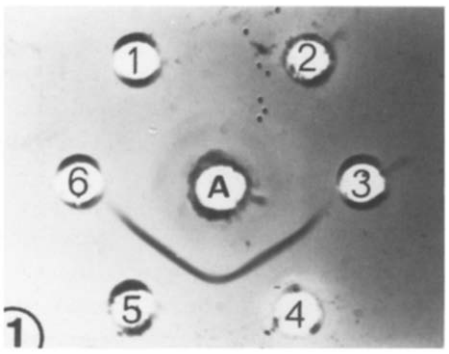
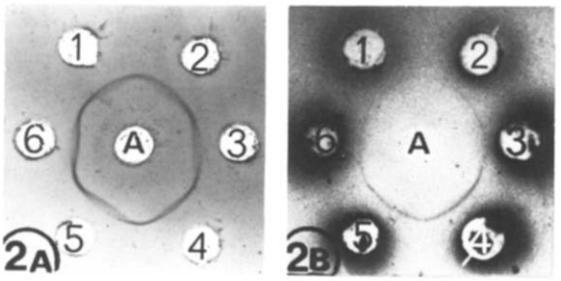


Fig.1. Immunodiffusion pattern of the drosophila Kc line cells treated, after 3 days of subculture, with 10^{-7} M β-ecdysone for 5 h (2), 16 h (3), 2 days (4), 3 days (5), against an immune serum. (A) Directed against an electrophoretic-separated protein specifically induced by 10^{-7} M β-ecdysone in a *Drosophila* Kc clone. (1,6) Control (untreated cells). Antigens well 1–6: 10 mg protein/ml.



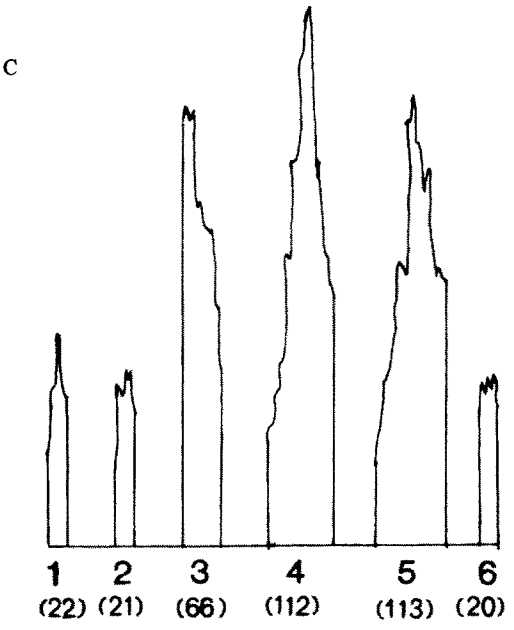


Fig.2. (A) Immunodiffusion pattern of the same extracts and serum as in fig.1. But all cell extracts (10 mg protein/ml) are radiolabelled with [^{14}C]leucine (0.5 μCi/ml culture medium). A cold 3 days β-ecdysone treated Kc line cell extract has been added in each well (10 mg protein/ml) as a carrier, which accounts for the presence of the precipitation line facing wells 1–6. (B) Autoradiogram of (A). Detection in control cells (well 1,6) of a threshold synthesis of the protein, just as in 5 h β-ecdysone treated cells (well 2). (C). Densitometric scanning pattern of the precipitation lines of the autoradiogram of (B). The peaks (1–6) correspond to the precipitation lines facing well 1–6 in (B). Numbers into brackets give the integration values of the corresponding peaks.

scanning pattern, fig.2C give the results, if β-ecdysone was added 3 days after subculture of the Kc line:

(i) Control cells (well 1,6) show a threshold level of synthesis of the protein which is of the same magnitude as that of the 5 h hormone-treated cells (well 2).

(ii) The rate of synthesis is increased 2–3-times in cells treated for 16 h by the steroid (well 3) and 5–6-times in cells treated for 48 h (well 4). The maximal level reached at 48 h is maintained 3 days after hormone treatment (well 5).

A similar experiment was performed and analogous results were obtained if β-ecdysone was added 1 h after subculture of the Kc line cells; the values of integration of the precipitation line peaks being: 14 for the control cells, 15 for 2 h of hormone treatment, 40 for 18 h, 83 for 2 days of treatment. Here too the specific protein synthesis is enhanced at 18 h of β-ecdysone action, being 2–3-times higher than that of the control, and at 2 days reaching 5–6-times that of the control. The phenomenon is thus similar if the steroid is added early in the exponential growth phase of the cells or near the plateau and is thus independent of the growth state of the cell population.

The clone 5284 derived from the Kc line was tested in the same way, β-ecdysone being added 3 days after subculture, and it gave similar results. The amplification by β-ecdysone of this specific protein synthesis does take place from 5–16 h after the steroid is added to the cells, and the maximal effect is reached at 48 h. It is thus also a late hormone effect.

4. Discussion

Here we have followed the time-course of synthesis of a specific protein characterized by its immune serum. This protein has been shown to exist throughout *Drosophila* development from early eggs, larvae, pupae and even in the imago [13].

By quantifying the immunoautoradiographic method used we have demonstrated that the protein increase is not an induction but a protein amplification and that it is a late effect of the ecdysteroid in *Drosophila* cells free of any earlier hormonal stimulation.

Studies of the timing of β-ecdysone stimulated increase of protein synthesis in in vitro *Drosophila* cells [3,4,12,14–16] reveal in one case only an early protein induction, claimed in a one-dimensional electrophoretic system [14]. With a two-dimensional electrophoretic separation, it was shown that the neosynthesis or the amplification of several proteins became visible in the Kc 0% cell line only after 15 h of β-ecdysone stimulation [15].

Very sensitive enzymatic techniques have shown too that, in in vitro *Drosophila* cells, ectysteroids produce a late effect. Induction of acetylcholinesterase [3,16] or β-galactosidase [12] becomes measurable only after 24 h hormonal treatment.

In contrast, if *Drosophila* cultures are first treated with a short β-ecdysone stimulus (10^{-6} M) [14] or with an infraliminar hormonal amount [17] after a second steroid stimulus the protein (enzyme) induction is very rapid (within a few hours).

We think that steroid stimulation experiments performed in vivo [18] or after organ explantation in vitro [19] reproduce this last scheme of action. In vivo cells are permanently submitted to, at least, a threshold level of steroid. Indeed it has been shown that throughout every developmental stages of *Drosophila* a non-negligible level of β-ecdysone is maintained [20,21]. The same seems true for in vivo steroid stimulation in mammalian cells, where a very rapid increase in protein synthesis is always demonstrated [22,23].

On the contrary in vitro, very comparable to our results, the steroid dexamethazone stimulated a 4-fold increase in tyrosine aminotransferase synthesis, only after 16 h of the glucocorticoid treatment of a mammalian hepatoma cell line in vitro [24].

So, with a hormonal virgin background found in cell lines or clones in vitro, what does this lag between the first hormonal signal and its effects mean? What cellular sites are concerned? Some clues thereto should come from a study of the relation of the protein amplification with the other primary effects of the steroid on the *Drosophila* cell lines, which all were shown to be late effects, appearing at >16 h after the hormonal stimulus [2,5,8,25]. For any effect to be induced, is this lag employed by the hormone to first amplify its receptors?

We think it worthy to pursue this study although it has been claimed that after β-ecdysone action, *Drosophila* cells should undergo lethal programmed changes [7]. Our observations [25] do not support this idea. After 4 days of 10^{-7} M β-ecdysone action we could observe that cells of the 5284 clone, agglutinated into compact cluster, remained viable as subcultures of the clumps after their dispersal succeeded in growing, giving first a sheet of morphological trans-

formed cells which gathered again into clusters later on. Moreover after withdrawal of β-ecdysone from the medium after 4 days of action on the clone 5284, neuromimetic transformed cells were seen to grow out of the clusters giving later a complete sheet of dividing cells.

References

[1] Echalier, G. and Ohanessian, A. (1969) CR Acad. Sci. 268, 1771–1773.
[2] Courgeon, A. M. (1972) Exp. Cell. Res. 74, 327–336.
[3] Cherbas, P., Cherbas, L. and Williams, C. M. (1977) Science 197, 275–277.
[4] Berger, E., Ringler, R., Alahiotis, S. and Frank, M. (1978) Dev. Biol. 62, 498–511.
[5] Courgeon, A. M. (1972) Nature New Biol. 238, 250–251.
[6] Wyss, C. (1976) Experientia 32, 1272–1274.
[7] Rosset, R. (1978) Exp. Cell Res. 111, 31–36.
[8] Munsch, N. and Cade-Treyer, D. (1980) in preparation.
[9] Best-Belpomme, M. and Courgeon, A. M. (1976) CR Acad. Sci. 282, 469–471.
[10] Best-Belpomme, M., Sykiotis, M. and Courgeon, A. M. (1978) FEBS Lett. 89, 86–88.
[11] Echalier, G. and Ohanessian, A. (1970) In Vitro 6, 162–172.
[12] Best-Belpomme, M., Courgeon, A. M. and Rambach, A. (1978) Proc. Natl. Acad. Sci. USA 75, 6102–6106.
[13] Cade-Treyer, D., Best-Belpomme, M. and Courgeon, A. M. (1980) unpublished.
[14] Cherbas, L., Cherbas, P., Savakis, C., Demetri, G., Manteuffel, M., Cymborovska, M., Yonge, C. D. and Williams, C. M. (1980) in: Vth Int. Conf. Invertebrate Tissue Culture (Kurstak, E. et al. eds) in press.
[15] Couderc, J. L. (1980) in preparation.
[16] Best-Belpomme, M. and Courgeon, A. M. (1977) FEBS Lett. 82, 345–347.
[17] Best-Belpomme, M., Courgeon, A. M. and Echalier, G. (1980) in: Progress in ecdysone research (Hoffman, J. A. ed) vol. 7, pp. 379–392, Elsevier/North-Holland, Amsterdam, New York.
[18] Lepesant, J. A., Kejzlarova-Lepesant, J. and Garen, A. (1978) Proc. Natl. Acad. Sci. USA 75, 5570–5574.
[19] Ashburner, M. (1971) Nature New Biol. 239, 9–14.
[20] Garen, A., Kauvar, L. and Lepesant, J. A. (1977) Proc. Natl. Acad. Sci. USA 74, 5099–5103.
[21] Berreur, P., Porcheron, P., Berreur-Bonnenfant, J. and Simpson, P. (1979) J. Exp. Zool. 210, 347–352.
[22] Barnea, A. and Gorski, J. (1970) Biochemistry 9, 1899–1904.
[23] Palmiter, R. D. (1975) Cell 4, 189–197.
[24] Tomkins, G. M., Levinson, B. B., Baxter, J. D. and Dethlefsen, L. (1972) Nature New Biol. 239, 9–14.
[25] Cade-Treyer, D. (1980) unpublished.