BINDING OF THYROTROPIN RELEASING HORMONE AND PROLACTIN RELEASE BY SYNCHRONIZED GH3 RAT PITUITARY CELL LINE

A. Faivre-Bauman, D. Gourdji, D. Grouselle and A. Tixier-Vidal
Groupe de Neuroendocrinologie Cellulaire, Laboratoire de Physiologie Cellulaire, Collège de France 75231 PARIS 05

Received July 29, 1975

Summary - GH3 cells were synchronized by growing them in a low serum concentration (1%). They were thereafter put back in normal medium (17.5% serum) (time 0 of synchronization). Four parameters were then examined every two hours for up to 40 hours : rate of [^{3}H] thymidine incorporation, cell number, binding of [^{3}H] Thyrotropin Releasing Hormone (TRH) after a 30 min exposure, and prolactin (PRL) content of culture medium and cell extract.

The rate of thymidine incorporation presented a 10-20 fold increase in S phase, beginning on 12-16 hours and lasting at 26 hours. The cell population was doubled at 28 hours. [^{3}H] TRH binding to attached cells was observed throughout the cell cycle, but presented a significant increase (40-80%) during the S phase. In contrast, the % increase of PRL release in response to TRH was optimum (300% of control) in G_1 phase. Variations of the PRL cell content as well as of the PRL spontaneous release ability of the cell do not account for the variations of TRH responsiveness. The discrepancy between the two parameters of the TRH-GH3 cells interaction strongly suggest a morphological or functional heterogeneity of the TRH-binding sites.

The GH3 rat pituitary cell line secretes both prolactin (PRL) and growth hormone (11, 9). Synthetic TRH (Thyrotropin Releasing Hormone) increases its prolactin production and modifies both the morphology and the ultrastructural organization of the cells but does not affect cell growth and protein synthesis (10, 2, 3, 4, 13).

Previous analysis of the interaction of [^{3}H]TRH with intact cells demonstrated : that the binding capacity increases linearly during the first 15 or 30 min, thereafter reaching a plateau (3, 4). This kinetics is closely similar to that of the TRH induced prolactin release (3, 6, 12, 13).

In contrast with in situ normal cells, pituitary cells in culture are continuously dividing. Moreover they do not divided synchronously. The mobilization of cell machinery involved in

mitosis may interfere with some features of TRH-cell interaction. It was therefore of great interest to analyze the binding capacity of GH3 cells for [^{3}H]TRH during the cell cycle, together with the induced prolactin release stimulation. This type of analysis requires synchronized cells.

Results reported here show that [^{3}H]TRH binding to attached synchronized GH3 cells was observed all through the cell cycle, but presented a significant increase (40-80%) during the S phase. In contrast the related biological response, that is the % increase of prolactin release after 30 min exposure to [^{3}H]TRH, was optimum (300% of the control) in the G_1 phase. This might suggest that only one part of the binding sites, as observed on intact cells, is involved in the short term PRL response.

MATERIALS AND METHODS

GH3 cells were grown in HAM F 10 medium supplemented with heat inactivated horse serum (15%) and fetal calf serum (2,5%) and antibiotic mixture (Penicillin 50 U/ml, Streptomycin (50 µg/ml), in 30 mm plastic Petri dishes (Nunclon 301 420) in an humidified atmosphere of 5% CO2 95% air at 37° C. Each dish was inoculated with 2-3 x 10^5 cells in 2 ml medium.

For synchronization the GH3 cells were grown for 36 hours on HAM F 10 with 1% serum, three days after seeding. They were thereafter put back in normal medium (17.5% serum) (time 0 of the synchronization) and four parameters were followed as described below.

1° Rate of [^{3}H]thymidine incorporation into DNA

The S phase duration was determined by measuring the incorporation of [^{3}H]thymidine (26 Ci/mM or 1 mCi/mM, 1 µCi/ml of Eagle MEM/ dish) into DNA, on attached cells for 15 minutes. after extensive washings with ice cold HAM F 10, the cold 10% TCA was added. The precipitate was dissolved in NaOH. One aliquot was counted in Toluene-Triton (toluene : 1 L, POPOP : 0.236 g, PPO : 5 g and Triton x 100 : 352 ml) in an Intertechnique SL 30. Another aliquot was used for protein determination according to Lowry (5).

2° The time of mitosis was determined by counting the cells per dish.

3° Binding of [^{3}H]TRH to synchronized GH3 cells

Attached synchronized cells were rinsed once with F 10 medium and incubated in 1 ml of regular culture medium in presence of 4 ng [^{3}H]TRH (1.3 x 10^{-8} M) (specific activity : 60 Ci/mM). After 30 min at 37° C, the cells were rinsed on ice four times with cold F 10 medium, scraped in distilled water and submitted to ultrasonic disruption for 10 sec. at 40 W (Sonimasse apparatus T 50). An aliquot of the cell sonicate was digested in NCS tissue solubilizer and counted as above, while protein determination was performed on another aliquot according to Lowry (5).

4° Prolactin assays

Prolactin (PRL) was measured by radio-immunoassay using the Rat PRL kit provided by NIAMDD rat hormone distribution program. The medium PRL content was examined : 1. before [3H] TRH binding experiments, 2. after 30 min incubation in fresh regular medium (spontaneous prolactin release) and 3. after 30 min exposure to [^{3}H] TRH (TRH biological response).

PRL cell contents were also measured after the cells were disrupted by sonication in PBS, freezing and thawing.

For each experiment these parameters were examined every two hours from 0 time up to 40 hours after the regular culture medium was added back. 2-3 dishes were used at each time interval. Each one of these 4 parameters was followed in at least 2 or 3 independant experiments.

Reagents - [^{3}H] TRH was obtained from Dr. Fromageot, CEA, Saclay, and ^{3}H thymidine purchaised from CEA, Saclay, NCS tissue solubilizer from Amersham-Searle.

HAM F 10 and horse serum were purchased from Eurobio, Eagle MEM from Mérieux and fetal calf serum from Sorga.

RESULTS

A. cell cycle analysis

The rate of [^{3}H] thymidine incorporation (fig. 1 A) increased by a factor 10-20 (from 1300-2000 cpm to 25000 cpm per aliquotes of 200 µg of precipitated protein) during the S phase which began on 12-16 hours depending on the experiment and was achieved at 26 hours in all experiments. The G_1 phase lasted about 10-12 hours while the G_2 phase seemed in all cases very short. Cell protein content increased by a factor 1.5 between 12 and 16 hours.

The cell population was doubled at 28 hours.

B. [^{3}H] TRH binding to GH3 cells

We chose the [^{3}H] TRH concentration of 4 ng/ml for two reasons : 1. it corresponds to the high affinity binding system of the GH3 cells (3, 12) and 2. it induces the half maximum increase of PRL release in 30 minutes.

Fig. 1 B shows two typical experiments. The [^{3}H] TRH binding, as expressed in % of the maximum dpm bound per mg cell protein, decreased in early G_1 phase, reached up its maximum in the S phase around 22-24 hours, and eventually decreased in G2 and mitosis. The increase in the S phase was between 40 to 80% of the minimum level ($P \ll 0.001$) with an average binding of 150,000 to 200,000 sites per cell.

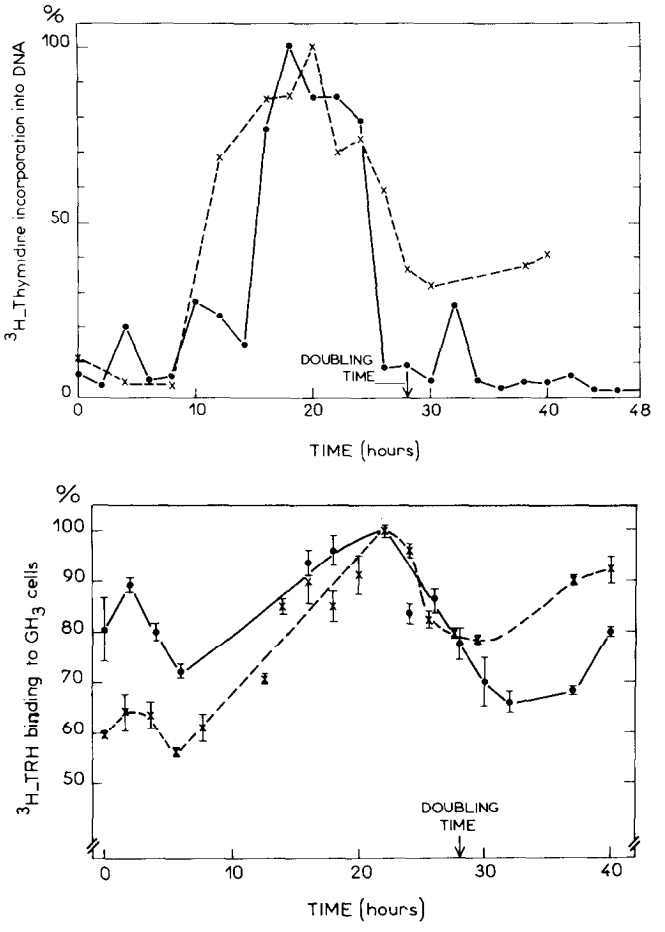

Fig. 1. 1 A - [^{3}H]thymidine incorporation into DNA throughout the cell cycle, expressed in % of maximum incorporation and per mg precipitated protein after 15 min incubation in two independant experiments.

. ——— . [^{3}H]thymidine 1 Ci/mM and x --- x 26 Ci/mM.
Each value is the mean of 2 dishes
The cell population doubled to reach 8 x 10^5 cells/dish (two dishes were counted at each time interval).

1 B - Change in [^{3}H]TRH binding to GH3 cells during the cell cycle. Results of two independant experiments (. ——— .) and (x --- x) are shown. Results are expressed in % of maximum dpm bound per mg protein, which corresponded respectively to 180 and 140 dpm per µg protein. Each value is the mean and standard error of three plates.

The [^{3}H] TRH binding exhibited ($P \ll 0.001$) a second minimum at the beginning of the second cycle, 6 to 8 hours after mitosis. This rules out a possible artefactual origin, due to the

synchronizing pretreatment, for the first minimum, in the early first G_1 phase.

C. Prolactin secretion

1° Intracellular content of prolactin (not shown) in GH3 cells did not significantly vary during the cell cycles ; the prolactin specific activity closely paralleled prolactin content per cell ; its mean varied depending on the experiment : around 300 ng/mg cell protein and 60 ng/mg cell prot.

2° PRL accumulation in the culture medium throughout the cell cycle

GH3 cells spontaneously released prolactin in the cell growth reinitiation culture medium which was renewed. Fig. 2 shows that this accumulation was linear during the G_1 phase. During the S phase, the rate of prolactin accumulation decreased and tended to a plateau, which persisted up to a few hours after mitosis.

3° PRL release in response to a 30 min exposure to [^{3}H] TRH

a) control medium

Changing the medium at different time intervals during the cell cycle, reinduced PRL release within 30 minutes. The evolution of the PRL release ability of the cells in response to a medium renewall did not followed the same time course than the spontaneous PRL medium accumulation (fig. 3). It did not display important fluctuations throughout the cell cycle.

b) [^{3}H] TRH

The addition of [^{3}H] TRH to the renewed medium induced an increase of PRL release in 30 minutes throughout the cell cycle (fig. 3). The percent PRL increase as compared to the control medium ($\frac{\text{TRH - control}}{\text{control}}$ %) was maximum during G_1 phase (up to 200%). It decreased at the beginning of S phase and during late S phase (25%) and mitosis.

DISCUSSION

Several specialized functions of differentiated eucaryotic cells have been reported to fluctuate during the cell cycle in synchronized cultures. Among these studies very few are dealing with hormone responsiveness. In melanoma cells both the receptors and the biological response to melanocyte stimulating hormone (MSH) were only seen in G_2 phase (14). In Neuroblastoma cells

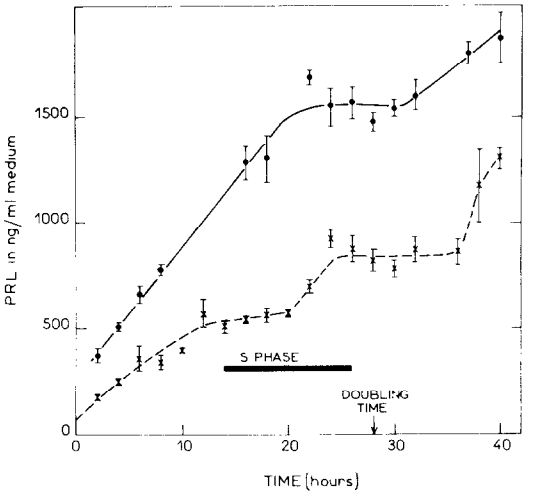

Fig. 2. Medium PRL release by GH3 cells during the cell cycle. Spontaneous accumulation in the medium during the cell cycle. Results are expressed in ng PRL found per ml medium at different times after cells resumed growth in normal medium. Each value is the mean of 3 dishes ± standard error in two independant experiments. ∘——∘ and x --- x. Effect of [^{3}H]TRH in 30 min after a medium renewal.

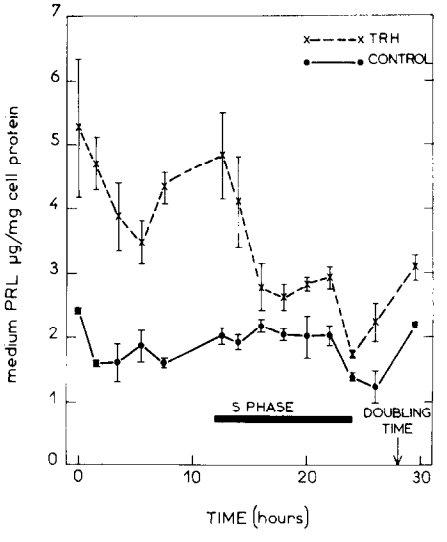

Fig. 3. Medium PRL release within 30 minutes after a medium renewall, either in control medium (.—— .) or in [^{3}H]TRH supplemented medium (4 ng/ml) (+— . — +). Results are in ng PRL per mg/cell protein. Each value is the mean of 3 dishes ± error standard.

(C. 1300) binding of nerve growth factor is maximum during the late G_1 and early S phases (8). In these two cases receptors are considered to be located on the plasma membrane. In hepatoma cells the inducibility of tyrosine amino-transferase by dexamethasone is only observed in late G_1 and in S phase (7).

In order to follow the [^{3}H]TRH binding capacity of GH3 cells and its related biological response (PRL release), we have synchronized the cells by lowering the medium serum concentration. The cell synchronization, as appreciated by a 10-20 fold increase of the [^{3}H]thymidine incorporation in S phase, seemed satisfactory.

[^{3}H] TRH binding was found to occur all through the cell cycle, but in contrast of the above cited systems, it increased (40% - 80%) during the S phase. Variations in the accessibility and/or in the synthesis of TRH receptors may account for this augmentation. Previous work (3,1,12) demonstrated that after a 30 min binding TRH entered GH3 cells (asynchronous cultures) without being degradated or modified, and was found associated to the nuclear fraction of the cells in a proportion of 16%. This is in agreement with the increase of TRH receptors during S phase and suggest the existence of nuclear receptors for TRH.

The increase of PRL release in response to a 30 min exposure to TRH varied throughout the cell cycle, following a different pattern than those of TRH binding capacity. Indeed it displayed its maximum during G_1 phase and began to decrease when the TRH binding sites were reaching their maximum. Nevertheless, after S phase and up to mitosis both biological response and TRH binding sites were at a low level. These variations of the cell responsiveness to TRH cannot be related to variations of intracellular PRL store, since the latter did not significantly vary during the cell cycle. For the same reason, they cannot be related either to the PRL spontaneous release ability of the cell in response to a medium change (fig. 3).

The discrepancy between these two parameters of the TRH-GH3 cells interaction strongly suggest an heterogeneity of TRH receptors. This heterogeneity may be related to other possible biological effects of TRH such as on PRL synthesis, GH release and synthesis (10) as well as on some, if any,

unknown parameter. It also may be related to variations of the TRH receptors efficiency in relation with other molecular factors involved in the biological response and which remain to be examined.

ACKNOWLEDGEMENTS : This work was supported by grants from the CNRS (ER 89) and from the INSERM (AT 35).

REFERENCES

1. Brunet, N., Gourdji, D., Tixier-Vidal, A., Pradelles, Ph. Morgat, J.L. and Fromageot, P. (1974) FEBS Letters, 38, 129-133.

2. Gourdji, D., Kerdelhué, B., and Tixier-Vidal, A. (1972) C.R. Acad. Sci. Ser. D, 274, 437-440.

3. Gourdji, D., Tixier-Vidal, A., Morin, A., Pradelles, Ph., Morgat, J.L., Fromageot, P., and Kerdelhué, B. (1973) Exp. Cell Res. 82, 39-46.

4. Hinkle, P.M., and Tashjian, A.H. Jr. (1973) J. Biol. Chem. 248, 6180-6186.

5. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1961) J. Biol. Chem., 193, 265-275.

6. Morin, A., Tixier-Vidal, A., Gourdji, D., Kerdelhué, B. and Grouselle, D. (1975) Molecular Cell Endocrinol., 3 (in press).

7. Martin, D., Tomkins, G.M. and Granner, D. (1969) Biochem. 62, 248-255.

8. Revoltella, R. Bertolini, L., Pediconi, M., Vigneti, E.(1974) J. Exper. Medicine, 140, 437-451.

9. Tashjian, A.H. Jr., Bancroft, F.C., and Levine, L. (1970). J. Cell Biol., 47, 61-71.

10. Tashjian, A.H. Jr., Barowsky, N.J. and Jensen, D.K. (1971) Biochem. Biophys. Res. Commun., 43, 516-523.

11. Tashjian, A.H. Jr., Yasumura, Y., Levine, L. and Sato, G. (1968) Endocrinology, 82, 342-352.

12. Tixier-Vidal, A., Gourdji, D., Pradelles, Ph., Morgat, J.L. Fromageot, P. and Kerdelhué, B. (1974) Intern. Symp. on Hypothalamic Hormones, Milano, October 16-18. Serono Symposia (in press).

13. Tixier-Vidal, A., Gourdji, D. and Tougard, C. (1975) Intern. Rev. Cytology, 41, 173-239.

14. Varga, J.M., Dipasquale, A., Pawelek, J. Mc Guire, J.S. and Lerner, A.B. (1974) Proc. Nat. Acad. Sci. USA, 71, 1590-1593.