Vol. 118, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 13, 1984 Pages 206-211

GONADOTROPIN RECEPTOR COMPLEXES AND FREE RECEPTORS IN PORCINE LEYDIG CELL CULTURES DURING RECOVERY FROM hCG STIMULATION

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Received November 30, 1983

ABSTRACT : Free and occupied gonadotropin receptors were studied in vitro in porcine Leydig cells culture maintained in chemically defined medium. Free receptors were evaluated by the binding capacity for ^{125}I-hCG. hCG bound molecules (or hCG receptor complexes) were evaluated using immunocytochemical visualization on fixed cells. Exposure to hCG for 16 hours (.5 to 50 ng/ml) induced the disappearance of free receptors. After removal of the hormone, the return to control levels was observed at 48 and 72 hours. Visualization of hCG bound at the cell surface indicates that, following continuous exposure to gonadotropins for 48 hours, hCG molecules are still present on the cell. Following short-time exposure (1 h) to hCG and 48 hrs washing the number of stained cells is very close to the initial value suggesting that the occupied sites (at 48 hours) represent the initial hormone receptor complexes. These results indicate that, during prolonged incubation, hCG binding is not reversible, that the half-life of some of the complexes at the cell surface is very long and that the receptors recovery is slow and is probably the result of a de novo synthesis.

Leydig cells have been shown to be the site of action of LH-hCG in the testis (1, 2). The level of hCG receptors on the Leydig cells has been shown to be regulated by the level of the gonadotropin (3-7). Incubation of cells with hCG induced the disappearance of free receptors (down regulation) in a time and dose-dependent fashion. However it is not clearly determined whether the apparent lack of free binding sites is the result of receptor occupancy or removal of the hormone-receptor complexes. Moreover, the return of receptors following down regulation has not been carefully studied.

Immunocytochemical approaches have shown that the hormone receptor complex is located at the cell surface and suggested that it might remain at the cell surface for some time (8-10). So it was of interest to study the maintenance of the hCG receptor complex at the cell surface under controled conditions of gonadotropin exposure.

The present paper describes the return of free receptors and the fate of the hCG receptor complexes following in vitro down regulation.

MATERIAL AND METHODS

Cell culture. Testes were collected on ice at the time of castration of 3 to 4 weeks old piglets. Cells were prepared for culture as previously described (7, 11). The cells (10^6 cells/plate) were plated on multiwell culture dishes (Linbro, 35 mm, Flow

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Laboratories) for binding studies and in multiplate and plastic cover slips (Scientific Corporation) for immunocytochemical reaction.

Cells were maintained at 35°C in an atmostphere of 5% CO_2 in F12/DME medium supplemented with 0.1 % calf serum and insulin (5 µg/ml), transferrin (5 µg/ml) and vitamin E (2 µg/ml). Culture medium was changed daily. According to morphological criteria, and 3 β-ol dehydrogenase activity and hCG receptor complexes visualization, the Leydig cells represent 40 to 60 % of the cell preparation (10).

Binding studies. Purified hCG (gift from Dr. R. Canfield) was iodinated with ^{125}I by the lactoperoxydase method, as previously described (12) with a specific activity close to 100 µCi/µg. Monolayer cultures (10^6 cells per plate) were incubated with 1.10^6 cpm for two hours at 37°C and specific binding measured as previously described (7).

Visualization of hCG by immunocytochemical reaction. Following hCG exposure and careful washing with PBS the cell cultures were fixed with glutaraldehyde solution 2.5 % in phosphate buffer 0.1 M, pH 7.4 for 1 hour at 4°C. Cells were washed three times with PBS and the immunocytochemical reaction was performed (10, 13, 14). The first antiserum was a rabbit anti-hCG serum (gift from Dr. M.P. Dubois, INRA, Nouzilly). The second antiserum was a goat antirabbit IgG conjugated with peroxydase (10). Peroxydase bound to cells was detected using .03 % substrate (H_2O_2) and 4 chloro-naphtol (20 mg/100 ml) as electron donor. After final washing, preparations were mounted in buffered glycerol and the staining of cells appeared as dark purple granulations at the cell surface. Endogenous Leydig cell peroxidases are not detected in this procedure. The reagents do not enter the fixed cell culture and the staining represents the hCG at the cell surface (10). Stained and unstained cells were counted (5 x 100 cells/plate) immediately following the immunocytological reaction. All results are expressed as mean + SD.

The relationship between staining and % of occupied receptors is not linear. However, up to 50 % receptor occupancy, 100 % of the Leydig cells are seen as stained. The number of stained cells is close to 100 % up to 15 % receptor occupancy as measured by binding studies. Below this rate of occupancy the number of stained cells is sharply decreasing but the staining itself cannot be precisely quantified.

RESULTS

Reappearance of free binding sites after down regulation. Fig. 1 shows that exposure to high concentrations of hCG (50 and 5 ng/ml) for 16 h produced a greater decrease in the level of free receptors than low concentration (.5 ng/ml). These results are in keeping with binding studies (7) showing that the disappearance of free receptors is almost complete following 16 h exposure to 5 and 50 ng/ml hCG while half of the receptors are still free following exposure to .5 ng hCG/ml.

Following removal of hCG the return of the free binding sites can be followed for several days, as indicated in fig. 1. The amount of ^{125}I-hCG bound increases with time and is a function of hCG concentration during the 16 hours of pretreatment. At 48 hours for the concentration of .5 ng/ml hCG the number of free receptors reached the control level whereas for the concentration of 50 ng/ml (or 5 ng/ml) the control level is not reached before 72 hours. The presence of cycloheximide (10 µg/ml) suppresses the reappearance of free receptors (unpublished results), suggesting that they might be newly synthetised.

Fate of bound hCG following hCG removal. Following exposure to ^{125}I-hCG (5 ng/ml) for 16 hours and subsequent washing (100 % of binding), the amount of radioactivity measured in the cell compartment is respectively 56 ± 5.4 % and 34 ±

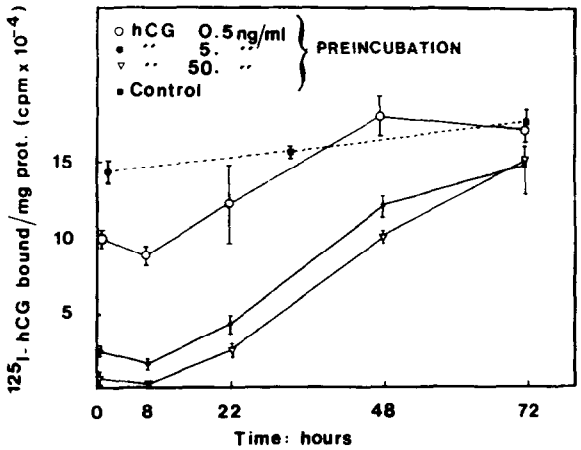

Fig. 1. Evolution of binding sites following exposure to hCG for 16 h and removal of hCG at time 0. Cells were incubated for 16 hours at various doses of hCG (0, .5, 5, 50 ng/ml). At 0 time, cells were washed and the binding of ^{125}I-iodo hCG was measured at 8, 22, 48, 72 hours. During this period the medium was changed daily.

1.3 % at 24 h and 48 h (Fig. 2). These cell-bound radioactive products migrate like hCG molecules in SDS gel electrophoresis (10).

Visualisation of hCG by the immunocytochemical reaction when the cells are maintained in the constant presence of hCG. When the cells are preexposed to hCG (1 h, 50 ng) a dark purple staining can be observed on the surface of some of the cells (10). In a typical experiment (Table 1), 40.3 ± 3 % of the cells were stained following one hour exposure to 50 ng hCG. This number of stained cells is maintained with time since at 48 h this value is not significantly different.

Following 48 h exposure to 5 ng/ml hCG which is also close to a saturating concentration (7) very similar results are observed. Following exposure to .5 ng/ml hCG (50 % receptor occupancy (7)) the percent of stained cells is 42.1 ± 4 % of the cell

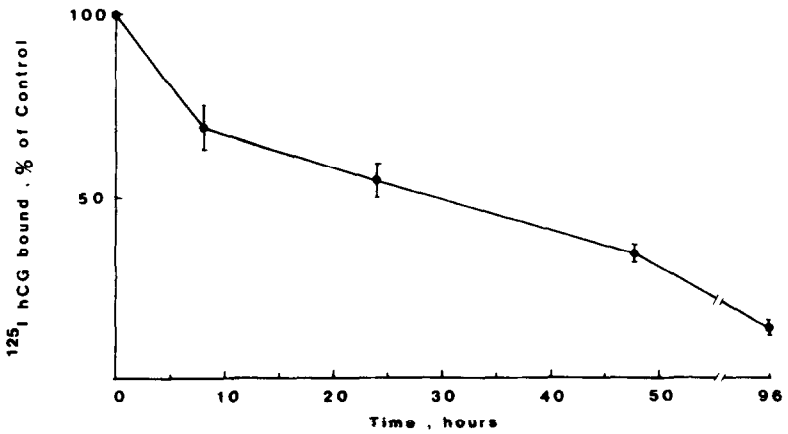

Fig. 2. Fate of bound hCG following hCG removal (time 0). Cells were incubated for 16 hours with ^{125}I-hCG (25 ng/ml). At time 0, cells were washed and the amount of radioactivity in the cell compartment was measured at 0, 8, 24, 48 and 96 hours.

Table 1

Percentage of stained cells (immunocytochemical reaction) following short and long term exposure to various hCG concentrations

	hCG ng/ml		
Time of exposure	50	5	.5
1 h	40.2 ± 3		
48 h	41.5 ± 4	42 ± 3.2	42.1 ± 2.4

population. As indicated in the paragraph "Methods", the staining procedure does not allow to discriminate and quantify the amount of occupied receptors. Between 50 and 100 % receptor occupancy, the intensity of the staining and the number of stained cells are consequently similar.

These results indicate however that hCG molecules are constantly present at the cell surface of the cells during prolonged exposure to high or low hCG concentrations.

Effect of hCG removal (24 or 48 hours) on the percent of stained cells (following hCG exposure : 1 hour). Table 2 indicates again that following one hour exposure to 5 or 50 ng hCG, 40 % of the cells are stained. Following hCG removal and culture in hCG free medium the number of stained cells observed is very similar at 24 or 48 h for both hCG concentrations.

Following short term exposure to concentrations of 5 or 50 ng/ml hCG the hormone-receptor complexes formed seem consequently to be extremely stable since they can be visualized as long as 48 hours following binding. Since the results obtained, under the same experimental condition using ^{125}I-hCG indicates that, following 24 h or 48 h washing, 56 % and 34 % of the radioactive hCG is still bound in the cellular compartment, this amount of hCG is sufficient for obtaining the immunocytochemical staining assuming that all hCG molecules are still at the cell surface.

DISCUSSION

We have previously shown that hCG induced a disappearance of the free hCG receptors in Leydig cells either in vivo (3, 4) and in vitro (4, 7). This disappearance is

Table 2

Percentage of stained cells (immunocytochemical reaction) following short-term exposure to various hCG concentrations and 24 h or 48 h washing

	hCG ng/ml	
Time of exposure	50	5
1 h	40.1 ± 2.6	40.2 ± 3.6
	hCG removal	
24 h	42.9 ± 3	34.9 ± 3.6
48 h	42.5 ± 0.5	42.6 ± 2.9

dependent on the concentration of hCG (1-7). Around 12 hours following hCG exposure, the levels of free receptors reach a steady state whatever the hCG concentration used (7) and consequently the time 16 hours has been chosen for our study. At that time the disappearance of the free receptors is almost total for the concentration of 5 and 50 ng/ml and in the order of 50 % for 0.5 ng/ml. It is not known however if this is the result of irreversible occupancy or removal of the hormone receptor complexes.

Figure 1 confirms that following hCG exposure, the number of free binding sites is reduced (time 0). However when hCG is removed the number of free receptors does not increase immediately. An increase in the number of free receptors is observed after 8 hours either for high or for low hCG pretreatment. Such a delay in the receptor recovery is observed in vivo (3, 4) and is partially due to the remainder of the hCG bolus. However, following careful washing in vitro this is not feasible and the reason for this lag time is not yet elucidated. Following exposure to a saturating concentration of hCG 72 hours are necessary for full recovery of receptor number. We can compare these results with those obtained in vivo, in the rat, following down regulation with high doses of hCG. It has been previously shown (3) that the increase in free receptors is not observed before 72 hours following the injection. Similar results have been obtained by others (15) in the adult rat while in the immature rat the speed of receptor recovery is much faster. However these in vivo results are the resultant of several phenomena and are particularly linked to the clearance of the hormone.

Several hormones have been visualized by immunochemical reaction (10, 13, 14). In the case of hCG molecules the staining of the cells is visible only after exposure to hCG and anti-hCG antibody. Moreover, the presence of unstained cells in the same culture is an assessment in favor of its specificity. The discrimination between stained and unstained cells is easy but a quantitative evaluation of the staining on each individual cell cannot be made. The staining is located on the cell surface by morphological criteria (10). Since the reagents do not enter the cells fixed in culture, intracellular hCG, if present, should not be revealed (10).

When the stained cells are counted in cultures in the continuous presence of hCG (at saturating concentions ; 50 and 5 ng/ml or half-saturating ; 0.5 ng/ml), it is of interest to observe that the number of stained cells is maintained for 48 hours. During the same time the binding studies clearly indicate that the number of the free receptors is negligible or reduced (7). The comparison of these results suggests that the disappearance of receptors is, in major part, the consequence of constant receptor occupancy. These results are in complete agreement with experiments obtained in vivo following hCG injection (8, 9, 17). The detection of hormone-receptor complexes on the rat Leydig cell surface is obvious at 24 h following the injection, indicating that a significant number of hCG molecules are still present on the Leydig cells (15, 16), in the corpus luteum membrane (17) or in the granulosa cells (18). This possibility has been clearly confirmed by experiments of binding of ^{125}I-hCG and analysis of the radioactive product after 24 or 48 hrs washing. These cells still contain significant

amount of large iodinated protein (10) compatible with the presence of undestroyed hCG for as long as 48 hours following binding.

Since following hCG removal the number of stained cells is identical at 24 and 48 hrs of washing as at 1 hr, it can be concluded that some of the complexes are the initial ones. This confirms the stability of the binding under long term incubation, as previously suggested (19) and indicates that most of the hCG molecules might still be located on the cell surface. These data differ from the results obtained with Leydig cell tumor (20) but might reflect the differences between tumoral or non tumoral cells.

In short term studies it has been shown that hCG bound can be more easily released by chemical treatment for short hCG exposure than for long exposure (7). This has been explained in term of aggregation and internalisation in the case of hCG (7, 21, 22). However the present data suggest that significant amount of irreversible complexes are maintained at the cell surface for longer periods than previously expected. The effects, on the cell biology, of these persistent hormone-receptors complexes remain to be determined.

REFERENCES

1. Mather, J.P., and Haour, F. (1981) Functionally differenciated cell lines, p. 93-108.
2. Tell, G.P., Haour, F.F., and Saez, J.M. (1978) Metabolism 27, 1566-1592.
3. Haour, F., and Saez, J.M. (1977) Mol. Cell. Endocrinol. 7, 17-24.
4. Haour, F., Dray, F., and Mather, J.P. (1982) The cell biology of the Testis, Ann. N.Y. Acad. Sci. 383, 231-248.
5. Haour, F., and Saez, J.M. (1978) Structure and Function of the Gonadotropins, Vol. 2, K.W. McKerns (ed.). pp. 497-516. Plenum Press, New York.
6. Sharpe, R.M. (1977) Biochem. Biophys. Res. Comm. 75, 711-717.
7. Mather, J.P., Saez, J.M., and Haour, F. (1982) Endocrinology 110, 933-940.
8. Rajaniemi, H., Karjalainen, M., Veijola, M., Ritanen-Kaivamo, S., Lekkokumpu, S., and Metsikko, K. (1981) Histochem. Cytochem. 29, 813-816.
9. Kinnunen, R., Kaijalainen, M., and Rajaniemi, H. (1981) J. Histochem. Cytochem. 29, 9-16.
10. Begeot, M., Mombrial, C., Dubois, P.M., Dubois, M.P., Dazord, A., and Haour, F. (1982) J. Histochem. Cytochem., 31, 893-904.
11. Mather, J.P., Saez, J.M., and Haour, F. (1981) Steroids 38, 35-44.
12. Haour, F., and Saxena, B.B. (1974) J. Biol. Chem. 269, 2195-2205.
13. Begeot, M., Dupouy, J.P., Dubois, P.M., and Dubois, M.P. (1981) Neuroendocrinology 32, 285-294.
14. Dubois, P.M., Begeot, M., Dubois, M.P., and Herbert, D.C. (1978) Cell Tissue Res. 191, 249-265.
15. Huhtaniemi, J.T., Ketikinemi, M., and Catt, K.J. (1981) Endocrinology 109, 588-595.
16. Hsueh, A.J.W., Dufau, M.L., Katz, S.I., and Catt, K.J. (1976) Nature 261, 710-711.
17. Zimnisk, S.S., Rorke, E.A., Sickel, M.A., and Vaitukaitis, J.L. (1982) Endocrinology 111, 625-634.
18. Robinson, M.S., Rhodes, J.A., and Albertini, D.F. (1983) J. Cell. Physiol. 117, 43-50.
19. Moyle, W.R. (1980) Oxford Review of Reproductive Biology, Vol. 2, C.A. Finn, ed. Clarendon Press, Oxford, p. 123-173.
20. Ascoli, M., and Puett, D. (1978) J. Biol. Chem. 253, 4892-4899.
21. Amsterdam, A., Berkowitz, A., Nimrod, A., and Kohen, F. (1980) Proc. Natl. Acad. Sci. USA 77, 3440-3444.
22. Amsterdam, A., Naor, Z., Knecht, M., Dufau, M.L., and Catt, K.J. (1981) Receptor-mediated binding and internalization of toxins and hormones, pp. 283-310. Academic Press, New York.