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NUCLEAR LOCALIZATION OF A GLUCOCORTICOID ANTAGONIST IN CULTURED HEPATOMA CELLS

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The binding characteristics of promegestone, a typical antagonist of glucocorticoid action, has been investigated. We localized [^{3}H] promegestone in the nuclei purified from steroid-treated HTC cells but the radioactivity specifically bound to the nucleus was much lower for the antagonist than for an inducer steroid, for instance dexamethasone. We attempted to define the nature of the "nuclear" activity and found that promegestone does not bind to the perinuclear membrane but is associated with the chromatin fraction.

Promegestone, 17 α ,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione (R 5020), has been found to antagonize the action of glucocorticoids (1). This synthetic progestin is able to bind to glucocorticoid receptors in the cytosol from hepatoma tissue culture (HTC) cells (2). From **in vitro** competition studies (3), it was concluded that antiglucocorticoids act by competition with inducer steroids for the cytoplasmic receptor binding sites and that the antagonist-receptor complex cannot undergo the conformational changes necessary for the translocation into the nucleus. Thus the antagonist has no proper action but prevents the formation of an active hormone-receptor complex and obviates the expression of the glucocorticoid activity (3).

In a previous paper (4), we have shown that this concept of antagonistic action is questionable for two reasons : (i) the necessity of an excess of the antagonist (at least 100 times the concentration of the inducer) to inhibit tyrosine aminotransferase induction; (ii) the treatment of HTC cells by two steroids whose effects are opposite allows only the second steroid administered to be effective. These experiments suggest that the antagonistic action, like the induction process, could occur at the nuclear level (4).
In the current study we have followed the behaviour of radiolabeled promegestone in HTC target cells comparative to that of dexamethasone, a potent inducer steroid , considering especially the nuclear binding of both steroids.

MATERIAL and METHODS

Incubation of HTC cells with radiolabeled steroids.
[^{3}H] Dexamethasone (20 Ci/mM) and [^{3}H] promegestone (87 Ci/mM) were purchased from New England Nuclear.

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HTC cells, grown in suspension as previously described by Thompson et al (5), were harvested in the log phase of growth at a cell density of 5.10^5 cells/ml. The incubation with the steroids was performed during 1 h at 37°C by magnetic stirring. In order to increase the steroid concentration in the culture medium, the cells were centrifuged and their density was raised to 12.10^6 cells/ml just before adding the steroid in a twenty fold smaller volume. Control experiments showed that, in the concentrated culture, the cells stay in the log phase of growth for another period of 2 hours. Moreover the higher cell density does not modify the penetration of the steroid into the cells (results not shown).

Isolation and purification of nuclei

Cells were rinsed in saline, resuspended for 15 min in 2 ml of hypotonic buffer (Tris-HCl pH 7.4, 1 mM) which allows the cells to swell, and incubated during another 15 min period in the presence of 5 % Nonidet P 40 (NP 40). This treatment aids the subsequent rupture of cell membranes in the 5 ml Braun Potter homogenizer (by 5 manual strokes). Sucrose, 300 mM, and $CaCl_2$, 3 mM, were added during the homogenization in order to protect the nuclei which are liberated.

To get rid of cytoplasmic contaminants, the nuclei were washed twice through a 1 M sucrose layer; the purified nuclei, collected by low speed centrifugation (4,000 rpm, 10 min), were suspended in 1 ml sodium phosphate buffer pH 7.85, 2 mM, sucrose 250 mM (buffer A). At each step, 100-200 µl samples were set aside. In one series of samples, we measured the activity of lactic dehydrogenase. The other series of samples were put in new glass vials with 10 ml Beckman Ready-Solve scintillation fluid and counted during 3 periods of 4 min each and the background was substracted; the counting efficiency was 10 %.

Purification of nuclear membranes

Nuclear membranes were purified according to the method described by Smith and von Holt (6). The nuclear pellet from 70.10^6 cells was resuspended at 4°C in 4 ml buffer A to give a final DNA concentration of about 250 µg/ml. Heparin (lithium salt, Sigma) was added slowly with stirring to give a DNA/heparin ratio of 1. In these proportions, the heparin solubilizes the chromatin. The solution was stirred gently for 1 h and then centrifuged for 50 min at 30,000 rpm in a Ti 50 Beckman rotor. The supernatant containing the chromatin fraction was discarded and the pellet corresponding to the crude nuclear membranes was taken up in 300 µl of sodium phosphate buffer pH 7.5, 2 mM, sucrose 250 mM (buffer B), layered over a 25-50% (w/v) sucrose gradient and centrifuged for 2 h at 40,000 rpm in a SW 41 Beckman rotor. Fractions (0.5 ml) were collected and the absorption at 280 nm was measured. Material banding at 37 % sucrose (d 1.18-1.20 g/ml) was diluted 5-fold with buffer B and pelleted by centrifugation at 50,000 rpm during 30 min in a Ti 50 Beckman rotor. This pellet was taken as pure nuclear membranes.

Lactic dehydrogenase assay

The amount of lactic dehydrogenase (LDH, EC 1.1.1.27) was measured by a spectrophotometric method (optimized Test-Combination, Boehringer, Mannheim) based on the rate of disappearance of the band at 340 nm in NADH. The method may be applied directly to crude extracts.

RESULTS

Examination of the nuclear preparations

In order to obtain information on a possible translocation of a cytoplasmic receptor-antagonist complex to the nucleus, it was essential to isolate nuclei suitable for such a study. Therefore our nuclear preparations were examined for purity and morphological integrity. We first followed, during the different purification steps, the disappearance of lactic dehydrogenase. This enzyme of the glycolysis is specifically cytoplasmic and

Table 1. Disappearance of lactic dehydrogenase during the purification of nuclei. At each step, the volume of the nuclear preparation is brought up to 2 ml and the enzyme activity is measured in 200 µl samples. In the homogenate and in the first supernatant, we noticed an apparent decrease of the enzyme activity (18 %) due to the presence of detergent (NP 40); the values in the table were corrected, taking into account this discrepancy.

		LDH activity (mU/ml)	%
Homogenate		2 360	100
1st wash	supernatant	2 000	82.2
	nuclei	190	8.0
2nd wash	supernatant	158	6.5
	nuclei	10	0.4

represents a good marker for cytoplasmic contaminations. In table 1, the results show the gradual elimination of LDH activity. After the second wash, only 0.4 % of the initial activity measured in the homogenate was left in the nuclear pellet. Integrity of the isolated nuclei was checked by phase contrast microscopy. The combined treatment of the cells by hypotonic shock and detergent permitted the isolation of nuclei which are clear of cytoplasmic contamination after the second wash; moreover the nuclear membrane appeared unaltered (fig. 1). A third wash of the nuclei did not give better results and frequently led to damages of the nuclear membrane.

Competition experiments between dexamethasone and promegestone

The ability of promegestone to compete for dexamethasone binding "**in vivo**" in the whole cell was tested by incubating HTC cells in the presence of

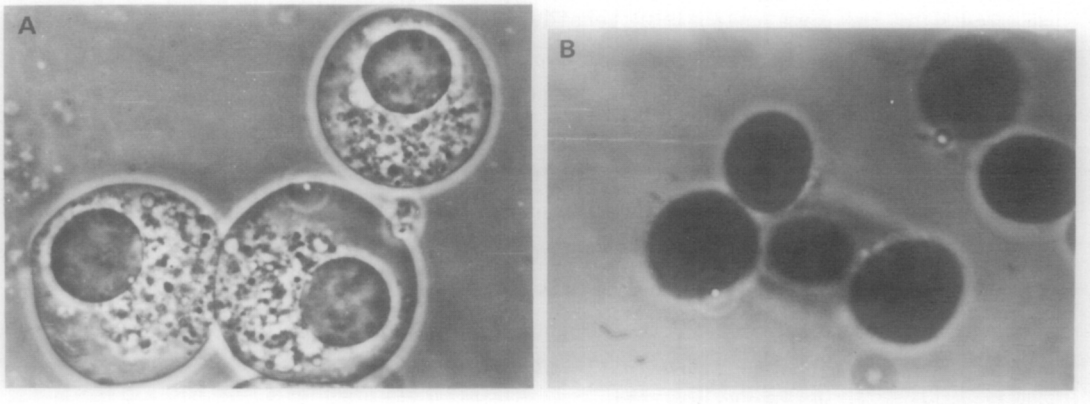

Figure 1. Phase contrast micrographs (x420) of HTC cells after hypotonic treatment in the presence of the detergent NP 40 (A) and of nuclei purified from those cells after the second wash (B).

Table 2. Effect of promegestone on dexamethasone binding. HTC cells (70.10^6 cells in 10 ml) were incubated with [^{3}H] dexamethasone (10^{-8}M) alone or with a 100 fold excess of non radioactive promegestone. The binding of dexamethasone in the whole cell and in the nucleus was assayed as described in Materials and Methods.

	[^{3}H]dexamethasone bound in the whole cell		nucleus	
	cpm	%	cpm	%
- promegestone	58 250	100	2 709	100
+ promegestone	19 600	33.6	700	25.8

tritiated dexamethasone with or without a 100-fold excess of unlabeled promegestone. When promegestone was added, the radioactivity located in the cell or in the nucleus was respectively 34 % or 26 % of that measured in presence of dexamethasone alone (table 2) ; this suggests that promegestone binds to dexamethasone receptors. In a previous paper we have reported that, in the same conditions, the activity of tyrosine aminotransferase was lowered to 28 % of the optimal activity (4). Taken together, these data support the hypothesis that the magnitude of a biological response is directly related to the number of steroid receptor complexes bound in the cell and in the nucleus.

Binding of [^{3}H]promegestone to the nucleus

In comparing the penetration of promegestone and dexamethasone into the cell, it becomes evident that the penetration of the antagonist is three times less than that of the agonist . Moreover, promegestone was detected in the nucleus but in a smaller proportion as compared to dexamethasone (table 3, upper part). In order to check if the antagonist binds specifically to the nucleus, the amount of tritiated steroid bound was measured in the presence of an excess of the same unlabeled steroid. As shown in fig. 2, the specific binding of promegestone represents about 30 % of that estimated for dexamethasone either in the whole cell (fig. 2A) or in the nucleus (fig. 2B). Considering the amount of promegestone taken up by the cell, its specific binding appears to be 10 times less than the specific binding of dexamethasone. Some points of similarity can be established between the differences measured here for the two steroids and the difference of affinity for the receptor already reported. As a matter of fact, the affinity of promegestone is about one fifteenth that of dexamethasone and the number of high affinity binding sites was estimated to be 20 % of the value corresponding to dexamethasone (2).

We have also observed that the non-specific binding of promegestone to the nuclei is twice that of dexamethasone.

Table 3. Localization of promegestone and dexamethasone in different cell compartments. Two sets of results are reported : (★) those of a typical experiment conducted with 10 ml cell culture containing 7.10^6 cells/ml and incubated for 60 min, and (★★) the mean value of 4 different experiments expressed as fmoles. The specific activity for **dexamethasone** and **promegestone** were respectively 4,660 and 20,275 cpm/pmole.

		Dexamethasone (10^{-8}M)			Promegestone (10^{-8}M)		
		cpm/70.10^6 cells (★)	fmoles/10^6 cells (★★)	%	cpm/70.10^6 cells (★)	fmoles/10^6 cells (★★)	%
radioactivity	in the whole cell	31 000	95	100	44 000	31	100
	in the nucleus	2 100	6.4	7	2 260	1.6	5.14
radioactivity bound to the chromatin		2 000	6.1	6.45	1 960	1.4	4.45
radioactivity bound to the nuclear membrane	crude	60	0.2	0.2	270	0.2	0.7
	purified	0	0	0	75	0.05	0.17

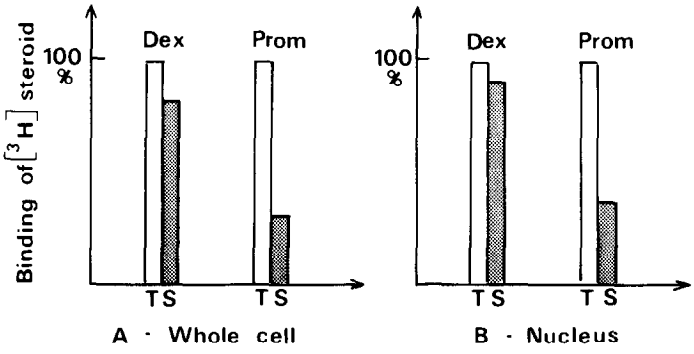

Figure 2. Specific binding of promegestone and dexamethasone. The total counts (T □) were measured in presence of the tritiated steroid at a 10^{-8} M concentration. The specific binding (S ▩) was estimated by substracting from T the radioactivity measured by adding simultaneously the labeled steroid and a high ratio of the same unlabeled steroid (10^{-5} M). The percentage of specific binding of dexamethasone and promegestone corresponds respectively to 5.39 and 0.63 fmoles/10^6 cells in the whole cell (A) and to 0.41 and 0.042 fmoles/10^6 cells in the nucleus (B).

In order to pinpoint the nature of the nuclear binding of promegestone, the nuclei were disrupted and the membranes were separated from the chromatin fraction. The data presented in table 3 show that almost all the radioactivity bound to the nucleus is located in the chromatin fraction, both for promegestone and for dexamethasone.

DISCUSSION

The principal importance of hormone antagonists lies in their clinical potential. However the mechanism of their behaviour at the cellular and the molecular level has not been identified. To date, only two antiglucocorticoids have been studied, essentially during the lysis of thymocytes **in vitro**. The data reported for progesterone and for cortexolone are quite different : whereas progesterone acts only in the cytoplasm (3, 7, 8), cortexolone is translocated into the nucleus but its binding to the chromatin is impaired (9, 10). These observations suggest that the mechanism of antagonism is more complex than expected and cannot be identified only on the basis of agonist studies (3).

The availability of a highly radioactive antagonist has greatly facilitated our study in whole HTC cells and in purified nuclei. We have been able to show unambiguously that promegestone binds to the nucleus; this binding, which is specific, proceeds via the glucocorticoid receptor and is dependent on the affinity of the antagonist for the receptor. Moreover the binding measured can be correlated to the tyrosine aminotransferase response (induction or inhibition of enzyme synthesis).

The nuclear fractionation provides further information on the localization of the anti-inducer : promegestone given directly to the cell does not bind to the perinuclear membrane which might be the target for specific cytoplasmic hormonal signals (6), but shows the involvement of the chromatin for the association of the nuclear receptor.

Our results support the idea that the appearance of promegestone in the nuclei may be the key event upon which depend all further biochemical steps leading to the mechanism of action of antiglucocorticoids, as is the case for anti-estrogens (11, 12). Obviously our preliminary experiments should be extended to additional antagonists and to new systems before considering the detailed function in gene regulation using the approach recently described for a few agonists (13, 14).

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REFERENCES

1. Raynaud, J.P., Bouton, M.M., Moguilewsky, M., Ojasoo, T., Philibert, D., Beck, G., Labrie, F. and Mornon, J.P. (1980) J. Steroid Biochem. 12, 143-157.
2. Nordeen, S.K., Lan, N.C., Showers, M.O. and Baxter, J.D. (1981) J. Biol. Chem. 256, 10503-10508.
3. Rousseau, G.G. and Baxter, J.D. (1979) in "Glucocorticoid Hormone Action" (Baxter, J.D. and Rousseau, G.G. Eds), Springer Verlag, Berlin, Vol. 12, pp. 49-77.
4. Giesen, E.M. and Beck, G. (1982) Horm. Metabol. Res. 14, 252-256.
5. Thompson, E.B., Tomkins, G.M. and Curran, J.F. (1966) Proc. Nat. Acad. Sci. US 56, 296-303.
6. Smith, P. and von Holt, K. (1981) Biochemistry 20, 2900-2908.
7. Jones, T.R. and Bell, P.A. (1980) Biochem. J. 188, 237-245.
8. Kaiser, N., Mayer, M., Milholland, R.J. and Rosen, F. (1979) J. Steroid Biochem. 10, 379-386.
9. Turnell, R.W., Kaiser, N., Milholland, R.J. and Rosen, F. (1974) J. Biol. Chem. 249, 1133-1138.
10. Naylor, P.H. and Rosen, F. (1982) in "Hormone Antagonists" (Agarwal, M.K. Ed.), De Gruyter, W., Berlin, pp. 365-380.
11. Katzenellenbogen, B.S., Katzenellenbogen, J.A., Ferguson, E.R. and Krauthammer, N. (1978) J. Biol. Chem. 253, 697-707.
12. Rochefort, H., Garcia, M. and Borgna, J.L. (1978) Biochem. Biophys. Res. Commun. 88, 351-357.
13. Payvar, F., Wrange, O., Carlstedt-Janke, J., Okret, S., Gustafsson, J.A. and Yamamoto, K.R. (1981) Proc. Nat. Acad. Sci. US 78, 6628-6632.
14. Robins, D.M., Paek, I., Seeburg, P.H. and Axel, R. (1982) Cell 29, 623-631.