KINETICS OF GLUCOCORTICOID INTERACTION WITH WILD-TYPE AND VARIANT RECEPTORS

M. SPINDLER-BARTH and U. GEHRING

Institut für Biologische Chemie, Universität Heidelberg, Im Neuenheimer Feld 501, 6900 Heidelberg, FRG

1. Introduction

According to our present knowledge about steroid hormone action specific receptors play a pivotal role in the biochemical mechanism of action (reviews [1–3]). Following its entry through the plasma membrane of a target cell the steroid is bound by intracellular receptors. Subsequently, the receptor–hormone complex is transferred to the cell nucleus and interacts with chromatin to bring about changes in specific gene expression. The receptor therefore has to be regarded as a molecular entity which comprises 2 active domains: one for steroid binding and the other for nuclear interaction [4,5].

The growth inhibitory effect elicited by glucocorticoids in certain cells of lymphatic origin has been used to select for unresponsive variant cells with alterations in their receptors (reviews [5,6]). Although the majority of these variants is characterized by negligible receptor binding activities, several variants have been obtained with roughly normal steroid binding but abnormal interaction of the receptor complexes with cell nuclei, chromatin or DNA. In one such phenotype the receptor–glucocorticoid complex is defective in nuclear binding (nt^-, nuclear-transfer deficient) while in another increased nuclear binding and increased affinity for DNA are observed (nt^i, increased nuclear transfer).

Here, we ask the question whether defects in the nuclear interaction domain of the receptor would also lead to alterations, albeit subtle, in the steroid binding site. The technique we applied was to measure the kinetics of receptor–steroid interaction. In one clone of nt^- phenotype we indeed found a significant difference in the dissociation of the receptor–steroid complex while in other variants the kinetics had wild-type characteristics.

2. Materials and methods

2.1. *Cell lines and culture conditions*

The mouse lymphoma line WEHI-7 [7] was kindly provided by Dr A. W. Harris. The S49.1 lymphoma sublines were those in [8,9]; their phenotypes are listed in table 1. Cells were grown and harvested as in [10] and stored frozen at $-90°C$.

2.2. *Cytosol preparations*

Frozen cell pellets were thawed and homogenized at $0°C$ in 20 mM tricine buffer (pH 7.4) containing 50 mM KCl, 5 mM dithioerythrol and 10% glycerol using a Dounce homogenizer. Following centrifugation at $100 000 × g$ for 90 min the clear supernatant was removed and used for binding studies. Throughout these experiments cytosols were prepared in such a way that 300 μl corresponded to 10^8 cells.

2.3. *Binding assays*

Samples (300 μl) of cytosol were incubated at $0°C$ with various concentrations of [^{3}H]dexamethasone (Amersham, 25 Ci/mmol) with or without a 1000-fold excess of non-radioactive dexamethasone as a competitor. Specific binding was assessed from the difference of hormone binding in non-competed and competed samples [11]. All samples were in duplicate. Following the addition of 50 μl dextran-coated charcoal (100 mg Norit A and 5 mg dextran T-500 (Pharmacia)/ml) the samples were agitated for 5 s and centrifuged for 5 min in a table-top centrifuge. Radioactivity was determined in the supernatant using a Triton X-100 containing scintillant and a Beckman LS 7000 liquid-scintillation counter at 43% efficiency. For equilibrium binding experiments incubations were for 15 h; the data were evaluated by the Scatchard method [12]. Association kinetics were measured

in cytosol preparations exposed to [^{3}H]dexamethasone at 10–30 nM; samples were removed at intervals and assayed as described. Association rate constants k_a were obtained as in [13] assuming receptor saturation after 3–4 h incubation. Dissociation kinetics were measured in cytosol preparations pre-equilibrated with 50 nM [^{3}H]dexamethasone for 90 min; following the addition of a 1000-fold excess of unlabelled steroid, specific binding was determined at intervals using the charcoal assay.

3. Results

3.1. Association of dexamethasone with receptors

Three glucocorticoid-sensitive sublines of the S49.1 mouse lymphoma were used here. Their receptors were compared to those of the WEHI-7 thymic lymphoma cell line which is known to contain roughly double the level of intracellular receptors of indistinguishable binding characteristics [14,15]. Three glucocorticoid-resistant clones of S49.1 were also investigated (table 1). The binding of radiolabelled dexamethasone to receptors contained in cytosol preparations is shown in fig.1A for 4 representative cell clones. Saturation of receptors was attained by 2–3 h. The kinetic data obtained for the initial 60 min incubation were evaluated from a linearized plot [13] assuming second-order kinetics (fig.1B). The results are compiled in table 1. The association rate constant k_a for the formation of the receptor—dexamethasone complex was ~1 × 10^8 min^{-1} . M^{-1} for all cell clones tested. This value is of the same order of magnitude as those reported for dexamethasone binding to receptors of various other cell types [16–20].

3.2. Dissociation of receptor–dexamethasone complexes

Dissociation of preformed receptor complexes with radiolabelled dexamethasone was measured by following the displacement by unlabelled steroid that had been added in excess. As depicted in fig.2 for 5 cell clones the kinetics was first order. Dissociation rate constants k_d calculated from the slopes of such semilogarithmic plots are listed in table 1. For all cell lines except one the rate constant was ~1 × 10^{-3} min^{-1} with half-times of dissociation of ~5 h. The complex of one of our nt$^-$ clones, S49.1TB.4.22R, however, dissociated much faster (fig.2, table 1); the half-life of the complex was found to be 76 min only.

3.3. Equilibrium dissociation constant

The kinetic constants for dexamethasone interaction with receptors were used to compute the equilibrium dissociation constant K_d for the complexes (table 1). For the nt$^-$ line S49.1TB.4.22R the K_d was ~3-fold different from those of the others. For comparison, K_d values were also determined by equilibrium binding experiments (not shown). In general, K_d-values obtained from association and dissociation kinetics are regarded to be more accurate than those measured by equilibrium binding [18,20,21] because of the instability of unbound glucocorticoid receptors during long incubation times and at low steroid con-
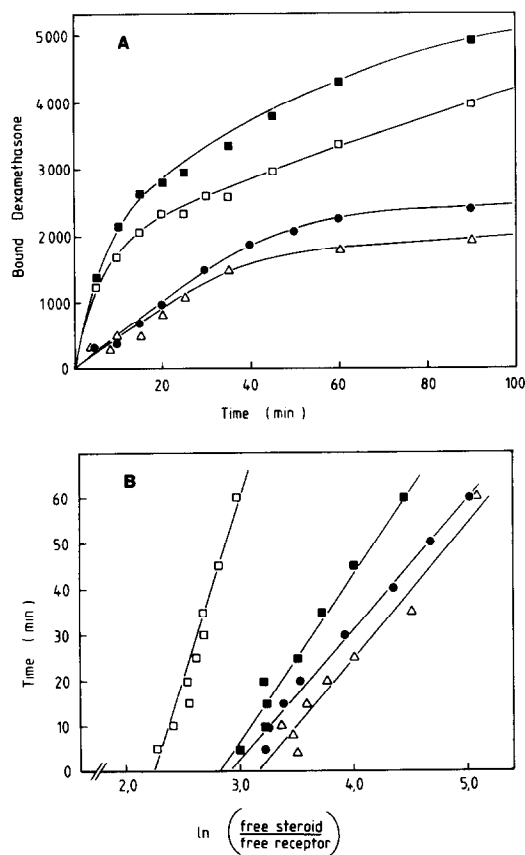

Fig.1. Kinetics of dexamethasone association with receptors. Cytosols of WEHI-7 (■), S49.1TB.4 (●), S49.1TB.4.22R (△) and S49.1TB.4.55R (□) were incubated with [^{3}H]dexamethasone at 10–30 nM. Samples were removed at intervals and assayed for binding. (A) Bound dexamethasone is expressed as occupied sites per cell. (B) The data are replotted for the initial 60 min as ln ([free dexamethasone]/[unoccupied receptor sites]). Total receptor concentrations were estimated from binding at 3–4 h incubation.

Table 1

Glucocorticoid responsiveness and kinetics of receptor—dexamethasone interaction

Cell line	Glucocorticoid response (phenotype)	Association rate constant k_a (min^{-1} . M^{-1})[a]	Dissociation rate constant k_d (min^{-1})	Half-life $t_{1/2}$ (min)[b]	Equilibrium dissociation constant K_d (nM)[c]
WEHI-7	Sensitive (wt)[d]	$1.18 \pm 0.23 \times 10^6$ (3)	$1.24 \pm 0.35 \times 10^{-3}$ (3)	243	1.05
S49.1	Sensitive (wt)		$1.21 \pm 0.13 \times 10^{-3}$ (2)	249	
S49.1TB.4	Sensitive (wt)	$1.38 \pm 0.31 \times 10^6$ (3)	$1.19 \pm 0.11 \times 10^{-3}$ (3)	253	0.86
S49.1G.3	Sensitive (wt)		$0.86 \pm 0.23 \times 10^{-3}$ (2)	350	
S49.1TB.4.55R	Resistant (nt^i)	$1.18 \pm 0.28 \times 10^6$ (2)	$0.87 \pm 0.07 \times 10^{-3}$ (4)	346	0.74
S49.1TB.4.22R	Resistant (nt$^-$)	$1.35 \pm 0.10 \times 10^6$ (2)	$3.95 \pm 0.62 \times 10^{-3}$ (4)	76	2.92
S49.1G.3.83R	Resistant (nt$^-$)	$0.82 \pm 0.05 \times 10^6$ (4)	$1.24 \pm 0.02 \times 10^{-3}$ (2)	243	1.51

[a] Mean values and standard deviations are given (number of separate experiments in parenthesis)
[b] $t_{1/2}$ was calculated from the dissociation rate constant
[c] Equilibrium dissociation constant was calculated from rate constants k_a and k_d
[d] wt signifies wild-type glucocorticoid sensitivity

centrations. Nevertheless, the computed equilibrium dissociation constants of table 1 compare quite well to those obtained from equilibrium binding studies for lines WEHI-7 and S49.1 [22].

4. Discussion

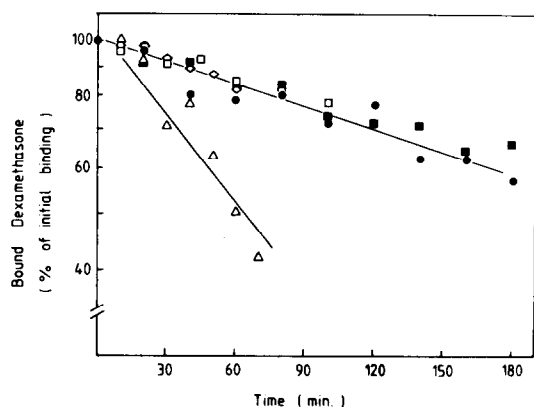A careful study of the thermodynamics of gluco-corticoid binding to the receptors of hepatoma cells

Fig.2. Kinetics of dexamethasone dissociation from receptor complexes. Displacement of [³H]dexamethasone from pre-labelled receptor complexes was measured as in section 2. The data are plotted as % of initial binding on a logarithmic scale. Symbols are as in fig.1 except that data for line S49.-1G3.83R (◇) are also shown.

[23] has led to the view that hydrophobic interactions between receptor and steroid account for most of the binding energy. The steroid binding site of the receptor can thus be viewed as a hydrophobic domain within the polypeptide structure. It is interesting in this regard that glucocorticoids of varying structures associate with the same rate constant with receptors of rat thymocytes and mouse L-cells [18,20]. Similarly, as shown here, the rate of association of dexametha-sone with receptors of several mouse lymphoma cell clones was the same independent of whether these clones were of wild-type glucocorticoid sensitivity or whether they contained receptors with altered nuclear binding properties. Thus the association of gluco-corticoids with receptors of different cells appears to be rather invariant.

However, the dissociation rate of receptor—gluco-corticoid complexes varies greatly with the structure of the steroid as has been shown for receptors of thymocytes [18] and L-cells [20]. There are also substantial differences in the dissociation rate con-stants of receptor complexes of different cell types with the same steroid, for example, dexamethasone [16—20]. Thus the dissociation rates of receptor com-plexes appear to be quite sensitive to alterations in both ligand and receptor structures. It is therefore significant that the dexamethasone complex with the receptors of one of our nt$^-$-resistant lymphoma cell variants (S49.1TB.4.22R) dissociates much more rapidly than that of the other clones. This clone, as

well as the other variant clones used here has been obtained by a single-step isolation procedure [24] which is likely to yield single rather than multiple mutations in a specific gene product. It is therefore probable that a single genetic event in clone S49.1 TB.-4.22R has affected both the steroid binding site and the nuclear interaction domain of the receptor molecule. Definite proof for this view, however, will require detailed protein analytical studies with pure receptors. Nevertheless, these data support the general idea of a bifunctional structure of steroid hormone receptors which comprise within a single molecular entity 2 active domains, one for steroid binding and the other for interaction with chromatin.

Acknowledgements

References

[1] Yamamoto, K. R. and Alberts, B. M. (1976) Annu. Rev. Biochem. 45, 721–746.

[2] Higgins, S. J. and Gehring, U. (1978) Adv. Cancer Res. 28, 313–397.

[3] Katzenellenbogen, B. S. (1980) Annu. Rev. Physiol. 42, 17–35.

[4] Gehring, U. and Tomkins, G. M. (1974) Cell 3, 301–306.

[5] Gehring, U. (1980) in: Biochemical Actions of Hormones (Litwack, G. ed) vol. 7, pp. 205–232, Academic Press, New York.

[6] Yamamoto, K. R., Gehring, U., Stampfer, M. R. and Sibley, C. H. (1976) Rec. Prog. Horm. Res. 32, 3–32.

[7] Harris, A. W., Bankhurst, A. D., Mason, S. and Warner, N. L. (1973) J. Immunol. 110, 431–437.

[8] Gehring, U. (1979) Adv. Enz. Reg. 17, 343–361.

[9] Andreasen, P. A. and Gehring, U. (1981) Eur. J. Biochem. 120, 443–449.

[10] Gehring, U. (1980) Mol. Cell. Endocrinol. 20, 261–274.

[11] Rousseau, G. G., Baxter, J. D. and Tomkins, G. M. (1972) J. Mol. Biol. 67, 99–115.

[12] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.

[13] Rousseau, G. G. and Baxter, J. D. (1979) in: Glucocorticoid Hormone Action (Baxter, J. D. and Rousseau, G. G. eds) Monogr. Endocrinol., vol. 12, pp. 49–77, Springer-Verlag, Berlin.

[14] Bourgeois, S. and Newby, R. F. (1977) Cell 11, 423–430.

[15] Pfahl, M., Sandros, T. and Bourgeois, S. (1978) Mol. Cell. Endocrinol. 10, 175–191.

[16] Baxter, J. D. and Tomkins, G. M. (1971) Proc. Natl. Acad. Sci. USA 68, 932–937.

[17] Koblinsky, M., Beato, M., Kalimi, M. and Feigelson, P. (1972) J. Biol. Chem. 247, 7897–7904.

[18] Bell, P. A. and Munck, A. (1973) Biochem. J. 136, 97–107.

[19] Shyamala, G. (1974) J. Biol. Chem. 249, 2160–2163.

[20] Pratt, W. B., Kaine, J. L. and Pratt, D. V. (1975) J. Biol. Chem. 250, 4584–4591.

[21] Arányi, P. (1980) in: Hormones and Cancer (Iacobelli, S. et al. eds) pp. 217–226, Raven, New York.

[22] Spindler-Barth, M., Ulrich, J. and Gehring, U. (1982) submitted.

[23] Wolff, M. E., Baxter, J. D., Kollmann, P. A., Lee, D. L., Kuntz, I. D., Bloom, E., Matulich, D. T. and Morris, J. (1978) Biochemistry 17, 3201–3208.

[24] Sibley, C. H. and Tomkins, G. M. (1974) Cell 2, 213–220.