J. Steroid Biochem. Molec. Biol. Vol. 38, No. 6, pp. 721–725, 1991
Printed in Great Britain. All rights reserved

0960-0760/91 $3.00 + 0.00
Copyright © 1991 Pergamon Press plc

UPTAKE OF CORTICOSTERONE INTO ISOLATED RAT LIVER CELLS: POSSIBLE INVOLVEMENT OF Na^+/K^+-ATPase

KLAUS-DIETER SPINDLER,* KAZUKO KANUMA and DIETER GROSSMANN
Institut für Zoologie, Lehrstuhl für Hormon- und Entwicklungsphysiologie, Heinrich-Heine-Universität Düsseldorf, 4000 Düsseldorf, Universitätsstr. 1, Deutschland

(Received 18 July 1990; received for publication 21 February 1991)

Summary—Isolated rat hepatocytes possess a saturable glucocorticoid uptake system with high affinity (K_d value $= 2.8 \pm 0.7 \times 10^{-8}$ M; $318{,}000 \pm 80{,}000$ binding sites per cell; 317 fmol/mg protein). The initial rates of uptake decrease by about 30–40% if the cells are incubated simultaneously with [^{3}H]corticosterone and either SH-reagents (*N*-ethylmaleimide and *p*-chloromercuriphenylsulphonate, 1 mM), metabolic inhibitors (2,4-dinitrophenol, 1 mM; and antimycin, 0.1 mM) or the Na^+/K^+-ATPase-inhibitors, ouabain and quercetine. These Na^+/K^+-ATPase-blockers exert half-maximal inhibition at 3×10^{-7} and 3×10^{-6} M, respectively. A slight increase in K^+ concentration and a corresponding decrease in Na^+ in the medium leads to a significant reduction in the initial uptake rate. The uptake system from the rat hepatocytes shows a clear steroid specificity, being different from the intracellular receptor. Corticosterone and progesterone are the strongest competitors, cortisol, 5α- and 5β-dihydrocorticosterone, 11-deoxycorticosterone, cortisone and testosterone have an intermediate effect and only weak competition is exerted by dexamethasone and by the mineralocorticoid, aldosterone. Estradiol and estrone sulphate as well as the synthetic glucocorticoid triamcinolone acetonide are unable to inhibit initial corticosterone uptake.

INTRODUCTION

The mode of entry of steroid hormones into cells is still a matter of debate (for reviews see Refs [1–7]) and there are contradictory results for a given steroid hormone, as summarized by Szego and Pietras [4]. Even using isolated plasma membranes from the same organ and studying the uptake of identical steroids, controversial results have been obtained [8, 9].

In three different systems there is now increasing evidence for a carrier-mediated uptake of steroid hormones, namely the uptake of glucocorticoids into isolated cells or plasma membranes from rat liver [10–15] and hypophysis [16–18] and for the uptake of a moulting hormone in the isolated hypodermis of crayfish [19–22].

The requirement or involvement of metabolic energy for a mediated transport and uptake process of steroid hormones has been repeatedly demonstrated, using metabolic inhibitors like cyanide, 2,4-dinitrophenol or antimycin A [11–13, 20].

The transport of small molecules like sugars or amino acids across biological membranes is often coupled to a Na^+-gradient [23]. This possibility has been only very occasionally examined in steroid hormone entry. Uptake of cortisol into isolated rat liver cells was influenced neither by changing the external Na^+ concentration nor by 1 mM ouabain [11], which was also without effect on the uptake of estradiol into rat uterus [24]. On the other hand, an inhibitory effect of ouabain has been demonstrated for the uptake of ecdysteroids in crayfish hypodermis [22]. An inhibition of estrone sulfate uptake into isolated rat liver cells by about 50% was reached using 0.5 mM ouabain or total replacement of external sodium by potassium [25]. Unfortunately, in the latter study no dose–response curves have been performed; in addition, the ouabain concentration used was very high and the viability of the cells after total replacement of sodium was not studied.

One of our aims was therefore to study in more detail and under controlled conditions the influence of ouabain on corticosterone uptake in isolated rat hepatocytes.

*To whom correspondence should be addressed.

Another characteristic of a recognition site for a steroid hormone is the specificity. Since contradictory results have been obtained concerning the specificity of uptake of corticosteroids in rat liver cells or plasma membranes [10, 13, 14] we were also interested in this problem; such an approach could perhaps in addition permit discrimination between different glucocorticoid binding sites.

EXPERIMENTAL

Isolation of hepatocytes

Hepatocytes from male Wistar rats were kindly provided by Drs Kolb-Bachofen and Schlepper-Schäfer (Institut für Biophysik und Elektronenmikroskopie, Düsseldorf). The isolation procedure has been described previously [26]. Immediately after the isolation procedure the cells were gassed with 95% O_2 and 5% CO_2 with continuous shaking. The cells were counted in a Bürker chamber and viability determined by exclusion of Trypan Blue (0.1%). Experiments were performed only if >95% of the cells were intact.

Measurement of uptake of corticosterone

Incubations were performed in a HEPES-buffer (143 mM NaCl, 6.72 mM KCl, 1.22 mM $CaCl_2$, 11 mM KH_2PO_4, 7 mM Na_2SO_4, 13.2 mM $MgCl_2$ and 10 mM HEPES, pH 7.4). Cells were incubated with [^{3}H]corticosterone (8.8 nM; sp. act. 76 Ci/mmol, Amersham–Buchler, code TRK 406) alone or in the presence of a 200-fold excess of unlabelled corticosterone. Usually about 10^5 cells in 30 μl were pipetted into 250 μl of the incubation buffer. After 30 s an aliquot of 250 μl was taken and the free hormone separated from the cells by filtration on wet glassfibre-filters (Whatman GF). The filters were washed 3 times with 5 ml of HEPES-buffer. The separation procedure was checked by filtration of cells incubated with [^{3}H]corticosterone and [^{14}C]inulin simultaneously. Bound [^{3}H]corticosterone remained constant even after a 4th or 5th wash, whereas [^{14}C]inulin was no more detectable. The incubation and all subsequent steps were performed at 4°C. The radioactivity of the filters was determined and corrected for adsorption of [^{3}H]corticosterone alone, which was always <5% of the bound radioactivity. Using this standard procedure the reproducibility within one set of experiments ($n = 3$) was between 0.5 and 6.5% and there was linearity in specific uptake up to 1.6×10^5 cells/assay. The absolute uptake per cell varied greatly from preparation to preparation and reproducibility was less good ($n = 18$, SD = 34%).

RESULTS

Basic characteristics of the glucocorticoid carrier

Some of the basic characteristics of the corticosterone uptake system were reinvestigated and compared with the already published data (Table 1). Uptake of corticosterone into isolated rat hepatocytes was highly temperature-dependent, preferentially at lower temperatures ($Q_{10} = 4$, between 5 and 15°C, but $Q_{10} = 1.7$, between 25 and 35°C). There were roughly 320,000 binding sites per cell with an affinity of about 30 nM, as determined by Scatchard plot analysis.

Influence of metabolic inhibitors and SH-reagents on glucocorticoid uptake

Uptake of corticosterone was also dependent on energy and on SH-groups. This was shown by reduction of uptake by the metabolic inhibitors, 2,4-dinitrophenol and antimycin A and the SH-blocking reagents *N*-ethylmaleimide and *p*-chloromercuriphenylsulphonate (Table 2).

Table 1. Some basic characteristics of corticosterone uptake; Allera *et al.* [14] used isolated plasma membranes from rat hepatocytes, whereas Rao *et al.* [13] and we used isolated rat hepatocytes

Parameter	Present results	Rao *et al.* [13]	Allera *et al.* [14]
K_d value ($\times 10^{-8}$ M) (component with highest binding affinity)	2.8 ± 0.7 ($n = 4$)	5.6 ± 3.2 ($n = 3$)	0.7 ± 0.2 and 23.4 ± 6.7 ($n = 4$)
Concentration of binding sites (fmol/mg protein)	317 ± 80 ($n = 4$)		180 ($n = 4$)
Q_{10} value for initial uptake	4	6	Not determined

The values are means ± SD, the numbers in parentheses represent *n*.

Table 2. Inhibitory effect of metabolic inhibitors and SH-reagents on uptake of corticosterone by isolated rat hepatocytes

Reagent (molarity)	Uptake (% of control)
2,4-Dinitrophenol (1 mM)	63 ± 2
Antimycin A (0.1 mM)	74 ± 3
N-Ethylmaleimide (1 mM)	65 ± 1
P-Chloromercuriphenylsulphonate (1 mM)	67 ± 11

Initial uptake after 15 s was measured. The metabolic inhibitors were added simultaneously with the hormone. $n = 4$, means ± SD.

Involvement of Na^+/K^+-ATPase on glucocorticoid uptake

Inhibition of Na^+/K^+-ATPase by ouabain led immediately to a decrease in uptake of corticosterone. The degree of inhibition was between 30 and 75%, depending on the cell preparation. Inhibition was only measurable within a relatively short time after the isolation of the cells and was no longer seen after about 1 h. In addition, the cells showed undiminished viability with dye exclusion as the criterion. Without preincubation of the cells with ouabain, the effect of this substance was immediately obvious (Fig. 1). Ouabain inhibited corticosterone uptake in a dose-dependent way (Fig. 2) reaching maximum values at 2×10^{-6} M and half-maximal inhibition at 3×10^{-7} M. In order to prove that inhibition of corticosterone uptake by ouabain is due to inhibition of Na^+/K^+-ATPase and not to its steroidal nature, quercetine (3,3′,4′,5,7-pentahydroxy-flavone) was also tested. This Na^+/K^+-ATPase-inhibitor [27] also decreased corticosterone uptake, but was less potent (maximal inhibition at about 10^{-5}) M, half-maximal value at 3×10^{-6} M; Fig. 2). Varying Na^+/K^+-ratios also influenced corticosterone uptake. An increase of 5 mM K^+ (and a corresponding decrease of 5 mM Na^+) led to an inhibition of 36% in glucocorticoid uptake into isolated rat hepatocytes.

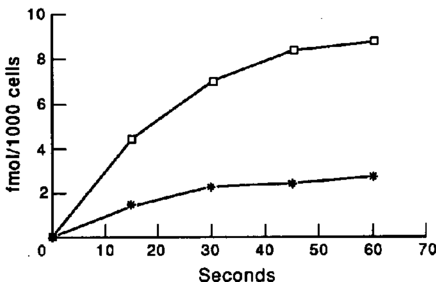

Fig. 1. The influence of ouabain (0.1 mM) on initial uptake of [³H]corticosterone into isolated rat hepatocytes. The cells were incubated with [³H]corticosterone (□) alone or in the presence of ouabain (∗). Experiments were performed in quadruplicate, SD < 6%.

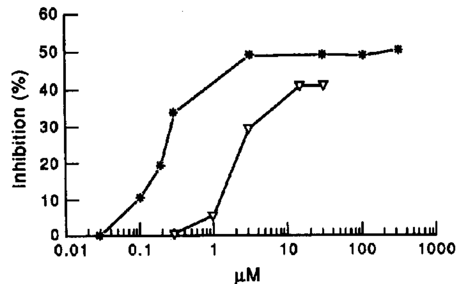

Fig. 2. Dose–response curve of the effects of ouabain (∗) and quercetine (▽) on the uptake of [³H]corticosterone, measured during the first 30 s of incubation. The cells were incubated simultaneously with the hormone and the inhibitors. The values are means of 4 independent measurements, SD < 6%.

Specificity of the corticosterone uptake system

In addition to the parameters described above, the specificity of uptake into isolated rat hepatocytes was also tested. The results of these tests are summarized in Fig. 3. Besides corticosterone, progesterone was the most effective competitor and no other steroid hormone inhibited corticosterone uptake so strongly. An intermediate degree of inhibition was also reached by some other glucocorticoids and by testosterone, whereas the mineralocorticoid aldosterone was only weakly active in this respect. Female sex hormones obviously do not interfere with the glucocorticoid uptake

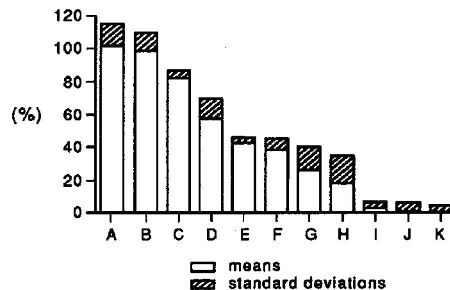

Fig. 3. The influence of various steroid hormones on the uptake of [³H]corticosterone (8.8 nM) into isolated rat hepatocytes. All hormones were applied in a 200-fold excess as compared with the radiolabelled glucocorticoid. The cells were incubated simultaneously with [³H]corticosterone and the corresponding competitors: A = corticosterone, B = progesterone, C = cortisol, D = 5α-dihydrocorti-costerone, E = testosterone, F = cortisone, G = aldosterone, H = dexamethasone, I = triamcinolone acetonid, J = estrone sulphate and K = 17β-estradiol. All experiments were performed in triplicate. The data are expressed as % competition of initial uptake; maximal competition by corticosterone is set as 100%.

system. The very potent glucocorticoids, dexamethasone and triamcinolone acetonide, which have a higher affinity for the intracellular glucocorticoid receptor than corticosterone itself, showed an extremely weak or even absent effect on corticosterone uptake, even if a 1000-fold excess of competitor was present (data not shown).

DISCUSSION

In the present investigation, the uptake of corticosterone into isolated rat hepatocytes was studied. Several features are indicative for a membrane bound recognition site for glucocorticoids, which is different from the intracellular receptor. The most pronounced differences concern the energy-requirement for binding and uptake and the steroid specificity.

Uptake of glucocorticoids into rat hepatocytes is a strongly temperature-dependent process, as documented by the Q_{10} values of 4 (this paper) or 6 [13], but it has also been described as not influenced by metabolic inhibitors [13], which is in contrast to our own results (see Table 2) and also to results of studies on the uptake of cortisol into rat hepatocytes [12]. This difference may be due to the fact that, depending on the preparation, the degree of inhibition of the initial uptake of corticosterone varied. Within one batch of cells there was great reproducibility in terms of the percentage of inhibition, but from batch to batch we found great variability. In a few preparations no effect was seen, whereas in most experiments there was an inhibition of 30–40% even without any preincubation of the cells with the corresponding inhibitor; a preincubation might slightly increase the degree of inhibition [22].

About the same level of inhibition was reached with SH-reagents (Table 2), which is in accordance with [13]. The effect of the SH-reagent *p*-chloromercuriphenylsulphonate, which does not penetrate the cell membrane within the short incubation times [28] can be completely overcome by adding an excess of dithiothreitol. The same finding has already been published for the uptake of corticosteroids into mouse hypophyseal cells [18] and also for the entry of ecdysteroids into crayfish hypodermis [20].

Ouabain is able to inhibit the uptake of corticosterone into rat hepatocytes (Figs 1 and 2) and of moulting hormones into crayfish hypodermis [22]. The negative results [11, 24] with this agent might be due to the quite obvious "instability" of the membrane-bound enzyme system. In recent experiments it has been shown that, depending on the substrate and the preparation, a dramatic decrease in the rates of uptake for certain substances can occur within rather short times of primary cultures of rat hepatocytes [29]. The possible involvement of a Na^{+}/K^{+}-ATPase in the uptake of glucocorticoids into rat hepatocytes obviously is no special feature for steroid hormones, since these cells also possess an energy- and sodium-dependent, ouabain-sensitive uptake mechanism for cholic acid [30] and for oleate [31].

The studies on the steroid specificity of the uptake clearly demonstrate that there exist glucocorticoid recognition sites of different specificity in addition to the well-known intracellular receptors. The localization of these binding sites on the outer cell membrane by electron microscopic techniques has been demonstrated recently [32]. The membrane-bound binding site preferentially binds corticosterone, progesterone and natural glucocorticoids, but not the synthetic glucocorticoids dexamethasone and triamcinolone acetonide, which is in contrast to the well known intracellular glucocorticoid receptor but which is in full agreement with findings on isolated vesicles from rat liver [5, 15] or dispersed pituitary cells [17, 33]. A comparable ligand specificity as compared to that of the hepatocyte membrane is known from glucocorticoid-binding globulins (transcortin) [34] and indeed, the pituitary cells of rats possess a transcortin-like material on cell membranes which is presumably involved in binding and uptake of glucocorticoids [17, 33]. On the other hand, the difference in ligand specificity between the membrane associated steroid recognition site and that of intracellular receptors is not only present in glucocorticoids but seems to be a general feature [6].

In summary, we have been able to add some new arguments in favour of a membrane-bound glucocorticoid binding site, though the physiological relevance of these recognition sites and their possible involvement in the expression of non-genomic steroid hormone actions is still not yet clear.

Acknowledgements—The support of these investigations by a grant from the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen is gratefully acknowledged. We also gratefully acknowledge the fruitful discussions of our results with Dr Allera (Bonn).

REFERENCES

1. Ballard P. L.: Delivery and transport of glucocorticoids to target cells. In *Glucocorticoid Hormone Action* (Edited by J. D. Baxter and G. G. Rousseau). Springer, Berlin (1979) pp. 25–48.
2. Giorgi E. P.: The transport of steroid hormones into animal cells. *Int. Rev. Cytol.* **65** (1980) 49–115.
3. Rao G. S.: Mode of entry of steroid and thyroid hormones into cells. *Molec. Cell. Endocr.* **21** (1981) 97–108.
4. Szego C. M. and Pietras R. J.: Membrane recognition and effector sites in steroid hormone action. In *Biochemical Actions of Hormones* (Edited by G. Litwack). Academic Press, New York (1981) pp. 307–463.
5. Allera A., Rao G. S. and Wildt L.: Transport von Steroidhormonen durch die Zellmembran. Ist die Zellmembran an der Wirkungsweise von Steroidhormonen beteiligt? *Aktuelle Onkologie* **31** (1986) 151–171.
6. Haukkamaa M.: Membrane-associated steroid hormone receptors. In *Steroid Hormone Receptors—Their Intracellular Localisation* (Edited by C. R. Clark). Verlag Chemie, Weinheim (1987) pp. 155–169.
7. Spindler K.-D. and Spindler-Barth M.: Uptake of ecdysteroids. In *Ecdysone—From Chemistry to Mode of Action* (Edited by J. Koolman). Thieme, Stuttgart (1989) pp. 245–249.
8. Pietras R. J. and Szego C. M.: Estrogen receptors in uterine plasma membrane. *J. Steroid Biochem.* **11** (1979) 1471–1483.
9. Muller R. E., Johnston T. C. and Wotiz H. H.: Binding of estradiol to purified uterine plasma membranes. *J. Biol. Chem.* **254** (1979) 7895–7900.
10. Suyemitsu T. and Terayama H.: Specific binding sites for natural glucocorticoids in plasma membranes of rat liver. *Endocrinology* **96** (1975) 1499–1508.
11. Rao M. L., Rao G. S., Hoeller M., Breuer H., Schattenberg P. J. and Stein W. D.: Uptake of cortisol by isolated rat liver cells—a phenomenon indicative of carrier-mediation and simple diffusion. *Hoppe-Seyler's Z. Physiol. Chem.* **357** (1976) 573–584.
12. Rao G. S. Schulze-Hagen K., Rao M. L. and Breuer H.: Kinetics of steroid transport through cell membranes: comparison of the uptake of cortisol by isolated rat liver cells with binding of cortisol to rat liver cytosol. *J. Steroid Biochem* **7** (1976) 1123–1129.
13. Rao M. L., Rao G. S., Eckel J. and Breuer H.: Factors involved in the uptake of corticosterone by rat liver cells. *Biochim. Biophys. Acta* **500** (1977) 322–332.
14. Allera A., Rao G. S. and Breuer H.: Specific interaction of corticosteroids with components of the cell membrane which are involved in the translocation of the hormone into the intravesicular space of purified rat liver plasma membrane vesicles. *J. Steroid Biochem.* **12** (1980) 259–266.
15. Allera A. and Rao G. S.: Characteristics and specificity of the glucocorticoid "carrier" of rat liver plasma membrane. In *Steroid Hormone Resistance* (Edited by G. P. Chrousos, D. Lynn Loriaux and M. B. Lipsett). Plenum Press, New York (1986) pp. 53–65.
16. Harrison R. W., Fairfield S. and Orth D. N.: Evidence for glucocorticoid transport into AtT-20/D-1 cells. *Biochemistry* **14** (1975) 1304–1307.
17. Koch B., Lutz-Bucher B., Briaud B. and Mialhe C.: Specific interaction of corticosteroids with binding sites in the plasma membranes of the rat anterior pituitary gland. *J.Endocr.* **79** (1978) 215–222.
18. Harrison R. W. and Yeakley J.: Evidence for glucocorticoid transport into AtT-20 cells. Selective effect of a sulfhydryl inhibitor on uptake. *Molec. Cell. Endocr.* **15** (1979) 13–18.
19. Daig K. and Spindler K.-D.: Uptake and retention of moulting hormones by the integument of crayfishes *in vitro*. II. Influence of metabolic inhibitors and sulfhydryl-group inhibitors. *Molec. Cell. Endocr.* **32** (1983) 73–79.
20. Daig K. and Spindler K.-D.: Uptake and retention of moulting hormones by the integument of crayfishes *in vitro*: I. Influence of temperature, hormone concentration and hormone structure. *Molec. Cell. Endocr.* **31** (1983) 93–104.
21. Spindler K.-D., Dinan L. and Londershausen M.: On the mode of action of ecdysteroids in crustaceans. In *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones* (Edited by J. Hoffmann and M. Porchet). Springer, Berlin (1984) pp. 255–264.
22. Spindler K.-D. and Grossmann D.: Uptake and retention of moulting hormones by the integument of crayfishes *in vitro*. III: The possible involvement of Na^+/K^+-ATPase. *Molec. Cell. Endocr.* **52** (1987) 81.
23. Hoefer M.: *Transport Across Biological Membranes.* Pitman, London (1981) p. 184.
24. Milgrom E., Atger M. and Baulieu E.-E.: Studies of estrogen entry into uterine cells and on estradiol-receptor complex attachment to the nucleus—is the entry of estrogen into uterine cells a protein-mediated process? *Biochim. Biophys. Acta* **320** (1973) 267–283.
25. Schwenk M. and Lopez del Pino V.: Uptake of estrone sulfate by isolated rat liver cells. *J. Steroid Biochem.* **13** (1980) 669–673.
26. Kolb H., Schudt C., Kolb-Bachofen V. and Kolb H. A.: Cellular recognition by rat liver cells of neuraminidase-treated erythrocytes. *Expl Cell Res.* **113** (1978) 319–325.
27. Linnett P. E. and Bechey R. B.: Inhibitors of ATP synthetase system. *Meth. Enzym.* **LV** (1979) 472–518.
28. Vansteveninck J., Weed R. I. and Rothstein A.: Localization of erythrocyte membrane sulfhydryl groups essential for glucose transport. *J. Gen. Physiol.* **48** (1965) 617–632.
29. Kukongviriyapan V. and Stacey N. H.: Comparison of uptake kinetics in freshly isolated suspensions and short-term primary cultures of rat hepatocytes. *J. Cell. Physiol.* **140** (1989) 491–497.
30. Ohkuma S., Tamura J. and Kuriyama K.: Characteristics of cholic acid uptake in primary cultured hepatocytes. *Steroids* **42** (1983) 205–215.
31. Stremmel W., Strohmeyer G. and Berk P. D.: Hepatocellular uptake of oleate is energy dependent, sodium linked, and inhibited by an antibody to a hepatocyte plasma membrane fatty acid binding protein. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 3584–3588.
32. Krahwinkel R., Kanuma K., Grossmann D. and Spindler K.-D.: Demonstration of glucocorticoid recognition sites on isolated rat hepatocytes. *J. Steroid Biochem.* **25** (1986) 300.
33. Koch B., Sakly M. and Lutz-Bucher B.: Modulation by transcortin-like binding sites of uptake and distribution of glucocorticoids by dispersed pituitary cells. *Molec. Cell. Endocr.* **22** (1981) 169–178.
34. Westphal U.: *Steroid–Protein Interactions II.* Springer, Berlin (1986) p. 604.