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ELECTRON MICROSCOPIC DEMONSTRATION OF GLUCOCORTICOID RECOGNITION SITES ON ISOLATED RAT HEPATOCYTES

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Summary—Ultrastructural evidence is presented for the presence of membrane-bound glucocorticoid recognition and binding sites. Corticosterone was derivatized at 3 different positions and coupled covalently to bovine serum albumin (BSA). All three derivatives competed for binding of [^{3}H]corticosterone by isolated rat hepatocytes. The most effective competitor, corticosterone–succinate–BSA (CSB), was adsorbed onto colloidal gold particles (CSB-gold, 17 ± 3 nm dia). When isolated rat hepatocytes or mouse pituitary tumor cells (AtT 20) are incubated with CSB-gold, specific binding in the microvilli-rich region of these cells is seen. This binding of CSB-gold is reduced by about 50% in the presence of unlabelled CSB or corticosterone.

INTRODUCTION

Target tissues and cells for all typical vertebrate as well as arthropod steroid hormones are characterized by the presence of intracellular receptors [1–3]. Although there is some debate about the localization, the mechanism of activation, transformation and translocation of the receptor [4, 5], there is quite clear evidence for the involvement of the receptor in regulation of gene expression. In addition, specific binding sites for steroid hormone receptors on steroid hormone regulated genes have been described [6–8].

On the other hand there is accumulating evidence for non-genomic steroid hormone effects, which are not mediated by intracellular receptors (summarized in [9]).

The plasma membrane seems to be an especially good candidate for such non-genomic steroid hormone effects, and several reviews have dealt with steroid–hormone plasma membrane interactions [10–13]. In addition, studies on the mode of entry of steroid hormones into cells have also contributed to this question, both in vertebrates [12, 14–17] and in arthropods [18]. From biophysical considerations, Alfsen [19] in a recent review concluded that "in the living cell, membranes constitute the ideal medium for reception, selection and transmission of a starting signal for many biological functions such as those of steroid hormones".

Nevertheless, a cytological demonstration of steroid hormone recognition sites on the plasma membrane has been rarely performed [20–22] and an ultrastructural localization of steroid hormone binding sites on the plasma membrane is not yet available. It was therefore the aim of our investigation to fill this gap, using isolated rat hepatocytes and corticosterone as a model system, where specific uptake has already been demonstrated [16, 23–26]. For comparison we also used a pituitary tumor cell line from the mouse (AtT 20), where specific glucocorticoid transport has also been demonstrated [27, 28]. In these studies binding and transport (uptake) of the steroid hormone were measured. Because of the high velocity of the processes, these two events could not be separated by the techniques used. In order to prevent transport and to study only the presumed membrane-bound glucocorticoid binding sites colloidal gold was used as an electron dense marker.

EXPERIMENTAL

Livers of male Wistar rats weighing 150–180 g were perfused with buffered collagenase-solution (Boehringer-Mannheim, Fed. Rep. Germany),

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and hepatocytes were isolated as described previously [29].

Preparation of corticosterone derivatives and coupling to bovine serum albumin (BSA)

Corticosterone–oxime was prepared [30] and purified by HPLC (reversed-phase, Bondapak C-18, eluant: bidistilled water to methanol = 6:4; 1.5 ml/min; 1800 psi). The yield was 60 to 70%. The oxime was then coupled to BSA [30]. The ratio of corticosterone to BSA was 10:1, as determined by the use of [^{3}H]corticosterone–oxime. The corticosterone-acid was synthesized [31] and kindly provided by Dr Spindler-Barth (Zoologisches Institut, Düsseldorf, Fed. Rep. Germany). The structure was confirmed by GC-MS analysis, kindly performed by Dr Mathiesen (Institut für Physiologische Chemie II, Düsseldorf, Fed. Rep. Germany). This derivative was coupled to BSA according to [32]. The ratio of corticosterone/BSA was 9:1, as determined by the use of [^{3}H]corticosterone-acid. Corticosterone–succinate–BSA was from Sigma (Deisenhofen, Fed. Rep. Germany), the degree of coupling for this compound was 24 molecules of corticosterone per molecule BSA. The BSA used was essentially fatty acid-free (Sigma).

Uptake of [^{3}H]corticosterone into isolated rat hepatocytes

Each batch of cells was first tested for specific uptake of corticosterone. Hepatocytes (10^5) were incubated for 30 s at 4°C in 8.8 nM [^{3}H]corticosterone alone or in the presence of a 200 fold excess of corticosterone in 300 μl of a Hepes-buffer (120 nM, pH 7.4. In a 250 μl aliquot bound and free hormone were separated by filtration on wet glassfibre filters (Whatman GF/C); the filters were then washed 3 times with 5 ml of Hepes-buffer and the radioactivity on the filters counted. The reliability of this procedure was demonstrated in a previous paper [26]. In all subsequent experiments cells were first incubated with BSA for 5 min. A final concentration of BSA of 2.5×10^{-7}M already reduces the uptake by about 50%. An increase in BSA concentration up to 5×10^{-5}M ml does not further reduce uptake. The influence of the different corticosterone–BSA-derivatives was tested as follows: the cells were first preincubated with BSA (2.5×10^{-7}M) for 5 min at 4°C, then preincubated with varying concentrations of the corticosterone–BSA-derivatives for 10 min and then initial uptake (30 s) of [^{3}H]corticosterone (8.8 nM) was measured.

Preparation of the electron-dense ligand

Colloidal gold granules of 17 nm were prepared according to [33]. The size and size distribution were determined by electron microscopy (17 ± 3 nm). BSA and corticosterone–BSA complexes were adsorbed onto gold particles by incubating the gold sol with protein (44 to 57 μg/ml gold sol) at a pH of 6.8 to 7.0 (phosphate-buffered saline) in the presence of a 0.02% polyethylene glycol (M_w 20,000) for 30 min at 4°C. Stabilization of the protein–gold complexes was scored visually after addition of NaCl to aliquots of the incubation mixture. The protein–gold complexes were washed twice by centrifugation (24,000**g**, for 30 min, at 4°C) and finally suspended in phosphate-buffered saline containing 0.05% polyethylene glycol and penicillin (100 IU/l) and streptomycin (100 mg/l). The concentrated stock solution contained about 10^{11} gold particles/ml.

Electron microscopy of ligand binding

Cells, 4×10^5 (in 100 μl) were first preincubated with BSA (2.5×10^{-7}M) for 5 min, followed by an incubation with either Hepes-buffer or corticosterone–BSA-complex in Hepes-buffer as competitor for 10 min and then incubated with the protein–gold complex for 10 min at 4°C. In all experiments the incubation was stopped by the addition of the same volume of ice-cold 0.2% glutaraldehyde in *S*-collidine-buffer (0.1 M *S*-collidine, 0.1 M saccharose, 0.1 M NaCl and 2 mM $CaCl_2$, pH 7.4). Cells were immediately spun down to 40**g** and resuspended in Hepes-buffer. The cell pellet was fixed for 10 min with 0.1% glutaraldehyde, washed twice in buffer, fixed for 40 min in 2% OsO_4 in the *S*-collidine-buffer at 4°C and finally washed twice with *S*-collidine-buffer. The samples were dehydrated with ethanol and embedded in epoxy resin [34]. Sections of about 100 nm thickness were contrasted with ethanolic uranylacetate for 10 min and with lead citrate for 10 min [35].

Estimation of the number of particles bound on hepatocytes

The mean diameter of the embedded hepatocytes was 21 ± 4 μm ($n = 27$). Random sections of embedded material were examined in the electron microscope. Only sections through the nuclear equator (both nuclear membranes clearly discernible) were used for the determi-

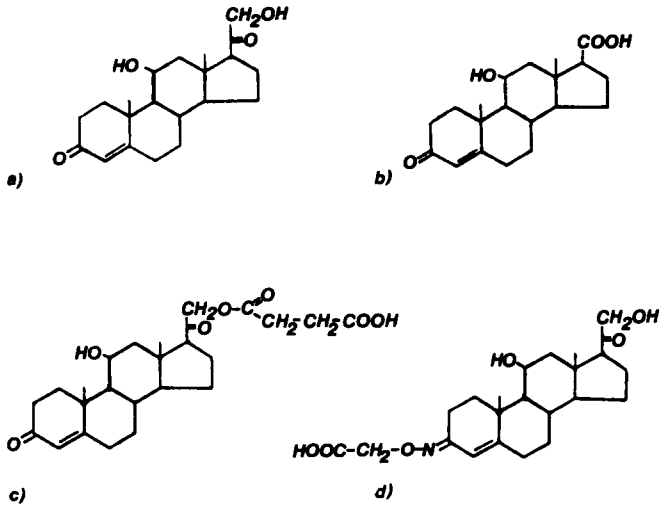

Fig. 1. Derivatives of corticosterone (a) used for the coupling to BSA. (b) = corticosteronic-acid, (c) = corticosterone–succinate and (d) = corticosterone–oxime.

nation of the particles bound to the plasma membrane. In addition to a determination of gold particles per section a determination of the number of gold particles per μm cell membrane was performed. For the quantitative analyses various sections from one experimental set were used. Variability between different experimental sets and embeddings were especially pronounced with the AtT 20 cells (0.3 up to 1.1 gold particles per μm cell membrane.

RESULTS

Since uptake of glucocorticoids in isolated rat liver cells [23, 36] or AtT 20 cells [27, 28] is a very rapid process being much faster than fixation for electron microscopical purposes, we first had to covalently couple corticosteroids to a macromolecule which would not enter the cell in such a short time to study the initial recognition event. Three different derivatives of corticosterone were synthesized (Fig. 1) and coupled to BSA. The binding and uptake of [^{3}H]corticosterone by isolated rat hepatocytes was then studied using these three corticosteroid–BSA-conjugates as competitors. As seen in Fig. 2 corticosterone–succinate–BSA was most effective in competing for the glucocorticoid recognition site. Modifying the corticosterone-molecule at the keto-group in the A-ring (oxime) or removing the side chain (corticosterone-acid) strongly reduces the effectiveness of these derivatives as competitors. For the electron microscopic demonstration of glucocorticoid binding sites corticosterone–succinate–BSA was therefore chosen as the ligand. Figure 3 schematically shows the strategy for this ultrastructural localization. Using this approach binding of the corticosterone–BSA-gold particles to microvilli of the hepatocytes can be demonstrated (Fig. 4). The gold particles preferentially bind to regions with microvilli (sinusoidal surface) and there is only very occasional binding at the smooth surfaced (lateral) membrane regions. BSA–gold particles show only background binding to hepatocytes (Table 1) and only 5 granules per section were detected, randomly distributed on the cell surface. If hepatocytes were preincubated with corticosterone–succinate–BSA the number of gold

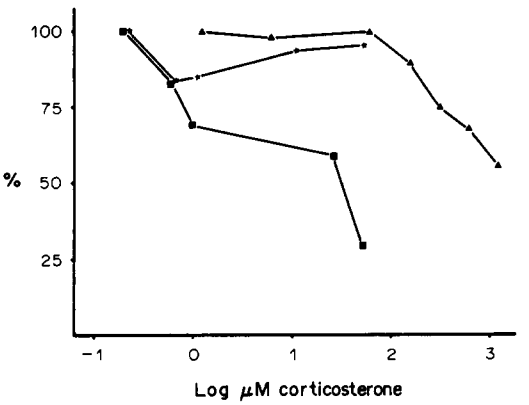

Fig. 2. Specificity of the corticosterone binding sites on the membrane of rat hepatocytes as demonstrated by the use of corticosterone-derivatives (see Fig. 1) coupled covalently to BSA. 10^5 cells/assay were incubated at 4°C for 5 min with BSA (2.5×10^{-7}M), followed by an incubation with corticosterone–succinate–BSA (■), corticosterone–oxime–BSA (▲) or corticosterone-acid–BSA (★) for 10 min. Initial uptake of [^{3}H]corticosterone (8.8 nM) was then measured (30 s) and the values expressed as % of the control (no competitor).

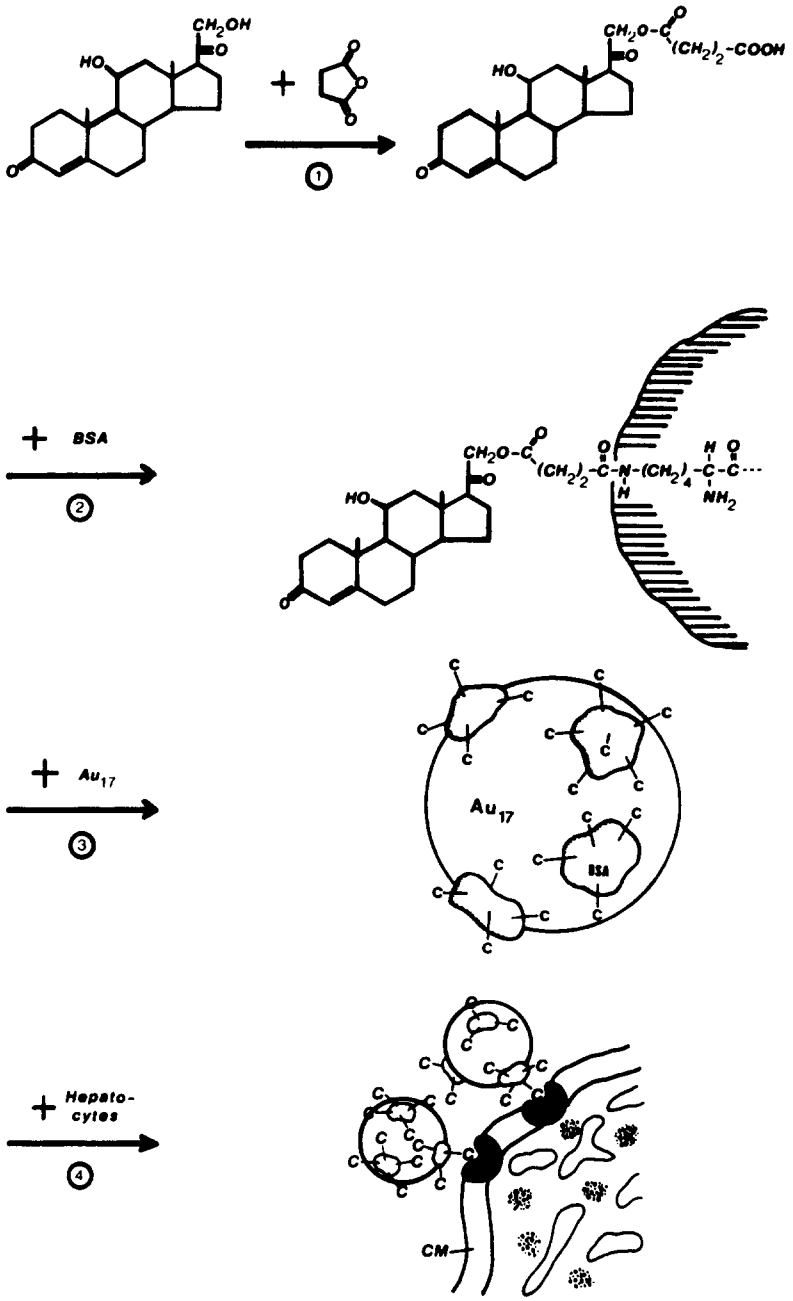

Fig. 3. Strategy for the electron microscopic demonstration of specific glucocorticoid binding sites on rat hepatocytes. (1) Derivatization of corticosterone to corticosterone–succinate; (2) coupling of this derivative to BSA (molar ratio = 24); (3) adsorption of the corticosterone–BSA-complex to colloidal gold (Au_{17}; approx. 60–70 molecules BSA/Au_{17}); and (4) incubation of rat hepatocytes with the corticosterone–BSA–gold particles. In the compeititon assay cells were preincubated with the corticosterone–BSA-complex. The sizes do not reflect the real size relationships. CM = cell membrane (glucocorticoid binding site).

granules is reduced (Fig. 4). The reduction of gold granules per section from 693 ± 346 to 366 ± 206 is statistically significant ($P < 0.01$), and accounts for 47% of binding (Table 1). From the number of gold granules per section, the thickness of the sections (100 nm), the radius (10.6 μm) and the length of plasma membrane (cell perimeter) the total number of gold granules per cell can be roughly calculated. Assuming the cells to be spherical and assuming further that one gold granule represents one glucocorticoid binding site, 72,000 specific binding sites per hepatocyte were calculated ($n = 28$).

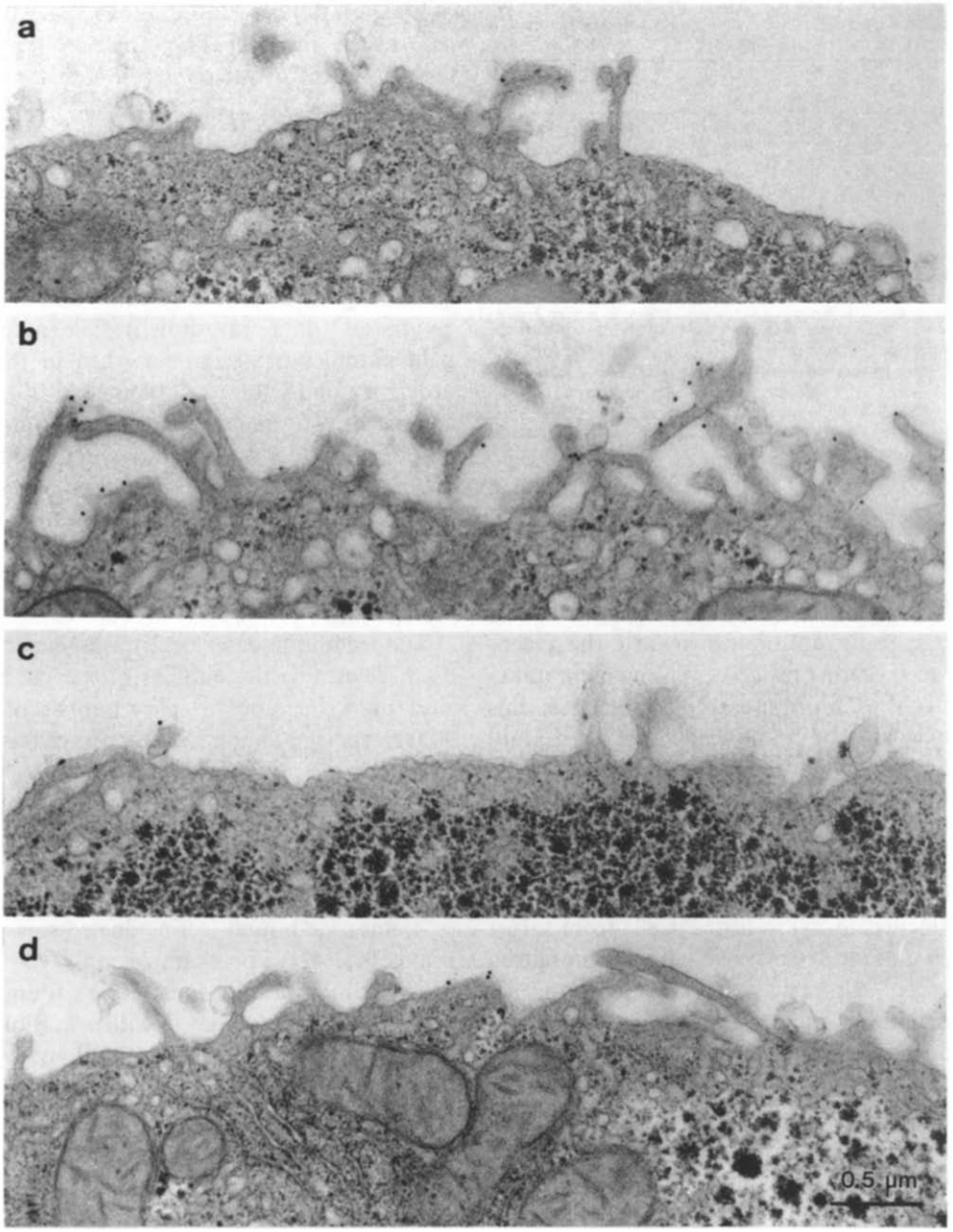

Fig. 4. Electron micrographs of isolated hepatocytes after incubation with colloidal gold-labelled ligand (Au_{17}). (a) Binding of the BSA–Au_{17} complex was minimal; (b) binding of the CSB–Au_{17} complex occurs all over the plasma membrane of the former space of Disse, on microvilli as well as on non-villous regions; (c) only background binding is seen at the smooth surfaced lateral region of hepatocytes; and (d) CSB–Au_{17} binding is blocked by the presence of excess CSB in the incubation mixture. Magnification of all micrographs: 25,600 × .

Using the same strategy but the tumorous pituitary cell line AtT 20 from the mouse we were also able to demonstrate specific membrane-bound glucocorticoid binding sites. The density of such binding sites is lower than with hepatocytes, but the degree of unspecific binding by BSA–gold particles is nearly the same. This also holds true for the degree of inhibition (52%), which not only can be achieved by corticosterone–succinate–BSA, but also by corticosterone (Table 1).

DISCUSSION

Colloidal gold granules used as a marker for cell surface receptors have already been used in 1975 by Horisberger [37] and since then applied successfully for ultrastructural localization of

Table 1. Quantitative determination of specific glucocorticoid recognition sites on rat hepatocytes (A) and mouse pituitary tumor cells AtT 20 (B)

Ligand	Competitor	n	Gold particles/μm cell membrane
(A)			
BSA–Au_{17}	BSA	10	0.07 ± 0.04
CSB–Au_{17}	BSA	28	10.40 ± 5.20
CSB–Au_{17}	BSA + CSB	29	5.50 ± 3.10
(B)			
BSA–Au_{17}	BSA	10	0.09 ± 0.08
CSB–Au_{17}	BSA	20	0.89 ± 0.54
CSB–Au_{17}	BSA + C	18	0.43 ± 0.26

Cells were incubated either with BSA–gold sol (BSA–Au_{17}) for the determination of unspecific adsorption of protein or with corticosterone–succinate–BSA–gold sol (CSB–Au_{17}). Competitors (BSA or CSB, corticosterone = C) were added at a 1000 fold excess. The differences between lines 1 and 2 and 2 and 3 are significant ($P < 0.01$). n = number of sections used for the determination.

proteins, carbohydrates, lectins and antibodies [37, 38]. In a recent paper [39] this technique has also been used for studies on the localization of intracellular steroid hormone receptors, using antibodies against the receptors. For a demonstration of plasma membrane-bound steroid hormone recognition sites this technique has not been applied to date. Until now cytological localization of such binding sites has been demonstrated by light-microscopic techniques. Pietras and Szego [20] elegantly demonstrated such binding sites using estradiol, covalently coupled to albumin and then attached to nylon fibres. Cells from target tissue bound to such covalently immobilized hormone in contrast to cells from non-target tissues. Binding of the cells was temperature-dependent. The same technique has also been applied to demonstrate estradiol binding sites on the cell membrane of hepatocytes [21]. Nenci *et al.* [22] used fluorescent-labelled estradiol–BSA conjugates and isolated breast cancer cells, which were able to bind these conjugates specifically. The advantage of these two methods is their usefulness for kinetic studies and the fact that there are quick and obvious results. On the other hand, an ultrastructural localization is impossible and quantification is at least very difficult. Furthermore the possibility cannot be excluded that the steroid ligands are internalized by the cell and subsequently bind to intracellular receptors.

Our results clearly demonstrate that glucocorticoid binding sites are indeed present on the outer surface of two target tissues and that binding is not due to intracellular receptors. In addition, we were able to localize these binding sites on one of the three membrane domains of the hepatocyte only [40] namely the microvilli-rich region that *in situ* forms the space of Disse next to the sinusoids and which is the physiologically relevant site of steroid hormone transport into the cell.

The importance of the site of derivatization of the ligand could also be demonstrated. If the corticosterone is modified in position 3 (as an oxime) this derivative is a weak competitor and if corticosterone–oxime–BSA is adsorbed by colloidal gold there is no specific binding of the complexes (data not shown). The number of gold granules per section is then in the same order as found for the binding of BSA–gold-complexes. The same conclusions about specificity of glucocorticoid binding sites and a clear distinction from membrane-bound estrogen binding sites can also be drawn by studying the initial kinetics of steroid hormone uptake by rat hepatocytes [26] or isolated membranes from rat liver [25].

The technique described in this paper allows us to determine the number of specific binding sites on a hepatocyte. The number of about 70,000 specific binding sites per cell is much lower than the 318,000 binding sites determined by kinetical studies using unmodified corticosterone [26]. This is not surprising, since with the colloidal gold technique lower values have been determined for example in determinations of membrane-bound lectins due to sterical hindrance [41, 42]. For example, only 10–15% of the lectin binding sites were found with gold–lectin compared with radiolabelled lectin [41]. In addition, we do not know whether the assumption on the one to one ratio of gold granules to binding sites is valid. Considering the size and the amount of hormone per gold granule makes it highly probable that one CSB–Au_{17} is bound by more than one receptor. An underestimate of binding sites due to internalization is according to our results not likely for the hepatocytes.

In addition to the "classical" intracellular glucocorticoid receptor the presence of binding sites on the surface of rat hepatocytes and mouse pituitary AtT 20 cells has been demonstrated unequivocally, but the physiological role of these additional binding sites still has to be elucidated. These binding sites may represent either glucocorticoid carriers or membrane-bound receptors being involved in non-genomic steroid hormone effects or both.

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