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TRANSFORMATION AND NUCLEAR TRANSLOCATION OF BRAIN TYPE I CORTICOSTEROID RECEPTORS COMPLEXED WITH THE MINERALOCORTICOID ANTAGONIST ZK 91587, ALDOSTERONE OR DEXAMETHASONE

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Summary—Type I corticosteroid receptors were determined in cytosol from hippocampus (HIPPO) and amygdala (AMYG), using [^{3}H]aldosterone (ALDO), [^{3}H]dexamethasone (DEX) or the mineralocorticoid antagonist [^{3}H]ZK 91587 as ligands. Incubations with the first two compounds also contained the pure glucocorticoid RU 28362 to block type II receptors. Binding of the three ligands was comparable in cytosol from HIPPO and it was slightly higher for [^{3}H]DEX in AMYG. However, after heat-induced receptor transformation, binding to DNA-cellulose was observed for [^{3}H]ALDO-receptor complex obtained from HIPPO or AMYG, whereas it was negligible for [^{3}H]ZK 91587. Receptors charged with [^{3}H]DEX or [^{3}H]ALDO showed similar retention on DNA-cellulose columns in the case of the AMYG, while binding to the polynucleotide was higher for [^{3}H]ALDO in the HIPPO. Finally, only [^{3}H]ALDO was taken up to a significant extent in purified cell nuclei prepared from slices of HIPPO and AMYG previously incubated with the three ligands. It is concluded that binding of a natural agonist steroid may be a prerequisite for type I receptor transformation and translocation from the cytoplasm into the nuclear fraction. DEX binding to type I receptors resembles a partial agonist with antagonist properties, whereas antagonists such as ZK 91587 are bound and retained in cytoplasm, without further translocation.

INTRODUCTION

It has been shown that the central nervous system (CNS) presents heterogeneity in receptors for adrenal corticosteroids [1]. In the case of the rat brain, two receptor systems for adrenal corticosteroids have been described, which differ in their regional localization as well as in steroid specificity [2, 3]. Type I receptor binds *in vitro* aldosterone (ALDO), corticosterone and dexamethasone (DEX) with high affinity [2, 4, 5] and localizes predominantly in the hippocampus (HIPPO), lateral septum and amygdaloid regions [6]. Type II receptor shows a widespread localization in the CNS, and displays high affinity for DEX, lower affinity for corticosterone (CORT) and still lower for ALDO [7].

Studies of these receptor types were carried out using natural or synthetic ligands. Among them, we and others have used ZK 91587, which is highly specific for type I sites [8, 9] and is a potent antagonist of mineralocorticoid action in peripheral tissues such as the kidney [10]. Three mechanisms have been proposed for antagonism of steroid action: first stabilization by the antagonist of a cytoplasmic form of the receptor impairing the nuclear translocation step; second, translocation of the antagonist-receptor complex to the nucleus with binding to non-specific DNA sites only; and third, binding of the complex to specific DNA, but without interaction with transcription factors needed for the expression of hormone action [11, 12].

We have reported the binding properties of [^{3}H]ZK 91587 in brain regions, concluding that it constitutes a useful ligand for putative mineralocorticoid receptors, due to its high affinity, stability and lack of cross reactivity with type II receptors [9]. Presently, we have studied the transformation of the [^{3}H]ZK 91587-receptor

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complex by measuring its binding to DNA-cellulose and its capacity for nuclear translocation *in vitro*. Furthermore, these properties were compared with those yielded with type I receptors labeled with the natural mineralocorticoid [^{3}H]ALDO and with [^{3}H]DEX, as the latter also shows high affinity for type I receptors *in vitro* [13, 14].

EXPERIMENTAL

Experimental animals

Male Sprague–Dawley rats (250–300 g) were bilaterally adrenalectomized under ether anesthesia. They were given 0.9% NaCl as drinking fluid for 3–5 days and Purina rat chow *ad libitum*. The day of the experiment, the animals were anesthetized with ether and perfused intracardially with 30 ml 10% (v/v) dimethylsulfoxide, a cryprotector used for assaying receptors in frozen tissues [15]. Brains were removed and kept at −70°C until used. The HIPPO and the amygdala (AMYG) were dissected out as described previously [6] and used for binding assays.

Cytosol binding assay for type I receptors

The macrodissected brain regions were homogenized in TEMG + MO buffer (10 mM Tris pH 7.4, 1.5 mM EDTA, 2 mM mercaptoethanol, 10% glycerol, 20 mM sodium molybdate) and centrifuged at 105,000 ***g*** during 60 min at 0–4°C. Aliquots of the supernatant (500 μl) were incubated with 5 nM [^{3}H]ZK 91587, [^{3}H]ALDO or [^{3}H]DEX, in the presence or absence of 1000-fold excess of non-radioactive ligand. In this and all subsequent incubations performed with [^{3}H]ALDO and [^{3}H]DEX, we added 1 μM of the glucocorticoid agonist RU 28362, to block binding to type II receptors [5]. This step was unnecessary for [^{3}H]ZK 91587, which does not cross react with type II receptors [9]. After 20 h of incubation at 0–4°C, bound and free hormones were separated on minicolumns of Sephadex LH 20. Radioactivity of the eluates, containing the bound fraction, was determined by liquid scintillation spectrometry, and results of specific binding were expressed in fmol bound per mg protein. Proteins were measured according to the method of Lowry *et al.* [16].

Nuclear uptake of [^{3}H]steroids in vitro

A modification of the method of De Kloet *et al.* [17] was used. Slices were prepared from HIPPO and AMYG regions, and placed into 1 ml Krebs-Ringer phosphate (KRP) buffer at pH 7.4. After a 5 min preincubation at 25°C, the buffer was discarded and new buffer containing 1 μCi [^{3}H]ZK 91587, [^{3}H]ALDO or [^{3}H]DEX was added. Incubation continued for 60 min at 25°C, with parallel incubations containing a 1000-fold excess of unlabeled ligand. After incubation, the tissues were washed twice with KRP and centrifuged at 800 g for 10 min. Purified cell nuclei were obtained from incubated slices according to Roy and McEwen [18]. Tissues were homogenized in 1.7 ml NI (0.32 M sucrose, 3 mM $MgCl_2$, 1 mM KH_2PO_4, 0.25% Triton X100, 10% glycerol, pH 6.5) and centrifuged at 800 ***g*** for 10 min; radioactivity was determined in aliquots of the supernatant. The precipitate was resuspended in 1 ml NII (NI without Triton X100), centrifuged as above, and washed twice in the same medium. Purified nuclei were finally suspended in 0.2 ml NII to which 1.1 ml NIII was added (2.39 M sucrose, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 10% glycerol). The tubes were vigorously mixed and centrifuged at 9000 ***g*** for 45 min at 0–4°C. The pellet was suspended in 1 ml pure ethanol and left overnight at room temperature to extract nuclear-bound radioactivity, and results were expressed as fmol [^{3}H]steroid extracted from the nuclear fraction per mg DNA.

Receptor transformation and DNA-cellulose binding

Cytosolic receptors bound to the radioactive ligands were transformed by heating at 23°C for 20 min [19]. Aliquots of the transformed receptors and control cytosols maintained at 0–4°C in the presence of 20 mM sodium molybdate, were layered on top of minicolumns containing 0.1 g DNA-cellulose suspended in TEMG buffer (without sodium molybdate) additioned of 200 μg/ml bovine serum albumin (BSA) and 10 mM NaCl [20]. After washing, DNA-bound receptors were eluted with TEMG containing 300 mM NaCl, and the radioactivity of the eluates was determined and converted into fmol [^{3}H]steroid bound per mg protein of the original cytosol sample.

Statistical analysis

Results represent the mean ± SE. Binding of tritiated steroids in the presence or absence of sodium molybdate was compared by Student's "*t*" test. One way ANOVA followed by *post hoc* comparison with the Newman-Keuls test was used to evaluate differences in binding of the

three ligands in cytosols, purified nuclei and DNA-cellulose columns.

Materials

[1,2,6,7-^{3}H]ALDO (sp. act. 80 Ci/mmol), (7α-methoxycarbonyl-15β,16β-methylene-3-oxo-17αpregn-4-ene-21,17 carbolactone) or [^{3}H]ZK 91587 (sp.act. 70 Ci/mmol), and [^{3}H]DEX (sp.act. 45.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). RU 28362 was the kind gift of Dr D. Philibert (Roussel-Uclaf, France), Non-radioactive steroids and reagents were purchased from Sigma (St Louis, MO, U.S.A.).

RESULTS

Binding of [^{3}H]ALDO, [^{3}H]ZK 91587 and [^{3}H]DEX to type I receptors was similar in cytosol of HIPPO incubated in the presence or absence of sodium molybdate, as shown in the upper graph of Fig. 1. However, differences were obtained with these ligands when binding to DNA-cellulose was assessed, as shown in Fig. 1 (lower graph). Thus, the percent of [^{3}H]ALDO-transformed receptor bound to DNA was higher ($P < 0.01$) than that of the non-transformed receptor. In contrast, receptors bound to [^{3}H]ZK 91587 were not retained by DNA-cellulose. In the case of [^{3}H]DEX, a slight increment in binding to DNA was observed after heat-induced transformation, which did not reach statistical significance.

Figure 2 (upper graph) shows that the AMYG binding to type I receptors was lower in comparison to the HIPPO. Binding of the three ligands in the absence of sodium molybdate revealed some differences; it was higher for [^{3}H]DEX ($P < 0.01$) than for [^{3}H]ALDO or [^{3}H]ZK 91587, both of which exhibited similar binding levels. In the presence of sodium molybdate, i.e. non-transforming conditions, values for the three ligands were not statistically different (Fig. 2, upper graph). The behavior of [^{3}H]ALDO and [^{3}H]ZK 91587 receptor complexes on DNA-cellulose columns partially resembled the studies described for the HIPPO, in that only [^{3}H]ALDO showed affinity towards DNA-cellulose after receptor transformation (Fig. 2, lower graph). However, while in the AMYG receptors bound to [^{3}H]ALDO and [^{3}H]DEX showed similar retention on DNA-cellulose columns, binding to the polynucleotide was higher for [^{3}H]ALDO than for [^{3}H]DEX in the HIPPO.

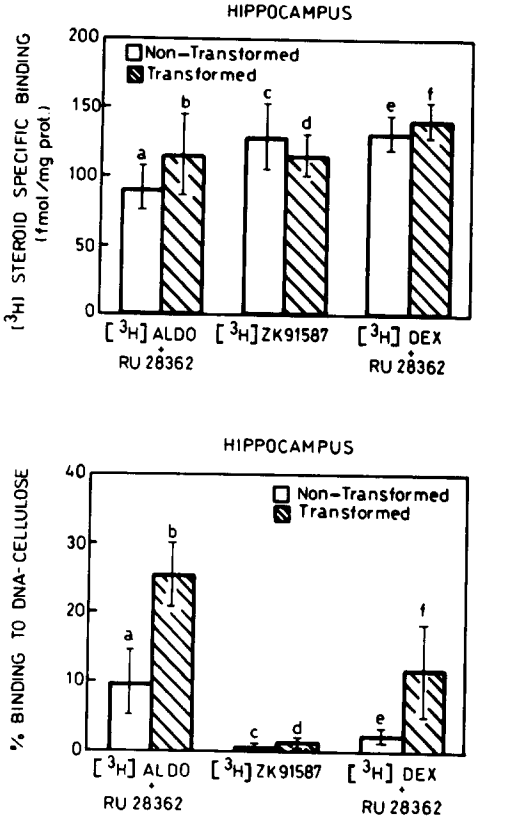

Fig. 1. *Upper graph*: binding of [^{3}H]ALDO, [^{3}H]ZK 91587 and [^{3}H]DEX to type I receptors in cytosol of HIPPO. Incubations with [^{3}H]ALDO and [^{3}H]DEX contained 1 μM RU 28362 to block binding to type II receptors. Open columns represent incubations under non-transforming conditions (i.e. 4°C in the presence of sodium molybdate), whereas hatched columns are incubations without sodium molybdate (heat-transformed receptors). *Lower graph*: percent binding to DNA-cellulose of cytosolic type I receptor complex from HIPPO obtained under transforming (hatched columns) or non-transforming conditions (open columns). Results in both graphs represent the mean ± SE of $n = 3$ experiments. Statistical analysis demonstrated that $a < b$ ($P < 0.05$), whereas c vs d and e vs f were not significantly different.

Figure 3 shows results of *in vitro* translocation experiments in which tissue slices were incubated with the tritiated ligands, and radioactive steroid in the cell homogenate or retained by purified cell nuclei was measured. The results with [^{3}H]ZK 91587 demonstrated a substantial amount of radioactivity in cell homogenates prepared from the HIPPO (upper graph) as well as the AMYG (lower graph). However, little radioactivity was present in the purified nuclear fraction after incubation with the mineralocorticoid antagonist or with [^{3}H]DEX. On the contrary, [^{3}H]ALDO was retained to a significant extent in purified cell nuclei from both regions, suggesting that binding of a natural mineralocorticoid agonist steroid may be a

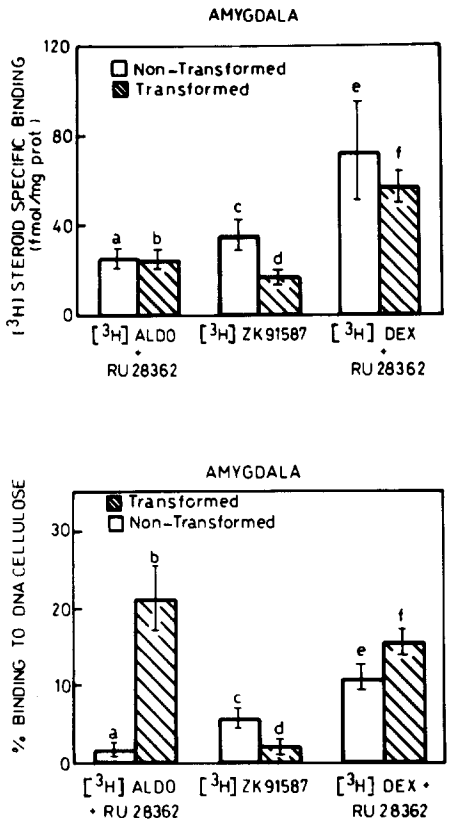

Fig. 2. *Upper graph*: binding of [^{3}H]ALDO, [^{3}H]ZK 91587 and [^{3}H]DEX in cytosol of AMYG under conditions specified in the legend to Fig. 1. Statistical analysis demonstrated that a, c and e were not significantly different, whereas f > b and d ($P < 0.01$). *Lower graph*: percent binding to DNA-cellulose of cytosolic type I receptor complex from AMYG, using the conditions specified in the legend to Fig. 1. Statistical analysis demonstrated that a < b ($P < 0.01$), whereas c vs d and c vs f were not significantly different.

prerequisite for type I receptor translocation from the cytoplasmic compartment into the nuclear fraction.

DISCUSSION

The present results confirm that type I receptors bound to a natural agonist steroid, namely [^{3}H]ALDO, can be transformed to show higher affinity for DNA-cellulose [21] and also translocate to cell nuclei. Thus, when incubations were carried out with [^{3}H]ALDO—in the presence of RU 28362 to prevent cross reaction to type II sites—we observed binding to the cytosolic fraction, to DNA-cellulose, and we found nuclear retention *in vitro* by slices of HIPPO and AMYG.

The purpose of using the HIPPO for such studies, is derived from many studies confirming the predominant localization of type I sites in this brain structure [1–6]. It should be noted that these type I receptors, while showing comparable high affinity for ALDO and CORT *in vitro* [1, 3], probably function predominantly as high affinity glucocorticoid receptors *in vivo*, concerned with specific functions such as changes in behavior and control of the hypothalamic–pituitary–adrenal axis [22, 23]. The AMYG, in turn, contains a moderate amount of type I sites [9], which may be part of a brain neuroendocrine system responding to mineralocorticoid treatment with development of salt appetite [24] and with reductions in the activity of Na,K-ATPase and the biosynthesis of the mRNA for the β subunit of this enzyme [6, 25]. Thus, it is possible that amygdaloid type I receptors may be one of the brain type I sites functioning as physiological mineralocorticoid receptors *in vivo*.

In contrast, after labeling with the mineralocorticoid antagonist [^{3}H]ZK 91587, cytosolic

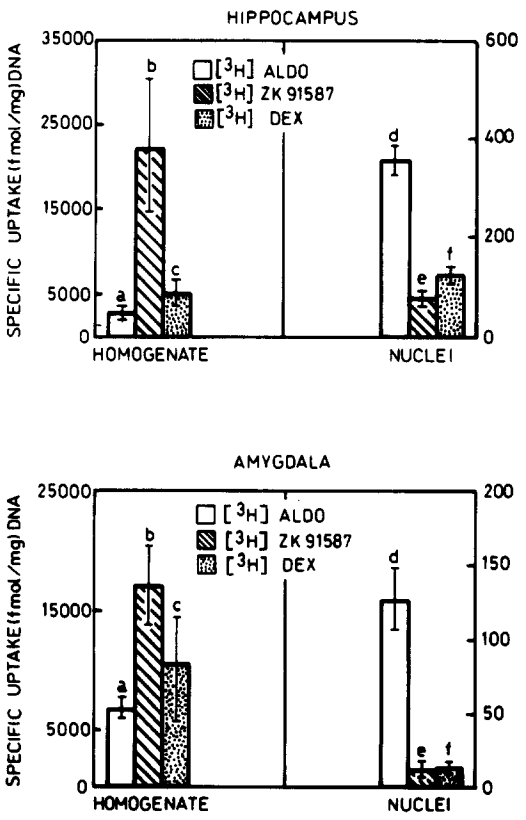

Fig. 3. *In vitro* uptake of [^{3}H]ALDO (open columns), [^{3}H]ZK 91587 (hatched columns) and [^{3}H]DEX (stippled columns) after incubation of these ligands with slices from HIPPO (upper graph) and AMYG (lower graph). Results expressed as the mean ± SE of $n = 4$ experiments performed with nuclei-free homogenates (left-hand graph) or purified nuclei (right-hand graph). Statistical analysis demonstrated, for both HIPPO and AMYG that b > a or c ($P < 0.05$ or less), and d > e or f ($P < 0.01$).

binding was highly efficient, but the resulting complex did not attach to DNA-cellulose, nor did it bind to the nuclear fraction of *in vitro* incubated slices. These results support the hypothesis that antagonism by ZK 91587 results from blockade of nuclear translocation of the cytosolic receptor, retention into the cytoplasm being accompanied by binding to HSP^{90} [11, 12]. Similar results have been reported for spironolactone [26], although in view of its low affinity for brain type I receptors, it may not be a suitable mineralocorticoid antagonist for receptor blockade [27].

When receptors were labeled with [^{3}H]DEX in the presence of RU 28362 to block type II sites, the results varied according to the tissue studied. In the HIPPO, cytosolic binding of [^{3}H]DEX was highly efficient, in agreement with previous reports revealing that type I receptor affinity for [^{3}H]DEX was in the range of that for [^{3}H]ALDO or [^{3}H]ZK 91587 [4, 13, 14]. Binding to DNA-cellulose, although lower than that shown by [^{3}H]ALDO, was still detectable in HIPPO. In the AMYG, however, cytosolic binding of [^{3}H]DEX prevailed over the other ligands, while binding to DNA-cellulose seemed non-specific, i.e. similar under both transforming and non-transforming conditions. To explain the discrepancies of [^{3}H]DEX binding between HIPPO and AMYG, we hypothesize that cell populations may differ, being mostly neuronal in the HIPPO while being mixed (glial/neuronal) in the AMYG. It is known that glial cells express a low quantity of type I sites [28]. This assumption needs to consider further heterogeneity of type I sites among cell populations in the brain. Considering the recently postulated heterogeneity in type II receptors in the nervous system [4, 29] this issue deserves further experimentation.

In conclusion, our results confirm that receptors bound to a natural mineralocorticoid are those capable of being transformed to a molecular species showing affinity for DNA or RNA and nuclear components [21]. Secondly, mineralocorticoid antagonists such as ZK 91587 are bound and retained in cytoplasm, without further nuclear translocation [30]. This property of ZK 91587 may be pharmacologically important to prevent some undesirable effects of mineralocorticoids in brain, which can result in development of salt appetite, high blood pressure and imbalance of fluid and electrolytes [31–33]. Third, [^{3}H]DEX binding to type I receptors resembles in some aspects binding of ZK 91587; nevertheless they were weakly transformed, in contrast to the lack of interaction of ZK 91587 with DNA-cellulose or nuclear components. Thus DEX binding to type I receptors resembles a partial agonist, with considerable antagonist properties. However, the poor transformation of [^{3}H]DEX bound type I receptors *in vitro* correlate and can explain the ineffectiveness of DEX in causing type I receptor activation *in vivo* [34, 35].

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