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PROPERTIES AND DISTRIBUTION OF BINDING SITES FOR THE MINERALOCORTICOID RECEPTOR ANTAGONIST [^{3}H]ZK 91587 IN BRAIN

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Summary—We have studied the binding of the synthetic antimineralocorticoid [^{3}H]ZK 91587 to soluble receptors in brain of adrenalectomized rats. It was observed that [^{3}H]ZK 91587 labeled a single receptor class with high affinity (K_d 1.3 nM) and low capacity (51.1 fmol/mg prot.) in cytosol of hippocampus (HIPPO). The ligand was efficiently displaced *in vitro* from the receptor by aldosterone (IC_{50} 2.0 nM) and corticosterone (2.3), while dexamethasone showed less potency (IC_{50} 5.1 nM) and the pure antiglucocorticoid RU 28362 competed weakly (161 nM). Furthermore, there was a widespread distribution of binding sites all over the brain for this compound, but with CA_1 and CA_3 regions of HIPPO, some amygdaloid nuclei and lateral septum containing most of the binding sites, as revealed by binding assays employing 16 different microdissected brain regions. Finally, the receptor labeled with [^{3}H]ZK 91587 was readily displaced by administration of aldosterone *in vivo* in physiological amounts, from 5 whole brain regions examined, but preferentially from preoptic area, amygdala and HIPPO. It is concluded that [^{3}H]ZK 91587 is a useful ligand for further studies on putative mineralocorticoid responsive cells in brain, due to its high affinity, stability and lack of cross reactivity with glucocorticoid receptors. Its brain distribution is similar to that previously obtained using [^{3}H]aldosterone in the presence of RU 28362 to block ligand binding to the glucocorticoid receptor.

INTRODUCTION

Labeling of a specific steroid receptor may be achieved with a ligand showing minimum cross reactivity with other steroid receptors present in the same tissue preparation. For glucocorticoid receptors, the antiglucocorticoid [^{3}H]RU 28362 meets this condition due to its very low affinity for mineralocorticoid receptors, as observed in peripheral organs as well as in the central nervous system [1, 2]. In the case of the mineralocorticoid binding site, lack of a specific agonist leads to some methodological difficulties, due to the fact that aldosterone (ALDO), the natural mineralocorticoid, binds in responsive tissues to two receptor types, distinguishable for their high affinity (type I sites) or low affinity (type II sites) for this hormone [3, 4].

Synthetic antagonists were also used to specifically label the mineralocorticoid site. However, these compounds apparently bind to a different receptor, in addition to competing for the binding site occupied by [^{3}H]ALDO [5, 6]. This dual behavior has been observed for [^{3}H]spirolactone and for [^{3}H]RU 26752 (7α-propyl-spirolactone) in target organs [5, 6]. Recently, a new antagonist referred to as ZK 91587, which does not give rise to this problem, became available in its tritiated form. In kidney, it showed no cross reactivity with the glucocorticoid receptor, while having high affinity for the mineralocorticoid or type I site [7, 8]. In brain, it reportedly binds to the so-called "corticosterone (CORT)-preferring receptor", similarly to ALDO [8]. We report here studies of the binding properties of [^{3}H]ZK 91587 in cytosol from macrodissected and microdissected brain regions. We compare these results with former experiments using [^{3}H]ALDO incubated in the presence of RU 28362 to block ligand binding to the glucocorticoid site [9, 10].

EXPERIMENTAL

Male Sprague–Dawley rats (200–250 g) were used. Bilateral adrenalectomy (ADX) was performed 2–3 days before killing; during this time rats were given 0.9% NaCl as drinking fluid.

After ether anesthesia, rats were perfused intracardially with 10% dimethylsulfoxide, a cryoprotective agent used for assaying steroid receptors in frozen tissues [9]. Brains were removed and kept either at −70°C or used immediately; in both cases brains were maintained at −8°C and placed on cryostat chucks and sections cut every 300 μ. Sections laid on

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microscope slides were kept at −70°C during 20 h. The punch-out method of Palkovits[10] was employed to remove areas from frozen sections. The following areas were dissected following anatomical limits indicated in the atlas of Pellegrino *et al.*[11]: septum lateral (SEPT-LAT), medial and lateral preoptic areas (POA-MED and LAT), anterior and lateral hypothalamic areas (HT-ANT and LAT), paraventricular (PVN), arcuate (ARC) and ventromedial (VMN) nuclei, subfornical organ (SFO), CA_1 and CA_3 areas of hippocampus (HIPPO), parietal cerebral cortex (CTX), anterior amygdaloid area (AAA), lateral (AL), medial (AME) and cortical amygdala (ACO). These areas were shown previously to contain binding sites for [^{3}H]ALDO [12].

To study occupation of putative mineralocorticoid receptors by ALDO, a group of 10 ADX rats was divided into two groups: the first group was left untreated, whereas a second group received s.c. implants of ALZET miniosmotic pumps containing 1 mg ALDO/ml propylene glycol; release rate of ALDO was 1 μg/μl/h. This dose was effective to reduce sodium appetite of ADX rats, as shown before [13]. Both groups were killed after 4 days and the brains were removed and kept at −70°C. After thawing, 5 regions were dissected for mineralocorticoid receptor assay: HIPPO, amygdala (AMYG), HT, POA and SEPT, as shown by Coirini *et al.*[14].

Mineralocorticoid receptor assay

When using whole brain regions, tissues 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* for 60 min at 0–4°C. The resulting supernatant was incubated with a single (5 nM) or a range of concentrations (0.1–20 nM) of [^{3}H]ZK 91587. The latter approach was employed for saturation analysis, whereas single ligand dose was used for competition assays. For *in vitro* competition studies, cytosol from whole HIPPO was incubated with 5 nM [^{3}H]ZK 91587 and competed with 0.5, 5, 20, 250 and 500 nM of non-radioactive ALDO, CORT, dexamethasone (DEX) or the antiglucocorticoid RU 28362. IC_{50} for these compounds was obtained after logit–log transformation of displacement curves produced by the competitors. For *in vivo* competition studies, cytosol from whole brain regions of ADX or ADX rats treated with ALDO, was incubated with 5 nM [^{3}H]ZK 91587, plus or minus a 1000-fold excess of unlabeled ALDO.

Areas punched out from frozen brain slices were homogenized and processed as previously described [12, 15]. Incubations contained (in TEMG + Mo buffer) 5 nM [^{3}H]ZK 91587 alone or in the presence of a 1000-fold molar excess non-radioactive ALDO, and were performed at 0–4°C during 20 h. After separation of bound and free hormones on minicolumns of Sephadex LH 20 [16], eluted radioactivity was determined and converted into fmol specifically bound steroid per mg protein.

Materials

(7α-methoxycarbonyl-15β,16β-methylene-3-oxo-17α pregn-4-ene-21,17 carbolactone) or [^{3}H]ZK 91587 (sp. act. 70 Ci/mmol) was purchased from New England Nuclear. RU 28362 was the kind gift of Dr D. Philibert, Roussel-Uclaf, France. All other steroids were obtained from Sigma. ALZET model 2001 miniosmotic pumps were purchased from ALZA Corp. (Palo Alto, Calif.).

RESULTS AND DISCUSSION

Time-course studies revealed that [^{3}H]ZK 91587 binding to soluble brain receptors was maintained up to 24 h of incubation (data not shown) in agreement with data from other laboratories demonstrating the pronounced stability of receptor complexes formed with [^{3}H]ZK 91587 [7, 8]. Data in Fig. 1 shows that in cytosol of HIPPO, an area highly favored in [^{3}H]ALDO binding sites in comparison with the rest of the brain, the antimineralocorticoid labeled a single set of sites with an affinity greater (K_d 1.3 nM) than that obtained with [^{3}H]ALDO (K_d 3.6 nM) [17], in confirmation with results of Sutanto and de Kloet [8]. Maximal number of sites in this tissue was equal to, or slightly lower than, that reported for [^{3}H]ALDO bound in the presence of excess RU 28362, which blocks ligand binding to glucocorticoid type II receptors [1, 2].

Data in Fig. 2 established that [^{3}H]ZK 91587 binding in cytosol of HIPPO was equally displaced by unlabeled ALDO or CORT, IC_{50} for these compounds being 2.0 and 2.3 nM, respectively. This high

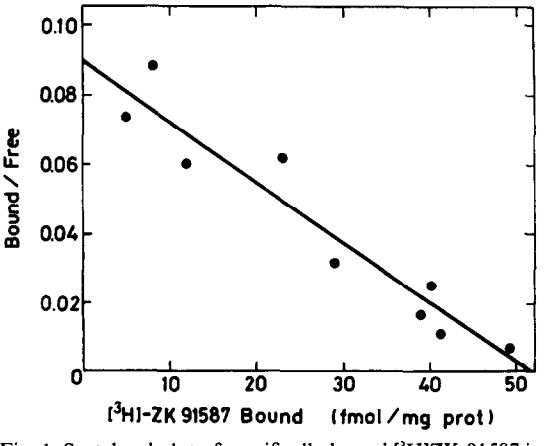

Fig. 1. Scatchard plot of specifically bound [^{3}H]ZK 91587 in cytosol of hippocampus. Cytosol was incubated with 0.1–20 nM tritiated ZK 91587, with parallel incubations in the presence of a 1000-fold molar excess of radioactive ALDO. K_d was 1.3 nM, whereas B_{max} measured 51.1 fmol/mg protein (for the slope, $r = 0.91$). Binding parameters were calculated by the method of Cressie and Keightley[25].

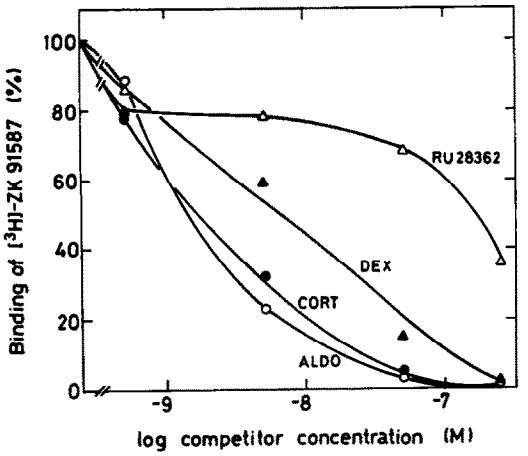

Fig. 2. Competition of [^{3}H]ZK 91587 binding in cytosol of hippocampus. 5 nM tritiated ligand was competed with 0.5–20 nM of aldosterone (ALDO, ○——○), corticosterone (CORT ●——●), dexamethasone (DEX ▲——▲) or RU 28362 (△——△). After logit–log transformation of displacement curves, IC_{50} values were calculated as the concentration of competitor required to inhibit binding of [^{3}H]ZK 91587 by 50%, and the values obtained were: ALDO 2.0, CORT 2.3, DEX 5.1 and RU 28362 161 nM, respectively.

affinity for ALDO differentiates this binding site occupied by [^{3}H]ZK 91587 from another mineralocorticoid antagonist receptor which binds [^{3}H]RU 26752 (7α-propyl-spirolactone) or [^{3}H]spirolactone but shows little affinity for ALDO in target organs [5, 6]. The similar affinity of CORT and ALDO for the type I receptor of the HIPPO or of the cloned receptor from kidney was already reported [17, 18]. This fact has made necessary reinterpretation of the physiological ligand occupying this site, in view of the much higher circulating levels of CORT than ALDO [20]. In this context, we have previously shown that ablation of the dorsal HIPPO, while removing most of the [^{3}H]ALDO binding sites, preserved ALDO suppression of salt intake [13], suggesting that the HIPPO binding site might not function as such *in vivo* [21]. On the other hand, this HIPPO binding site for [^{3}H]ZK 91587 showed less preference for glucocorticoids, as IC_{50} for DEX was greater than that for ALDO or CORT (5.1 nM) and that for the pure glucocorticoid receptor marker RU 28362 was much higher (161 nM, Fig. 2). The relative potency of DEX can be explained on the basis of its lower, still considerable, affinity towards the type I site [17, 20].

In view of the high affinity and specificity of [^{3}H]ZK 91587 for the hippocampal type I site, we considered important to examine the distribution of this compound in microdissected brain areas, previously observed to contain variable quantities of binding sites for the natural ligand [^{3}H]ALDO, resistant to blockade by RU 28362 [12]. We thought this experiment of value for identification of putative mineralocorticoid receptors outside the HIPPO. As shown in Fig. 3, highest concentration of sites for the antimineralocorticoid were found in CA_1 and CA_3

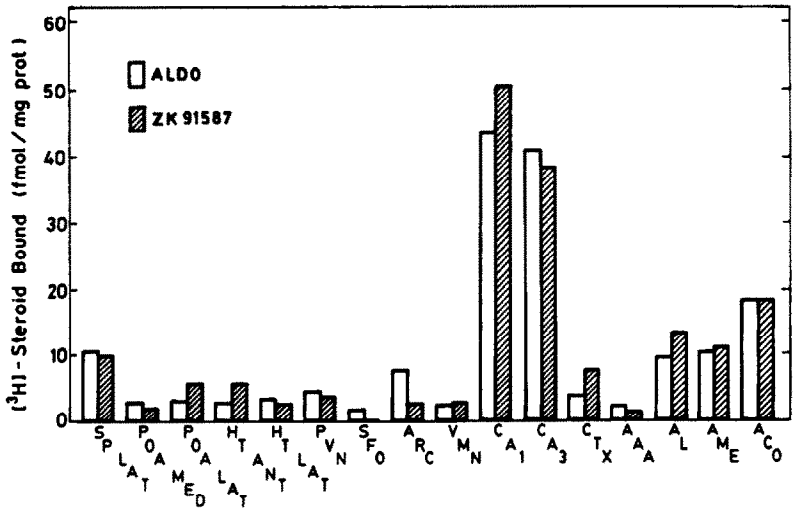

Fig. 3. Binding of [^{3}H]ZK 91587 to microdissected brain regions (hatched columns). Specific binding was calculated after subtraction from total binding (5 nM) radioactivity obtained in the presence of a 1000-fold molar excess ALDO. For comparison, previously published data for 5 nM [^{3}H]ALDO in the presence of 500 nM excess RU 28362 are included (open columns, Ref. [12]). ANOVA followed by a multiple range analysis [26] showed that the groups were significantly different (F: 6.534 [15, 16] $P < 0.003$) and that highest binding was obtained in CA_1 and CA_3 areas of HIPPO, followed by ACO. Although the latter contained similar binding values to AL = AME = SEPT-LAT = CTX, it was higher than the rest of the areas studied. *Abbreviations*—SFO, subfornical organ; POA-MED and LAT, medial and lateral preoptic areas; HT-MED and LAT, medial and lateral hypothalamic areas; VMN, ARC and PVN, ventromedial, arcuate and paraventricular nuclei of the hypothalamus, CTX, cerebral cortex; SEPT-LAT, lateral septum; AAA, AME, AL and ACO, anterior, medial, lateral and cortical nuclei of the amygdala; CA_1 and CA_3, areas of the hippocampus (HIPPO).

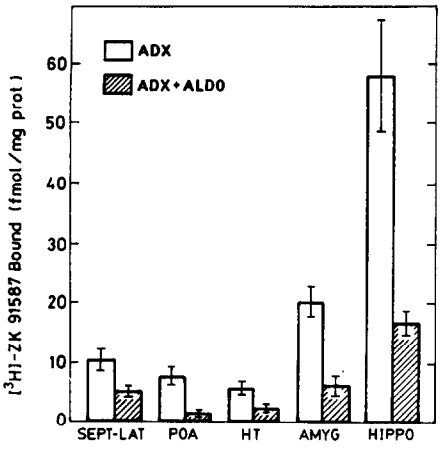

Fig. 4. Binding of [^{3}H]ZK 91587 in whole brain regions from ADX rats (open columns) and ADX rats receiving minipumps releasing 1 μg/μl/h of ALDO during 4 days (hatched columns). Results represent the mean $\pm$ SE ($n = 5$ rats per group). ALDO-treated rats showed significantly lower binding in lateral septum (SEPTUM-LAT, $P < 0.025$), preoptic area (POA, $P < 0.005$), hypothalamus (HT, $P < 0.01$), amygdala (AMYG, $P < 0.001$) and hippocampus (HIPPO, $P < 0.005$) by Student's t-test.

areas of HIPPO; other areas rich in binding sites were AL, AME, ACO and SEPT-LAT, with the rest of the regions showing modest to very low binding levels. This profile closely approximates that obtained *in vitro* with [^{3}H]ALDO and also depicted in Fig. 3 for the purposes of comparison, or that obtained during *in vivo* cell nuclear labeling after systemic administration of the hormone [21]. We considered the possibility that binding sites in amygdaloid areas are worthy of further analysis regarding their role as transducers of mineralocorticoid signals in brain, because we have found changes in the enzyme Na,K-ATPase in these areas following high dose treatment with mineralocorticoids, without changes in HIPPO [12]. That the AMYG, besides other brain regions, may be part of the neural circuit controlling salt appetite in rats, is supported by demonstrations that damage to these regions decreases salt appetite promoted by mineralocorticoids [22].

In order to ascertain whether the physiological mineralocorticoid of the rat—ALDO—would occupy the binding site labeled with [^{3}H]ZK 91587, as it did efficiently *in vitro* (Fig. 3), we implanted minipumps into ADX rats releasing ALDO in doses capable of eliciting a biological response such as the suppression of salt intake elevated as a consequence of ADX [13, 21]. Figure 4 shows that animals perfused during 4 days with 1 μg/μl/h of ALDO showed a statistically significant reduction in [^{3}H]ZK 91587 binding in five anatomical regions of the brain: SEPT-LAT (55% inhibition, $P < 0.025$), POA (76.6%, $P < 0.005$), HT (61%, $P < 0.01$), AMYG (71.4%, $P < 0.001$) and HIPPO (71.4%, $P < 0.005$, by unpaired Student's t-test).

From the above mentioned experiments, the following conclusions were drawn: (a) [^{3}H]ZK 91587 labels a single binding site in brain with considerable high affinity; (b) the tritiated antagonist was significantly displaced by type I site preferring ligands such as ALDO and CORT, but weakly or not at all by type II site ligands such as DEX or RU 28362; (c) there was a widespread distribution of binding sites all over the brain but with HIPPO, AMYG nuclei and SEPT-LAT accounting for most of the binding, and (d) the site labeled with [^{3}H]ZK 91587 was readily occupied by exogenously administered ALDO in physiological doses, with greater occupancy observed in POA, AMYG and HIPPO.

These results expand previous data of Sutanto and de Kloet [8] who reported binding properties of this compound exclusively in HIPPO, by providing additional data on brain distribution of binding sites, using whole brain regions and microdissected areas obtained by the punch-out method. Our study may be useful for pointing out the actual target cells being responsive to mineralocorticoids, due to the fact that it is difficult to distinguish "mineralocorticoid-responding cells" by means of binding assays, since type I receptor shows comparable affinity for ALDO and natural glucocorticoids [18–20]. In this context, it has been recently reported that recognition between a mineralo and a glucocorticoid at the cellular level may be enzyme, but not receptor, mediated [23]. Thus, degradation of CORT to 11-dehydrocorticosterone by the activity of 11β-hydroxy-dehydrogenase prevents CORT from reaching the receptor, thereby allowing the binding of ALDO. Since ZK 91587 does not have an 11β hydroxy group in its molecule, it will be preserved from enzymatic attack. Additionally, [^{3}H]ZK 91587 may be used alternatively to ALDO due to the binding of the latter to type II or glucocorticoid receptor, unless competitors of type II sites such as RU 28362 or RU 26988 are added to the incubations. This latter and widely used procedure may have its drawbacks, however, as competitors could affect allosterically ligand binding [24]. The pronounced stability of the receptor complex formed by [^{3}H]ZK 91587 also makes it a useful ligand, possibly due to the enhanced affinity towards this antagonist, which doubles that reported for ALDO [7, 8, 17].

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