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CHARACTERIZATION OF SPIRONOLACTONE BINDING SITES DISTINCT FROM ALDOSTERONE RECEPTORS IN RAT KIDNEY HOMOGENATES

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Abstract—The binding of [^{3}H]spironolactone to kidney homogenates from adrenalectomized rats was studied by dextran-charcoal absorption methods. [^{3}H]Spironolactone binds with high affinity and low capacity (K_D = 12.9 ± 0.6 nM; B_{max} = 93.4 ± 3.8 fmoles/mg protein) at low temperatures (0°–2°). Its hormone specificity, as measured by relative binding affinity (RBA) is spironolactone > prorenone > methyltrienolone > testosterone > progesterone > aldosterone > dexamethasone. In the same tissue preparation, specific spironolactone binding sites and classical mineralocorticoid receptor sites labelled with [^{3}H]aldosterone differ in their thermal stability, binding parameters and hormone specificities, whereas their tissue distributions are similar. In conclusion, [^{3}H]spironolactone binds specifically to kidney homogenates from adrenalectomized rats and these binding sites, apparently, are different from the classical mineralocorticoid receptors. The theoretical and practical aspects of this finding are discussed.

Spironolactone belongs to a family of compounds, the spirolactones, which are believed to exert their potent antimineralocorticoid effect by competitively antagonizing the binding of aldosterone to its renal cytoplasmic receptors [1–3]. The molecular mechanism of this inhibitory effect is assumed to involve some sort of inactivation of the steroid–receptor complex, which is then unable either to enter the nucleus or to attach to nuclear acceptor sites or to initiate the transcriptional event if attachment should take place [4, 5]. Spirolactones have been shown to displace [^{3}H]aldosterone from its specific receptors *in vitro* [2, 6–8] but there is only scanty information so far about the binding characteristics of these compounds [3, 7]. Claire *et al.* studied the binding of [^{3}H] prorenone and demonstrated that even though this agent probably did bind to mineralocorticoid receptors, as suggested by cross-competition experiments, its interaction with the receptor was somewhat different from that of [^{3}H]aldosterone [7]. This finding fits well with the general allosteric model of differential interaction of mineralocorticoid and antimineralocorticoid with the same cytoplasmatic receptor proposed by Feldman *et al.* [4]. On the other hand, although spironolactone has been so far employed for a variety of clinical situations, its binding to renal mineralocorticoid receptor has not been throughly investigated, probably due to a lack of a suitable labelled form. Since it is now available a high specific activity tritium labelled spironolactone, obtained from the synthetic pathway suggested by Dr G. Winters from our Organic Chemistry Department, it was decided to investigate (a) how [^{3}H] spironolactone binds to kidney homogenates from adrenalectomized rats; (b) whether its binding differs from that of [^{3}H]aldosterone; (c) whether the labelled antagonist might be used to discriminate between aldosterone- and antialdosteron-like agents *in vitro* for the search of new potential antimineralocorticoids.

MATERIALS AND METHODS

Chemicals. [1,2-^{3}H]Aldosterone (41 Ci/mmole) and [1,2(n)-^{3}H]spironolactone (44 Ci/mmole), prepared by reduction of the Δ^1-double bond of the corresponding $\Delta^{1,4}$-3-keto steroid synthesized in our Chemistry Department, were produced from Amersham International Limited, Amersham, England and checked for radiochemical purity before use. Prorenone was a gift from G. D. Searle & Co., Chicago, IL, U.S.A. All other non-radioactive steroids were purchased from commercial sources. The following systematic names are used: spironolactone, 3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl)-propionic acid γ-lactone; prorenone, 3-(17β-hydroxy-6β,7β-methylen-3-oxo-4-androsten-17α-yl)-propionic acid γ-lactone; methyltrienolone, 17α-methyl-19-nor-$\Delta^{4,9,11}$-androstatrien-17β-ol-3-one.

Animals. Male Sprague–Dawley rats (Charles River, Italy) weighing 75–100 g were adrenalectomized and maintained on 0.9% saline instead of drinking water until used (4–5 days after adrenalectomy).

Binding studies. Animals were killed by decapitation; the organs were removed and washed free of blood. Tissues were homogenized with 3 volumes of ice-cold Krebs–Ringer phosphate buffer, pH 7.4 with a Polytron PT 10-35 (Kinematica, Luzern, Switzerland). Aliquots (0.6 ml) of homogenate were incubated in triplicate with [^{3}H]aldosterone or [^{3}H] spironolactone, with or without excess unlabelled steroids. Saturation studies were performed with concentrations of labelled steroids from 0.03 to 27.9 nM for [^{3}H]aldosterone and from 0.1 to 22.6 nM

for [^{3}H]spironolactone, in the presence or absence of 8 μM unlabelled aldosterone or spironolactone. Competition assays were carried out with [^{3}H]aldosterone at 0.3 or 2.5 nM and [^{3}H]spironolactone at 3 nM, whereas the concentrations of unlabelled steroids ranged from 0.8 to 8000 nM. At the end of incubation (20 min at 25° for [^{3}H]aldosterone, 60 min at 0°–2° for [^{3}H]spironolactone), homogenates were centrifuged at 1000 *g* for 10 min. Aliquots (0.3 ml, 9–10 mg protein) of supernatant were collected in tubes to which 0.3 ml dextran-coated charcoal (0.2% Dextran T 70, Pharmacia, Sweden and 2% Norit A, Serva, West Germany, in Krebs–Ringer phosphate buffer) was added. After 10 min at 0°–2°, the tubes were centrifuged at 2000 *g* and 0.4 ml of supernatant was transferred into vials containing 10 ml of a scintillation cocktail (Biofluor, New England Nuclear, Boston, U.S.A.). Radioactivity was measured by liquid scintillation spectrometry (counting efficiency 35–40%). Protein concentrations were determined by the method of Lowry [9], using bovine serum albumin as standard. Specific binding of both labelled steroids was calculated by subtracting non-specific binding (labelled steroid bound in the presence of excess unlabelled steroid) from total binding (labelled steroid bound in the absence of excess unlabelled steroid). Non-specific binding accounted for 5–10% of the total binding for both [^{3}H]aldosterone and [^{3}H]spironolactone.

Specific binding data were analysed according to Scatchard [10]. In competition studies, data were expressed as relative binding affinity (RBA) with respect to spironolactone or aldosterone, arbitrarily set at 100. RBA for each steroid was calculated from the following relation:

$$\frac{\text{concentration of unlabelled spironolactone or aldosterone at 50\% displacement}}{\text{concentration of unlabelled competitor at 50\% displacement}} \times 100.$$

For each component and concentration, at least three determinations were performed in triplicate.

RESULTS

Binding parameters

Specific [^{3}H]spironolactone binding to kidney homogenate was of the high-affinity, low-capacity type, with a single class of binding sites (apparent K_D = 12.9 ± 0.6 nM; B_{max} = 93.4 ± 3.8 fmoles/mg protein, mean ± S.E. of five separate plots). An example of data that fit this model is shown in Fig. 1A. When specific [^{3}H]aldosterone binding to the same homogenate was analysed, a more complex interaction was apparent, suggesting the existence of two separate sets of binding sites (Fig. 1B). These two components were resolved by a computer-assisted hyperbolic iterative curve-fitting model [11] and their parameters were: K_{D1} = 0.28 ± 0.04, K_{D2} = 5.6 ± 0.4 nM, B_1 = 4.4 ± 0.05 and B_2 = 62.6 ± 5.9 fmoles/mg protein (mean ± S.E. of four separate plots).

Temperature dependence of the binding

The time-course of specific [^{3}H]spironolactone and [^{3}H]aldosterone binding to renal homogenates at 0°–2° and 25° are compared in Fig. 2. At 0°–2° [^{3}H]spironolactone rapidly reached its maximal binding after 1 hr incubation, then quickly declined to about one-third within 6 hr. [^{3}H]Aldosterone binding, on the other hand, was slower, reaching its maximum only after 4–6 hr. The persistence of the binding was not further evaluated (Fig. 2A). The overall pictures were rather different at 25°. [^{3}H] Spironolactone binding was very labile; after 5 min incubation, it was only 60% of the corresponding maximum after 1 hr at 0°–2°. After 2 hr incubation specific binding was almost undetectable. [^{3}H]Aldosterone binding was more stable, reaching a plateau within 10 min and remaining high for at least 30 min, after which it began to decrease (Fig. 2B).

Competition studies

In order to further characterize spironolactone binding sites in rat kidney homogenates, competition

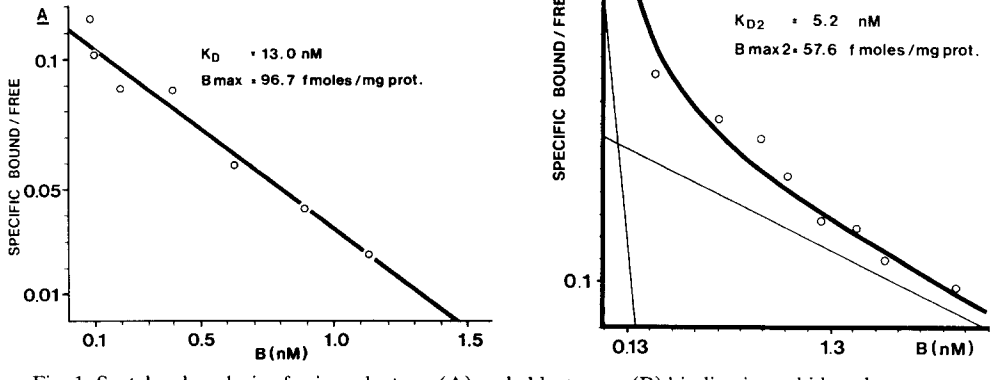

Fig. 1. Scatchard analysis of spironolactone (A) and aldosterone (B) binding in rat kidney homogenates. All data are expressed as specific binding.

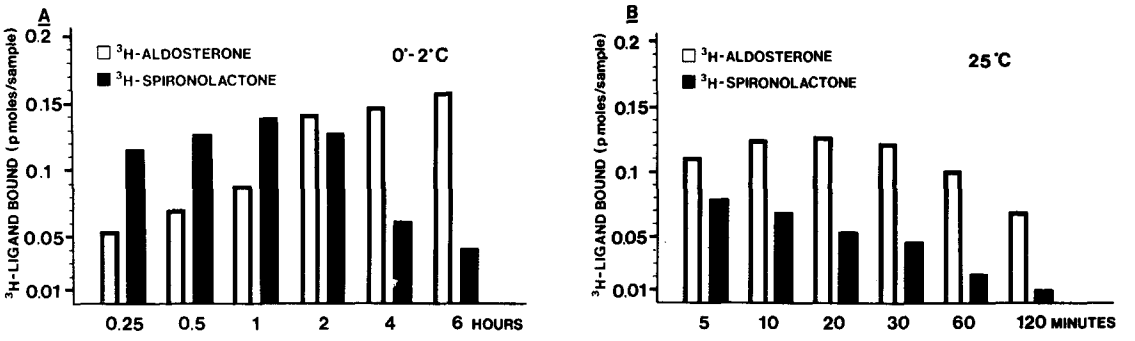

Fig. 2. Time-course of specific [³H]spironolactone and [³H]aldosterone binding to kidney homogenates at 0°–2° (A) and at 25° (B).

experiments were carried out with [³H]spironolactone and [³H]aldosterone. For each ligand their respective optimal binding conditions were used (60 min incubation at 0°–2°, 3 nM ligand concentration for [³H]spironolactone; 20 min incubation at 25°, 0.3 and 2.5 nM ligand concentration for [³H]aldosterone to differentiate between the two binding components). Table 1 shows the relative binding affinities (RBA) (see Methods) of various steroids for [³H]spironolactone binding. The specificity of [³H]spironolactone binding was remarkable, since only prorenone, a known spironolactone-like agent [12], significantly displaced [³H]spironolactone from its binding sites. The interference of other hormonal steroids was poor (methyltrienolone and testosterone: RBA = 10 and 6 respectively; progesterone: RBA = 3; estradiol 17β: RBA $\ll$ 0.2). Very surprisingly, aldosterone was also a very weak competitor of [³H]spironolactone binding (RBA = 2). There appeared to be no glucocorticoid component of the binding since dexamethasone was totally ineffective in displacing [³H]spironolactone. When the same steroids were tested against [³H]aldosterone, a completely different picture was obtained (Table 2, left column). Dexamethasone was a good competitor, since its RBA is about one-third of that of aldosterone, indicating the presence of a considerable glucocorticoid component in the binding. Classical antialdosterone agents like spironolactone and prorenone and also progesterone, which is supposed to be an antagonist for renal mineralocorticoid receptors [13], had somewhat lesser impacts on aldosterone receptors (RBA = 14 for spironolactone, 11 for prorenone and 17 for progesterone). Testosterone was almost inactive (RBA = 1). When competition studies were carried out in the presence of a higher ligand concentration (2.5 nM) than that previously employed, the results were essentially the same, with dexamethasone perhaps a little more active in displacing [³H]aldosterone, indicating more pronounced interaction with the glucocorticoid component of the binding (Table 2, right column).

Tissue distribution of specific [³H]spironolactone and [³H]aldosterone binding

The localization of spironolactone and aldosterone binding sites in eight different organs of the adrenalectomized rat was studied (Fig. 3). The concentration of binding sites was highest for both ligands in the kidney with levels 2–3 times higher than those found in the lung and brain: lower levels were seen in testis, spleen, heart, liver and thymus. However, no important differences in the distribution of the two ligands specific binding among the tissues considered were seen.

DISCUSSION

Our studies demonstrate that there are specific spironolactone binding sites in homogenates of kidneys from adrenalectomized rats as well as, although at lesser concentrations, in other tissues, including the lung and brain. Even though the distribution of these sites is similar to that of [³H]aldosterone, there are several differences that distinguish the spironolactone binding sites from aldosterone receptors.

Table 1. Relative bonding affinities (RBA) of various steroids for [³H]spironolactone receptors. [³H]spironolactone concentration: 3 mM; incubation for 60 min at 0°–2°

Steroid	RBA
Spironolactone	100
Prorenone	30
Methyltrienolone	10
Testosterone	6
Progesterone	3
Aldosterone	2
Estradiol 17β	$\ll$0.2
Dexamethasone	$\ll$0.2

Table 2. Relative binding affinities (RBA) of various steroids for [³H]aldosterone receptors. [³H]aldosterone concentrations: (a) 0.3 nM; incubation for 20 min at 25°, (b) 2.5 nM; incubation for 120 min at 0°–2°

Steroid	RBA a	RBA b
Aldosterone	100	100
Dexamethasone	28	39
Progesterone	17	18
Spironolactone	14	15
Prorenone	11	8
Testosterone	1	1

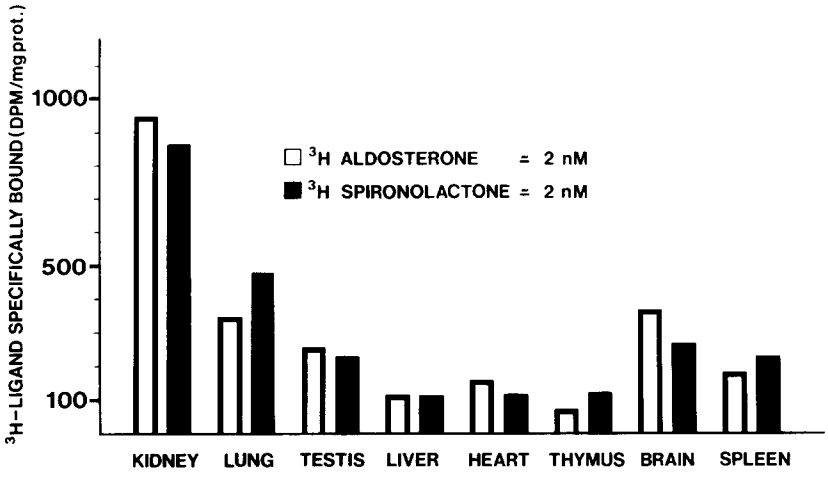

Fig. 3. Typical tissue distributions of specific [^{3}H]spironolactone and [^{3}H]aldosterone binding in various organs of the adrenalectomized rat. The experiment was repeated three times. Dispersion of the data was minimal (less than 10%).

First of all, the characteristics of spironolactone binding in kidney homogenates, analysed according to Scatchard, indicate a single type of saturable specific binding sites, whereas for aldosterone binding, a model implying the existence of two types of binding sites better fits the experimental data. This latter finding is consistent with the well-documented presence of two types of aldosterone receptors, the higher-affinity type I sites with K_D in the subnanomolar range and the lower-affinity type II sites with K_D at least ten times higher [13–15]. The K_D value for [^{3}H]spironolactone binding is not far from the K_{D2} value for [^{3}H]aldosterone, so the two ligands might seem to share at least one set of binding sites. That this is not the case is shown by the results of the cross-competition studies. In fact the displacements of [^{3}H]spironolactone and of [^{3}H]aldosterone binding by reference steroids are completely different, not only when the assay is carried out at optimal conditions for each ligand, which are different, but also when they are compared under the same binding conditions (compare Table 1 with Table 2, right column). The rank order of potencies of various steroids in displacing [^{3}H]spironolactone is spironolactone > prorenone > methyltrienolone, testosterone, progesterone and aldosterone ≫ estradiol 17β and dexamethasone; whereas for [^{3}H] aldosterone it is aldosterone > dexamethasone > progesterone, spironolactone and prorenone > testosterone. The spironolactone binding sites, therefore, are characterized by noteworthy selectivity for antimineralocorticoids, by a relatively low sensitivity to aldosterone and by a complete lack of interference by dexamethasone.

On the contrary, the aldosterone binding sites have less marked selectivity, since they are greatly affected by dexamethasone and, although to a lesser extent, by progesterone and antimineralocorticoids. Another important difference between spironolactone binding sites and aldosterone receptors is a distinct temperature-dependence of the binding. The binding of both ligands at low temperature (0°–2°) is relatively stable, for at least a few hours, but equilibrium for [^{3}H]spironolactone at 0°–2° is attained more rapidly than that of [^{3}H]aldosterone. At 25° the binding of [^{3}H]spironolactone is very elusive, unlike that of [^{3}H]aldosterone which is rather stable. The problem of the stability of the binding is important when dealing with mineralocorticoid binding. It has been reported that unless a series of precautions are taken to preserve the stability of aldosterone receptors, such as the addition of ligand and stabilizing agents and low temperature during manipulation, most of the binding observed may be primarily that to the glucocorticoid receptor [16]. Our experimental conditions, which are similar to those used by Ojasoo and Raynaud [17], seem to preserve the aldosterone receptor quite satisfactorily. As for the spironolactone binding sites, we cannot exclude that some kind of degradation during the assay did occur, given its lability even at low temperatures. In any case the types of binding site we label with spironolactone seem to be different from those labelled with aldosterone.

Our studies with labelled spironolactone suggests, therefore, that spironolactone binds to specific high affinity binding sites which are distinct from aldosterone receptors.

Recently several reports have provided evidence for a binding site for antiestrogens that is distinct from the estrogen receptor, and these may have a role in regulating the effects of non-steroidal antiestrogens [18, 19]. The same could be true for spironolactone. In addition to the well-documented fact that spironolactone competitively inhibits [^{3}H] aldosterone binding at its specific binding sites, spironolactone probably also binds to a new type of binding site which is different from the aldosterone receptor. One can also speculate that the close contiguity of these two types of binding sites, as suggested by the similar tissue distributions of the two ligands, might even indicate a possible modulatory effect of the spironolactone binding sites over the biological function of aldosterone receptors.

Strangely enough, [^{3}H]spironolactone binding sites are different from those recognized by [^{3}H] prorenone. According to Claire *et al.* [7], [^{3}H]prorenone does not discriminate between aldosterone-

and antialdosterone-like agents, since their RBA are rather similar. On the contrary, aldosterone is a very weak competitor of [^{3}H]spironolactone. At present it is difficult to explain these discrepancies, which, however, would deserve further studies. In conclusion, our data suggest that, in addition to classical mineralocorticoid receptors, there is also a specific high affinity binding site for spironolactone which is different from the aldosterone receptor. A practical consequence of this is that labelled spironolactone can be used to discriminate between aldosterone- and antialdosterone-like agents *in vitro*, which we feel will be an important improvement in the search for new potential antimineralocorticoids.

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