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Aldosterone antagonists destabilize the mineralocorticosteroid receptor

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To elucidate the mechanism of action of aldosterone antagonists, we studied the interaction of spironolactone with the chick mineralocorticosteroid receptor (MR). Intestinal cytosol contains specific spironolactone-binding sites ($K_d \sim 3$ nM; max. no. of binding sites ~ 100 fmol/mg of protein) that have been identified as MRs by competition experiments with steroid ligands and with the monoclonal anti-idiotypic antibody H10E that interacts with aldosterone-binding domain of the MR. Binding studies indicate that aldosterone and spironolactone bind to the MR through a common site that encompasses the epitope recognized by H10E. At 4 °C, spironolactone dissociates much more rapidly from the cytosol 8–9 S form of MR ($t_{\frac{1}{2}}$ 38 min) than does aldosterone ($t_{\frac{1}{2}}$ 3240 min). A high dissociation rate was also observed for progesterone, a natural aldosterone antagonist ($t_{\frac{1}{2}}$ 84 min). The covalent linkage of the 90 kDa heat shock protein (hsp90) to the ligand-binding subunit of MR with dimethyl pimelimidate did not notably modify the rate of dissociation of spironolactone from the receptor ($t_{\frac{1}{2}}$ 96 min), excluding the possibility that the rapid dissociation rate of the antagonist was related to hsp90 release. The effects of aldosterone and the two anti-mineralocorticosteroids on the 8–9 S hetero-oligomeric structure of the MR differed strikingly. Using low-salt density-gradient centrifugation analysis, aldosterone-labelled receptors were recovered as 8–9S complexes, whereas 4 S entities were detected after spironolactone and progesterone binding. This indicated that, under the experimental conditions used, aldosterone antagonists facilitate hsp90 release and thus do not stabilize the non-DNA-binding 8–9S form of MR. We propose that the combination of rapid dissociation of the ligand and a weakened hsp90–receptor interaction is involved in the anti-mineralococorticosteroid activity of aldosterone antagonists.

INTRODUCTION

Spirolactones, synthetic steroids which are anti-mineralocorticosteroid compounds, act mainly by antagonizing the effect of aldosterone at the target cell level [1]. Spirolactones are widely used as anti-hypertensive agents; however, the molecular basis of their effects remain unclear. It has been proposed that they exert their effects by competitively inhibiting the binding of aldosterone to its intracellular receptor. Marver *et al.* [2] and Claire *et al.* [3] reported that tritiated spirolactones bind to the cytosol mineralocorticosteroid receptor (MR). Unlike aldosterone–receptor complexes, spirolactone-labelled receptors were not detected in the nucleus after subcellular fractionation of prelabelled renal tissues. On the basis of these observations, it has been proposed that spirolactone-labelled receptors are unable to be translocated into the nucleus and/or to bind to specific nuclear acceptor sites [2,3]. However, specific nuclear binding of spironolactone in rabbit kidney cells has recently been detected by autoradiography [4]. It was therefore of interest to determine the steps, in the cascade of events from steroid binding to transcriptional activation, responsible for the antagonist activity of spirolactones.

Steroid receptors are regulatory transcriptional factors that are composed of a steroid-binding unit which is found associated with other cellular components, most notably the 90 kDa heat-shock protein (hsp90) [5–7]. In its hetero-oligomeric structure, the receptor is unable to interact with specific DNA sequences. Activation of the receptor to its DNA-binding state is accompanied by dissociation of hsp90 from the steroid-binding unit [8]. The biological role of the hsp90–receptor association in receptor function is still poorly understood. It has been proposed that hsp90 caps the DNA-binding site of the receptor [8]. Hsp90 has also been shown to protect the receptor against proteolysis, to stabilize the conformation of the functional hormone-binding domain, and thus to be involved in signal transduction [9–12].

In a previous study we have shown that the MR of the chick intestine is associated with hsp90 [13]. To gain further insight into the mechanism of action of aldosterone antagonists, we characterized the binding to the chick intestine MR of spironolactone, the only spirolactone which is used for treatment of high blood pressure. We also determined whether the spironolactone-binding site was the same as the one for aldosterone. For this purpose, we took advantage of a monoclonal anti-idiotypic antibody that specifically recognizes the aldosterone-binding site of the MR [14]. In order to detect putative differences between agonist and antagonist in their interaction with the MR, we also examined the binding kinetics of aldosterone, spironolactone and progesterone, a natural aldosterone agonist. Finally, we analysed the effects of agonist and antagonists on the hetero-oligomeric structure of the MR to determine whether the ligand is a key modulator of the hsp90–receptor interaction, and to determine if the biological activity of a steroid can be correlated with its effects on the oligomeric structure of the receptor.

MATERIALS AND METHODS

Chemicals

[1,2-^{3}H]Aldosterone (40–60 Ci/mmol) and [1,2-^{3}H]progesterone (40–60 Ci/mmol) were provided by Amersham International, Amersham, Bucks., U.K. [1,2-^{3}H]Spironolactone was a gift from Searle Laboratories. Non-radioactive aldosterone, dihydrotestosterone (DHT), oestradiol and progesterone were from Sigma. RU486 was from Roussel-Uclaf. Spironolactone (SC9420) was

Abbreviations used: hsp90, heat-shock protein of 90 kDa; RU486, 11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(prop-1-ynyl)oestra-4,9-dien-3-one; spironolactone (SC9420), 3-(7α-acetylthio-17β-hydroxy-3-oxoandrost-4-en-17α-yl)propionic acid lactone; DHT, dihydrotestosterone; MR, mineralocorticosteroid receptor.

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from Searle Laboratories. Dextran T70 and charcoal (Norit A) were purchased from Pharmacia, Uppsala, Sweden. Sodium tungstate was from Sigma. All other products were from Merck. To avoid steroid adsorption, the steroid solutions prepared in ethanol were dried and resuspended in 50% poly(ethylene glycol) 300 prepared in TEG buffer (see below) to give a 5% final concentration of PEG 300 in the cytosol.

Buffers

TE buffer contained 20 mM-Tris/HCl and 1 mM-EDTA; TEW buffer was TE buffer containing 20 mM-sodium tungstate. TEG buffer consisted of 20 mM-Tris/HCl, 1 mM-EDTA and 10% (v/v) glycerol, and TEGW buffer was TEG containing 20 mM-sodium tungstate. All buffers were adjusted to pH 7.4 (25 °C).

Antibodies

The monoclonal anti-idiotypic antibody H10E, a mouse IgG_1 immunoglobulin, was used as diluted ascites fluid [14]. The rat monoclonal antibody BF4, which reacts with hsp90, was prepared as described previously [15].

Intestinal cytosol preparation

Chickens of the Warren strain (6–8 weeks old) were decapitated, and the intestine was removed, slit longitudinally and washed successively with ice-cold 0.9% NaCl and TEG or TEGW buffer. The tissue was cut into small pieces and homogenized in TEG or TEGW buffer (2 ml/g) by using an Ultra Turrax tissue homogenizer. The homogenate was centrifuged at 5000 *g* for 10 min at 4 °C, and the resulting supernatant was centrifuged at 105000 *g* for 60 min at 4 °C. The final supernatant was frozen in liquid nitrogen until further use.

Covalent cross-linking

The cross-linking conditions used have been described before [16]. To the chick intestine cytosol was added 0.1 vol. of 2.2 M-triethanolamine (pH 8) to adjust the pH. Then 0.2 vol. of 0.1 M-dimethyl pimelimidate, freshly prepared in 0.2 M-triethanolamine (pH 8), was added. The reaction was allowed to proceed for 30 min at 10 °C.

Spironolactone binding characteristics at equilibrium

Cytosol, prepared in TEGW buffer, was incubated with 1 μM-RU486 for 2 h at 4 °C. Increasing amounts of [^{3}H]spironolactone (0.1–500 nM) were added to 50 μl aliquots of cytosol. After 4 h at 4 °C, total radioactivity was counted. Bound (B) and unbound (U) steroid were separated by the dextran/charcoal method described previously [13]. The evolution of bound as a function of unbound steroid was analysed by a previously described computer method [17]. Increasingly complex models of interaction were tested, comprising one class of specific sites [$B = NU/(K_d + U)$], one class of specific sites plus one type of non-specific binding [$B = NU/(K_d + U) + \beta U$] and two classes of specific sites [$B = N_1U/(K_{d_1} + U) + N_2U/(K_{d_2} + U)$]. N represents the number of specific binding sites, K_d their dissociation constants at equilibrium and β the constant of non-specific binding.

Competition experiments

Cytosol, prepared in TEGW buffer, was first incubated with 1 μM-RU486 for 2 h at 4 °C to saturate the glucocorticosteroid receptors (GRs). Aliquots of RU486-preincubated cytosol (50 μl) were incubated with 10 nM-[^{3}H]spironolactone in the absence or presence of various concentrations of unlabelled competitors (1–100 nM). After 4 h of incubation at 4 °C, bound and free steroid were separated by the dextran/charcoal method. A further competition experiment was performed to investigate whether agonist and antagonist bind to the same site. Cytosol was incubated with 10 nM-[^{3}H]aldosterone or -[3H]spironolactone and 1 μM-RU486 in the absence or presence of either H10E (ascites fluid diluted 1:100) or the corresponding unlabelled steroid. After 4 h at 4 °C, bound and free steroids were separated by the dextran/charcoal method.

Kinetic experiments

Cytosol, prepared in TEGW buffer and treated or not with dimethyl pimelimidate, was incubated with 10 nM-[^{3}H]steroid (spironolactone, aldosterone or progesterone) in the presence of 1 μM-RU486 for 4 h at 4 °C to reach steroid-binding equilibrium. The cytosol was divided into several samples. One was maintained at 4 °C to measure the stability of the steroid-binding sites, and the others were incubated with 1 μM unlabelled steroid or with the monoclonal anti-idiotypic antibody H10E (1:100 diluted ascites fluid) for various periods of time. After each incubation period, bound and free steroid were separated by the dextran/charcoal method. Parallel incubations containing [^{3}H]steroid in the presence of a 100-fold excess of the corresponding unlabelled steroid were carried out to calculate the non-specific binding.

The dissociation rate constant (k_{-1}), and the half-life of the steroids–receptor complexes ($t_{\frac{1}{2}}$) were calculated from the equation $B(t) = B(0)\ e^{-k_{-1}t}$, where $B(0)$ and $B(t)$ represent the specific steroid binding at times zero and t respectively of the dissociation period. $B(t)$ is corrected by taking into account the stability of steroid binding at each dissociation time point.

Glycerol gradient centrifugation

Cytosol prepared in TEG or TEGW buffer was incubated for 4 h at 4 °C with 10 nM-[^{3}H]steroid and 1 μM-RU486 in the presence or absence of the corresponding unlabelled steroid. Samples (usually 100 μl) of charcoal-treated cytosol were layered on top of 15–40% (v/v) glycerol gradients prepared in TE or TEW buffer. Gradients were prepared as six discontinuous layers, and were maintained at 4 °C for at least 2 h prior to use. Gradients were centrifuged for 18 h at 257000 *g* in a Beckman SW 60 rotor. Two-drop fractions were collected, by piercing the bottom of each tube, and were counted for radioactivity. Myoglobin (2 S), BSA (4.6S) and aldolase (7.9S) (from Sigma) were used as external standards. Gradient analysis performed in a VTi 80 rotor was run at 354000 *g* for 3 h at 4 °C. For this experimental procedure, a 5–20% sucrose gradient prepared in 20 mM-Tris/HCl/1 mM-EDTA/20% glycerol, pH 7.4, was used.

Miscellaneous

The protein concentration in the cytosol was determined by the Bradford method [18], using BSA as standard. In all experiments the cytosolic protein concentration was between 10 and 15 mg/ml. Radioactivity was measured in a Packard liquid scintillation spectrometer after addition of 5 ml of Picofluor-15 (counting efficiency 50%).

RESULTS AND DISCUSSION

To clarify the molecular mechanism of action of aldosterone antagonists, we first characterized the interaction of [^{3}H]-spironolactone with MR.

Characterization of spironolactone binding

The binding of [^{3}H]spironolactone to the MR was characterized in chick intestinal cytosol. In order to focus our study on the MR, all the experiments were performed in the presence of 1 μM-RU486, which prevents aldosterone binding to the glucocorticosteroid receptor [13]. Table 1 shows the [^{3}H]-

Table 1. Binding parameters at equilibrium of spironolactone and aldosterone in chick intestine cytosol

[^{3}H]Spironolactone and [^{3}H]aldosterone binding at equilibrium was measured after a 4 h incubation at 4 °C with the chick intestine cytosol, presaturated with 1 μM-RU486. The binding parameter values N (maximum number of specific binding sites), K_d (dissociation constant at equilibrium), β (constant of non-specific binding) and their confidence limits (intraexperimental S.D.) were calculated according to the computerized method described in [17]. A model with one class of specific binding sites and non-specific binding [$B = NU\ (K_d + U) + \beta U$] best describes the experimental data.

	N of (fmol/mg of protein)	K_d (nM)	β
Spironolactone	108±6	3.02±0.22	0.0280±0.0190
Aldosterone	164±3	0.69±0.05	0.0031±0.0002

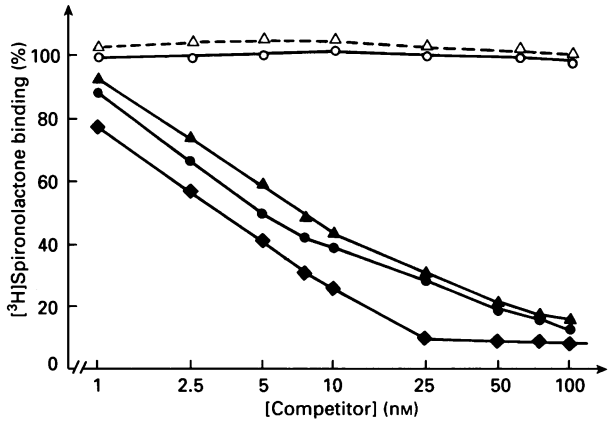

Fig. 1. Specificity of [^{3}H]spironolactone binding in chick intestine cytosol

Cytosol, prepared in TEGW buffer, was incubated with 1 μM-RU486 for 2 h at 4 °C to saturate glucocorticosteroid receptors. Aliquots of the RU486-presaturated cytosol were incubated with 10 nM-[^{3}H]spironolactone in the absence or presence of various concentrations of different steroids: aldosterone (◆), spironolactone (●); progesterone (▲), DHT (○) and oestradiol (△). Bound and free steroid were separated by dextran/charcoal treatment. Results are expressed as a percentage of the binding measured with [^{3}H]-spironolactone alone (~ 103 fmol/mg of protein). The values represent the means of the three separate experiments.

spironolactone and [^{3}H]aldosterone binding parameters at equilibrium. [^{3}H]Spironolactone binds to a single class of receptor with a K_d value of 3.02 nM and a maximum number of binding sites of 108 fmol/mg of protein. In addition, non-specific binding was also detected (see β value on Table 1). The maximum number of spironolactone-binding sites is close to that for aldosterone (164 fmol/mg of protein). The dissociation constant of spironolactone was approx. 5 times greater than that of aldosterone (K_d 0.69 nM).

The competition experiments reported in Fig. 1 show that aldosterone and progesterone, a natural aldosterone antagonist, were nearly as potent as spironolactone in inhibiting [^{3}H]-spironolactone binding. In contrast, DHT and oestradiol showed no affinity for [^{3}H]spironolactone-binding sites. These results complement our previous observation that another spirolactone (RU26752) has a high affinity for the chick intestine MR [13]. Our data are also in good agreement with those obtained in rat kidney [1,3] and in toad bladder [19].

Immunological identification of antagonist-binding site

It is difficult to conclude from the binding competition experiments whether agonist and antagonist bind to the same site or to distinct but interacting sites. Indeed, an allosteric model, in which the antagonist-binding site is different from that of the agonist, has been proposed for steroid receptors [20–22]. A monoclonal anti-idiotypic antibody (H10E) which interacts with aldosterone-binding domain of the rabbit MR [14] was an appropriate probe to map the binding site of aldosterone antagonists. As shown in Table 2, H10E specifically inhibited aldosterone binding to avian MRs. The cross-reactivity of H10E for the avian MR is not surprising since H10E, as an internal image of aldosterone, theoretically should interact with aldosterone-binding sites of the MR regardless of species. As can be observed in Table 2, [^{3}H]spironolactone binding to MR was also inhibited by H10E, suggesting that aldosterone and spironolactone bind to a common site that includes the epitope recognized by H10E.

The interaction of aldosterone and H10E with spironolactone-binding sites was further indicated by chase experiments, involving dissociation kinetic studies at 4 °C. As shown in Fig. 2, aldosterone was as effective as spironolactone in causing dissociation of [^{3}H]spironolactone from the MR. H10E was also able to displace [^{3}H]spironolactone from the receptor, with dissociation kinetics similar to those induced by unlabelled steroids. These results indicate that the inhibitory activity of H10E was not due to steric hindrance, but rather to direct binding to the ligand-binding site. Altogether, our results indicate that spironolactone and aldosterone interact with at least a common region of the ligand-binding domain of the receptor.

Dissociation kinetics of ligand–receptor complexes: comparison between agonist and antagonist

Despite the likely identity of the aldosterone and anti-mineralocorticosteroid binding sites on the MR, there may be discrete differences between the binding of antagonists and agonists within the steroid-binding domain, which could account in part for the antagonist properties of anti-mineralocorticosteroids. It has been proposed that the steroid-binding domain folds to form a hydrophobic pocket to which steroids bind [23]. Several amino acid residues of the hydrophobic surface interact with functional groups of the steroid backbone through a number of linkages to ensure appropriate binding [24–27]. With respect to the MR, it has been proposed that the binding of a steroid is the result of a tight association between the receptor and the steroidal A ring [28]. Steroid substitution studies have also indicated that the C-7 and the C-17 groups play a fundamental role in the interaction of the steroid with the receptor [29]. The antagonist properties of spirolactones may be due to steric interference caused by the lactone group. It is likely that agonists and antagonists differ by at least one of the linkages implicated in the interaction with the receptor. This should be revealed by differences in kinetic parameter values. We performed dissociation kinetic studies with aldosterone and two antagonists of different steroidal structure: spironolactone and progesterone. Their dissociation rate constants (k_{-1}) at 4 °C are reported in Table 3. As can be observed, the half-life of the aldosterone–MR complex ($t_{\frac{1}{2}}$ 3240 min) was about 50–100-fold greater than that of spironolactone ($t_{\frac{1}{2}}$ 38 min) and progesterone ($t_{\frac{1}{2}}$ 84 min). From these results, and from the comparison of the chemical structures of the ligands, it is likely that the C-21 hydroxy group and the C-18 aldehyde group of aldosterone, which are absent from both progesterone and spironolactone, are involved in the tight

Table 2. Effect of the anti-idiotypic antibody H10E on steroid binding to the MR

Cytosol was incubated for 4 h at 4 °C with 10 nM-[^{3}H]aldosterone or [^{3}H]spironolactone ([^{3}H]SC9420) plus 1 μM-RU486 in the absence or presence of H10E (1:100 diluted ascites fluid) or the corresponding unlabelled steroid (1 μM). After dextran/charcoal treatment, bound steroid was counted for radioactivity.

Addition	[^{3}H]Steroid binding (d.p.m./50 μl of cytosol)	
	[^{3}H]Aldosterone	[^{3}H]SC9420
None	11044±519	7663±329
H10E	2220±43	392±13
Corresponding unlabelled steroid	348±16	296±30

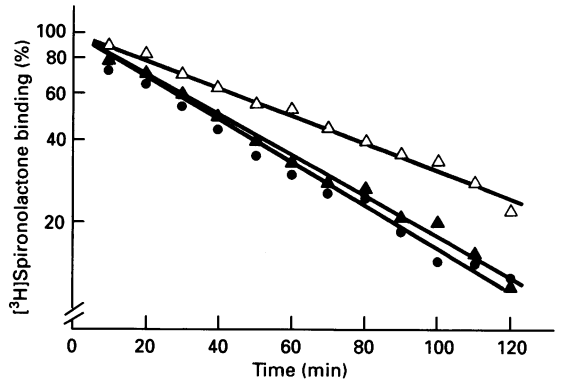

Fig. 2. Dissociation kinetics of [^{3}H]spironolactone–MR complexes

Cytosol prepared in TEGW buffer was incubated for 4 h at 4 °C with 10 nM-[^{3}H]spironolactone in the presence of 1 μM-RU486. This time represents the zero time for the kinetic study. [^{3}H]Spironolactone-labelled cytosol was divided into four aliquots. The first one was maintained at 4 °C to measure the stability of the receptor, and the three others were incubated with 1 μM-spironolactone (●), 1 μM-aldosterone (▲) or the anti-idiotypic antibody H10E (△) (diluted 1:100). Bound [^{3}H]spironolactone was measured at various periods of time after separation of bound and free steroids by dextran/charcoal treatment. Non-specific binding was measured for each incubation time period. Results were corrected by taking into account the dissociation of the receptor due to its instability, and expressed as a percentage of the binding measured at zero time (~ 90 fmol/mg of protein).

Table 3. Kinetic parameters of dissociation of aldosterone and anti-mineralocorticosteroids in chick intestine cytosol

Cytosol, prepared in TEGW buffer, was incubated for 4 h at 4 °C with 10 nM-[^{3}H]steroid ([^{3}H]aldosterone, [^{3}H]spironolactone or [^{3}H]progesterone) and 1 μM-RU486. The zero time for kinetic studies is taken after the 4 h incubation period. The labelled cytosol was either maintained at 4 °C in the absence of the further addition of steroids as a control for steroid–receptor stability, or with 1 μM of the corresponding unlabelled steroid. Dextran/charcoal treatment was performed after various incubation times periods. $t_{\frac{1}{2}}$ and k_{-1} were calculated as described in the Material and methods section. Results are means±S.D. except for progesterone, where $n = 1$.

	$t_{\frac{1}{2}}$ (min)	$10^{-2} \times k_{-1}$ (min^{-1})
Aldosterone ($n = 2$)	3240±169	0.021±0.001
Spironolactone ($n = 3$)	38±5	1.82±0.24
Progesterone	84	0.82

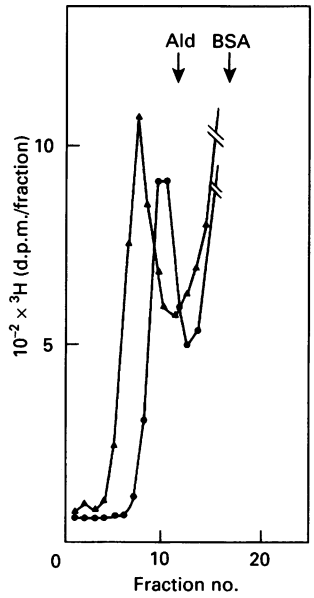

Fig. 3. Reactivity of the monoclonal antibody BF4 with spironolactone–MR complexes

Cytosol prepared in TEGW buffer was incubated for 4 h at 4 °C with 10 nM-[^{3}H]spironolactone and 1 μM-RU486. Aliquots of labelled cytosol (100 μl) were further incubated for 4 h at 4 °C with 100 μl of either BF4 (▲) or non-immune rat IgG (●). Charcoal-treated samples were then layered on a 15–40% glycerol gradient prepared in TEW buffer. Gradients were centrifuged for 18 h at 257000 *g* in a SW 60 rotor at 4 °C. Sedimentation markers are indicated for aldolase (Ald, 7.9S) and BSA (4.6S).

association between aldosterone and the MR. From our results it is tempting to propose that the anti-mineralocorticosteroid activity of an aldosterone antagonist could be related to its rapid dissociation kinetics. Such a mechanism has already been put forward to account for the anti-glucocorticosteroid activity of several compounds, including 17β-carboxamide derivatives of dexamethasone [30,31]. However, RU486 behaves differently, since it dissociates very slowly from the glucocorticosteroid receptor as compared with the agonist [32].

Hsp90 is part of the hetero-oligomeric structure of untransformed receptors. Its association with the receptor has been shown, at least for the glucocorticosteroid receptor [10], to be necessary to ensure a competent steroid binding site. To confirm that the rapid dissociation of [^{3}H]spironolactone was not secondary to the release of hsp90, we measured the dissociation rate of spironolactone from the MR in cytosol treated with dimethyl pimelimidate, which induces a covalent bond between hsp90 and the steroid-binding unit [13]. Under these conditions the half-life of the [^{3}H]spironolactone–receptor complex was not notably modified ($t_{\frac{1}{2}}$ 96 min), and remained far from that of aldosterone ($t_{\frac{1}{2}}$ 3240 min).

Influence of the ligand on the hsp90–receptor interaction

Our results indicated that agonists and antagonists bind to a common site but differ with respect to their dissociation kinetics. The question which remains is how the formation of a rapidly dissociating antagonist–receptor complex can lead to an inactive form of the receptor. Possibilities include a modification of the receptor conformation leading to an inactive transcriptional

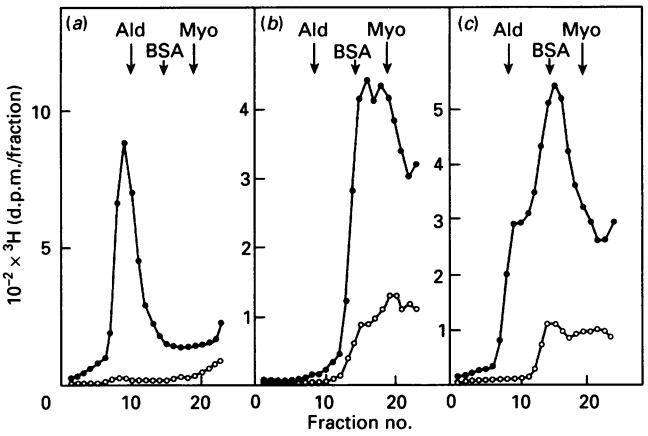

Fig. 4. Sedimentation gradient analysis of MR labelled with aldosterone and anti-mineralocorticosteroids

Cytosol prepared in TEG buffer was incubated for 4 h at 4 °C with 10 nM-[^{3}H]aldosterone (*a*), [^{3}H]spironolactone (*b*) or [^{3}H]progesterone (*c*), with 1 μM-RU486 to saturate glucocorticosteroid receptors, in the absence (●) or presence (○) of a 100-fold excess of the respective unlabelled steroid. After dextran/charcoal treatment a 100 μl aliquot of each cytosol preparation was layered on a 15–40% glycerol gradient prepared in TE buffer. Gradients were centrifuged for 18 h at 257000 *g* in a SW60 rotor at 4 °C. Sedimentation markers are indicated for aldolase (Ald, 7.9S), BSA (4.6S) and myoglobin (Myo, 2S).

complex, or stabilization of the receptor as a non-DNA-binding form. Such a mechanism has already been proposed for RU486 [33,34] and other non-steroidal anti-glucocorticosteroids [35]. To answer this question we examined the MR structure upon agonist and antagonist binding.

In a previous paper [13] we reported that, in sodium tungstate-containing buffer, the aldosterone–receptor complex was recovered associated with hsp90. A hetero-oligomeric structure of MR could be also observed under the same conditions when the receptor was labelled with spironolactone. This was evidenced by a 8.9S → 10.4S shift of the spironolactone–receptor complex induced by BF4, a monoclonal anti-hsp90 antibody (Fig. 3).

The effect of ligand on hsp90–receptor interaction was further examined using glycerol gradients under free oxyanion conditions. When the cytosol and the glycerol gradient buffers were prepared in the absence of sodium tungstate, aldosterone-labelled receptors sedimented in the 8–9S region (Fig. 4*a*). In contrast, both [^{3}H]spironolactone– and [^{3}H]progesterone–receptor complexes sedimented as specific 4S peaks (Figs. 4*b* and 4*c*). The release of hsp90 from the antagonist-bound MR was time-dependent, since it was observed after 18 h (centrifugation in a SW60 rotor) but not after 3 h (centrifugation in a VTi 80 rotor) (results not shown). This hsp90 release is inhibited by oxyanions (compare Figs. 3 and 4*b*). Our results indicate that, in contrast to aldosterone, spironolactone and progesterone are unable to maintain the hetero-oligomeric structure even under low-salt conditions. They also strongly support a major role of the ligand in modulating the interaction between hsp90 and MR, and suggest that the steroid-binding domain of the MR is implicated in the interaction with hsp90. This is in good agreement with studies on the glucocorticosteroid receptor [36–39], the progesterone receptor [40] and the oestrogen receptor [41], which have reported that the ligand-binding domain of the receptor is involved in the interaction with hsp90.

The aim of the present study was to gain a better understanding of the molecular basis of the anti-mineralocorticosteroid actions of aldosterone antagonists. In the chick intestine cytosol, spironolactone and aldosterone bind to a common site, as indicated by inhibition of [^{3}H]spironolactone binding by aldosterone and H10E antibody. Kinetic studies revealed a clear-cut difference in the dissociation rate between aldosterone and its antagonists. The half-life of antagonist–MR complexes was ~ 100-fold shorter than that of aldosterone–MR complexes. Finally, spironolactone and progesterone induce a weaker interaction between hsp90 and the steroid-binding subunit than does aldosterone.

In conclusion, aldosterone antagonists dissociate rapidly from MR, and *in vitro* their binding facilitates the release of hsp90 from the receptor complex. Whether these events occur *in vivo*, and how they are involved in inactivating the receptor to promote gene transcription, remain to be established. Deprived of both antagonist and hsp90, the receptor may undergo inactivation as a result of transconformational and/or proteolytic mechanisms. This may result in turn in a defect in binding to DNA hormone response elements, an inability to form active homodimers, and/or inappropriate interactions with other constituents of the transcriptional machinery.

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