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Correlation between pregnanesteroid conformation, receptor affinity, and anti-natriuretic effect

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Abstract

The aim of this study was to correlate mineralocorticoid action and steroid structure. Inasmuch as Na^+ retention follows a parabolic dose–response curve for most pregnanesteroids, the second-order coefficient of the function was used as a representative factor for this bipartite biological effect. The C_3=O/D angle of the ligands was correlated with both Na^+-retaining activity and binding affinity for the mineralocorticoid receptor. Because some steroids exhibit identical functional groups and different conformational structure, we also postulate that the flat conformation of a pregnanesteroid determines its Na^+-retaining capacity in vivo. No correlations were found in vitro, which demonstrates the multifactorial nature of the second-order coefficient determined in vivo under more complex and interactive conditions that include various pre-receptor variables. These findings may allow the estimation of the putative biological activity of a given steroid simply by knowing its conformational structure, which may be important for designing compounds in a pharmaceutical setting.

Keywords: Mineralocorticoid receptor; Natriuresis; Aldosterone; Steroid conformation

1. Introduction

Mineralocorticoids are steroid hormones that enhance the reabsorption of Na^+ and also affect the transport of hydrogen and potassium ions, although these two effects appear to be independent of Na^+ transport (Young, 1988; Bastl and Hayslett, 1992) and are quantitatively less significant. The most potent of the naturally occurring corticosteroids, aldosterone, exerts its action by binding to the mineralocorticoid receptor. Native rat kidney mineralocorticoid is a phosphoprotein (Galigniana, 1998) primarily localized in the cytoplasmic compartment of renal cells in the absence of steroid, as is also the case for the human mineralocorticoid receptor (Alnemri et al., 1991; Robertson et al., 1993). Upon ligand binding, the mineralocorticoid receptor rapidly translocates to the nucleus ($t_{1/2} \approx 6-7$ min) (Galigniana, 2000) in a process where serine/threonine phosphatases seem to play a crucial role (Piwien-Pilipuk and Galigniana, 1998). In the nucleus, the steroid–receptor complex mediates its biological action by binding to the promoter region of target genes and recruitment of other factors (reviewed in Farman and Rafestin-Oblin, 2001).

Ligand bioavailability is important for receptor activation. Most steroids bind to plasma proteins, which constitute a transport system and also a reservoir diminishing the bioavailability of free ligand. Nonetheless, binding to these large-capacity and low-affinity acceptors cannot explain per se the specificity of mineralocorticoids as evidenced by using both in vivo (Sheppard and Funder, 1987) and in vitro models (Lombès et al., 1994). It became evident that the bioavailability of ligands for the mineralocorticoid receptor is mainly regulated by metabolic, pre-receptor factors. Thus, glucocorticoid hormones can also behave like mineralocorticoids in epithelial tissues if they are not excluded from competition for the receptor by the inactivating action of the enzyme 11β-hydroxysteroid dehydrogenase (Funder et al., 1988). Despite the fact that this enzyme plays a key role in discriminating glucocorticoid from mineralocorticoid ligands, a growing body of evidence (Lombès et al., 1994; Funder, 1998, 2000) raises the

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question of whether this enzyme alone can fully determine the activity of ligand-bound mineralocorticoid receptor, which suggests that other regulatory mechanisms may also exist. Moreover, the selective recruitment of co-activators or co-repressors by the steroid-activated receptor is also a relevant modulatory mechanism for selective transcription of specific genes (Farman and Rafestin-Oblin, 2001). Such an intricate and complex molecular mechanism of action for the mineralocorticoid receptor has made the structural requirements of an ideal mineralocorticoid agonist extremely difficult to define, and to date no relationship between ligand structure and biological activity has ever been demonstrated.

Nonetheless, the first step in the molecular mechanism of action of any ligand is the binding to its cognate receptor, and certain structural properties of the hormone are required to properly activate the receptor. The observation that typical glucocorticoids show a more angled steroid nucleus at the A/B ring junction than aldosterone led to postulate that mineralocorticoids may require a flat conformation for optimal activity (Lantos et al., 1981). Based on that premise, the highly planar pregnanesteroid 11,19-oxidoprogesterone and its bent isomer 6,19-oxidoprogesterone were synthesized to study their mineralocorticoid properties. 11,19-Oxidoprogesterone is a selective mineralocorticoid receptor ligand and as potent a mineralocorticoid as 11-deoxycorticosterone, whereas its bent counterpart, 6,19-oxidoprogesterone, is devoid of both biological activity and affinity for the mineralocorticoid receptor (Galigniana et al., 1993).

It is classically accepted that certain critical functional groups enhance mineralocorticoid potency, for example, a C_{21}-hydroxyl (Koshida et al., 1990) or a functional C_{18}-methyl as in the case of 18-vinylprogesterone [Souque et al., 1995). Interestingly, 11,19-oxidoprogesterone lacks those functional groups, its main characteristic being overall conformational planarity. On the other hand, the biological properties of some pairs of compounds such as 11,19-oxidoprogesterone (**II**)/6,19-oxidoprogesterone (**XXII**) or 5α-di*H*-progesterone/5β-di*H*-progesterone (**XIII**) are dissimilar (Burton et al., 1995; Fig. 2). Because these compounds possess exactly the same functional groups but differ in their conformational properties, it suggests that a flat conformation of a given ligand may be more important than certain functional groups for the acquisition of mineralocorticoid activity. To explore this hypothesis, in a previous study, we extended the analysis to a group of eight 21-deoxysteroids and compared them to aldosterone and 11-deoxycorticosterone (Burton et al., 1995). Even though these steroids show a tendency to improve the mineralocorticoid biological effect with increasing planarity, such a tendency was not entirely convincing, in particular, for the second most potent physiological mineralocorticoid, 11-deoxycorticosterone. In part, this may be due to the limited number of steroids studied, which made the analysis uncertain.

The question then arose whether the tentative "planarity rule" suggested for 21-deoxysteroids applies for most 21-deoxypregnanesteroids and might also be extended to 21-hydroxypregnane-steroids. The potential inclusion of the latter group of compounds is of particular interest since the presence of a 21-hydroxyl group is thought to be a critical requirement for the mineralocorticoid effect. Therefore, in this work, we analyzed a wide variety of natural and synthetic 21-deoxy and 21-hydroxysteroids. We measured the Na^+-retaining capacity and the affinity for the renal mineralocorticoid receptor of 33 pregnanesteroids with diverse geometrical parameters and functional groups, and correlated all these properties with the overall planarity of the steroidal skeleton.

2. Materials and methods

2.1. Chemicals

[1,2-^{3}H]Aldosterone (specific activity = 59.0 Ci/mmol) was from NEN Life Science Products (Boston, MA). RU28362 was a kind gift from Roussel-Uclaf (Romainville, France). Oxidopregnanes were a kind gift from Dr.Gerardo Burton (Brachet-Cota and Burton, 1990; Veleiro et al., 1995). All other steroids were from either Sigma (St. Louis, MO) or Steraloids (Newport, RI).

2.2. Geometry optimization for steroids

Geometry optimizations were carried out with the program PCMODEL (Serena Software). Semiempirical calculations were performed with HyperChem release 6.02 (Hypercube), using the AM1 method and the Polak–Ribiere optimization algorithm. All geometries were reoptimized twice to ensure convergence. Conformational searches were conducted to ensure that the most stable conformer was obtained. Best planes for groups of atoms were those defined by the primary and secondary inertial axes of the atoms involved; only carbon atoms were considered for the calculation of inertial axes.

2.3. Cell transfection and luciferase activity

African green monkey (*Cercopithecus aethiops*) COS-7 renal fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. The medium was replaced by Opti-MEM medium and, after 2 h of incubation, the cells were co-transfected for 1.5 h with 1 μg/ml phMR3750, 4 μg/ml MMTV-Luc, and 0.1 μg/ml RSV-β-galactosidase. The transfection mixture was prepared with Trans-Fast reagent (Promega, Madison, WI). The transfection medium was replaced by Opti-MEM medium supplemented with 3% v/v charcoal-stripped serum, and the cells were grown for an additional 48-h period. Steroids were added to the medium and both luciferase and β-galactosidase activities were measured in cell lysates after 4 h of incubation with hormone. Luciferase activity was normalized to β-galactosidase activity.

2.4. Bioassays

Animal handling followed the ethical guidelines approved by the Institutional Animal Care Committee of the University of Buenos Aires. Male Sprague–Dawley rats weighing 200–250 g underwent adrenalectomy 48 h prior to the experiments and were maintained on Purina chow (Diet 1), and 0.9% NaCl and fresh water ad libitum. Food was removed the previous night and liquids were removed 4 h prior to the experiment. Steroids were dissolved in ethanol/propylene glycol/0.9% NaCl (3:3:34) and injected into the thigh. Rats were simultaneously given subcutaneous injections of 3 ml of 0.9% NaCl. After 3 h, blood and urine samples were taken and the excretion rates for electrolytes and creatinine measured. Only vehicle was injected into the control group.

2.5. Binding assays

Adrenalectomized rats were sacrificed and bled by heart puncture. Ice-cold saline solution was perfused through the aorta and the renal artery until the kidneys were completely blanched. The organs were excised, decapsulated, and homogenized in two volumes of ice-cold buffer (25 mM Tris, 10 mM EDTA, 25% glycerol, 10 mM β-mercaptoethanol, 20 mM Na_2MoO_4, 0.1 mM phenylmethylsulfonyl fluoride, 2 IU/ml aprotinin, 30 μg/ml trypsin–chymotrypsin inhibitor, pH 7.4). The same buffer was used when COS-7 cells expressing mineralocorticoid receptor were used as a source of receptor. The homogenates were centrifuged at $67{,}000 \times g$ for 30 min at 0 °C, and the supernatant is referred to as cytosol. Binding assays were performed as previously described in detail (Galigniana and Piwien-Pilipuk, 1999). The relative binding affinity for steroid receptors was measured at 0 °C by competition between 5.0 nM [^{3}H]aldosterone and increasing concentrations of unlabeled steroids, in the presence of 1.0 μM RU28362 to prevent the possible binding of steroids to the glucocorticoid receptor. The nonspecific binding (~ 20–25% of total binding) was determined with 1000-fold excess of unlabeled aldosterone and subtracted from the total binding.

2.6. Statistical tests

Data were analyzed by one-way nonparametric analysis of variance followed by Kruskal–Wallis test.

3. Results

3.1. Structure of the steroids

Fig. 1 depicts the planar molecular structure for all steroids used in this work. As can be seen, a wide variety of compounds were tested. These include natural and synthetic steroids with known and dissimilar mineralocorticoid potency, as well as compounds without a previously studied biological effect. Thus, many of the steroids of the latter group were chosen based on their geometrical parameters after we performed a preliminary analysis of the structure–activity trend observed with the steroids of the former group. This approach allowed us to select steroids that covered a wide range of conformations and, even more importantly, it allowed us to estimate a priori the putative Na^+-retaining activity of the ligands.

In order to establish a representative parameter to evaluate steroid planarity, we calculated the angles A/D, A/BCD, and C_3 = O/D for each steroid according to the planes defined by the primary and secondary inertial axes of the corresponding carbon atoms, as follows: C_3, C_4, C_5, and C_{10} for A ring; C_5 to C_{17} for BCD rings; and C_{14} to C_{17} for D ring. Values are listed in Table 1.

3.2. Na^+-retaining activity

In order to evaluate the Na^+-retaining activity, we treated adrenalectomized male rats with several types of steroids in the range of 0.01–500 μg/100 g. Fig. 2 shows the dose–response curves for the anti-natriuretic effect of the compounds. For the sake of clarity, the curves were arbitrarily plotted on several panels to avoid overlapping of data. To group the compounds based on their biological activity, we analyzed all these curves by a multifactorial analysis of variance with two factors (ligand and dose) as shown in Table 2. The first group includes the most potent physiological mineralocorticoid, aldosterone (**I**), which reaches a maximum effect at a dose of approximately 1 μg/100 g, and the 21-hydroxysteroids 11-deoxycorticosterone (**IV**), 5α-di*H*-aldosterone (**V**), Δ^{11}-11-deoxycorticosterone (**VI**), and 19-*nor*-11-deoxycorticosterone (**VII**). Interestingly, two 21-deoxysteroids such as 21-*deoxy*-aldosterone (**III**) and 11,19-oxidoprogesterone (**II**) also cluster this group of potent Na^+-retaining steroids.

A second group of steroids, composed of the 21-deoxysteroids 5α-di*H*-progesterone (**XIII**) and 5β-di*H*-progesterone (**XIV**), shows maximum Na^+ retention at a dose of 10 μg, this maximum effect being undistinguishable from the saturating effect measured with aldosterone. However, there are important differences between these two groups at low doses, where the reduced progesterone derivatives exhibit a weaker effect. In addition, there are also differences between both groups at high doses. Thus, the maximum effect obtained with 5α-di*H*-progesterone (**XIII**) appears to reach a plateau between 10 and 75 μg, and a clear reversion of the Na^+-retaining effect is observed at doses higher than 100 μg. For the 5β-isomer, the maximum effect coincides with the 5α-isomer, but the reversion can be already observed at a dose of 30 μg. It should be noted that the tendency to reverse the Na^+ retention effect at higher doses, although less evident, is also present in the most active steroids that belong to the first group.

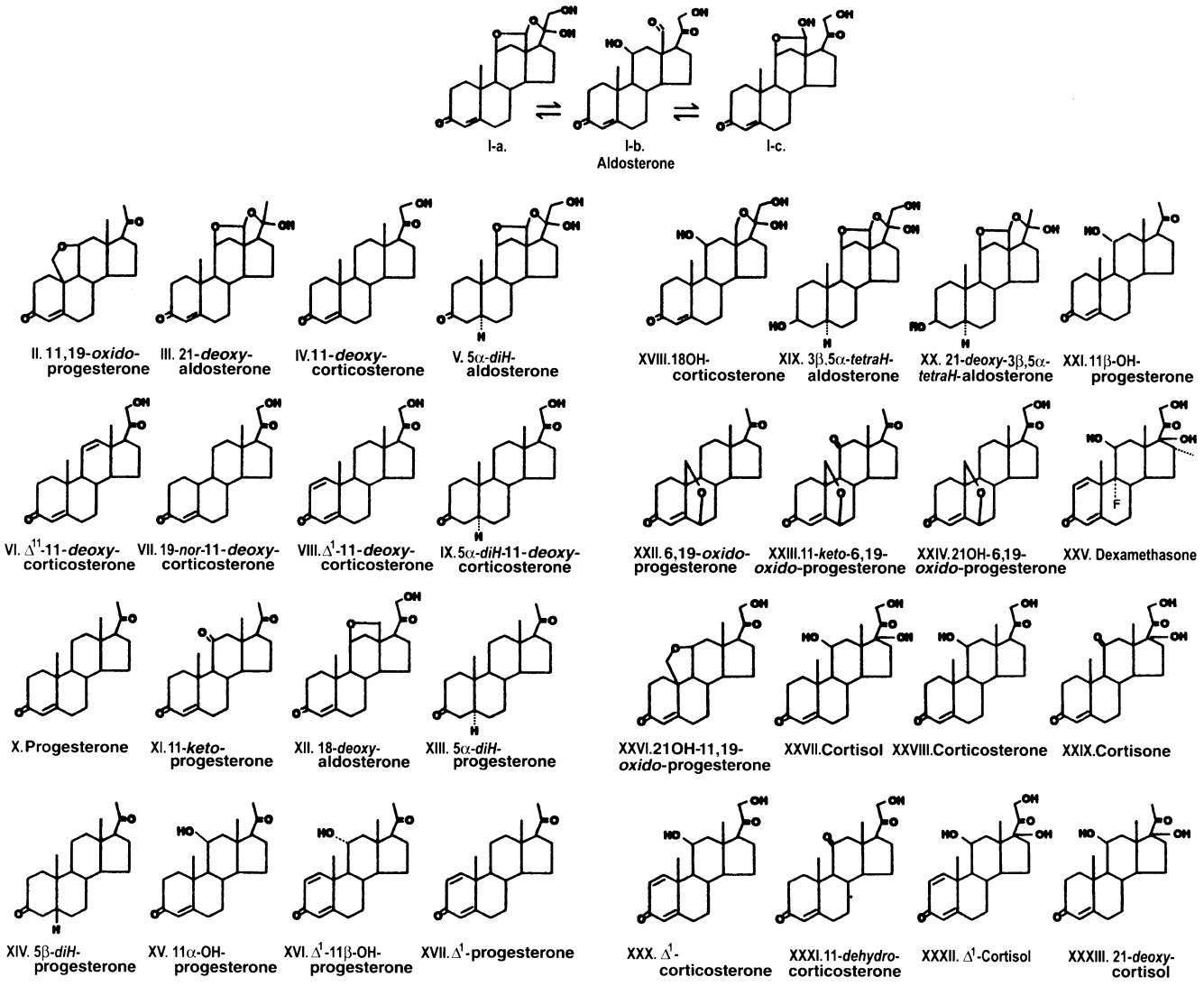

Fig. 1. Structure of the steroids assayed in this work.

A third group of steroids with a moderate potency includes Δ^1-11-deoxycorticosterone (**VIII**), 5α-di*H*-11-deoxycorticosterone (**IX**) and 18-*deoxy*-aldosterone (**XII**). These steroids show a clear bipartite function that reaches a maximum effect at 100 μg. Like the former group, a rapid loss of activity is also observed at higher doses.

A fourth group of compounds can be obtained by clustering steroids with poor mineralocorticoid action, so that the maximum biological effect cannot be reached. As a common feature, these steroids exhibit a dramatic parabolic function and also an almost complete reversion of the biological effect at high doses. The steroids that belong to this group are progesterone (**X**), 11-*keto*-progesterone (**XI**), 11α-OH-progesterone (**XV**), Δ^1-11β-OH-progesterone (**XVI**), Δ^1-progesterone (**XVII**), 18-OH-corticosterone (**XVIII**), 21OH-6,19-oxidoprogesterone (**XXIV**), 11β-OH-progesterone (**XXI**), 11-*keto*-6,19-oxidoprogesterone (**XXIII**), 6,19-oxidoprogesterone (**XXII**), 3β,5α-tetra*H*-aldosterone (**XIX**), and 21-*deoxy*-3β,5α-tetra*H*-aldosterone (**XX**).

Finally, a fifth group of steroids includes those steroids that do not exhibit the characteristic parabolic dose–response function observed by most of the others in the range of doses studied here, i.e. 21OH-11,19-oxidoprogesterone (**XXVI**), or steroids without substantial Na^+-retaining activity, i.e. dexamethasone (**XXV**), cortisol (**XXVII**), corticosterone (**XXVIII**), cortisone (**XXIX**), Δ^1-corticosterone (**XXX**), 11-*dehydro*-corticosterone (**XXXI**), Δ^1-cortisol (**XXXII**), and 21-*deoxy*-cortisol (**XXXIII**). Because the dose–response curves for steroids **XXX**, **XXXI**, and **XXXIII** are similar to those exhibited by corticosterone (**XXVIII**) and cortisol (**XXVII**), we do not show them in Fig. 2. It should be pointed out that, even though we could classify the steroids in these five groups, the statistical test based on multiple-range analysis (95% LSD intervals) did not clearly distinguish groups two and three.

Both plasma electrolyte concentration and endogenous creatinine clearance were not affected by the treatment with steroids. Because the creatinine clearance was con-

Table 1
Geometric parameters of the steroids used in this work, concavity of polynomial log dose–response curves, and relative affinity for the renal mineralocorticoid receptor

Steroid	Angle			*a*	RBA (nM)	
	A/D	A/BCD	C_3=O/D		−HAP	+HAP
(I) Aldosterone	−8.7	−21.1	−14.6	0.048	4±1	3±1
(II) 11,19-oxidoprogesterone	4.4	−3.7	8.9	0.015	56±2	45±5
(III) 21-*deoxy*-aldosterone	−8.7	−21.1	−15.1	0.029	6±4	4±2
(IV) 11-deoxycorticosterone	−22.2	−24.8	−28.3	0.030	6±1	4±1
(V) 5α-di*H*-aldosterone	11.4	−0.7	−12.1	0.035	7±2	5±3
(VI) $\Delta^{11,12}$-deoxycorticosterone	−15.2	−19.3	−21.3	0.051	10±4	6±2
(VII) 19-*nor*-deoxycorticosterone	−16.1	−19.7	−24.8	0.072	6±2	8±1
(VIII) $\Delta^{1,2}$-deoxycorticosterone	−33.9	−33.2	−31.3	0.155	30±3	17±2
(IX) 5α-di*H*-deoxycorticosterone	−7.5	−33.2	−32.0	0.231	21±4	14±2
(X) Progesterone	−21.6	−24.4	−27.3	0.295	40±2	31±5
(XI) 11-*keto*-progesterone	−21.3	−24.6	−26.5	0.479	30±2	25±7
(XII) 18-*deoxy*-aldosterone	−16.6	−24.2	−23.1	0.526	38±2	33±3
(XIII) 5α-di*H*-progesterone	−7.9	−8.7	−32.4	0.577	31±4	26±2
(XIV) 5β-di*H*-progesterone	−68.6	−69.8	−44.0	0.680	70±8	62±7
(XV) 11α-OH-progesterone	−21.5	−25.1	−29.9	0.766	89±6	92±9
(XVI) $\Delta^{1,2}$-11β-OH-progesterone	−35.0	−34.5	−32.2	0.950	66±7	45±6
(XVII) $\Delta^{1,2}$-progesterone	−31.2	−29.8	−27.0	0.999	39±4	33±6
(XVIII) 18-OH-corticosterone	−28.6	−38.4	−36.8	1.862	269±24	244±37
(XIX) 3β,5α-tetra*H*-aldosterone	8.9	−3.2	32.9[a]	2.012	1025±202	901±245
(XX) 21-*deoxy*-3β,5α-tetra*H*-aldosterone	8.4	−3.2	32.3[a]	2.402	1705±331	1378±233
(XXI) 11β-OH-progesterone	−24.1	−32.1	−29.8	2.438	501±50	71±9
(XXII) 6,19-oxidoprogesterone	−57.8	−57.6	−55.2	2.939	3206±367	2413±451
(XXIII) 11-*keto*-6,19-oxidoprogesterone	−57.5	−57.7	−54.6	3.713	2886±814	2264±334
(XXIV) 21OH-6,19-oxidoprogesterone	−57.6	−54.4	−53.0	4.571	24,155±1221	23,050±2222
(XXV) Dexamethasone	−35.6	−37.1	−35.0	N.D.	100±15	85±6
(XXVI) 21OH-11,19-oxidoprogesterone	4.4	0.7	8.9	N.D.	4742±780	4985±921
(XXVII) Cortisol	−26.9	−35.4	−34.2	N.D.	29±5	10±4
(XXVIII) Corticosterone	−26.2	−27.7	−33.5	N.D.	61±8	6±3
(XXIX) Cortisone	−23.5	−32.7	−30.2	N.D.	17±1	14±2
(XXX) $\Delta^{1,2}$-corticosterone	−36.6	−36.4	−35.1	N.D.	13±2	8±2
(XXXI) 11-*dehydro*-corticosterone	−22.4	−31.2	−28.7	N.D.	40±3	24±5
(XXXII) $\Delta^{1,2}$-cortisol	−37.4	−37.2	−36.2	N.D.	89±9	70±6
(XXXIII) 21-*deoxy*-cortisol	−26.7	−35.0	−34.2	N.D.	1233±218	777±102

Angles for the most stable conformers were obtained from AM1 calculations after projection onto a reference plane defined by the secondary and tertiary axes of atoms C_5 to C_{17}. Log dose–response curves for the steroids were fit to the second-order polynomial of the function $y=ax^2+bx+c$. The second-order coefficient *a* is representative of the concavity of the function exhibited by the biological effect in vivo. The relative binding affinity (RBA) for the mineralocorticoid receptor was measured by competition curves of each steroid with [^{3}H]aldosterone in crude kidney cytosol (−HAP) or in CBG-free preparations obtained by cytosol adsorption on hydroxylapatite gels (+HAP).
N.D.: not determined.
[a] Indicates the C_3-OH angle.

stant in all cases, the observed variations on Na^+ elimination cannot thereby be assigned to changes in the glomerular filtration rate by any given steroid. On the other hand, we also measured the kaliuretic effect of the steroids (not shown), and significant increases were found for the most potent Na^+ retainers (e.g. aldosterone, 11-deoxycorticosterone, 11,19-oxidoprogesterone, 5α-di*H*-progesterone, etc.). However, dispersions were generally higher than those measured for the natriuretic effect and no parabolic response functions could be demonstrated, so that no correlations were found for this effect. These properties agree with the relative independence of K^+ elimination with respect to the anti-natriuretic effect (Lantos et al., 1981; Bastl and Hayslett, 1992; Yang, 1988).

3.3. Biological activity and structural properties of the steroids

The biphasic function of the dose–response curves obtained in vivo did not allow the use of a classical EC_{50} value to quantify the biological effect because it does not consider the multiple parameters involved in the parabolic function, such as doses at which the maximal retention is achieved, the magnitude of this maximal response, the minimal active dose, and more importantly, the reversion of the effect observed at higher doses.

The dose–response curves show a trend indicating that the decreased Na^+-retaining activity for a given steroid parallels the presence of a counter-effect. This is mainly evidenced in dose–response curves of intermediate Na^+

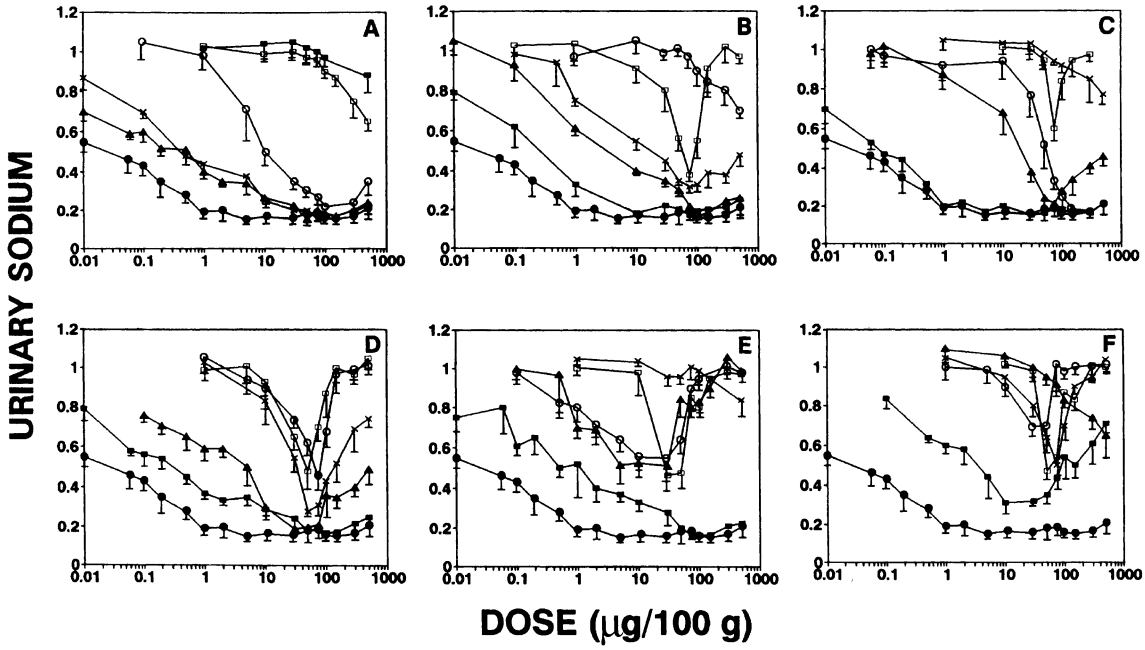

Fig. 2. Dose–response curves. Urinary Na^+ elimination was measured after injecting adrenalectomized male rats with the indicated doses of steroid expressed in μg of steroid per 100 g of rat body weight. Results are normalized to steroid/vehicle Na^+ elimination ratio. Each point is the mean ± S.E.M. of three experiments, in which one, 8–12 animals were used per dose. Aldosterone (●) was included in all panels for comparative purposes. Symbols represent the following steroids: A: (○) 5α-di*H*-11-deoxycorticosterone; (□) cortisol; (■) Δ^1-cortisol; (▲) 11-deoxycorticosterone; (×) 5α-di*H*-aldosterone. B: (○) cortisone; (□) 18-OH-corticosterone; (■) 19-*nor*-11-deoxycorticosterone; (▲) Δ^1-11-deoxycorticosterone; (×) 3β,5α-tetra*H*-aldosterone. C: (○) 21OH-11,19-oxidoprogesterone; (□) 21OH-6,19-oxidoprogesterone; (■) Δ^{11}-11-deoxycorticosterone; (▲) 18-*deoxy*-aldosterone; (×) dexamethasone. D: (○) Δ^1-11β-OH-progesterone; (□) 11β-OH-progesterone; (■) 11-OP; (▲) 5α-di*H*-progesterone; (×) 11α-OH-progesterone. E. (○) 11-*keto*-progesterone; (□) 11-*keto*-6,19-oxidoprogesterone; (■) 21-*deoxy*-aldosterone; (▲) progesterone; (×) 21-*deoxy*-cortisol. F. (○) Δ^1-progesterone; (□) 6,19-oxidoprogesterone; (■) 5β-di*H*-progesterone; (▲) corticosterone; (×) 21-*deoxy*-tetra*H*-aldosterone. For comparative purposes, the same dose–response curve for aldosterone was plotted in each panel.

retainers, whereas strong Na^+ retainers exhibit curves whose concavities are incipient at very high doses. On the other hand, the weakest Na^+ retainers lack concavities, although for a different reason, they are ineffective mineralocorticoids up to doses of the drug under these standard experimental conditions.

Therefore, in four of the five groups, such a deviation from a classical drug–response curve is strongly linked to a

Table 2
Multiple range analysis for in vivo response to steroid

Steroid	Homogeneous group	Steroid	Homogeneous group
(I) Aldosterone	a	**(XV)** 11α-OH-progesterone	d
(VI) $\Delta^{11,12}$-11-deoxycorticosterone	a	**(XVI)** $\Delta^{1,2}$-11β-OH-progesterone	d
(II) 11,19-oxidoprogesterone	a, b	**(XVII)** $\Delta^{1,2}$-progesterone	d
(III) 21-*deoxy*-aldosterone	a, b	**(XXI)** 11β-OH-progesterone	d
(IV) 11-deoxycorticosterone	a, b	**(XXII)** 6,19-oxidoprogesterone	d
(V) 5α-di*H*-aldosterone	a, b	**(XXIII)** 11-*keto*-6,19-oxidoprogesterone	d
(VII) 19-*nor*-11-deoxycorticosterone	a, b	**(XXIV)** 21-OH-6,19-oxidoprogesterone	d
(XIII) 5α-di*H*-progesterone	a, b, c	**(XXVI)** 21-OH-11,19-oxidoprogesterone	d, e
(VIII) $\Delta^{1,2}$-11-deoxycorticosterone	b, c	**(XXV)** Dexamethasone	f
(IX) 5α-di*H*-11-deoxycorticosterone	b, c	**(XXVII)** Cortisol	f
(XII) 18-*deoxy*-aldosterone	b, c	**(XXVIII)** Corticosterone	f
(XIV) 5β-di*H*-progesterone	b, c	**(XXIX)** Cortisone	f
(XIX) 3β,5α-tetra*H*-aldosterone	c, d	**(XXX)** $\Delta^{1,2}$-corticosterone	f
(XX) 21-*deoxy*-3β,5α-tetra*H*-aldosterone	c, d	**(XXXI)** 11-*dehydro*-corticosterone	f
(XVIII) 18-OH-corticosterone	c, d	**(XXXII)** $\Delta^{1,2}$-cortisol	f
(X) Progesterone	d	**(XXXIII)** 21-*deoxy*-cortisol	f
(XI) 11-*keto*-progesterone	d		

The dose–response curves shown in Fig. 2 were analyzed by multifactorial analysis of variance, with the response as a variable and the dose and steroid as factors. Multiple-range analysis was by 95% LSD intervals. Homogeneous group of steroids is shown with the same letter.

reversion of the effect, or perhaps more accurately, a suppression of the stimulant effect trigged by the agonist. Whatever the reason is, the shapes of the dose–response curves are biphasic for most of the ligands. Thus, the salt-retaining response can be adjusted to the second order polynomial of the function defined by the equation $y = ax^2 + bx + c$. The dose range for the fitting was selected so that the individual responses differed significantly from controls, exhibiting a correlation coefficient greater than 0.85. The values obtained for the second-order coefficient *a* (see Table 1) are a direct measure of the concavity of the polynomials which, in turn, represent the biopharmacological parameters of the dose–response curves obtained with each steroid.

Fig. 3 shows the correlation between various parameters representing the steroid geometry and the biological effect expressed as coefficient *a*. We plotted the A/D angle (Fig. 3A), the A/BCD angle (Fig. 3B), and the C_3=O/D angle (Fig. 3C) against the coefficient *a* calculated from the dose–response curves depicted in Fig. 2. Regardless of the functional groups present in the molecule, the steroids exhibit a clear tendency to improve the Na^+-retaining activity with the overall planarity of the ligand which, in turn, was one of the goals of this work. However, some steroids escape from this correlation and the data appear dispersed if either A/D (Fig. 3A) or A/BCD (Fig. 3B) angles are considered. This is particularly obvious in panels 3A and 3B for the highly bent progesterone derivative 5β-di*H*-progesterone (**XIV**). Nevertheless, all steroids align in a linear function when the C_3=O/D angle is plotted (Fig. 3C). Attempts to fit the linear function shown in Fig. 3C into a regression line reveal a highly significant correlation factor (r=0.75) for an in vivo response. When 11,19-oxidoprogesterone, a steroid that exhibits a slightly positive angle bending toward the α face, is omitted from this regression, the correlation factor r is 0.82 (the implications of considering this synthetic steroid will be discussed later). Importantly, no significant differences for such a linear correlation were found when 21-deoxysteroids (r=0.75) were compared to 21-hydroxysteroids (r=0.85).

The correlation shown by these plots suggests a relevant role for the orientation of the C_3=O group in recognition of the ligand by the specific receptor protein and, most likely, the importance of this group for anchoring the ligand in the hormone-binding pocket of the mineralocorticoid receptor. The findings of Fig. 3 provide a conclusive answer to our original question. A generalization for the "planarity rule" suggested for some 21-deoxysteroids may be extended to a wide variety of steroids, including the 21-hydroxylated ligands.

The notion stated above may even be applied (within certain limits) to the behavior of compounds possessing a C_3-OH group. Not shown in Fig. 3 are the corresponding 3β,5α-dihydro-derivatives of aldosterone (compounds **XIX** and **XX**). These steroids possess a framework highly bent towards the α face, so they have a positive rather than a

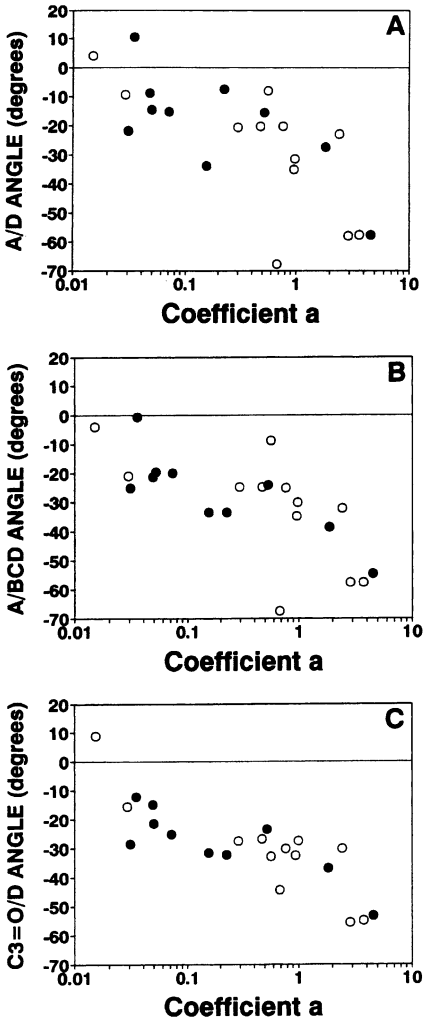

Fig. 3. Geometric parameters of the steroids vs. the second order coefficient calculated from the log dose–response curves depicted in Fig. 2. White circles represent 21-deoxysteroids and black circles represent 21-hydroxysteroids.

negative C_3=O/D angle (see Table 1). Interestingly, the second-order coefficient *a* calculated for these reduced aldosterone-derivatives approaches the values of the coefficient *a* calculated for 18-OH-corticosterone (**XVIII**) and 11β-OH-progesterone (**XVIII**). The latter pair of steroids bends towards the β face with a negative angle similar to the positive angle of compounds **XIX** and **XX**. In contrast to the other steroids analyzed herein, flexion towards the opposite face of the steroid is sterically possible for both compounds. Consequently, the pairs **XVII**/**XVIII** and **XIX**/**XX** show the same weak mineralocorticoid effect because the required planarity cannot be attained to efficiently trigger a significant biological effect.

3.4. Steroid geometry and relative affinity for the mineralocorticoid receptor

Fig. 4 depicts the relative binding affinities (RBA) measured in kidney cytosol (panels A, C, and E) or

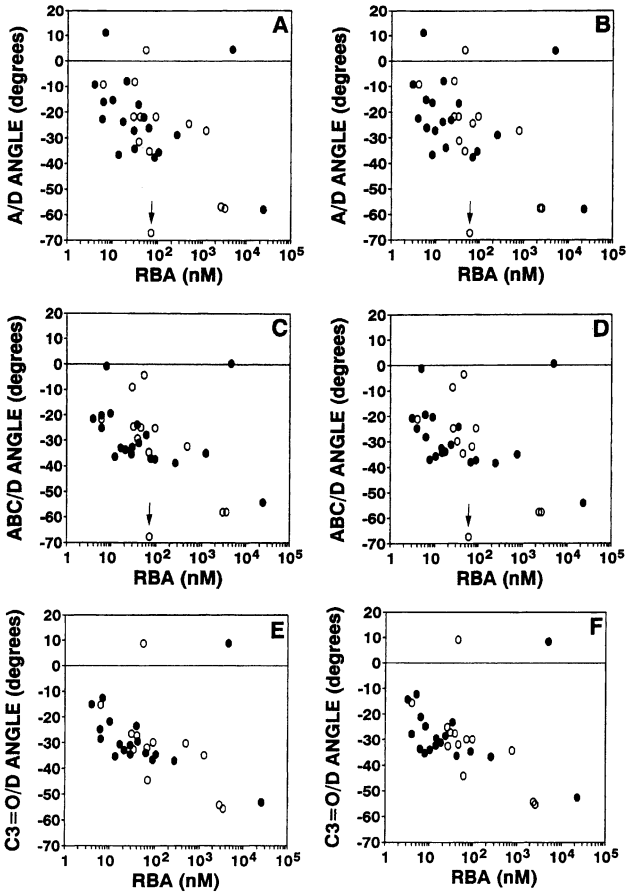

Fig. 4. Geometric parameters of the steroids vs. the relative binding affinity (RBA) for the renal mineralocorticoid receptor. Panels A, C, and E show the RBAs measured in untreated kidney cytosol, whereas panels B, D, and F show the RBAs measured in transortin (CBG)-free kidney cytosol. White circles are 21-deoxysteroids and black circles are 21-hydroxysteroids. The arrow in panels A to D shows the position of 5β-di*H*-progesterone (**XIV**).

transcortin (CBG)-free cytosol (panels B, D, and F) by competition of unlabeled steroid with [^{3}H]aldosterone. Paralleling the results obtained in Fig. 3, it can be seen that the affinity for the mineralocorticoid receptor increases with an increased planarity. Again, the $C_3=O/D$ angle (Fig. 4E and F) is as a better parameter than the A/D (Fig. 4A and B) and A/BCD (Fig. 4C and D) angles to align the steroids' geometry with their relative affinity for the mineralocorticoid receptor. Consistent with the data in Fig. 3, the highly bent 5β-di*H*-progesterone (**XIV**) clearly does not correlate in Fig. 4A and C (arrow), whereas it perfectly fits into such correlation if the $C_3=O/D$ angle is considered (Fig. 4E).

If the binding assay is performed in a CBG-free medium (Fig. 4B,D,F), similar conclusions can be reached regarding compound **XIV**. In this cytosol, some steroids undergo a pronounced shift to the left due to the absence of CBG competition. The most obvious relative binding affinity shift was observed for corticosterone (**XXVIII**) and 11β-OH-progesterone (**XXI**) (see Table 1).

Fig. 4E and F shows two clear exceptions from the correlation: the potent Na^+ retainer 11,19-oxidoprogesterone (**II**) and its weak 21-hydoxy derivative (**XXVI**). Both steroids are easily identified in the plot because of their positive angles. Inasmuch as we measure the *relative* affinity of each unlabeled steroid by competition with [^{3}H]aldosterone for binding to mineralocorticoid receptor, the exclusion of these two compounds from the correlation is not a surprise because these oxidopregnanes bind to mineralocorticoid receptor on either a second binding site different from the aldosterone binding site or, most likely, in the aldosterone-binding pocket but with a different orientation than the natural ligand (Galigniana et al., 2000; Piwien-Pilipuk et al., 2001). Therefore, if the binding of the competing steroid does not take place with the same positioning as the tracer, it may be entirely possible that the competition cannot be fully effective and the real affinity of both synthetic steroids for the receptor may be underestimated.

3.5. The logarithm of the biological effect is proportional to the mineralocorticoid receptor occupancy

We next plotted the second-order polynomial coefficient for Na^+ retention against the steroids' relative binding affinity for the renal mineralocorticoid receptor. Fig. 5A

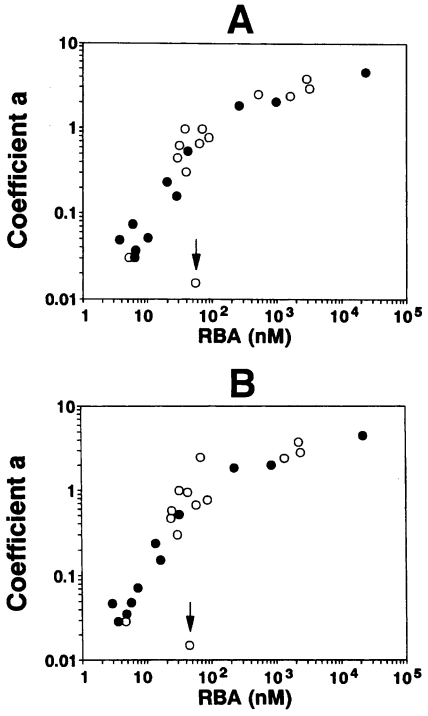

Fig. 5. Second-order coefficient calculated from the log dose–response curves vs. the relative binding affinity (RBA) measured in untreated cytosol (panel A) or transcortin (CBG)-free cytosol (panel B). The arrow shows the position of the synthetic steroid 11,19-oxidoprogesterone. White circles are 21-deoxysteroids and black circles are 21-hydroxysteroids.

and B (untreated cytosol and CBG-free cytosol, respectively) shows a clear hyperbolic relationship between the biological effect and the affinity for the receptor, where the more potent biological effect (lower *a* value) correlates with the higher affinity for the mineralocorticoid receptor. This function involves all the steroids listed in Table 1 that exhibit a measurable coefficient *a*, except 11,19-oxidoprogesterone (**II**), a steroid that is clearly excluded from the correlation (arrow). Again, the exclusion of compound **II** from the general function obtained for the other ligands can be explained by the fact that 11,19-oxidoprogesterone exhibits nonconventional binding property to the mineralocorticoid receptor. Nonetheless, this exemption is relevant because it somehow confirms the observation that the oxidopregnane does not bind to mineralocorticoid receptor like aldosterone does.

If we exclude the particular case of 11,19-oxidoprogesterone, all the steroids exhibiting normal competition properties with the tracer fit into the function. Importantly, this observation is valid for both natural and synthetic ligands, regardless of the presence of a C_{21}-hydroxyl group.

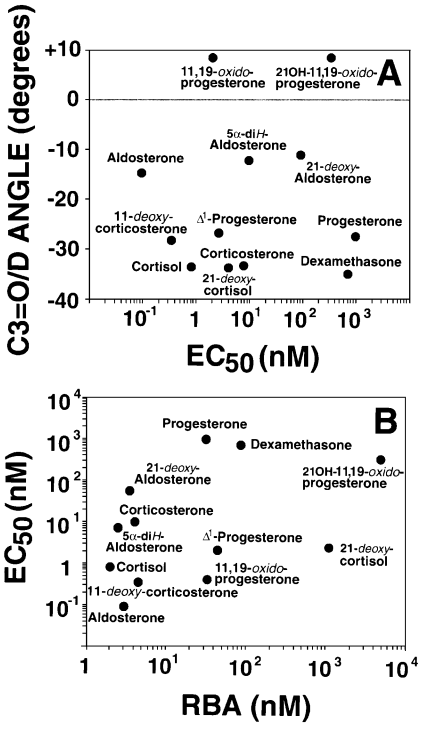

Fig. 6. Induction of luciferase activity. COS-7 cells were co-transfected with full-length human mineralocorticoid receptor, MMTV-Luc and β-galactosidase. Luciferase activity was induced in duplicate cultures by the indicated concentration of steroid, and its activity was measured in duplicate samples of each dish and normalized to β-galactosidase activity. The EC_{50} was plotted against the C_3=O/D angle of the ligand (panel A) and the relative binding affinity (RBA) for human mineralocorticoid receptor overexpressed in COS-7 cells (panel B).

3.6. Steroid conformation and mineralocorticoid receptor-dependent luciferase activity in COS-7 cells

We measured luciferase activity upon stimulation of COS-7 cells expressing full-length human mineralocorticoid receptor with various ligands. The concentration curves were carried out in the 10^{-12} to 10^{-7} M steroid range, and the EC_{50} values of the sigmoidal curves were plotted against the C_3=O/D angle (Fig. 6A) and the relative binding affinity for mineralocorticoid receptor (Fig. 6B). Two steroids also studied, Δ^1-11β-hydroxy-progesterone (**XVI**) and 6,19-oxidoprogesterone (**XXII**), were not included in these plots because their EC_{50} were $\gg 10^{-6}$ M. We would like to emphasize that, as expected, the EC50 values measured with transfected receptor were not different from those values measured with hydroxylapatite-adsorbed kidney cytosol.

In contrast to the in vivo correlations observed in Figs. 3C and 5, no relationship between ligand structure and biological response or receptor affinity was evidenced by the induction in vitro of the MMTV-Luc-gene reporter upon steroid-binding to mineralocorticoid receptor.

4. Discussion

There have been a wide variety of studies on the relationship between structure and activity of steroids (see Teutsch et al., 1995, and references therein). Nevertheless, little is known about the mechanism of binding of steroid agonists to the mineralocorticoid receptor and subsequent activation of the biological response. Thus, similar to the studies performed here with COS-7 cells, the transfection of CV-1 cells with mineralocorticoid receptor showed no correlation between the biological effect and the ligand structure (Grassy et al., 1997). In agreement with this observation, several other studies performed in vitro and in vivo have led to the conclusion that affinity for mineralocorticoid receptor cannot predict agonist or antagonist potency (reviewed in Agarwal and Mirshahi, 2000).This statement is correct because it was based on data analyzed by conventional methods. However, neither an in vitro experimental system nor a conventional method to analyze data obtained in vivo is suitable to study structure–activity relationships of mineralocorticoids. This may explain why no structure–activity correlations were found previously.

To solve the problem, we adjusted the shape of the log dose–response curves to a second order polynomial coefficient, and a correlation between steroid conformation, binding to mineralocorticoid receptor, and biological effect can now be evidenced. Therefore, a significant achievement of this study is the estimation for a given steroid of its affinity for mineralocorticoid receptor and/or its Na^+-retaining capacity by knowing the ligand structure. We extended to a large and heterogeneous population of

ligands the original notion that the C_3=O/D angle is the most suitable geometric parameter to predict the mineralocorticoid properties of a ligand among all of those parameters assayed, including the steroid hydrophobicity, hydration sphere, length of the molecule, total surface area, van der Waals radius, electronic density, etc. (not shown).

It should be emphasized that several factors are involved in the regulation of the biological response, i.e. binding to carrier proteins, metabolism to inactive and more active compounds, excretion rate, half life, etc., and all of them influence the final biological response. All these factors are implicitly considered when the coefficient *a* is calculated, which may explain why a correlation can be evidenced when several pre-receptor factors affect the steroid availability to the key switch that turn on the biological response, the mineralocorticoid receptor. Most of these pre-receptor factors are absent in the in vitro conditions used in the experiments shown in Fig. 6, as well as in similar studies carried out by other laboratories. Therefore, while a simplified assay system is certainly useful for dissecting the individual steps of the molecular mechanism of activation of the mineralocorticoid receptor, it is not suitable to evaluate the complex in vivo biological response. In this regard, a speculative but interesting observation is that if we approach the linear function followed by the steroids in Figs. 3C and 4E (angle vs. biological effect and angle vs. RBA, respectively) to the *y*-intercept, the value would represent the optimal angle of an ideal steroid which exhibits both optimal binding affinity for mineralocorticoid receptor and maximum Na^+ retention. The angle obtained from our data is -12.5 ± 3.7, this angle being similar to the C_3=O/D angle of the ketal form of aldosterone (**Ia**). If this speculation is valid, the coincidence between the geometry of an ideal steroid and the value exhibited by the most potent mineralocorticoid in nature would not be inappropriate from an evolutionary perspective.

An important conclusion extrapolated from this study is that the steroid conformation rather than certain functional groups appears to be a determining factor in terms of its Na^+-retaining properties. Of course, it is not possible to completely dissociate the presence of certain functional groups from the steroid conformation, but there are examples in which the conformers share identical chemical groups and dissimilar biological activity (e.g. 11,19-oxidoprogesterone (**II**)/6,19-oxidoprogesterone (**XXII**) and 5α-di*H*-progesterone (**XIII**)/5β-di*H*-progesterone (**XIV**)). Therefore, an explanation for this feature can be found in the overall conformation of the ligand. On the other hand, it should be pointed out that the divergent features of some pairs of compounds also undermine the functional importance of the C_{21}-OH function. Thus, progesterone (**X**) is a weak mineralocorticoid whereas its 21-hydroxylated derivative, 11-deoxycorticosterone (**IV**), is a strong one. However, 11,19-oxidoprogesterone (**II**) exhibits potent Na^+-retaining properties (comparable to 11-deoxycorticosterone, and even to aldosterone at higher doses), whereas the introduction of a 21-hydroxyl group (**XXVI**) greatly reduces the biological activity.

From the data shown in Fig. 4, it can be inferred that not only is the presence of a C_3-carbonyl group critical for optimal anchoring of the ligand in the steroid-binding pocket, but even more importantly, its orientation is essential for the optimal activation of the mineralocorticoid receptor (Fig. 3C). Because binding greatly improves when the torsional angle of the C_3-carbonyl flatters with respect to the D-plane, it appears that this contacting area is a tight cavity. The relevance of the steroid's C_3-carbonyl agrees with recent reports indicating that Gln-776 and Arg-817 residues in the human mineralocorticoid receptor interact with the 3-keto group of the steroid (Fagart et al., 1998).

On the other hand, the α face of the steroid appears to interact with a more spacious area within the steroid binding pocket. This is implied by the observation that several compounds, which exhibit good mineralocorticoid properties, also possess a relatively bulky group, e.g. the extra rings present in the ketal form of aldosterone (**Ia**) or the extra ring generated by the ether bridge of 11,19-oxidoprogesterone (**II**). Nevertheless, this region of the receptor appears to be less essential in activating the receptor because some 11-deoxysteroids also show good mineralocorticoid potency, e.g. 11-deoxycorticosterone (**IV**) and 5α-di*H*-progesterone (**XIII**).

As stated earlier, the C_{21}-hydroxyl group is not always essential for conferring mineralocorticoid properties. Although its importance seems evident when progesterone (**X**) is compared to 11-deoxycorticosterone (**IV**), the acquisition of Na^+-retaining properties of progesterone upon 5α-dihydrogenation (**XIII**) or, even more dramatically, the substantial loss of biological activity of the strong agonist 11,19-oxidoprogesterone upon 21-hydroxylation (**XXVI**) argue against the requirement of a C_{21}-OH group to improve the mineralocorticoid effect. On the other hand, all the previous concepts support the hypothesis that the overall conformation of the ligand is more critical than the presence of certain chemical groups.

Although our findings may be useful for drug design, some restrictions apply to the model. For example, (a) some steroids exhibit good steroid binding ability in a cell-free system, but they are totally devoid of biological effect in vivo in the wide range of doses used here, for example, corticosterone (**XVIII**) and cortisol (**XVII**). Both steroids are substrates for the enzyme 11β-hydroxysteroid dehydrogenase, which excludes them from competition with the receptor; (b) a steroid such as dexamethasone (**XXV**), which exhibits very weak affinity for the mineralocorticoid receptor and, consequently, is also a poor mineralocorticoid; (c) other steroids exhibit certain Na^+-retaining properties that can certainly be measured, but a counter-effect is not seen at the range of doses assayed here (e.g. 21OH-11,19-oxidoprogesterone (**XXVI**)), and so the second order coefficient *a* cannot be calculated. In spite of these limitations, the

alignments observed for most of the compounds in Figs. 3C, 4E, and 5A are clear and reflect a similar tendency for both 21-deoxy and 21-hydroxypregnanesteroids.

An interesting point to analyze is the effect of oxidation of the C_{11}-OH group of 11β-OH-progesterone (**XXI**), which transforms the compound into a more effective mineralocorticoid ligand. The relative binding affinity of 11-*keto*-progesterone (**XI**) is 16-fold higher than the hydroxylated form because the steric limitations enforced by the presence of the hydroxyl group are greatly reduced. Therefore, a higher flexibility in the 11-*keto* derivative allows the steroid to adapt better into the ligand-binding pocket of the receptor because the repulsion with C_{19} is greatly decreased. As a consequence, the ligand becomes flatter. Consistent with its higher affinity for mineralocorticoid receptor, 11-*keto*-progesterone also exhibits better Na^+-retaining properties. Interestingly, both steroids can be interconverted in a reversible fashion by 11β-hydroxysteroid dehydrogenase, and are then capable of inhibiting the normal metabolism of glucocorticoids, such that corticosterone (**XXVIII**) acquires potent Na^+-retaining activity in vivo (Galigniana et al., 1997).

Our studies are not focused on an important factor, the flexibility of the molecules, which may affect the ligand's adaptability to the receptor binding site; however, some predictions can be made. Like aldosterone, the structures of both oxidopregnanes (**II** and **XXII**) and their 21-hydroxylated derivatives (**XXIV** and **XXVI**) certainly predict a rigid steroidal frame. Therefore, the capacity of these steroids to adjust into the steroid binding pocket is limited by rigidity. Such rigidity (similar to aldosterone) associated to its flat conformation may explain the high specificity of 11,19-oxidoprogesterone (**II**) for the mineralocorticoid receptor, and why its 21-hydroxylated derivative (**XXVI**) behaves as a weak mineralocorticoid agonist. It is interesting to point out that the total length of 11,19-oxidoprogesterone is 11.38 Å (O_3–O_{21}), shorter than natural agonists, e.g. 12.45 Å for aldosterone and 12.30 Å for 11-deoxycorticosterone. Even though 11,19-oxidoprogesterone is certainly a flat steroid, it does not have the optimal C_3=O/D angle exhibited by aldosterone. However, its shorter length would allow the rigid frame of 11,19-oxidoprogesterone to adjust into the steroid binding pocket more easily than its 21-hydroxy derivative partner (12.23 Å). As a consequence, 11,19-oxidoprogesterone behaves as a strong mineralocorticoid, whereas 21OH-11,19-oxidoprogesterone (**XXVI**) is a weak Na^+ retainer despite possessing similar length and functional groups as natural agonists.

Although difficult to establish a reason beyond doubt, another interesting question to address is the shape of the log dose–response curves. A possible explanation may be the putative effect of activation of the glucocorticoid receptor at high concentrations of steroid, which may mitigate the salt-retaining effects of mineralocorticoid receptor activation. However, this hypothesis is unlikely in view of the fact that many steroids that exhibit a counter-effect at high doses are not efficient glucocorticoid receptor ligands (e.g. 5α-di*H*-11-deoxycorticosterone (**IX**), $\Delta^{1,2}$-progesterone, and 5α-di*H*-progesterone (**XIII**) in the 0.3–0.8 μM range), or they are not glucocorticoid receptor ligands even at concentrations higher than 2 μM (e.g. 6,19-oxidoprogesterone (**XXII**), 21OH-11,19-oxidoprogesterone (**XXVI**), 11,19-oxidoprogesterone (**II**), etc.). Moreover, no differences were observed in the shape of the dose–response curves when the anti-glucocorticoid RU486 was coinjected (data not shown) with steroids that exhibit an evident counter-effect (e.g. 18-OH-corticosterone (**XVIII**), 11β-OH-progesterone (**XXI**), 5 β-di*H*-progesterone (**XIV**), and 11,19-oxidoprogesterone (**XXII**)). Inasmuch as the biphasic effect is not unusual and can also be observed with other types of hormones under both in vivo and in vitro conditions, and due to the wide variety of steroids that exhibited this property in our bioassays (Fig. 2), the reason for such an effect may reflect a more basic and general mechanism. Recent studies have suggested a strong link between histone acetylation, chromatin remodeling, and gene regulation (reviewed by Struhl, 1998). Nuclear receptors induce a dramatic hyperacetylation of histones at the promoters of target genes in vivo. Interestingly, this hyperacetylation is transient and coincides with attenuation of hormone-induced gene activation even when hormone is still present in the medium (Chen et al., 1999). Therefore, an excess of hormone is capable of attenuating and even abolishing its own stimulatory effect. Whether or not these observations apply to the dose–response curves shown in Fig. 2 is uncertain and merely speculative at the present time.

Clearly, the endocrine system is an issue of evolution that has prompted today's biochemists to revise the old hypothesis that the hormone and its receptor could have been preexisting structures, the interaction of their cornerstones being necessarily the result of evolution itself. Indeed, the information for hormonal regulation is written not only in the hormone structure, but also in the receptor, so that both components function as a unit. In higher organisms, the nuclear receptor superfamily bears a close resemblance to its primordial predecessor. On the other hand, signaling molecules seem to have acquired their present role in a long evolutionary process, which may well sharp the separation between, for example, glucocorticoids and mineralocorticoids. Thus, it was key to mineralocorticoid physiology the emergence of aldosterone synthase (CYP11B2) since ketal/hemiketal groups are not substrates for 11β-hydroxydehydrogenase 2. Notably, the enzyme involved in the last step of aldosterone synthesis, aldosterone synthase, is highly homologous to the enzyme that catalyzes the last step in the production of cortisol, 11β-hydroxylase (CYP11B1). A pathological resemblance of this evolutionary process may be seen in the Glucocorticoid-Remediable Aldosteronism Syndrome (Dluhy, 2001).

Based on the structure–activity relationships found in our work, one may speculate that gradual changes in the

ligand conformation may have led to the acquisition of a specific mineralocorticoid effect during the transition process of adaptation to terrestrial life by changing the torsion of the steroid and/or the particular orientation of the C_3=O/D group with respect to the D ring. In same cases, these conformational changes may have been a critical requirement to generate a ''novel'' molecule sufficiently distinct to be recognized by separate receptors without a substantial alteration of the chemical structure. It might also be possible that some of the ligands resemble primordial ligands that are currently extinct or serve different function today.

In the facts, no single factor can be held solely responsible for the observed correlation between steroid structure and biological effect, as clearly seen in this study when the results obtained in vitro and in vivo are compared. Nonetheless, the evaluation of the entire dose–response curves by using the complex and multifactorial second-order coefficient *a* helps in the understanding of such an integrative phenomenon. Even when the elucidation of its causes and mechanisms at diverse levels still requires many interdisciplinary studies, it seems that the coefficient *a* measured from dose–response curves in vivo is the most representative parameter of the complete sequence of biological events leading to the final Na^+-retaining effect.

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