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Molecular mechanism of activation and nuclear translocation of the mineralocorticoid receptor upon binding of pregnanesteroids

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Abstract

The mineralocorticoid receptor (MR) is primarily localized in the cytoplasm of the cell in the absence of ligand. The first step in the genomic-dependent mechanism of action of mineralocorticoids is the binding of steroid to the MR, which in turn triggers MR nuclear translocation. The regulation of hormone-binding to MR is complex and involves a multifactorial mechanism, making it difficult to determine the optimal structure of a steroid for activating the MR and promoting its nuclear translocation. Here we review the structure–activity relationship for several pregnanesteroids that possess various functional groups, and suggest that a flat conformation of the ligand rather than the presence of particular chemical groups is a critical parameter for the final biological effect in vivo. We also discuss how the MR undergoes differential conformational changes according to the nature of the bound ligand, which in turn affects the dynein-dependent retrograde rate of movement for the steroid/receptor complex.

Keywords: Aldosterone; 11,19-Oxidoprogesterone; Natriuresis; Trafficking; Dynein; Immunophilins

1. The mineralocorticoid receptor

Genomic mineralocorticoid effects are mediated by the mineralocorticoid receptor (MR), which belongs to one of the most abundant classes of transcriptional regulators in metazoans, the nuclear receptor superfamily (Evans, 1988). As such, the MR not only functions as a receptor for a given ligand, but also as a transcriptional regulator.

The MR is expressed at the greatest abundance in the sodium-transporting epithelia such as the distal part of the nephron and the distal colon, as well as in sweat and salivary glands, the cardiovascular system, the central nervous system (particularly in the hippocampus), brown adipose tissue, and at a lower abundance in other tissues. The most potent natural mineralocorticoid agonist is aldosterone, its main chemical property being the presence of a hemiketalic ring that involves its aldhehyde in C_{18} (from which the name aldosterone derives). In epithelial tissues, aldosterone enhances the reabsorption of sodium and also affects the transport of hydrogen and potassium ions, although these two effects are known to be mechanistically independent of the anti-natriuretic action (Young, 1988; Bastl and Hayslett, 1992) and are quantitatively less significant.

Based on the sequence alignaments and phylogenetic analysis of both the DBD (DNA binding domain) and LBD (ligand binding domain), the members of the nuclear receptor superfamily were classified in a consensus tree defined by six subfamilies of receptors (Laudet, 1997). Thus, the receptors with steroid-binding ability comprise the same subfamily of highly homologous members, i.e. oestrogen receptor (ER), oestrogen-related receptor (ERR), androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor. In turn, based on their binding to a consensus DNA sequence, ERR and ER belong to the so-called ER-subgroup (they bind to an AGGTCA P-box sequence), whereas the GR, MR, AR and PR belong to the GR-subgroup (they bind to an AGAACA P-box sequence). Among the members of the nuclear receptor superfamily, the MR shows the highest percentage of sequence homology with its subgroup partner,

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the GR, not only when their DBDs are compared (94%), but also their LBDs (57%) (Evans, 1988). As a consequence, cross-reactions with ligands and hormone-response elements are expected.

Given the above-described scenario, it is not surprising the existence of cross-talks between "specific" ligands for a determined receptor and other members of the subfamily, as well as cross-talks of ligand/receptor complexes with the "specific" hormone-response elements. Therefore, it becomes evident that the specificity of the biological response must also be achieved by the combined effect of factors other than ligand/receptor and receptor/DNA recognition.

The MR is primarily located in the cytoplasm in the absence of hormone. The cytoplasmic form of the MR exists as a large heterocomplex that preserves the receptor in a transcriptionally inactive form and maintains its high-affinity state for the ligand. To a large extent, our knowledge of the heteromeric structure of most members of the steroid receptor subfamily is derived from early studies on the GR and the ER, both receptors being the first members to be cloned. It is now well established (Pratt and Toft, 1997; Morimoto, 2002) that steroid receptors are capable of forming heterocomplexes with the 90 and 70 kDa heat shock proteins (hsp90 and hsp70, respectively), the acidic protein p23, and proteins that posses a tetratricopeptide repeat sequence (TPR) such as FKBP52, FKBP51, Cyp40, PP5 or Hop/p60.

Independent of their primary localization in the cell, steroid receptors are not confined to one intracellular compartment in a static manner, but are capable of passing dynamically through the nuclear pore of hormone-free cells (DeFranco, 2002; Black et al., 2001; Vicent et al., 2002). Thus, when the equilibrium of this nucleocytoplasmic shuttling favours one or another compartment, it is said that the receptor is primarily located in either cytoplasm or nucleus. The classical theory to explain receptor trafficking was to assign the movement to a simple diffusion process. However, recent evidence supports the notion that the receptors may traffic towards the nucleus in an active manner, as we shall discuss later.

2. The mineralocorticoid effect is a multifactorial event

The mineralocorticoid effect on epithelial cells is an extremely complicated network of biochemical regulations that leads to the maintenance of electrolyte homeostasis. Two key protagonists in this plot are the agonist hormone, that broadcasts the information, and the receptor, that functions as a receiver and transducer, but the responsibility for the final biological effect is not limited entirely to them. The MR represents an important part of the story, whereas the ligand is another relevant part, so it can be stated that the information for hormonal regulation is written neither in the hormone nor in the receptor exclusively, but in both components of a complex functional unit. In turn, this functional unit may be subjected to other kinds of non-hormonal- and/or non-receptor-dependent regulations.

Despite being cloned 16 years ago (Arriza et al., 1987), many basic features of MR function and its regulation have yet to be fully characterised. One important question is how the MR can be selective for aldosterone when the circulating levels of glucocorticoids are two or three orders of magnitude greater. Given the fact that glucocorticoids exhibit high affinity for the MR, it was difficult to reconcile the specific biological effects shown in vivo by aldosterone in the presence of much higher circulating concentrations of glucocorticoids. An answer to this conundrum appeared to lie in the inactivating action of the enzyme 11β-hydroxysteroid dehydrogenase-2 (11βHSD-2) (Funder et al., 1988; Edwards et al., 1988). This enzyme plays a key role in discriminating glucocorticoids from mineralocorticoids by metabolising the former compounds to their inactive 11-dehydro-derivatives.

Nevertheless, a series of questions regarding the specificity of the MR remain still unanswered. It is known that the 11βHSD-2 does not co-localize with the MR in the hippocampus, such that the MR should be "unprotected" and presumably overwhelmingly occupied by glucocorticoids. However, specific aldosterone-dependent effects can still be seen. In addition, occupancy of the MR by cortisol or corticosterone does not mimic the effects of aldosterone in extraepithelial tissues where the receptor does not co-localize with the enzyme (Gómez-Sánchez et al., 1990; Sato et al., 1995; Young et al., 1994; Funder and Miles, 1996).

All of the above-described observations lead to the obvious conclusion that other factors must also be involved in the regulation of the mineralocorticoid effect. To begin with, the MR itself has intrinsic properties that discriminate between aldosterone and glucocorticoids. For example, even when the dissociation constant (K_d) for cortisol and aldosterone are the same, aldosterone/MR complexes show a more prolonged half-life than glucocorticoid/MR complexes due to a higher dissociation rate constant (k_{-1}) (Lombès et al., 1994). Moreover, the MR is capable of forming heterodimers with the GR, which can modulate transcription in a manner that is distinct from the GR and MR homodimers (Liu et al., 1995; Trapp et al., 1994). Also, the aldosterone-dependent effect can be antagonized by the PR (McDonnell et al., 1994) and the thyroid hormone receptor (Lim-Tio and Fuller, 1998), although it is still uncertain whether or not the MR can form heterodimers with these receptors.

The intranuclear distribution of the MR and the GR in hippocampal neurons shows a non-homogenous distribution, i.e. many clusters exclusively contain either MR or GR, although a number of domains were found to contain both receptor types (Van Steensel et al., 1996). In addition, interactions with specific co-activators and/or co-repressors may regulate differentially the transcriptional activity of both corticosteroid receptors. In this regard, it was recently reported that a ligand-selective regulation of the MR exists (Kitagawa et al., 2002), such that aldosterone-binding, but not cortisol-binding, recruits the RNA helicase A/

CREB-binding protein complex (RHA/CBP) to the AF-1a region and allows the cooperative potentiation of the MR transcriptional activity. Similarly, by chromatin immunoprecipitation assay it was demonstrated that aldosterone-binding, but not cortisol-binding, recruits the RHA/CBP complexes to native MR target gene promoters (Kitagawa et al., 2002).

Such intricate and complex molecular inter-relations have made the elucidation of the properties of an ideal ligand very difficult to determine, as well as making the comprehension of the molecular mechanism of regulation of the MR difficult.

3. Structure–activity relationship for the mineralocorticoid biological effect

As a consequence of the above-described intricate physiological and biochemical mechanism of action for the MR, the analysis of the structural requirements needed for an ideal mineralocorticoid agonist have always been extremely difficult to define. Actually, it is accepted that no correlation exists between ligand structure and biological effect. 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 must be required to properly activate the receptor. The observation that aldosterone possesses a poorly angled steroid nucleus at the A/B-ring junction led to postulate that mineralocorticoids may require a flat conformation for optimal activity in vivo (Lantos et al., 1981). To provide a graphic example, Fig. 1 depicts the structures of the most stable conformers for some of the pregnanesteroids we describe here. The most potent natural agonist, aldosterone, possesses an overall flat conformation as compared to others pregnanesteroids with a more angled steroid nucleus towards the α-face. Based on that premise, the highly planar pregnanesteroid 11,19-oxidoprogesterone (11-OP) and its bent isomer 6,19-oxidoprogesterone (6-OP) were synthesised to study their mineralocorticoid properties (Brachet-Cota and Burton, 1990). As expected, the flat steroid 11-OP is a selective MR ligand (as potent a mineralocorticoid as 11-deoxycorticosterone (DOC)), whereas its bent counterpart, 6-OP, is devoid of both affinity for the MR and sodium-retaining capacity (Galigniana et al., 1993; Burton et al., 1995; Piwien-Pilipuk et al., 2002a; and Fig. 2).

It is classically accepted that certain critical functional groups enhance mineralocorticoid potency, for example, a C_{21}-hydroxyl. Interestingly, 11-OP lacks those functional groups, its main characteristic being its overall conformational planarity. A similar statement can be made for the biological potency of other pairs of compounds such as the flat steroid 5α-diH-progesterone (a stronger sodium-retainer) and its bent isomer 5β-diH-progesterone. 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. Then, the question arises whether the tentative "planarity rule" also applies for most 21-deoxypregnanesteroids and might also be extended to 21-hydroxypregnanesteroids. To answer this hypothesis,

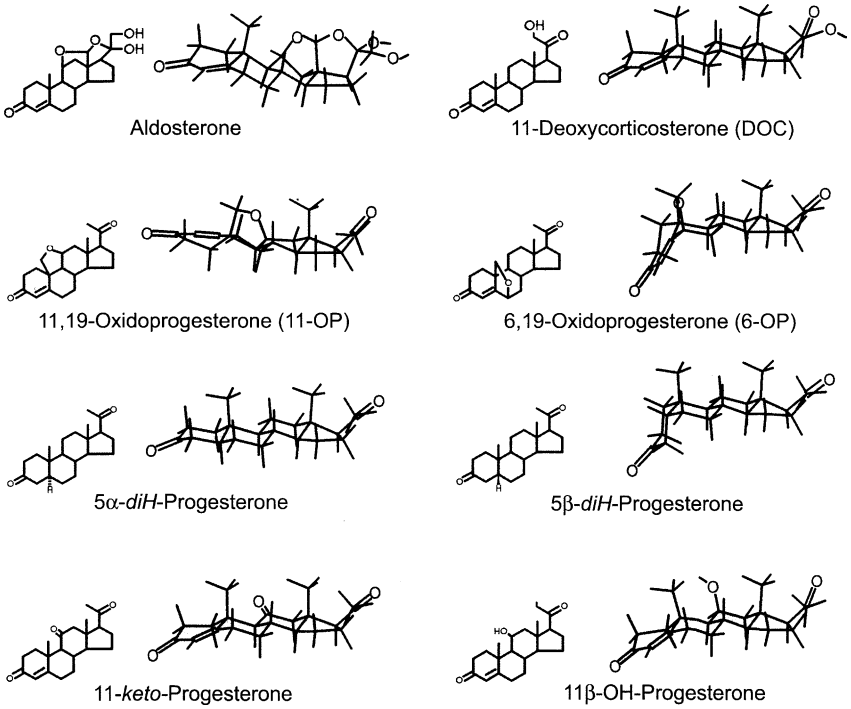

Fig. 1. Structures of some steroids analysed in this work.

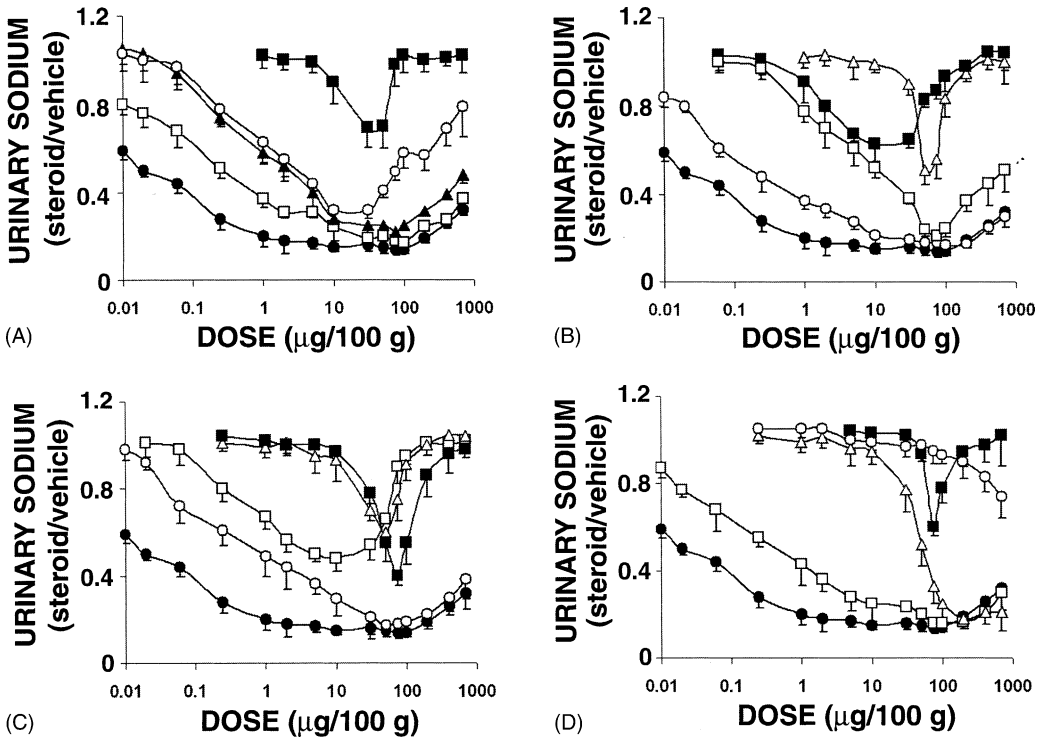

Fig. 2. Dose–response curves for some representative steroids. Urinary sodium elimination was measured after injecting adrenalectomised male rats with the indicated doses of steroid expressed in μg of steroid per 100 g of rat body weight. Results are normalized to steroid/vehicle sodium elimination ratio. Each point is the mean ± S.E.M. of three experiments, in which one 8–12 animals were used per dose. For the sake of clarity, the curves were arbitrarily plotted on several panels to avoid overlapping of data. Aldosterone (●) was included in all panels for comparative purposes. Symbols represent the following steroids: (A) (○) 5β-diH-progesterone, (■) Δ^1-progesterone, (□) DOC, (▲) 5α-diH-progesterone; (B) (○) 11-OP, (■) progesterone, (□) 18-deoxy-aldosterone, (△) 6-OP; (C) (○) 19-nor-DOC, (■) 18-OH-corticosterone, (□) 11-keto-progesterone, (△) 11β-OH-progesterone; (D) (○) cortisol, (■) 21-OH-6-OP, (□) 21-deoxy-aldosterone, (△) 21-OH-11-OP.

a series of 33 pregnanesteroids with diverse geometrical parameters and functional groups was recently studied, where the Na^+-retaining capacity and relative binding affinity (RBA) for the MR were analysed with respect to the overall planarity of the steroidal skeleton (Piwien-Pilipuk et al., 2002a). As described below, this study shows that a relationship between the steroid structure and its biological activity can be demonstrated if the sodium-retaining effect is analysed in toto.

In this review we have included some examples (Fig. 2) that depict the dose–response curves in the large range of 0.01–500 μg/100 g of rat body weight for some natural and synthetic compounds, many of them without a previously studied mineralocorticoid effect. Many of the "unstudied" steroids were chosen based on their geometrical parameters after a preliminary analysis of the structure–activity trend observed in a previous publication (Burton et al., 1995). This approach allowed us to select ligands that covered a wide range of conformations to estimate a priori the putative sodium-retaining activity of the molecules. Intriguingly, most steroids exhibit a parabolic function. A maximal anti-natriuretic effect, which varies according to the steroid, is shown at certain doses, whereas a clear reversion of the effect is observed at higher doses. The tendency to reverse the Na^+-retaining effect, although less evident than for other steroids, can also be observed for the most active compounds, including aldosterone, at the highest doses. Such a biphasic function of the dose–response curves does 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. On the other hand, this observation also reflects the fact that the mineralocorticoid effect is not a linear one.

The problem can be partially solved by correlating the Na^+-retaining response with the second-order polynomial of the function defined by the equation $y = ax^2 + bx + c$. Thus, the second-order coefficient '*a*' is 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. The individual values for each steroid studied in Piwien-Pilipuk et al. (2002a) are summarized in Table 1, along with the relative affinity for the MR and some geometrical parameters.

When the A/D angle, the A/BCD angle, and the C_3 = O/D angle are plotted against the coefficient *a*, the latter parameter is the best to demonstrate a correlation between the biological effect and both the geometry of the steroids

Table 1
Geometric parameters of the steroids, concavity of polynomial log dose–response curves, and relative affinity for the renal MR

Steroid	Angle			*a*	RBA (nM)	
	A/D	A/BCD	$C_3 = O/D$		−HAP	+HAP
Aldosterone	−8.7	−21.1	14.6	0.048	4 ± 1	3 ± 1
11-OP	4.4	−3.7	8.9	0.015	56 ± 2	45 ± 5
21-Deoxy-aldosterone	−8.7	−21.1	15.1	0.029	6 ± 4	4 ± 2
DOC	−22.2	−24.8	28.3	0.030	6 ± 1	4 ± 1
5α-diH-aldosterone	11.4	−0.7	12.1	0.035	7 ± 2	5 ± 3
$\Delta^{11,12}$-DOC	−15.2	−19.3	21.3	0.051	10 ± 4	6 ± 2
19-Nor-DOC	−16.1	−19.7	24.8	0.072	6 ± 2	8 ± 1
$\Delta^{1,2}$-DOC	−33.9	−33.2	31.3	0.155	30 ± 3	17 ± 2
5α-diH-DOC	−7.5	−33.2	32.0	0.231	21 ± 4	14 ± 2
Progesterone	−21.6	−24.4	27.3	0.295	40 ± 2	31 ± 5
11-Keto-progesterone	−21.3	−24.6	26.5	0.479	30 ± 2	25 ± 7
18-Deoxy-aldosterone	−16.6	−24.2	23.1	0.526	38 ± 2	33 ± 3
5α-diH-progesterone	−7.9	−8.7	32.4	0.577	31 ± 4	26 ± 2
5β-diH-progesterone	−68.6	−69.8	44.0	0.680	70 ± 8	62 ± 7
11α-OH-progesterone	−21.5	−25.1	29.9	0.766	89 ± 6	92 ± 9
$\Delta^{1,2}$-11β-OH-progesterone	−35.0	−34.5	32.2	0.950	66 ± 7	45 ± 6
$\Delta^{1,2}$-Progesterone	−31.2	−29.8	27.0	0.999	39 ± 4	33 ± 6
18-OH-corticosterone	−28.6	−38.4	36.8	1.862	269 ± 24	244 ± 37
3β,5α-TetraH-aldosterone	8.9	3.2	32.9[a]	2.012	1025 ± 202	901 ± 245
21-Deoxy-3β,5α-tetraH-aldosterone	8.4	3.2	32.3[a]	2.402	1705 ± 331	1378 ± 233
11β-OH-progesterone	−24.1	−32.1	29.8	2.438	501 ± 50	71 ± 9
6-OP	−57.8	−57.6	55.2	2.939	3206 ± 367	2413 ± 451
11-Keto-6-OP	−57.5	−57.7	54.6	3.713	2886 ± 814	2264 ± 334
21-OH-6-OP	−57.6	−54.4	53.0	4.571	24155 ± 1221	23050 ± 2222
Dexamethasone	−35.6	−37.1	35.0	ND	100 ± 15	85 ± 6
21-OH-11-OP	4.4	0.7	8.9	ND	4742 ± 780	4985 ± 921
Cortisol	−26.9	−35.4	34.2	ND	29 ± 5	10 ± 4
Corticosterone	−26.2	−27.7	33.5	ND	61 ± 8	6 ± 3
Cortisona	−23.5	−32.7	30.2	ND	17 ± 1	14 ± 2
$\Delta^{1,2}$-Corticosterone	−36.6	−36.4	35.1	ND	13 ± 2	8 ± 2
11-Dehydro-corticosterone	−22.4	−31.2	28.7	ND	40 ± 3	24 ± 5
$\Delta^{1,2}$-Cortisol	−37.4	−37.2	36.2	ND	89 ± 9	70 ± 6
21-Deoxy-cortisol	−26.7	−35.0	34.2	ND	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}. The 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 exhibit by the biological effect in vivo. The relative binding affinity to the MR was measured by competition curves of each steroid with [^{3}H]ALDO in crude kidney cytosol (−HAP) or in hydroxylapatite preadsorbed cytosol (+HAP). ND: unable to be determined.

[a] Indicates the C_3–OH angle.

(Fig. 3A) and ligand-binding to the MR (Fig. 3B). Therefore, a relevant role for the orientation of the $C_3 = O$ group may be inferred for the recognition of the ligand by the hormone-binding pocket of the MR. Interestingly, Fig. 3C shows that there is a tendency to increase the mineralocorticoid effect (lower coefficient *a* value) with a higher affinity for the MR. It should be emphasized that all of these observations are valid for both 21-deoxysteroids and 21-hydroxysteroids.

Therefore, the analysis of the whole dose–response curve measured in vivo allows the calculation of the coefficient *a*, which seems to be the most representative factor to semi-quantify, and perhaps predict, the mineralocorticoid effect for a given steroid according to its geometry. Nonetheless, one of the limitations of this model is that the second-order coefficient '*a*' cannot be measured for those steroids that show no parabolic function in the range of doses assayed, i.e. steroids that exhibit a weak mineralocorticoid effect in vivo.

We have unsuccessfully attempted to find correlations by using several other factors, among them steroid hydrophobicity, hydration sphere, length of the molecule, total surface area, van der Waals radius, electronic density, etc. Importantly, no straight correlation has ever been found when the "mineralocorticoid effect" was studied in vitro, i.e. the MR-dependent activity of a gene-reporter in transiently transfected cell lines (Grassi et al., 1997; Agarwal and Mirashi, 2000; Piwien-Pilipuk et al., 2002a; Quinkler et al., 2002), which evidences the enormous differences that can be obtained by working with an integrated biological effect under in vivo conditions. It is noteworthy that the structure–activity correlation can only be obtained when the in vivo biological effect is considered as a whole, regardless of the number and nature of the regulatory mechanisms

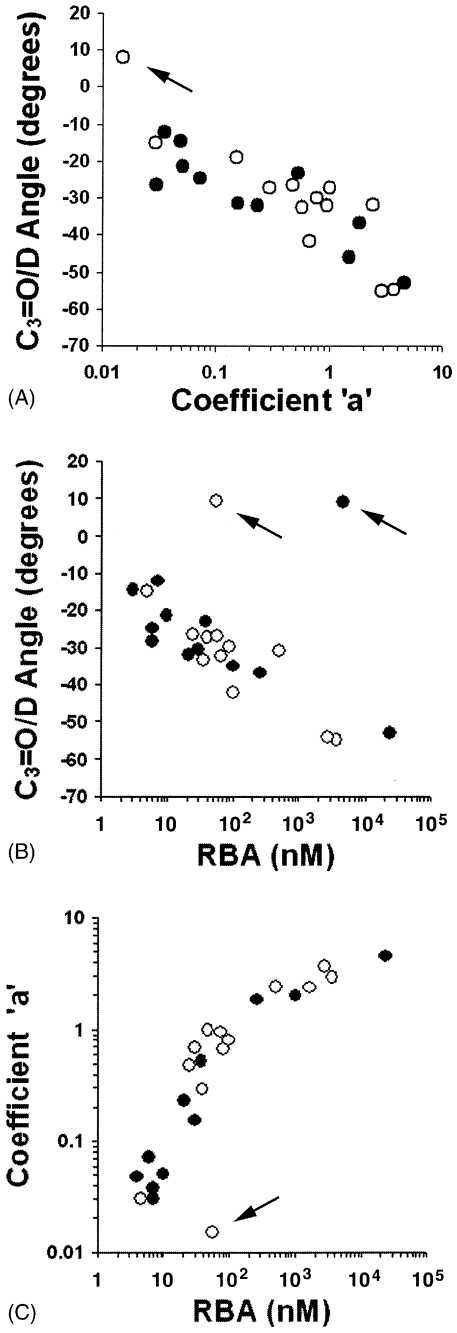

Fig. 3. Structure–activity relationships: (A) correlation between the C_3 = O/D angle of the steroids (geometric parameter) and the second-order coefficient '*a*' (biological parameter for the biphasic sodium-retaining effect); (B) geometric parameter of the steroids versus the relative affinity for the MR; (C) correlation between the biological effect and binding to the MR. Note that the coefficient '*a*' includes all the variables that affect the final biological response. Arrows identify 11-OP ligands. Symbols: (○) 21-deoxypregnanesteroids, (●) 21-OH-pregnanesteroids.

involved in the resultant mineralocorticoid action. There is a particular case that will be analysed later, 11-OP. This potent synthetic agonist (and its 21-hydroxy-derivative) is excluded from the correlations depicted in Fig. 3B and C, but not from the correlation shown in Fig. 3A (see arrows). As it is discussed further in Section 6, the reason for these exclusions is due to the fact that both 11-OP (and its 21-hydroxy derivative) may bind to the MR at an alternative binding site.

4. The particular features of some steroids

One of the most important derivations of the above-described studies is the demonstration that the steroid conformation rather than the presence of certain functional groups is the determining factor for sodium retention. Of course, it is not possible to completely dissociate the presence of certain functional groups from the steroid conformation, but there are some examples in which the conformers share identical chemical groups and dissimilar biological activity (e.g., 11-OP versus 6-OP and 5α-diH-progesterone versus high doses of 5β-diH-progesterone). Therefore, an explanation for this feature can be found in the overall conformation of the ligand. Thus, flat steroids such as those named in first term are more potent mineralocorticoids than their respective bent counterparts. Indeed, 6-OP is an almost inert steroid even though the chemical groups of its molecule are identical to those of highly active agonist 11-OP.

Another interesting case to point out is the two steroids 11β-hydroxyprogesterone and its 11-keto-derivative (see Fig. 2C). Almost a decade ago we described that these steroids are a shuttle pair of metabolites for the enzyme 11βHSD (Galigniana et al., 1994; Galigniana et al., 1997). Classically, 11-keto-derivatives were thought to be inactive compounds; however 11-keto-progesterone exhibits almost 17-fold higher affinity for the MR and is substantially more active as a Na^+-retainer than 11β-hydroxyprogesterone in the 0.1–50 μg/100 g dose-range. Like most of the other steroids, both compounds elicit an identical mineralocorticoid reversal effect above 50 μg/100 g, until Na^+-retention is totally abolished at doses near to 100 μg/100 g. The particular biological effect of 11-keto-progesterone may be assigned to the combined action of several factors, i.e. the lack of the 11β-hydroxy group that partially flattens the steroidal frame (favouring binding to the MR) combined with a substantial loss of affinity for transcortin (10-fold), which permits the concentration of free steroid available to bind the MR to increase significantly.

It is important to emphasize that both steroids (11β-hydroxy-and 11-keto-progestereone) are potent inhibitors of the enzyme 11βHSD, such that the co-injection of corticosterone elicits a strong aldosterone-like effect (Souness et al., 1995; Galigniana et al., 1997). From many decades it has been well established that under physiological conditions, the secretion of 11β-hydroxyprogesterone is substantial under stressful conditions, to the point that it surpasses the level of secretion of aldosterone by the adrenal gland (Heap et al., 1966; Kraulis et al., 1973). Inasmuch as the secretion of both 11β-hydroxyprogesterone and 11-keto-progesterone

is high during the last trimester of gestation as to reach similar levels as the K_i for 11βHSD, and because both steroids are also found at high concentration in amniotic fluid and umbilical blood, we postulate that it is entirely possible that both metabolites may contribute to the hypertensive disorder observed during pregnancy.

Due to similar reasons as those discussed for 11-keto-progesterone, cortisone and 11-dehydro-corticosterone (flatter molecules than cortisol and corticosterone, respectively) also improve their binding affinity for the MR. However, they are as poor mineralocorticoids in vivo as their reduced partners due to a more efficient and highly systemic reversal metabolism via 11βHSD-1 (K_m in the nM order), so these steroids are easily converted to their C_{11}-hydroxylated forms and no substantial Na^+-retaining effect was observed for the range of doses studied here.

We would like to emphasize that the divergent features of some pairs of compounds undermine the functional importance of the C_{21}-OH function. Thus, progesterone is a weak mineralocorticoid whereas its 21-hydroxylated derivative, DOC, is a strong one, and 21-deoxy-aldosterone loses activity with respect to aldosterone. On the other hand, 11-OP exhibits potent sodium-retaining properties (comparable to DOC, and even to aldosterone at higher doses), whereas the introduction of a 21-hydroxyl group greatly reduced the biological activity. In all these examples, the overall geometry of the molecules does not change significantly. However, there are additional factors to consider when these cases are analysed such as the length and flexibility of the molecule.

5. Length and flexibility of the steroidal frame

The flexibility of the molecules greatly affects the adaptability of the ligand to the binding site in the receptor. Like aldosterone, the structures of the oxidopregnanes 11-OP and 6-OP and their 21-hydroxylated derivatives 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-OP for the MR, and also explains why its 21-hydroxylated derivative behaves as a weak mineralocorticoid agonist. In this regard, it is interesting to point out that the total length of 11-OP is 11.38 Å (O_3–O_{20}), which is substantially shorter than the O_3–O_{21} length of natural agonists—12.45 Å for aldosterone and 12.30 Å for DOC. Even though 11-OP is certainly a flat steroid, it does not have the optimal $C_3 = O/D$ angle exhibited by aldosterone ($+8.9^\circ$ versus -14.6°, respectively). However, its shorter length would may allow the rigid frame of 11-OP to adjust into the steroid binding pocket more easily than its 21-hydroxy derivative partner (12.23 Å). As a consequence, 11-OP may behave as a strong mineralocorticoid, whereas 21OH-11-OP is a weak MR ligand and sodium-retainer in spite of possessing similar length and functional groups as natural agonists. Similarly, the lack of a 21-hydroxyl group in progesterone allows this steroid to bind to the MR in vitro with a relatively good affinity, but its agonist activity is poor. Accordingly, studies recently performed with hMR (which progesterone binds to with the same affinity as aldosterone) have linked the lack of agonistic effect to the inability of progesterone to establish contact with the Asn^{770} residue found in the steroid-binding pocket (Fagart et al., 1998).

6. 11,19-Oxidoprogesterone as a tool for studying the MR molecular mechanism of action

As shown in Fig. 3B and C, the relative affinity of 11-OP for the MR is lower than that expected due to its biological properties. There are a number of possible biopharmacological variables that may account for the biological effect of 11-OP, such as a longer half-life, stronger in vivo binding to the renal MR, non-genomic effects, etc. All of them were studied and ruled out (Galigniana et al., 2000). This includes a possible in vivo 21-hydroxylation because that putative metabolite, as discussed above, is even less active than 11-OP (Fig. 2D).

Interestingly, a competition curve of radioinert aldosterone by [^{3}H]aldosterone bound to the MR surprisingly revealed that the simultaneous presence of an equimolar concentration of 11-OP with respect to the tracer decreased the ability of unlabelled aldosterone to displace bound steroid (Fig. 4A). This effect depends on the concentration of 11-OP. A detailed analysis of the competition curves shows that a 10-fold excess of unlabelled aldosterone (50 nM) totally competes with the tracer specifically bound to the MR. However, this displacement decreases, respectively, to 22 and 35% if 5 or 30 nM of 11-OP is also present. This observation led us to the speculation that 11-OP may stabilise an “active” receptor conformation, which in turn, abrogates unlabelled aldosterone to compete with [^{3}H]aldosterone already bound to the MR. Accordingly, the dissociation rate constant of aldosterone from the MR is decreased three-fold in the presence of a concentration of 11-OP that fails to compete per se with aldosterone (Fig. 4B). This property was not observed when DOC or the isomer 6-OP was tested.

A treatment with saturating doses of aldosterone or 11-OP injected either individually or jointly, yields a maximum mineralocorticoid effect (Fig. 4C). Because no addition or potentiation of the effect is observed by co-injection of both agonists, a common pathway of activation can be inferred. Since the effect is efficiently antagonized by SC9420-spironolactone, such a shared pathway is MR-mediated. Interestingly, a potentiation of the mineralocorticoid effect is obtained when a suboptimal dose of 0.06 μg/100 g of aldosterone (∼50% of maximum) is co-injected with as low of a dose of 11-OP as 0.6 ng, which is inactive per se, such that a full mineralocorticoid effect is achieved. Similar results were obtained when the

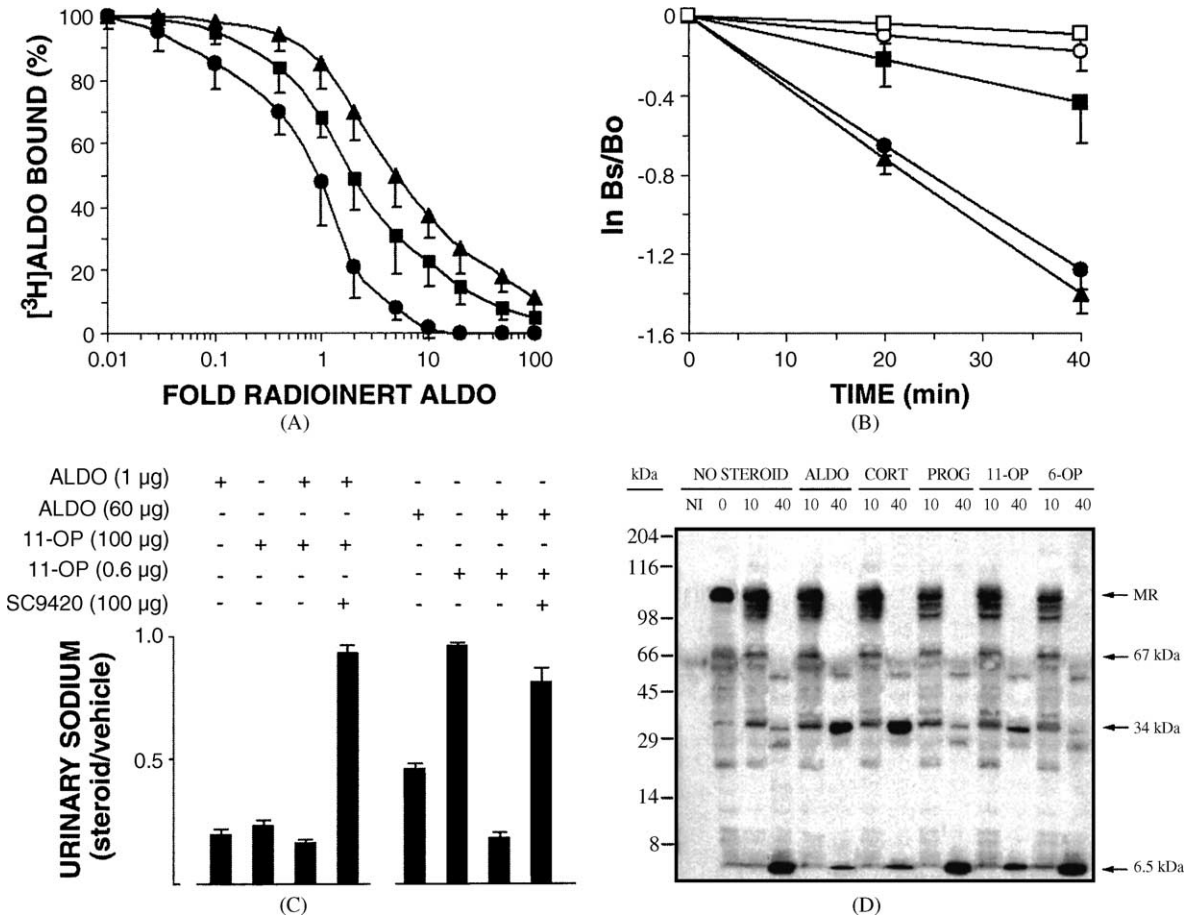

Fig. 4. 11-OP potentiates the aldosterone effect: (A) competition curves. [^{3}H]Aldosterone-bound MR was incubated with increasing concentrations of unlabelled aldosterone (ALDO) in the absence (●) or presence of 11-OP ((▲) 5 nM; (■) 30 nM). (B) Dissociation rate of aldosterone. [^{3}H]Aldosterone-bound MR complexes were incubated at 20 °C in the absence (Bo) or presence (Bs) of the following unlabelled steroids: (▲) 0.5 μM aldosterone, (○) 2.5 nM DOC, (□) 5 nM 11-OP, (●) 0.5 μM aldosterone and 2.5 nM DOC, and (■) 0.5 μM aldosterone and 5 nM 11-OP. (C) potentiation of the Na^+-retaining effect. Adrenalectomised male rats were injected with the doses shown in parenthesis (per 100 g of body weight) of aldosterone (ALDO), 11-OP and/or SC9420-spironolactone. Results are expressed as the ratio between the natriuresis measured in steroid-injected vs. vehicle-injected rats. (D) Limited chymotrypsinisation of liganded MR. Immunopurified MR bound to either aldosterone (ALDO), corticosterone (CORT), progesterone (PROG), 11-OP or 6-OP was incubated with α-chymotrypsin (10 or 40 U/ml). NI is the non-immune antibody.

21-hydroxy-derivative of 11-OP was used instead of 11-OP (Galigniana et al., 1994).

Based on these observations, we suggest that the MR possesses a different binding site for 11-OP from that of aldosterone. This may explain the experimental observations described above. Inasmuch as Scatchard plots performed with [^{3}H]aldosterone always show a single slope, the putative "11-OP site" is not recognised by the natural agonist. On the other hand, 11-OP is capable of competing with aldosterone only at higher concentrations than those required to potentiate aldosterone-binding. Therefore, it is possible that this (regulatory?) alternative-binding site may function as a stabiliser of an activated "aldosterone-like" form of the MR, which in turn decreases the off-rate of the natural ligand. That this may be the case is supported by the observation that the affinity of aldosterone by immunopurified MR is significantly increased from 0.99 ± 0.10 to 0.28 ± 0.03 nM in the presence of 1 nM 11-OP (Galigniana et al., 2000). In contrast, higher concentrations of 11-OP such as 30 and 100 nM (both capable of competing with aldosterone) decreased the affinity of the MR for aldosterone two-fold (1.95 ± 0.25 nM) and three-fold (3.54 ± 0.42 nM), respectively. This observation suggests that 11-OP is anchored to the aldosterone-binding pocket, which is permissive for flat molecules. A rigid model between steroid and protein would certainly be sufficient to account for the differential binding of ligands, as stated by the classical model which considers that the receptor switches from an inactive to an active form upon ligand binding. However, there is no reason to think that all ligands should be positioned in the same way or in exactly the same binding pocket. It is more likely that we have to deal with a subtler repositioning, taking into account several factors in addition to the functional groups. Therefore, an all or nothing event is unlikely in view of the fact that the ligand-binding is an adaptative process, so the structure of the receptor is accordingly influenced by

the nature of the ligand. As a consequence, 11-OP behaves like the endogenous agonists. The availability of radiolabelled 11-OP in the future may help to definitively answer these speculations.

However, we can certainly state without hesitation that the mineralocorticoid properties of 11-OP occur by binding to the MR, and that such binding is not equivalent to that of aldosterone. Thus, Fig. 4D shows that limited chymotrypsinisation of the rat MR yields several proteolytic fragments, among them, a 34 kDa key peptide. Under certain experimental conditions, this peptide is fully degraded in unliganded receptor samples, whereas it is preserved from degradation in both aldosterone-bound and corticosterone-bound MR complexes. On the other hand, antagonists such as progesterone were incapable of protecting this fragment from proteolysis. Interestingly, the synthetic agonist 11-OP cannot be grouped with any of those two classes of ligands because the 11-OP/MR complex yields an intermediate pattern of degradation between agonists and antagonists, suggesting again that the conformational change generated in the MR is not equivalent to that induced by natural agonists (Piwien-Pilipuk et al., 2000).

7. Cytoplasmic trafficking towards the nucleus

The MR resides predominantly in the cytoplasm of the cell in the absence of steroid (Robertson et al., 1993; Lombès et al., 1994; Piwien-Pilipuk and Galigniana, 1998; Nishi et al., 2001), although an equal distribution between cytoplasm and nucleus has also been reported (Fejes-Tóth et al., 1998; Pearce et al., 2002). Upon steroid-binding, the MR rapidly translocates into the nucleus (Galigniana, 2000). The nuclear, untransformed MR fraction is loosely bound to the nuclear matrix; it rapidly shuttles into the cytoplasm compartment and is easily recovered in cytosolic fractions during the cell fractionation, whereas agonist-transformed MR is tightly bound to chromatin and must be eluted by drastic extraction methods (detergents, high ionic-strength, treatment with DNase, etc.).

One of the still unknown steps in the mechanism of action of the MR is its trafficking in and out the nucleus. It has always been assumed that simple diffusion was the driving force for moving soluble proteins, which become "trapped" in their sites of action by protein–protein or nucleic acid–protein interactions. Alternatively, protein solutes may utilise a trafficking machinery (which may well operate bidirectionally), in which case, movement would be likely to involve molecular motors and cytoskeletal tracts (similar to vesicle transport) (Pratt, 1993). Recent evidence suggests that this may be the case (Galigniana et al., 2001). Thus, Fig. 5A shows that when the GR is immunoprecipitated from cytosol of mouse fibroblasts, dynein intermediate chain (DIC) co-immunoprecipitates along with hsp90 and the immunophilin FKBP52. Fig. 5B shows that when the receptor is stripped of associated proteins with high ionic-strength and then incubated with reticulocyte lysate, the native DIC-containing heterocomplex is reconstituted unless the hsp90-disrupting agent geldanamycin (GA) is present. These results indicate that hsp90 is required for DIC-recruitment to the heterocomplex. When the reconstitution of the GR with reticulocyte lysate is performed in the presence of a TPR-protein, the binding of both DIC and immunophilin is abolished, but not the binding of hsp90, which indicates that DIC is bound to FKBP52 rather than to the GR or hsp90. Importantly, both hsp90 and FKBP52, but no DIC are recovered in the complex when an excess of the rotamase domain of FKBP52 is added to the reconstitution mixture (the rotamase domain is also called the PPIase domain due to its peptidylproline *cis–trans* isomerase activity).

The model depicted in Fig. 5C summarize this experimental sequence. The association of FKBP52 to hsp90 occurs via the TPR domain present at the C-terminal end of the immunophilin and the TPR-binding site of the chaperone, most likely with a stoichiometry equal to one molecule of FKBP52 per dimer of hsp90 (Silverstein et al., 1999), whereas DIC-binding occurs via the rotamase domain present at the N-terminal end of FKBP52. Recent experimental evidence suggests a direct binding of DIC to FKBP52 (Galigniana et al., 2002). Importantly, the overexpression of the rotamase domain of FKBP52 prevented the steroid-dependent movement of the GR, as shown in Fig. 5D. It is important to emphasize that similar protein–protein interactions were also found for the MR and the hsp90-bound form of p53 (unpublished results).

Immunosuppressant drugs such as FK506 and rapamycin inhibit the enzymatic activity of immunophilins by binding to the cognate rotamase domain. Because this domain is evolutionary conserved, it has been inferred that it must be critical for basic cellular functions such as protein folding (Marks, 1996). In our hands, FK506 did not affect DIC-binding or receptor trafficking. It is interesting to point out that, to date, there is no evidence that the rotamase activity can modify the conformation of proteins in vivo, but only affects oligopeptides in vitro. Moreover, there is still no clear demonstration for the biological function of FKBP52. According to the findings summarised here, it is clear that in the cell the rotamase domain of this high molecular weight immunophilin is involved in protein–protein interactions.

The classical dogma maintains that upon ligand-binding, the hsp90-based heterocomplex is dissociated from the receptor as to allow the nuclear translocation of the receptor and transcriptional activation. If so, the question then arises about what role the proteins of the heterocomplex play in receptor trafficking and why the heterocomplex recruits a molecular motor protein. In view of these exciting findings, a major question is how the binding of ligand affects the function and balance between the proteins in the steroid/receptor heterocomplex.

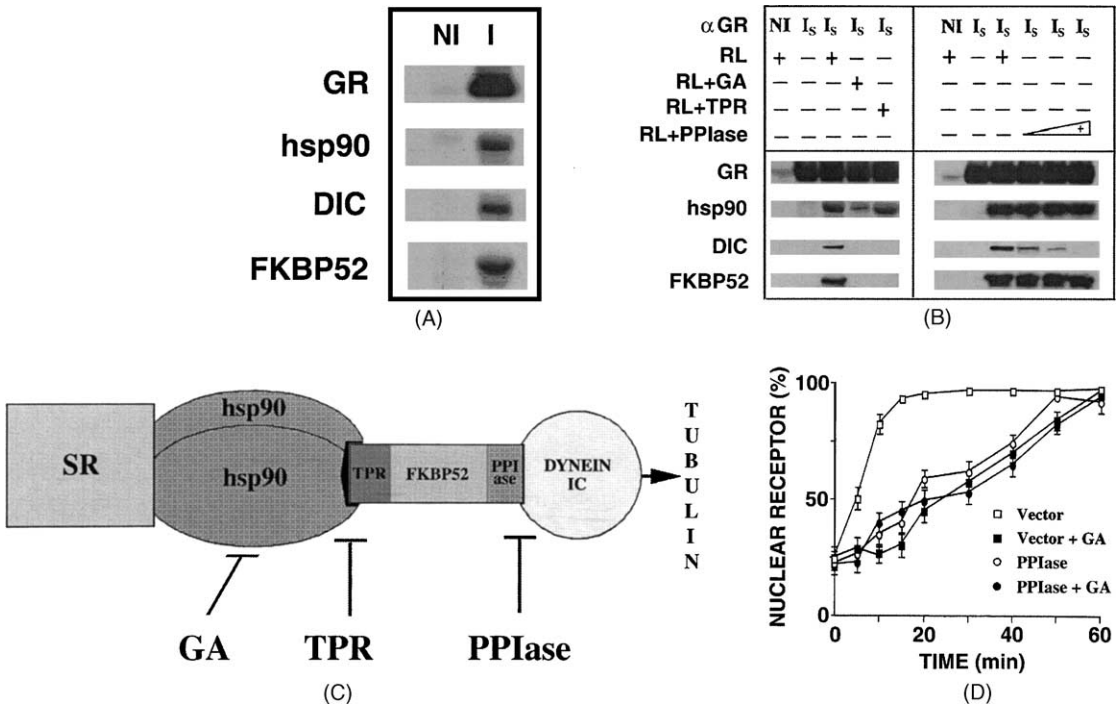

Fig. 5. The DIC·IMM complex is involved in retrograde trafficking of cytoplasmic steroid receptors: (A) DIC co-immunoprecipitates with the GR·hsp90·FKBP52 complex. The GR was immunopurified from cytosol of L929 fibroblasts and Western blotted for GR, hsp90, DIC and FKBP52. NI is the non-immune antibody, I the immune antibody against the GR. (B) Reconstitution of the heterocomplex. The GR was immunopurified and stripped (Is) of associated proteins by treatment with 0.5 M NaCl. The heterocomplex was then reconstituted by incubating stripped GR with reticulocyte lysate (RL) that had been preincubated with either the hsp90-disrupting agent geldanamycin (GA), the TPR peptide of rat PP5 (TPR), or the rotamase domain of FKBP52 (PPIase). (C) Model deducted from the competition experiments described in C. The lines show where GA, TPR or the rotamase (PPIase) peptide disrupts the heterocomplex. SR is the steroid receptor. (D) Overexpression of the rotamase domain (PPIase) impairs steroid receptor trafficking to the nucleus. Transfected cells with empty vector or the PPIase domain of FKBP52 were first incubated with steroid at 0 °C (to allow binding only), and then in the presence or absence of geldanamicyn (GA). At the end of these preincubations, the temperature was shifted to 37 °C (zero time) to trigger the nuclear translocation of the receptor.

8. Nuclear retention as determining factor for the biological effect

Upon ligand-binding, the nuclear translocation of the native rat MR expressed in renal cells is complete after ∼15 min at 37 °C (Galigniana, 2000). Nuclear trafficking of rat MR is sensitive to phosphatase inhibitors (Galigniana, 1998; Piwien-Pilipuk and Galigniana, 1998) and redox potential milieu (Piwien-Pilipuk and Galigniana, 2002b; Galigniana, 2000). The inhibitory effect observed under conditions of acute oxidative stress occurs despite the fact that the ligand is bound to the receptor, which suggests that the inhibition may take place at the trafficking system level and/or at the nuclear translocation step. Ultimately, chronic oxidative stress also affects the synthesis of the MR at the elongation/termination step (Piwien-Pilipuk et al., 2002c), which amplifies renal mineralocorticoid dysfunction associated with a disrupted MR-dependent signalling pathway.

When the rat MR (either flag-tagged MR transfected in cultured cells or endogenous receptor isolated from rat tissues) is immunoprecipitated, all the proteins described above for the GR (i.e. hsp90, hsp70, p23, IMMs, etc.) are also co-immunoprecipitated, which indicates that the heterocomplex associated to the MR is the same as that described for the GR. Dynein intermediate chain, dynein heavy chain and tubulin are also co-immunoprecipitated when a buffer that preserves tubulin polymerisation is used (Piwien-Pilipuk and Galigniana, unpublished results). Taken together, these observations suggest that the MR associates to cytoskeleton tracts and moves to the nucleus using the tubulin-associated motor protein dynein, as depicted in Fig. 5C.

Interestingly, the nuclear translocation rate of 11-OP/MR complexes is two-fold slower than the translocation rate of aldosterone/MR complexes. This is not surprising since it agrees with the argument that the MR undergoes a differential conformational change upon 11-OP binding. Therefore, it can be expected that the recruitment of some of the proteins to the MR·hsp90 heterocomplex may be affected by 11-OP. That this is the case is supported by the observation that the cytoplasmic aldosterone/MR complex recruits more FKBP52 and DIC to the MR·hsp90 heterocomplex than the 11-OP/MR complex (Piwien-Pilipuk and Galigniana, unpublished results). The inability of the receptor to associate with the cytoplasmic trafficking machinery may be the consequence of the particular conformation adapted by the MR upon 11-OP binding, and may also explain the

slower nuclear translocation rate of the liganded receptor. On the other hand, the nuclear export rate of the 11-OP/MR complex measured in digitonin-permeabilised cells is faster than that for aldosterone-bound receptor, and the subnuclear distribution of the former complex does not show the punctuated pattern exhibited by the latter (unpublished results). These recent findings parallel those obtained with certain synthetic ligands bound to the GR (Vicent et al., 2002) in which we proposed that the differential conformation of the receptor may consequently lead to a differential interaction of GR with other nuclear factors and/or chromatin, so the dynamic exchange of liganded receptor is likely to have significant consequences for the observed physiological responses triggered by different ligands that bind to the same receptor.

9. Considerations about the ligand/receptor interaction

Steroids can be found in plants and fungi, but not their cognate receptors as we know them in metazoans. These facts suggest an independent gain of ligand-binding capacity during evolution. On the other hand, it is interesting to point out that, once a given receptor has acquired the capacity to bind a particular type of ligand, it is not greatly modified during evolution, perhaps due to the relative advantage provided by ligand-dependent activation of the receptor. Given the complexity of the metabolic pathways for ligand synthesis, adaptation of an ancestral receptor for the binding of such molecules seems more reasonable than adaptation of a whole biosynthetic pathway of the ligand to the receptor. Nonetheless, both components, ligand and receptor, always function as a unit.

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 pre-existing structures that evolved independently, it is now clear that their interaction may necessarily be the result of evolution itself. The information for hormonal regulation at the gene level is unquestionable written in the receptor structure, which bears a close resemblance with its primordial predecessor. On the other hand, hormonal signalling molecules seem to have acquired their present role in a long evolutionary process, which may have determined the separation between, for example, glucocorticoids and mineralocorticoids. Thus, it was a key to mineralocorticoid physiology the emergence of aldosterone synthase (CYP11B2) since ketal/hemiketal groups are not substrates for 11βHSD. 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 described in this review, 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 some 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 compounds resemble primordial ligands that are currently extinct or serve different function today. Clearly, no single factor can be held solely responsible for the observed correlation between steroid structure and biological effect, as clearly seen when the results obtained in vitro and in vivo are compared.

It should be emphasized that several factors are involved in the regulation of the mineralocorticoid 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 influence the steroid availability to the MR. Most of these pre-receptor regulatory factors are absent under in vitro conditions. Therefore, while a simplified assay system is certainly useful for dissecting the individual steps of the molecular mechanism of activation of the MR, it is not suitable to evaluate the complex in vivo biological response.

Based on the experimental data described here, the calculated value that would represent the optimal angle of an ideal steroid that exhibits both optimal binding affinity for mineralocorticoid receptor and maximum sodium retention is $-12.5 \pm 3.7^{\circ}$, this angle being similar to the $C_3 = O/D$ angle of the ketal form of aldosterone. If this speculation were valid, the coincidence between the geometry of an ideal ligand and the value exhibited by the most potent mineralocorticoid in nature would not be inappropriate from an evolutionary perspective.

10. Considerations about the steroid-bound MR/DIC interaction

The near completion of the human genome sequencing project has revealed that many human diseases are due to defects in intracellular trafficking. To date, more than 150 diseases are related with failures in the transport of macromolecules to the proper compartment of the cell (Aridor and Hannan, 2002). The eukaryotic genome also shows that there are thousands of genes that have no counterparts in prokaryotes (Hutter et al., 2000). We do not know the exact sources for these innovations, but certainly many novel protein domains are, in fact, old ones that have been modified to the point that their origin cannot be easily recognized. However bacteria, which do not possess known

dynein or dynein-like motor proteins, do express highly homologous immunophilins (e.g., TcFK), which resembles human FKBP12 (Iida et al., 1998). Interestingly, FKBP12 (and by extrapolation, its bacterial partners) is unable to interact with DIC in spite of possessing a highly homologous rotamase domain with FKBP52 (Galigniana et al., 2001). This, and the fact that dynein is associated with a number of structures, proteins and physiological process, makes it unlikely that dynein/IMM function could have emerged spontaneously during evolution. More likely, the initial function of dynein was limited. For example, in budding yeast, the major function of dynein seems to be in linking microtubules to the cell cortex, and it is tempting to speculate that this could be the simplest role for dynein. The biological function of high molecular weight IMMs in general, and FKBP52 in particular, is still uncertain. However, the finding that FKBP52 is linked to DIC and tubulin and, on the other hand, it also associates to steroid receptors, provides a new challenging insight about the role of dynein as motor protein for soluble, non-vesicle associated proteins. Also, it is of a great interest for the understanding of the mechanism of receptor trafficking due to the finding that binding of different ligands to the MR promotes a differential conformation change of the receptor, such that it promotes the recruitment of variable amounts of FKBP52 and DIC to the heterocomplex. As a consequence, the translocation rate of the steroid/receptor complex is affected accordingly. Whether this is one of the possible cytoplasmic check-points for the regulation of the MR mechanism of action is still unknown. It is also uncertain what the possible implication of those interaction in the nucleus and a putative role of the proteins associated with the heterocomplex have on regulation of transcription. In this regard, it is noteworthy that, unlike aldosterone/MR complexes, 11-OP/MR complexes do not follow a nuclear pattern of enrichment in foci and are more rapidly excluded from the nucleus. Therefore, it still remains unanswered why 11-OP behaves as a potent sodium-retainer compound. These many questions will be presumably the future challenges in understanding the molecular mechanisms of mineralocorticoid function.

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