2

ACTIVATION OF THE LIGAND– MINERALOCORTICOID RECEPTOR FUNCTIONAL UNIT BY ANCIENT, CLASSICAL, AND NOVEL LIGANDS. STRUCTURE– ACTIVITY RELATIONSHIP

MARIO D. GALIGNIANA AND G. PIWIEN PILIPUK

The University of Michigan Medical School
Departments of Pharmacology and Molecular and Integrative Physiology
Ann Arbor, Michigan 48109

The mineralocorticoid effect on epithelial cells is the resultant of an intricate net of biochemical regulations that ultimately leads to the maintenance of electrolyte homeostasis. Two key protagonists in this plot are the ligand, which broadcasts the information, and the receptor, which functions as a receiver and transducer. Therefore, the responsibility for the final biological effect is not limited to each individual component but to both of them, so they constitute a functional unit. In addition, several prereceptor regulatory mechanisms are also determinant factors for the final biological response. Because steroids are present in both animals and plants and are derived from common precursors, it is intriguing how these simple molecules have acquired specialization to shape biological development and differentiation. This is particularly true for the function of aldosterone in mammals, which is mimicked by glucocorticoids or progesterone in some particular cases. Inasmuch as the most potent mineralocorticoid in nature, aldosterone, shows a poorly angled steroid nucleus at the A/B-ring junction, and because steroids that possess identical functional groups and different steroidal frames elicit different mineralocorticoid effects, we postulate that a planar conformation of the ligand is a key requirement to acquire potent sodium retention properties. The model takes into consideration all the mechanisms involved in the regulation of the final biological effect, although it does not provide a definitive answer to the original question. It is also discussed how the use of novel mineralocorticoid ligands may shed light on the still obscure mechanism of action of the mineralocorticoid receptor. © 2004 Elsevier Inc.

I. INTRODUCTION

Mineralocorticoids are so named for their role in the maintenance of the electrolyte balance, aldosterone being the most potent mineralocorticoid ligand in nature. Aldosterone is produced in the *zona glomerulosa* of the adrenal cortex, and its secretion is increased by diverse stimuli such as the level of pituitary adrenocorticotropic hormone (ACTH), kidney angiotensin II, hyponatremia, hyperkalemia, and dopamine agonists, and inhibited by atrial natriuretic peptide, dopamine, somatostatin, fatty acids, and so on.

(reviewed by Agarwal and Mirashi, 2000). Since a long time ago (Vander *et al.*, 1958), the antinatriuretic and kaliuretic effects of aldosterone and its effects on blood pressure were thought to be the result of genomic effects in the distal portion of the nephron. Rapid nongenomic effects of aldosterone were subsequently described for sodium exchange in erythrocytes (Spach and Streeten, 1964). However, the lack of suitable cell mammalian cell lines that exhibit vectorial sodium transport and express a good concentration of mineralocorticoid receptor (MR) and no glucocorticoid receptor (GR) has undoubtedly slowed progress in this field as compared with other steroid receptors. Nonetheless, a number of advances have been made during the last few years. Among them is the characterization that the serine–threonine kinase sgk-1 is one of the main proteins directly induced by aldosterone via the MR, which enhances the amiloride-sensitive epithelial sodium channel-dependent sodium current by phosphorylation of channel subunits (Chen *et al.*, 1999; Náray-Fejes-Tóth *et al.*, 1999; Shimkets *et al.*, 1998).

Binding of steroid to its cognate receptor constitutes a primordial first step to trigger a series of events that lead to the genomic biological effect. Because the proper recognition of both components of the functional unit is essential, ligands should exhibit some structural properties to allow an efficient activation of the steroid–receptor complex. As a first approach, Duax and colleagues (1978, 1982) summarized some minimal conformational requirements on ring A of steroid hormones for optimal binding to different receptors; in the case of the MR, the ideal conformation would be, according to those studies, a 1α-envelope to a $1\alpha,2\beta$-half-chair containing the 3-*keto*-4 *ene* function. Higher affinity ratios for the MR were also obtained when substituents that tend to bend the A-ring toward the α face of the steroid molecule were eliminated, for example, steroids lacking the C_{19}-methyl group or the C_{11}-hydroxy function (Yamakawa *et al.*, 1986). A similar effect is observed upon introduction of ketalic bridges, which flatten the overall structure, as in aldosterone and related 18-oxygenated analogues.

Although several compounds have been synthesized for all the other types of steroid receptors (and replaced the natural ligands in many clinical treatments), only one synthetic agonist showing no cross-reaction with the other members of the steroid receptor subfamily is currently available to study the mineralocorticoid function—11,19-oxidoprogesterone. This limitation may be explained if we take into consideration that the multifactorial regulation of the mineralocorticoid effect has always made the structural requirements of an ideal ligand very difficult to determine. In fact, it is accepted that no correlation between ligand structure and biological effect exists. On the other hand, a considerable effort has been invested on the development of antimineralocorticoid compounds, particularly during and after the RALES (Randomized Aldactone Evaluation Study) and EPHESUS (Eplerenone Post-AMI Heart Failure Efficacy and Survival Study) trials

(see Funder, 2003, for an updated editorial). There are several reports about these trials, as well as qualified review articles that referred to each particular topic in the mineralocorticoid field. This is why we decided not to address classical subjects with extreme detail. Our aim was to provide an integrated and original vision of the ligand-dependent mineralocorticoid action (i.e., from the possible origin of the mineralocorticoid function to the particular mechanistic aspects recently evidenced, thanks to the use of novel ligands): In this regard, we attempted to emphasize all those aspects that make each steroid–receptor complex a unique functional unit. We review recent findings in the structure–activity field and overview some facets of the regulatory mechanisms that may have shaped the selection of those structural features of the ligand. We propose that the overall planarity of the steroidal frame may be critical to confer a steroid mineralocorticoid activity *in vivo*.

II. THE NUCLEAR RECEPTOR SUPERFAMILY

One of the most abundant classes of transcriptional regulators in metazoans is the nuclear receptor superfamily. As such, the MR not only functions as a receptor for a given ligand, but also as a transcriptional regulator. Nuclear receptors are phylogenetically related proteins clustered into a large superfamily (Evans, 1988), which includes receptors for hydrophobic molecules such as steroid hormones (e.g., estrogens, progesterone, androgens, glucocorticoids, mineralocorticoids, vitamin D, ecdysone, oxysterols, bile acids), retinoic acids (all-*trans* and 9-*cis* isoforms), thyroid hormones, dioxin, sterols, fatty acids, leukotrienes, and prostaglandins.

All nuclear receptors share common structural and functional domains (Fig. 1). A canonical receptor possesses five or six such functional domains named A through E–F (Mangelsdorf *et al.*, 1995). The best conserved is the central portion of the molecule, known as region C or DNA-binding domain (DBD), which facilitates sequence-specific interaction with the major groove of the DNA double helix. The DBD is composed of two highly conserved zinc-fingers that set the nuclear receptors apart from other DNA-binding proteins (Klug and Schwabe, 1995). The E region is located toward the C-terminal half of the receptor and possesses a moderately conserved domain, the ligand-binding domain (LBD), which is separated from the C-terminal amino acid by a short and nonconserved domain named region F. The function of region F remains relatively unknown for most members of the superfamily, although for some of them, the F domain has been implicated in conferring ligand specificity and influence on transcriptional activation (Montano *et al.*, 1995). In spite of the fact that all members of the superfamily have a LBD, the specific endogenous ligands are still to be identified for many receptors, so they are generically referred to as "orphan receptors."
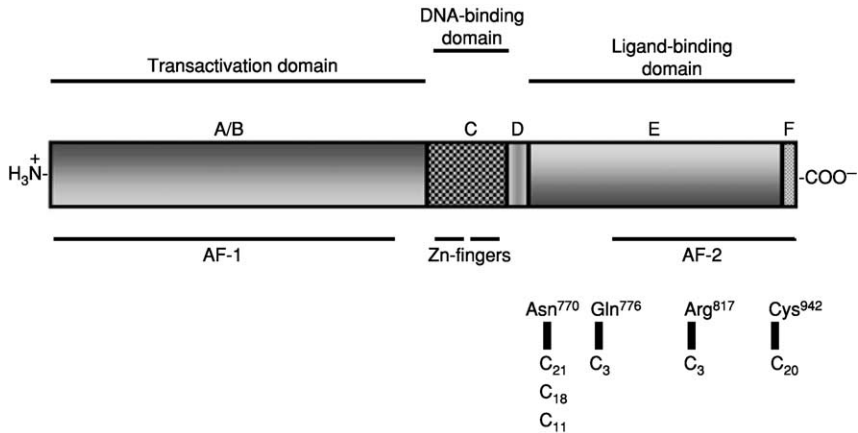
FIGURE 1. Schematic representation of a nuclear receptor. All nuclear receptors share a common set of domains characterized by a central DNA-binding domain flanked by the ligand-binding domain at the C-terminal half and the transactivation domain at the N-terminal half. Some functional regions of the molecule are commonly named with letters. The figure also shows essential amino acids of the hMR that are in contact with functional groups of aldosterone that radiate from the carbons indicated below the black bars. Based on studies by Fagart *et al.*, 1998; Lupo *et al.* 1998; and Rafestin-Oblin *et al.*, 2002.

Within the LBD, the residues used in receptor dimerization and the ligand-dependent activation function, AF-2, can be identified. Regions C (DBD) and E (LBD) are separated by a nonconserved hinge (region D). The N-terminal half corresponds to the hypervariable A–B domain and represents the ligand-independent activating function domain, AF-1. AF-1 functions in a cell-context- and promoter-specific manner and cooperates with AF-2 in regulation of transcription (Leid *et al.*, 1992). Although the A–B region displays no sequence conservation between different members of the superfamily, it has been well conserved through evolution for a given receptor.

Two possible evolutionary histories may have led to such a particular intramolecular organization of nuclear receptors. Different domains may have different origins, such that those related to the regulation of metabolism became fused to a DNA-binding motif to produce a transcription factor. On the other hand, a multidomain precursor that initially mediated a simple signal transduction mechanism may have acquired increasingly complex functions. Based on protein sequence comparison and evolutionary analysis, the second model is more accepted nowadays. Regardless of how the organization of nuclear receptors had taken place during evolution, they represent only a part of the story. As we shall address later, the ligands are the other 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

subject of other kinds of non-hormonal- and/or non-receptor-dependent regulations.

III. STEROID RECEPTORS

Based on the alignment of both DBD and LBD and due to phylogenetic analysis, the members of the superfamily were classified in a consensus tree defined by six subfamilies of receptors (Laudet, 1997). Thus, the receptors with steroid-binding ability comprised the same subfamily of highly homologous members, that is, estrogen receptor (ER), estrogen-related receptor (ERR), androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR). 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). It is thought that mutations in the ancestral ER P-box sequence favored the selection of receptors belonging to the GR-subgroup, which were adapted to recognize the novel site for anchorage (Zilliacus et al., 1994).

The other members of the superfamily are more distant from those related to the adrenal and sex steroid receptors (Baker, 1997). An important observation is that the human sequences of steroid receptors are closely related to their homologous forms in amphibians and fish. Thus, it is possible that divergence of this subfamily from other nuclear receptors occurred before the appearance of fishes. It has been predicted that a putative ancestral steroid receptor appeared in the primitive chordate Amphioxus (Baker, 1997; Owen and Zelent, 2000), perhaps resembling the features of the ER, the most representative member of the ER-subgroup.

To better understand the molecular mechanism of action of the steroid–receptor unit, we have to understand the context in which both components of the signaling pathway have evolved. The irruption of steroid receptors as transducers of physiological responses mediated by adrenal and sex steroids provided early vertebrates with an advantage in competing with the diverse organisms that evolved during the Cambrian explosion and lacked either some or all of these receptors. It is thought that the members of the GR-subgroup are derived from an ancestral receptor that underwent genome duplication to give GR–MR and AR–PR ancestors (Escriva et al., 1997). This process was then followed by a new diversification step to give the four separate receptors. Steroidogenesis studies from fish interrenal tissues (an organ equivalent to the adrenal cortex of mammals) could not demonstrate that aldosterone can be synthesized, so the general consensus is that most fish do not produce this steroid, and mineralocorticoid effects are mainly

managed by glucocorticoids (Wendelaar Bonga, 1997). Moreover, due to the absence of the MR in fish, it was thought that this receptor might have been the last of the nuclear receptors to have been brought under tight evolutionary control. Interestingly, a recent report described the presence of a mineralocorticoid-like receptor in the rainbow trout that favors cortisol binding over aldosterone binding (Colombe *et al.*, 2000).

On the other hand, the other characters of this plot, the ligands, share the general property of being small and hydrophobic molecules. Many of them are derived from common precursors, which are also found in plants. However, there are no equivalent receptors between both kingdoms (Clouse, 2002). This is interesting because it raises the question of how similar and simple compounds have been assigned to shape the biological development and differentiation in metazoans. The existence of common molecules may be the indication that common biosynthetic pathways and/or identical precursors were used as a tool during the evolutionary process to create signaling molecules. This may be particularly true for some ligands of the nuclear receptor superfamily such as steroids, retinoids, or prostaglandins, which are the same in plants and animals and are derived from similar chemical precursors. The use of common precursors certainly constrains the structure of the hormones, but it also means that their structures have inherent features that make them appropriate signaling molecules regardless of the organism where they function. Perhaps, this property may be related to the hypothesis that the primigenial membranes in protozoans were not the fatty-acid bilayers of today, but were probably terpenoid-based compounds. Thus, steroids and retinoids, which are terpenoid derivatives, may have evolved to signaling molecules when the organism needed to collect information from its outside world.

IV. THE MINERALOCORTICOID EFFECT

It is thought that life originated in the sea, so the circulating fluids of all animals may resemble the composition of the sea water of some millions of years ago, when life began. Since that time, the composition of that primitive Archean Ocean has been gradually changing due to precipitation of certain compounds on the sea bed and the washing-down of others by river action. The animals of today are clearly the consequence of evolutionary adaptation to the new environment during those millions of years; however, despite the great biological divergence, the composition of their blood is remarkably alike in ionic composition. This indicates that the conditions for life are very restricted and have remained relatively constant during evolution. Therefore, animal life has necessarily been attended by mechanisms that maintained an environment with the properties required for the continued life of its cells.

When the life forms abandoned the sea water, they faced the additional difficulty of keeping the osmotic pressure of their blood above that of the surrounding fresh water. Moreover, as soon as animals began to move on from water toward the land, far-reaching changes in the regulatory mechanisms became extremely necessary because the limits of tolerance are even narrower due to the additional influence of other new factors, such as evaporation and perspiration. The late developing of the MR, along with CYP11B2 (aldosterone synthase), offered a clear answer to specifically preserve the intracellular milieu from permanent environmental changes without influencing other biological functions.

The MR is thus far the largest member of the nuclear receptor superfamily. It shows the highest percentage of homology with its subgroup partner, the GR, not only when their DBDs are compared (94%), but also their LBDs (57%) (Evans, 1988). Consequently, cross-reactions with ligands and hormone-response elements are expected. In epithelial tissues, the classical mineralocorticoids aldosterone and 11-deoxycorticosterone (DOC) (Fig. 2) enhance the reabsorption of sodium and affect the transport of hydrogen and potassium ions, although these two effects are known to be mechanistically independent of the antinatriuretic action (Bastl and Hayslett, 1992; Lantos
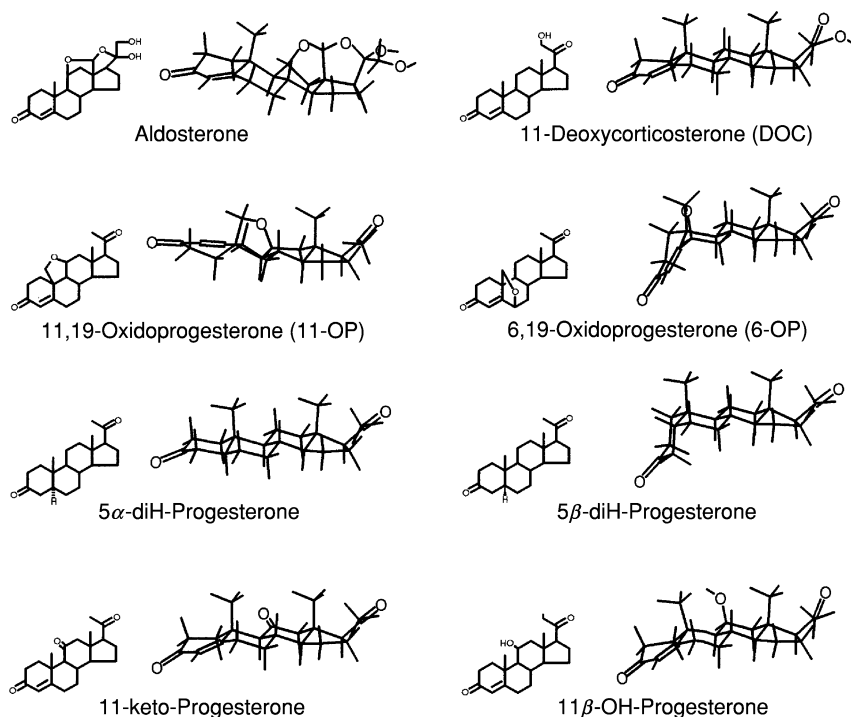

FIGURE 2. Structures and most stable conformation of some steroids discussed in this chapter.

et al., 1981; Young, 1988) and are quantitatively less significant. 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 lower abundance in other tissues. The most potent natural mineralocorticoid agonist is aldosterone (Fig. 2), its main chemical property being the presence of a hemiketalic ring that involves its aldehyde in C_{18} (from which the name aldosterone derives).

Despite being cloned several years ago (Arriza et al., 1987), many basic features of the MR function and its regulation have yet to be fully characterized; among them, how it 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 higher 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-hydoxysteroid dehydrogenase-2 (11βHSD-2) (Edwards et al., 1988; Funder et al., 1988). This enzyme plays a key role in discriminating glucocorticoids from mineralocorticoids by metabolizing the former compounds to their 11-dehydro derivatives. Thus, in cells coexpressing both the MR and 11βHSD-2, the receptor should be protected from the permanent occupancy of the highly abundant glucocorticoid steroids. The major role of 11βHSD-2 can be evidenced by situations where the enzyme is inactive due to mutations (apparent mineralocorticoid excess syndrome) or due to its inhibition (licorice derivatives). Accordingly, 11βHSD2$^{-/-}$ mice develop hypertension, polyuria, hypokalemia, and hyponatruria and show low plasma levels of aldosterone and renin activity (Kotelevtsev et al., 1999).

However, a series of questions still deserve an answer. It is known that the 11βHSD-2 does not colocalize with the MR in the hippocampus, such that the MR should be ''unprotected'' and presumably overwhelmingly occupied by glucocorticoids. Nevertheless, 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, if not antagonizes them, in extraepithelial tissues where the receptor does not colocalize with the enzyme (Funder and Miles, 1996; Gómez-Sánchez et al., 1990; Sato et al. 1995; Young et al., 1994).

All of the previously 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 mineralocorticoid and glucocorticoid ligands. For example, even when the dissociation constant (Kd) 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 to form heterodimers with the GR (with which it coexists in many tissues) that 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 TR (Lim-Tio and Fuller, 1998), although it is still uncertain whether the MR can form heterodimers with these receptors.

The intranuclear distribution of the MR and the GR in hippocampal neurons shows a nonhomogenous distribution; that is, many clusters exclusively contain either MR or GR, although a number of nuclear domains were found to contain both receptor types (Van Steensel et al., 1996). Finally, interactions with specific coactivators and/or corepressors may regulate differentially the transcriptional activity of both corticosteroid receptors. In this regard, it was recently reported that there exists a ligand-selective regulation of the MR (Kitagawa et al., 2002), so 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, a chromatin immunoprecipitation assay demonstrated that aldosterone binding, but not cortisol binding, recruits the RHA–CBP complexes to native MR target gene promoters (Kitagawa et al., 2002).

Inasmuch as it is thought that the MR has evolved more recently than other members of the nuclear receptor family, the fact that the MR shows no specificity to bind ligands when it is assayed in an isolated system is somehow surprising. Thus, primigenial steroids such as ''ancient'' progestins, which are also present in plants and yeast (Geuns, 1978), are capable of binding to one of the more recently evolved members of the nuclear receptor superfamily without showing significant differences in affinity with respect to ''classical'' glucocorticoid and mineralocorticoid ligands. During the evolutionary process, this inconvenience was resolved in part by adding a number of prereceptor checkpoints, which have preserved ''ancient'' ligands for their specific biological actions. On the other hand, the synthesis of ''novel'' (now classical) ligands such as aldosterone during that evolutionary period enhanced the specificity for the mineralocorticoid effect. Such an intricate and complex physiological and molecular interrelation has made the elucidation of the structure for an ideal sodium-retainer ligand very difficult to determine, as well as making the comprehension of the molecular mechanism of regulation of the MR difficult.

V. STRUCTURE–ACTIVITY RELATIONSHIP FOR THE MINERALOCORTICOID EFFECT

Taken together, all of the previously described scenarios make not surprising the existence of cross-talks between ''specific'' ligands for a determined receptor and many members of the subfamily of steroid receptors.

Similarly, cross-talks of ligand–receptor complexes with the ''specific'' hormone response elements can also be predicted. Therefore, it is 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.

Even though the analysis of the structural requirements needed for an ideal mineralocorticoid agonist have always been extremely difficult to define, 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 postulation that mineralocorticoids may require a flat conformation for optimal activity *in vivo* (Lantos *et al.*, 1981). To provide a graphic example, Fig. 2 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 with other pregnanesteroids, with a more angled steroid nucleus toward 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 synthesized *ex profeso* to study their mineralocorticoid properties. As expected, the flat steroid 11-OP is a selective MR ligand and 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 (Burton *et al.*, 1995; Galigniana *et al.*, 1993; Piwien-Pilipuk *et al.*, 2002b).

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, a series of 33 pregnanesteroids with diverse geometrical parameters and functional groups was recently studied, where the Na^+-retaining capacity and relative binding affinity for the MR were analyzed with respect to the overall planarity of the steroidal skeleton (Piwien-Pilipuk *et al.*, 2002b). In that study, it was shown that a trend that correlates the steroid structure and its biological activity may be achieved if the sodium-retaining effect is analyzed *in toto*.

In this chapter, we have included some examples where 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 are depicted, many of them without a previously studied mineralocorticoid effect (Fig. 3). Intriguingly, most steroids exhibit a parabolic function; that is, a maximal antinatriuretic effect, which varies according to the steroid, is shown at certain doses, whereas a clear reversion of the effect is observed at higher doses. Although less evident than for other steroids, the tendency to reverse the Na^+-retaining effect can also be observed for the most active compounds, including aldosterone, at the highest doses. Such a biphasic function of the dose–response curves makes unsuitable the use of the ED_{50} value to quantify the biological effect. Thus, a classical ED_{50} does not consider the multiple parameters involved in the parabolic
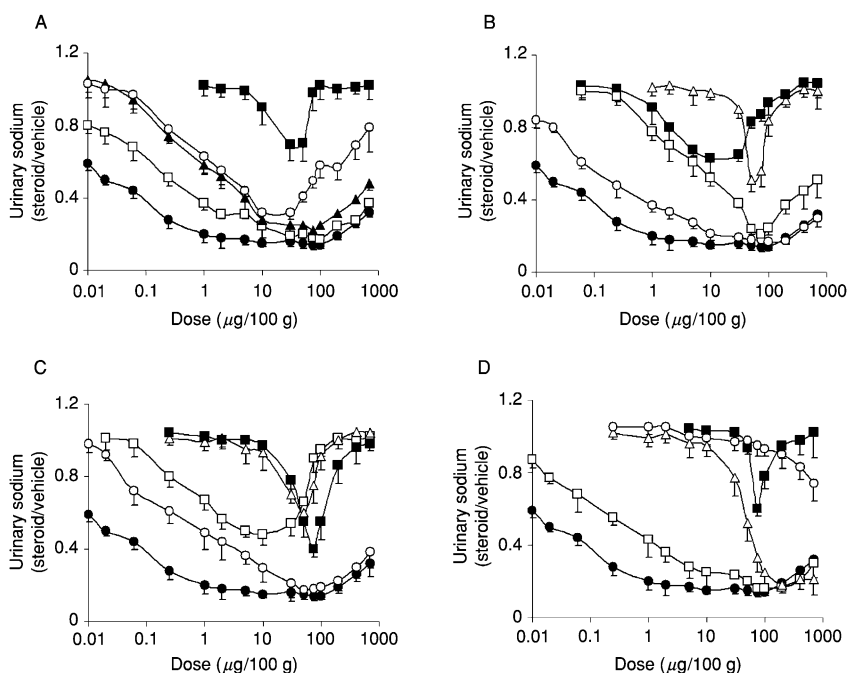
FIGURE 3. Dose–response curves for some representative steroids. Urinary sodium elimination was measured after injecting adrenalectomized male rats with the indicated doses of steroid expressed in μg of steroid per 100 grams of rat body weight. Results were normalized to steroid/vehicle sodium elimination ratio. These plots were modified from original data published by Piwien-Pilipuk *et al.*, 2002b. For the sake of clarity, only a few dose–response curves were reproduced here and arbitrarily grouped on 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.

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 problem could be partially solved by correlating the sodium-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 represent in turn the biopharmacological parameters of the dose–response curves obtained with each steroid. The study was only focused on sodium-retaining action because the kaliuretic effect is also affected by many factors other than mineralocorticoids.

When the A/D angle, the A/BCD angle, and the $C_3 = O/D$ angle were plotted against the coefficient "a," the latter parameter showed to be the best to demonstrate a correlation between the biological effect and both the geometry of the steroids (Fig. 4A) and ligand binding to the MR (Fig. 4B). 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. 4C 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.

Later, we shall analyze with more detail a particular case, 11-OP. This potent synthetic agonist is excluded from the correlations depicted in Fig. 4B and C (see arrows), but not from the correlation shown in Fig. 4A, where a pure *in vivo* measurement is shown. As discussed further in Section IX, the reason for these exclusions is because 11-OP (as well as its 21-hydroxy derivative) may bind to the MR at an alternative-binding site or because these ligands may accommodate their steroidal frames with a different positioning in the LBD of the receptor.

Because the coefficient "a" includes all the variables that affect ligand availability for the MR *in vivo*, this correlation is not surprising, but it should not be confused with the oversimplified conclusion that affinity potency for the receptor *in vitro* can predict ligand potency. 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 semiquantify, 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 ligands that show no parabolic function in the range of doses assayed, that is, steroids that exhibit a weak mineralocorticoid effect *in vivo* (see Section VI).

Attempts to find correlations by using several other parameters were unsuccessful (e.g., steroid hydrophobicity, hydration sphere, length of the molecule, total surface area, van der Waals radius, electronic density). Importantly, no straight correlation has ever been found previously when
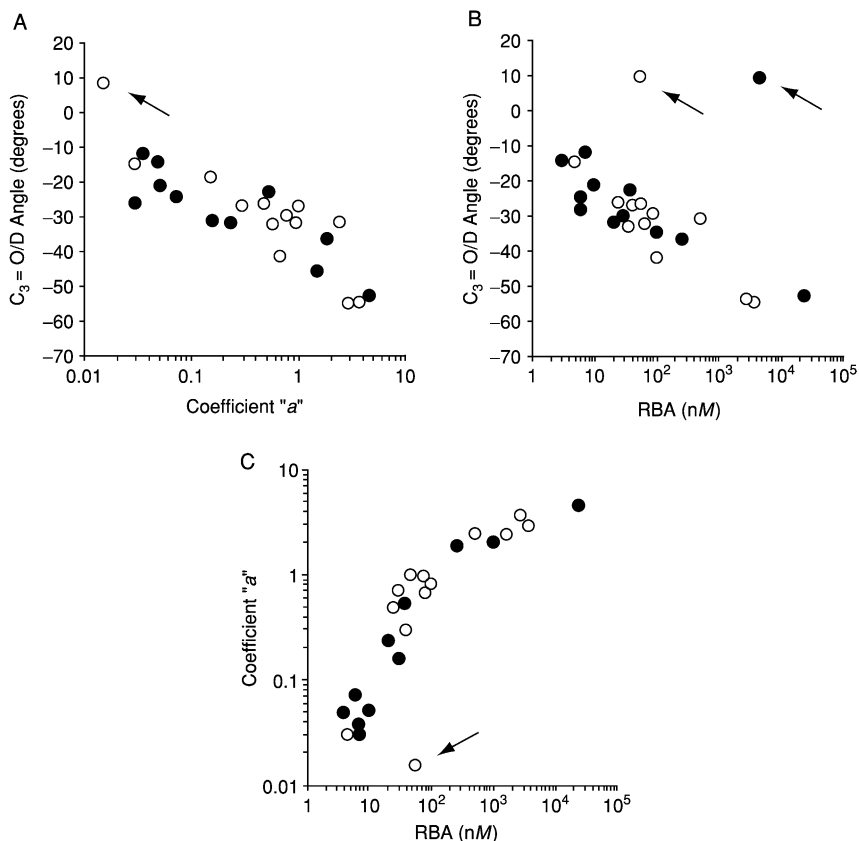
FIGURE 4. Structure–activity relationships. (A) Correlation between the $C_3 = O/D$ angle of the steroids (geometric parameter) and the second-order coefficient "a" (biological parameter for the biphasic sodium-retaining effect). (B) Geometric parameter of the steroids versus the relative affinity for 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 are: (○), 21-deoxypregnanesteroids; (●), 21-OH-pregnanesteroids. (Data from Piwien-Pilipuk et al., 2002b).

the "mineralocorticoid effect" was studied *in vitro*, that is, the MR-dependent activity of a gene–reporter in transiently transfected cell lines (Agarwal and Mirashi, 2000; Grassi *et al.*, 1997; Piwien-Pilipuk *et al.*, 2002b; 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 involved in the resultant mineralocorticoid action.

One of the most important derivations of the previously 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 (for example, 11-OP vs 6-OP or 5α-*diH*-progesterone vs 5β-*diH*-progesterone, at high doses). Therefore, an explanation for this feature can be found in the overall conformation of the ligand (i.e., flat steroids such as those named in first term are more potent mineralocorticoids than their respective bent counterparts). This postulate is highly notorious for 6-OP, which is an almost inert steroid even though the chemical groups are identical to those of the highly active agonist 11-OP (see Figs. 2 and 3).

It is interesting to point out that the divergent features of some pairs of compounds used in those studies undermine the functional importance of the C_{21}-OH function. Thus, progesterone is a weak mineralocorticoid and its 21-hydroxylated derivative, DOC, is a strong one. 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 (Fig. 4D). In contrast, 21-deoxy-aldosterone loses activity with respect to aldosterone, but even so, the biological effect of 21-deoxy-aldosterone still remains strong (i.e., similar to DOC). As discussed later, there are also additional factors to consider when these cases are analyzed, such as the length and flexibility of the molecule.

VI. LIMITATIONS OF THE MODEL

Although this model may be useful for drug design, it does not provide an absolute answer to the original question and some restrictions apply for many cases. For example: (1) some steroids exhibit good steroid binding ability in a cell-free system, but they are totally devoid of biological effect *in vivo* in a wide range of doses, such as corticosterone and cortisol, both steroids being good substrates for the enzyme 11β-hydroxysteroid dehydrogenase; (2) a steroid such as dexamethasone, which exhibits a relatively weak affinity for the mineralocorticoid receptor and poor mineralocorticoid activity, does not show a parabolic function; (3) other steroids may exhibit good sodium-retaining properties at certain doses, but a countereffect is not seen and the second-order coefficient "a" cannot be calculated (e.g., 21OH-11,19-oxidoprogesterone). In spite of these limitations, the alignments observed for most of the compounds are clear and reflect a similar tendency for both 21-deoxy and 21-hydroxypregnanesteroids.

As mentioned before, these studies do not address an important factor that greatly affects the adaptability of a given ligand to the binding site in the

receptor: the flexibility of the steroidal frame. However, some speculations may be considered. Like the classical agonist aldosterone, the structures of the novel 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 (there are no cross-reactions with other steroid receptors), 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, which is substantially shorter than the 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 ($+8.9°$ vs $-14.6°$, respectively). However, its shorter length could 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 (it is fully active at doses $\geq 75 \mu g$) in spite of possessing similar length and a C_{21}-OH functional group as the 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 agonistic activity is poor. Indeed, progesterone is an antagonistic steroid. Recent studies performed with hMR (which progesterone binds to with similar 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).

VII. PROGESTERONE AND PROGESTERONE DERIVATIVES

Progesterone binds to the MR with relatively high affinity, but confers only weak sodium-retaining activity per se and is an antagonistic ligand for full agonists. However, a family carrying a mutation in the MR where Leu is substituted for Ser at the 810 position has recently been described, which leads to a receptor that can be activated by aldosterone and progesterone and causes an early onset of hypertension that is markedly exacerbated during pregnancy (Geller *et al*., 2000).

Our understanding of how steroid receptors are activated was greatly improved by the elucidation of the crystal structures of several nuclear receptors (Bourget *et al*., 1995, 2000; Brzozowski *et al*., 1997; Matias *et al*., 2000; Renaud *et al*., 1995; Wagner *et al*., 1995; Williams and Sigler, 1998). The main difference between the unliganded receptor and the agonist-bound

nuclear receptor seems to be the positioning of the H12 helix that harbors the ligand-activated transactivation. Thus, the H12 helix protrudes away from the receptor in the unliganded state and is folded toward the core of the LBD in the agonist-associated receptor. This repositioning of H12 helix in the presence of ligand is accompanied by the bending of the H3 helix, which in turn creates an interface suitable for the binding of coactivators (Bourget *et al.*, 2000). Interestingly, the 21-hydroxyl group of a mineralocorticoid agonist seems to be not required when an H3 helix–H5-helix interaction is established by substituting Leu for Ser at the 810 position of the hMR, which may explain why progesterone becomes a potent mineralocorticoid agonist in this pathology (Geller *et al.*, 2000).

Progesterone plasma level increases significantly during the luteal phase of the menstrual cycle and peaks at very high concentration at the end of the third trimester of pregnancy (Johansson and Johansson, 1971). In contrast, aldosterone increases only slightly during the luteal phase and late pregnancy (Nolten *et al.*, 1978). In view of the high affinity of progesterone for hMR, it is unclear how aldosterone can maintain its function as an effective agonist in the presence of progesterone in physiological conditions. One possible explanation may be the strong binding of progesterone to plasma proteins as compared with aldosterone, so free progesterone is 3% of the total plasma concentration versus 30% for aldosterone. The effective metabolism of progesterone may constitute another additional mechanism to exclude this ''ancient'' ligand from competition—a highly effective metabolism of progesterone in renal cells (Bumke-Vogt *et al.*, 2002; Quinkler *et al.*, 2003). Notwithstanding evidence that progesterone can antagonize the action of aldosterone, there are studies indicating that aldosterone is responsible for maintaining sodium balance at a level appropriate for the stage of pregnancy (Bay and Ferris, 1999), so when sodium balance is perturbed, immediate and appropriate changes in aldosterone plasma concentration and sodium excretion are promoted to restore electrolyte balance. In this regard, it is interesting to emphasize that, in spite of the high plasma level of progesterone, the administration of mineralocorticoids to pregnant women still stimulates sodium retention and an increase in body weight. This demonstrates that the MR is capable of responding to the agonist and that the signaling mechanism that leads to the activation of the epithelial sodium channel is still active (Ehrlich and Lindheimer, 1972).

Interestingly, many of the metabolites of progesterone themselves have mineralocorticoid effect. A good example is 5α-*diH*-progesterone, a steroid that is produced by renal cells from progesterone (Quinkler *et al.*, 2001) and possesses a strong sodium retaining activity (Burton *et al.*, 1995; Piwien-Pilipuk *et al.*, 2002b). As emphasized earlier, 5α-*diH*-progesterone lacks 21-hydroxyl group and exhibits a flat conformation. Interestingly, 5α-reduced metabolites of progesterone, but not 5β, are potent inhibitors of the 11β-hydroxysteroid dehydrogenase activity (Latif *et al.*, 1997), which

in turn may exacerbate glucocorticoid-mediated sodium retention when progesterone levels are high.

11β-Hydroxyprogesterone and its 11-*keto*-derivative (see structures in Fig. 2) are another couple of steroids that are interesting to analyze with more detail. Almost a decade ago, it was described that these two compounds constitute a shuttle pair of metabolites for the enzyme 11βHSD (Galigniana *et al.*, 1994, 1997). Classically, 11-*keto*-derivatives are 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 (see Fig. 3). Like most of the other steroids, both compounds elicit an identical mineralocorticoid reversal effect above 50 μg/100 g, until sodium retention is totally abolished at doses close to 100 μg/100 g. The particular biological effect of 11-*keto*-progesterone may be assigned to the combined action of several factors, that is, the lack of the 11β-hydroxy group that partially flattens the steroidal frame 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.

Both steroids (11β-hydroxy- and 11-*keto*-progesterone) are potent inhibitors of the enzyme 11βHSD, such that the coinjection of corticosterone elicits a strong aldosterone-like effect (Galigniana *et al.*, 1997). In the early 1980s, the presence of a dialyzable, heat-stable 11βHSD inhibitor in human fetal tissues such as the chorium frondosum and the fetal zone of the adrenal gland had already been reported, as well as in the umbilical cord serum at term (López-Bernal *et al.*, 1979; Murphy, 1981). More recently, Souness *et al.* (1995) showed that 11β-hydroxyprogesterone and its 11α isomer are among these inhibitors. For 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 as high during the last trimester of gestation as to reach a concentration similar to the *Ki* for 11βHSD, and because both steroids are also found at high concentration in amniotic fluid and umbilical blood, it could be possible that both progesterone metabolites may contribute to the hypertensive disorder observed during pregnancy.

It was recently demonstrated in an elegant work (Rafestin-Oblin *et al.*, 2002) that the docking of 11β-hydroxyprogesterone within the LBD of hMR is caused by contacts between the 11β-hydroxy group of the ligand and the Asn at position 770; thus 11β-hydroxyprogesterone acquires agonistic activity as compared with progesterone. This study confirmed that the MR-dependent biological response is still possible, even in the absence of the 21-hydroxyl group.

VIII. EXTRA-ADRENAL MINERALOCORTICOID AGONISTS

Over half a century ago, it was already known that collagen deposition during tissue repair is largely governed by corticosteroids (Selye, 1946). Using classic methods of physiology and pharmacology, several studies have shown that cardiovascular tone may be modified by mineralocorticoids (reviewed by Ullian, 1999) (i.e., by promoting collagen deposition and enhancing tissue repair). On the other hand, glucocorticoids function as anti-inflammatory and antifibrotic agents. Until recently, it was assumed that these steroids were derived solely from adrenal glands. However, there is now convincing evidence demonstrating that cells of the heart and vasculature express genes responsible for the formation of aldosterone and corticosterone and that they produce these steroids. Vascular endothelial and smooth muscle cells express CYP11B1 and CYP11B2, genes responsible for 11β-hydroxylase and aldosterone synthase, respectively. Furthermore, smooth muscle cells also synthesize aldosterone. Nonetheless, the function of locally generated ligands remains to be fully elucidated; however, it would appear highly likely from what is already known that cardiovascular tissue-derived aldosterone does not contribute significantly to the circulating pool of aldosterone. Because cardiac MR may be occupied by locally produced agonists on a concentration basis, the local production of steroids might preclude the need for pre-receptor-mediated mechanisms of protection (such as a high activity of 11β-hydroxysteroid dehydrogenase).

Although the primary site of pharmacological action of aldosterone is at the MR, rapid (30 sec to 10 min) effects of the steroids have also been described (reviewed by Losel and Wehling, 2002 and Losel et al., 2002). These effects are not inhibited by spironolactone and are insensitive to actinomycin D and cycloheximide, so they are assigned to a plasma membrane receptor. Rapid actions of aldosterone were described in several tissues and cell types such as the distal tubule of the kidney, myocardiocytes, vascular smooth muscle cells, mononuclear leucocytes, brain, aortic endothelial cells, and liver (reviewed in Falkenstein et al., 2000).

In vascular smooth muscle cells, aldosterone (as well as fluodrocortisone) induces the activation of the sodium–proton exchanger, and affects sodium influx, cell volume, intracellular pH, and calcium concentration. These effects, which cannot be antagonized by carenone, are corelated with the rapid (30 sec) generation of inositol-1,4,5-triphosphate and diacylglycerol. On the other hand, classical glucocorticoids such as dexamethasone and cortisol show a weak agonist activity at suprapharmacological concentrations only. Because the EC_{50} for aldosterone is similar to the concentration of free aldosterone in human plasma, a physiological role of the rapid aldosterone-induced effects was hypothesized (Falkenstein et al., 2000).

At the present time, it is unknown whether a relationship between the ligand structure and its capability to activate the plasma membrane receptor exists. Also, it is highly possible that, if such correlation exists, it would not parallel that described for the conventional MR. As a matter of fact, the effects of aldosterone are not antagonized by spironolactone or carenone, and are not triggered in isolated cell systems by cortisol and other glucocorticoids unless supramicromolar concentrations are used. This is in high contrast with respect to the nonselective properties showed by the classical MR under similar experimental conditions.

The discovery of nongenomic actions for steroids raises the possibility that a two-step mechanism of action may be responsible for the magnitude and intensity of the final biological effect of a mineralocorticoid ligand (i.e., a rapid, nongenomic stimulation of signaling cascades, followed by a delayed, traditional genomic response): It is possible that ligands may exert a type of self-control of their own genomic effects through a nongenomic action. Again, based on these observations and those described earlier, it become evident that no single factor can be solely responsible for the magnitude and intensity of the final mineralocorticoid effect (i.e., ligand, the classic MR, the putative plasma membrane receptor, metabolizing enzymes, carrier proteins, specific cofactors), but all of them in an integrated manner. Consequently, the proposed second-order coefficient "a" measured from a whole dose–response curve obtained *in vivo* would provide, within the boundaries of its own limitations, the most integrated and accurate parameter to evaluate the structure–activity relationship of the complex and multifactorial mineralocorticoid effect.

IX. A NOVEL SYNTHETIC MINERALOCORTICOID— 11,19-OXIDOPROGESTERONE

As shown in Fig. 4B and C, the relative affinity of 11-OP for the MR is lower than that expected due to its structural and 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, nongenomic effects, etc. All of them have already been studied and ruled out (Galigniana *et al.*, 2000). This includes a possible *in vivo* 21-hydroxylation because that putative metabolite, as discussed earlier, is even less active than 11-OP (see Fig. 3D).

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 unlabeled aldosterone to displace bound steroid (Galigniana *et al.*, 2000). Importantly, this effect depends on the concentration of 11-OP.

This observation led to the speculation that 11-OP may stabilize an ''active'' receptor conformation, which, in turn, may abrogate unlabeled aldosterone to compete with the [³H]aldosterone already bound to the MR. Accordingly, the dissociation rate constant of aldosterone from the MR is decreased 3-fold in the presence of a concentration of 11-OP that fails to compete per se with aldosterone.

A treatment with saturating doses of aldosterone (1 μg/100 g) or 11-OP (100 μg/100 g), injected either individually or jointly, yields a maximum mineralocorticoid effect (Fig. 5). Because no addition or potentiation of the effect is observed by coinjection of both agonists, a common pathway of activation can be inferred. Because the effect is efficiently antagonized by SC9420-spironolactone, such a shared pathway should be MR-mediated. Interestingly, a potentiation of the mineralocorticoid effect is obtained when a suboptimal dose of 0.06 μg/100 g of aldosterone ($\sim$50% of maximum) is coinjected 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 an inactive dose (1 μg/100 g) of 21OH-11-OP was used.

Based on these observations, it was suggested that the MR possesses a different binding site for 11-OP (and its 21-hydroxy derivative) from that of aldosterone. This may explain the experimental observations described earlier. Inasmuch as Scatchard plots performed with [³H]aldosterone always show a single slope, the putative ''11-OP site'' is not recognized by the natural
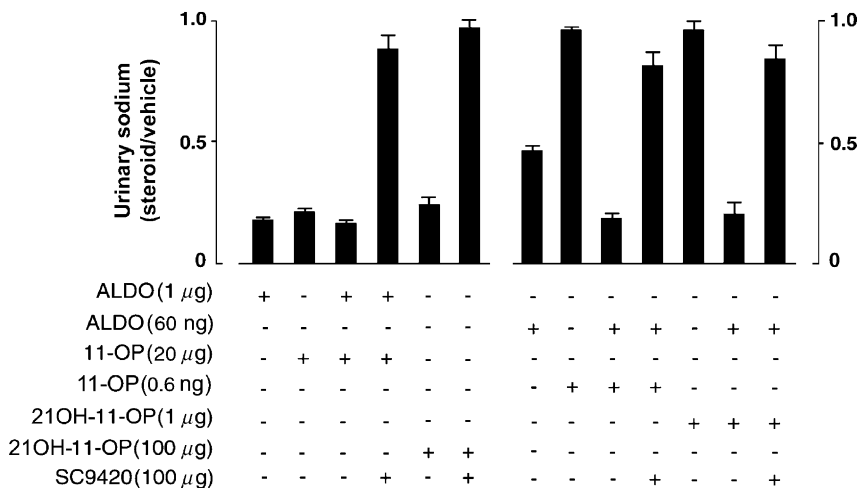
FIGURE 5. 11,19-Oxidoprogesterone and its 21-hydroxy derivative potentiate the mineralocorticoid effect of a suboptimal dose of aldosterone. Adrenalectomized male rats were injected with saturating doses (left panel) or suboptimal doses (right panel) of agonist ligands, and urinary sodium was measured 3 h after. The effects of aldosterone (ALDO), 11-OP, and 21OH-11-OP (injected alone or coinjected as indicated) were always antagonized by SC9420-spironolactone.

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 stabilizer 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 nM 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 nM and 100 nM (both capable of competing with aldosterone) decreased the affinity of the MR for aldosterone 2-fold (1.95 ± 0.25 nM) and 3-fold (3.54 ± 0.42 nM), respectively.

These observations suggest that 11-OP may be anchored to the aldosterone-binding pocket, which is permissive for flat molecules. In this regard, 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 adaptive process, so the structure of the receptor is influenced accordingly by the nature of the ligand. As a consequence, 11-OP may behave like an endogenous agonist.

Limited chymotrypsinization of the rat MR yields several proteolytic fragments, among them, a 34-kDa key peptide (Piwien-Pilipuk *et al.*, 2000). Under controlled experimental conditions, this peptide is fully degraded in unliganded receptor samples, whereas it is preserved from degradation when natural agonists are bound to MR. On the other hand, antagonists were incapable of protecting the 34-kDa fragment from full 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).

X. LIGAND-DEPENDENT CYTOPLASMIC TRAFFICKING OF THE MR

Our knowledge of the molecular mechanisms of action of steroid receptors is derived to a large extent from early studies on the GR and the ER, both receptors being the first members to be cloned. It is now well

established (Cheung and Smith, 2000; Morimoto, 2002; Picard, 2002; Pratt and Toft, 1997) that steroid receptors are capable to form heterocomplexes with the 90-kDa and 70-kDa heat shock proteins (hsp90 and hsp70, respectively), the acidic protein p23, and proteins that posses a tetra-tricopeptide repeat sequence (TPR) such as FKBP52, FKBP51, CyP40, PP5, or hop–p60. The model that prevails is that association to the hsp90–hsp70-based heterocomplex preserves steroid receptors in their transcriptionally inactive and high-affinity ligand-binding conformation, whereas dissociation of the heterocomplex upon binding of steroid promotes receptor dimeriza-tion, nuclear translocation (for cytoplasmic receptors), chromatin binding, and *trans*-activation or *trans*-repression. However, this model makes uncertain how the cytoplasmic form of the receptor moves throughout the cytoplasm toward the nucleus. A similar concept is also valid for primarily nuclear receptors (i.e., ER and PR) because, like the other members of the subfamily, they 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 (Black *et al.*, 2001; DeFranco, 2002; Vicent *et al.*, 2002). Thus, when the equilibrium of that nucleocytoplasmic shuttling favors one or another compartment, it is said that the receptor is primarily located in either cytoplasm or nucleus. Therefore, the simplest explanation for 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, that is, by using the microtubule-associated motor protein dynein (Galigniana *et al.*, 2001). If this novel model is correct, one may postulate that the newest members of the subfamily (i.e., the MR) may add more efficient check-points to regulate the biological response (e.g., anchoraging cytoplasmic factors, trafficking regulators, nuclear translocation factors) as compared with those members that are constitutively nuclear in the absence of ligand.

Most nuclear proteins, including steroid receptors, are highly mobile and are able to cross the nucleus in a few tens of seconds. However, subnuclear positioning of transcriptionally active loci appears confined to certain subnuclear regions only; so, it is thought that the ability of entire chromatin filaments to move throughout the nucleus must be compatible with it (Chubb and Bickmore, 2003). If this postulate is correct, it is logical to think that during the evolutionary process the interchromatin compart-ment should have avoided traffic congestion of proteins. Moreover, it is accepted that nuclear dynamics are also influenced by both nuclear size and nuclear volume (Gasser, 2002; Heun *et al.*, 2001). Therefore, it may be entirely possible that, in contrast to ancient and constitutively nuclear receptors, recently emerged members of the subfamily (such as GR and MR) had been excluded from the nuclear compartment to facilitate intranuclear trafficking of other macromolecular complexes and/or chromatin filaments.

Accordingly, the MR resides predominantly in the cytoplasm of the cell in the absence of steroid (Lombès *et al.*, 1994; Nishi *et al.*, 2001; Piwien-Pilipuk and Galigniana, 1998; Robertson *et al.*, 1993), although an equal distribution between cytoplasm and nucleus has also been observed (Fejes-Tóth, *et al.*, 1998). Upon steroid binding, the MR rapidly translocates into the nucleus (Galigniana, 2000). The nuclear fraction of untransformed MR 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 (e.g., detergents, high ionic-strength, treatment with DNase). It is still uncertain whether the presence of ligand can affect the intranuclear movement of the receptor or its dissociation from chromatin, and when ligand dissociation occurs.

Another still unknown step in the mechanism of action of the MR is its trafficking in and out of 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 use a trafficking machinery (which may well operate bidirectionally), in which case, movement would be likely to involve cytoskeletal tracts, similar to vesicle transport (Pratt, 1993). Recent evidence suggests that this may be the case (Galigniana *et al.*, 2001). It has been shown that the intermediate chain of the motor protein dynein (DIC) coimmunoprecipitates with the glucocorticoid receptor (GR), along with hsp90 and the hsp90-binding immunophilin FKBP52.

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 (also called PPIase domain due to its peptidylproline *cis/trans*-isomerase activity). Recent experimental evidence suggests a direct binding of DIC to FKBP52 (Galigniana *et al.*, 2002). As expected, the overexpression of the rotamase domain of FKBP52 prevented the steroid-dependent movement of the GR in intact cells (Galigniana *et al.*, 2001). Importantly, similar protein–protein interactions were also found for the MR (Piwien-Pilipuk and Galigniana, unpublished results), which suggests that the MR also associates to cytoskeleton tracts and moves to the nucleus using the tubulin-associated motor protein dynein.

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). However, FK506 does not affect DIC binding or receptor

trafficking, which suggest that protein trafficking is independent of the enzymatic activity. According to the findings summarized here, it is clear that the rotamase domain present in high molecular weight immunophilins is likely to be involved in protein–protein interactions in the cell.

The classical dogma maintains that upon ligand binding, the hsp90-based heterocomplex is dissociated from the receptor. This allows 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. This classical model was first challenged when it was proposed that hsp90 may function in transportation of proteins to the nucleus (Koyasu *et al.*, 1986). In view that the findings described earlier are in conflict with the classical dogma and seem to support the latter hypothesis, the next conundrum to solve is how the binding of a given ligand affects the function and balance between the proteins in the steroid–receptor heterocomplex.

It is known that upon ligand binding, the nuclear translocation of the native MR expressed in rat renal cells is completed after ~15 min at 37 °C in a phosphatase inhibitor- and redox potential-sensitive manner (Galigniana, 1998, 2000; Piwien-Pilipuk and Galigniana, 1998, 2000). Having depicted the novel picture for the molecular mechanism of action of steroid receptors, it is opportune to point out that the nuclear translocation rate of 11-OP–MR complexes is 2-fold slower than the translocation rate of aldosterone–MR complexes. This observation agrees with the argument that the MR undergoes a different conformational change upon binding of the oxidopregnanesteroid. Interestingly, it has also been demonstrated (Galigniana *et al.*, 2000) that the nuclear translocation of 11-OP–MR complexes is not abrogated in rat renal cells treated with the phosphatase inhibitor tautomycin, as is the case for aldosterone–MR complexes (Piwien-Pilipuk and Galigniana, 1998). Therefore, it may be possible that the recruitment of some proteins to the MR–hsp90 heterocomplex is differential upon binding of 11-OP. That this is the case is supported by the observation that aldosterone binding to the MR promotes the recruitment of more FKBP52 and DIC to the hsp90-based heterocomplex than does 11-OP binding. In conclusion, the inability of the receptor to associate with the cytoplasmic trafficking machinery may be the consequence of a particular conformation adopted by the MR upon 11-OP binding, which may also explain its slower nuclear translocation rate.

On the other hand, the nuclear export rate of the 11-OP–MR complex measured in digitonin-permeabilized cells is faster than that measured for aldosterone-bound receptor, and the subnuclear distribution of the former complex does not show the punctuated pattern exhibited by the latter (Piwien-Pilipuk and Galigniana, unpublished results). These findings parallel a recent work (Vicent *et al.*, 2002) reporting that synthetic ligands

bound to the GR induce a particular receptor conformation, which ultimately leads to a different interaction of the receptor with other nuclear factors and/or chromatin. Thus, the receptor may be targeted to *trans*-activation and/or *trans*-repression, eliciting pleiotropic responses in the same tissue. In conclusion, the dynamic exchange of liganded receptor is likely to have significant consequences in the observed physiological responses triggered by different ligands bound to the same receptor in the same cellular context. Moreover, this postulate potentiates the concept that both ligand and transducer constitute a functional unit able to induce various biological responses by using the same cellular machinery.

XI. REDOX MILIEU REGULATES LIGAND BINDING TO THE MR AND RECEPTOR BIOAVAILABILITY

Oxidative stress and cellular redox provide additional regulatory mechanisms for gating signal tranduction. In contrast to the conventional idea that reactive oxygen is mostly a trigger for oxidative damage of biological structures, today we know that physiological concentrations of reactive oxygen species regulate a variety of molecular mechanisms linked to essential cellular processes (e.g., transcriptional regulation, immune response, cell adhesion, aging, metabolism, apoptosis, cell proliferation) (Forman *et al.*, 2002; Galigniana, 2000; Mannick and Schonoff, 2002; Marshall *et al.*, 2000; Rusnak and Reiter, 2000; Sen, 1998). A simplistic definition of the cellular redox state is the degree of oxidation or reduction of various redox-active compounds present at a particular subcellular location at any given moment. Note that the concept of subcellular compartmentalization is essential in this tentative definition. Several compounds and enzyme systems are responsible for the maintenance of a stable intracellular redox state. The most abundant and widely distributed intracellular redox-active species are cysteine and glutathione (GSH), both present in the mM range.

A partial inhibition of ligand binding to the MR was observed after administering young mice a transition state inhibitor of γ-glutamyl-cysteine synthase, L-buthionine-(S,R)-sulfoximine (Piwien-Pilipuk and Galigniana, 2000). The consequent GSH depletion paralleled the inhibition of the mineralocorticoid biological responses to the same extent in the short term (up to 5 days of treatment) without affecting the expression of the MR, whereas a chronic treatment also affected the mechanism of transcription of the MR (Piwien-Pilipuk *et al.*, 2002a). Interestingly, not all proteins related to the MR molecular mechanism of action were equally affected upon chronic treatment, and compensatory mechanisms were triggered upon the onset of oxidative stress, for example, an increased total activity of the Na,K-ATPase as a consequence of increasing the number of pumps,

although the specific activity decreases due to the fact that pumps are also redox-sensitive (Piwien-Pilipuk and Galigniana, 2000). Again, life holistically opens its path by counterbalancing basic physiological processes, besides complicating our capacity of interpreting our reductionist assay systems.

The harmful effect of oxidative stress on the MR-dependent effect agrees with the observation (Lupo et al., 1998) that cysteines 849 and 942 of the hMR are crucial for steroid binding, the latter cysteine being involved in a direct contact with the 20-keto group of the ligand (see Fig. 1). Similarly, the toxic effects observed in vivo for some metal ions on the mineralocorticoid biological effect were also related to modification of cysteyl groups (Galigniana and Piwien-Pilipuk, 1999), which impaired ligand binding to the renal MR.

Even though it is incapable to bind steroid, it appears that the "thiol-blocked" form of the MR is capable of resisting harmful conditions that normally lead to receptor degradation in the "thiol-reduced" forms of MR. Because steroid binding could be fully recovered when the redox milieu was restored, it was postulated that changes in the intracellular redox potential may regulate the pools of active and inactive MR, which may constitute a mechanism to modulate receptor availability and the final biological response (Galigniana, 1996, 2000). This theory agrees with the observation that intact (not cleaved) immunoreactive ER can be extracted from primary human breast cancer tumors that produce high concentrations of reactive oxygen. However, this intact ER shows lower affinity for the steroid and does not recognize estrogen response elements due to oxidation of the zinc-fingers located in the DBD. However, this inhibitory effect is fully reversed when the receptor is incubated in vitro with reducing agents (Liang et al., 1988).

Covalent labeling of the MR with radioactive ligand allowed us to demonstrate that cytoplasmic trafficking and nuclear import of the MR was also impaired upon the onset of oxidative stress (Galigniana, 2000). Because the ligand is covalently bound to the receptor, abrogation of receptor trafficking may be the consequence of damaged trafficking machinery. The microfilament network may be one of the earliest targets of oxidative stress in many cell types, particularly the vascular endothelial cells, which are constantly exposed to many stimuli and stresses. A similar impairment of the mineralocorticoid function may take place during a wide variety of situations involving overproduction of reactive oxygen, such as chronic renal failure, sepsis, cardiovascular disorders, inflammation, during hemodialysis procedures, and the physiologically normal process of aging (Ceballos-Picot et al., 1996; Nath and Salahudeen, 1990; Piwien-Pilipuk et al., 2002a; Spittle et al., 2001; Yoshika et al., 1990).

Another example where steroid action is affected by an unfavorable redox potential is the S-nitrosylation of the GR, which prevents ligand binding. This inhibition does not occur if the receptor is previously bound to

the ligand or when the low redox potential milieu is restored (Galigniana et al., 1999). This observation agrees with the lack of beneficial effects of glucocorticoid hormones unless they are administered before the onset of the septic shock, a condition where the production of nitric oxide is greatly increased (Rees, 1995). Inasmuch as the MR appears to be more sensitive to an oxidative environment than the GR, it is entirely possible that the putative S-nitrosylation of MR may prevent ligand binding and contribute to amplify the renal mineralocorticoid dysfunction associated with that condition. The critical Cys^{942} of the hMR, which contacts the 20-ketone function of the ligand, may be modified under pathophysiological oxidative stress conditions, so steroid binding is abrograted. As a result of a logical feedback mechanism, it is expected that the consequent loss of capacity to maintain the electrolyte balance increases both aldosterone production and secretion. This may worsen the situation, since it was demonstrated (Ikeda et al., 1995) that a high level of aldosterone is associated to endothelial dysfunction, perhaps by inhibiting nitric oxide release (a self-protective mechanism to prevent the S-nitrosylation of MR?). Ideally, the development of novel ligands that can avoid the thiol-dependent anchorage to the MR may abolish the deleterious side effects generated during the course of pathologies associated to oxidative stress by classical mineralocorticoids. This advance might be particularly useful in those pathophysiological cases listed earlier. Of course, these beneficial ligands do not exist yet, and today it is difficult to accept the idea about the existence of steroids whose 20-ketone function is modified without affecting the mineralocorticoid response, such as a biologically active C_{21}-deoxysteroid as 11-OP was in the past.

XII. ENVOY

Steroids can be found in plants and fungi, but not their cognate receptors, as we know them in metazoans. This suggests 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 because of 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. Clearly, the endocrine system is an issue of evolution that has prompted biochemists today to revise the old hypothesis that the hormone and its receptor could have been preexisting structures that evolved independently. It is now clear that their interaction may necessarily be the result of evolution itself, and regardless of their origin, both ligand and receptor always function as a unique functional unit.

The information for hormonal regulation at the gene level is certainly written in the receptor structure, which bears a close resemblance with its primordial predecessor. On the other hand, hormonal signaling 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. 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). Importantly, the emergence of aldosterone synthase (CYP11B2) was also a key to mineralocorticoid physiology, since ketal–hemiketal groups are not substrates for 11β-HSD.

Based on the structure–activity relationships described in this chapter, one may speculate that gradual changes in the steroid conformation may have favored the acquisition of a specific 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. However, as repeatedly stated here, no single factor can be held solely responsible for the observed correlation between steroid structure and biological effect, as clearly seen when the mineralocorticoid responses obtained *in vitro* and *in vivo* are compared.

Today, it is clear that several mechanisms 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, different half-life, alternative binding sites, competition among various ligands), and all of them influence the final biological response. Importantly, all these convergent factors (and those we still do not know) are implicitly considered when the second-order coefficient ''a'' is calculated from a whole dose–response curve for sodium retention *in vivo*. The empiric nature of this coefficient may therefore explain why a correlation can be evidenced when several prereceptor factors influence the steroid availability to the MR. Most of these prereceptor regulatory factors are absent under *in vitro* conditions. Therefore, although 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 in Fig. 4, 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 $-12.5° \pm 3.7°$, 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.

It is possible that steroid hormones (i.e., those compounds we know as such today and perhaps others more ancient and currently extinct) may have played a primordial role as ligands capable of triggering nongenomic effects (for example, oxysterols, prostaglandins, fatty acyl-CoA esters, estrogens). When the most ancient nuclear receptors emerged some hundred million years ago, ligands were forced to encounter its cognate transducer (e.g., ER, ERR, TR, RXR, LXR) in the nucleus of the living forms, which was not a problem in view of their lipophilic properties. As discussed in Section X, the further need for excluding macromolecular complexes from the nucleus may have forced "novel" receptors to associate with heterocomplexes that anchored them in the cytoplasmic compartment (e.g., GR, MR, AR). Therefore, the association to a machinery responsible for nucleocytoplasmic shuttling should have occurred simultaneously, most likely by using preexisting motor protein complexes. However, the fingerprints of their ancestors are still present in the novel generation of nuclear receptors, particularly in the MR, so the development of a complex net of regulatory mechanisms was essential to ensure specificity of action.

On the other hand, the loss of torsion of the ligand's frame may have favored the mineralocorticoid response over the glucocorticoid effect. Thus, glucocorticoid ligands, which act as mineralocorticoids in species where aldosterone is not produced (i.e., fish) and in mammals (if no compensatory mechanisms would exist), were replaced by a flat steroid with weak glucocorticoid action, aldosterone. Therefore, the gradual straightening of ligands toward the β face of the steroid may represent the latest evolutionary innovation to confer mineralocorticoid specificity to ligands. Remarkably, the novel synthetic mineralocorticoid 11-OP shows no binding capacity to other steroid receptors and is a highly specific mineralocorticoid that does not require the additional support of metabolizing enzymes. Interestingly, the biochemical properties of the 11-OP–MR complex differ from those shown by aldosterone–MR complexes (e.g., association to the trafficking machinery, subnuclear distribution, nuclear retention, dissociation rate from the receptor). In other words, it is possible that the 11-OP–MR complex constitutes a novel type of steroid–receptor unit. If so, the existence of this novel functional complex raises the possibility that it may undergo a different regulation at several levels (e.g., cytoplasmic trafficking, transloca-tion through the nuclear pore, nuclear rearrangement, regulatory signaling cascades) with respect to classical steroid–receptor complexes.

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 to 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. Nonetheless, bacteria, which do not possess known dynein or dynein-like motor proteins, do express highly homologous immunophilins (e.g., TcFK), which resemble human FKBP12 (Iida *et al.*, 1998). Interestingly, FKBP12 (and by extrapolation, perhaps 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 processes, makes it unlikely that dynein–immunophilin 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.

Interestingly, the plant immunophilins FKBP73 and FKBP77, which are homologous to FKBP52, also exhibit the capability to bind mammalian DIC (Harrell *et al.*, 2002), indicating that immunophilins are highly conserved molecules. Nonetheless, the biological role of those two novel immunophilins in plants is unknown. Actually, the biological function of high molecular weight immunophilins 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, that it is associated to steroid receptors, provides a new challenging insight about the role of dynein as a motor protein for soluble, non-vesicle-associated proteins. This is also of great interest for our understanding about the mechanism of receptor trafficking. In effect, one encouraging observation is that the binding of a particular ligand promotes a different conformation change of MR, which recruits lower amounts of FKBP52 and DIC and, consequently, slows the nuclear translocation rate of the steroid–receptor complex. Whether this is one of the possible cytoplasmic check-points for the regulation of MR mechanism of action is still unknown. It is also uncertain what the possible implications of those particular interactions in the nucleus are. It is noteworthy that, unlike aldosterone–MR complexes, 11-OP–MR complexes do not follow a nuclear pattern of enrichment in foci and the receptor is more rapidly exported from the nuclear compartment. Interestingly, this property is also observed when MR is bound to antagonists. Therefore, it still remains unanswered why 11-OP behaves as a potent sodium-retainer ligand.

These many questions presumably will be the future challenges in understanding the molecular mechanisms of action of mineralocorticoid steroids.

In this regard, the development of novel specific mineralocorticoid ligands may be extremely useful not only to unravel the still unknown mechanistic aspects of the MR in particular, but it may also be useful to bring insight into the cell biology of intracellular trafficking and the regulatory mechanisms that influence the activity of other members of the nuclear receptor superfamily.

REFERENCES

Agarwal, M. K., and Mirashi, M. (2000). General overview of mineralocorticoid action. *Pharmacol. Ther.* **84,** 273–326.

Aridor, A., and Hannan, L. A. (2002). Traffick jams II: An update of diseases of intracellular transport. *Traffic* **3,** 781–790.

Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., and Evans, R. M. (1987). Cloning of human mineralocorticoid receptor complementary DNA: Structural and functional kinship with the glucocorticoid receptor. *Science* **237,** 268–275.

Baker, M. E. (1997). Steroid receptor phylogeny and vertebrate origins. *Mol. Cell. Endocrinol.* **135,** 101–107.

Bastl, C. P., and Hayslett, J. P. (1992). The cellular action of aldosterone in target epithelia. *Kidney Int.* **42,** 250–264.

Bay, W. H., and Ferris, T. F. (1999). Factors controlling plasma rennin and aldosterone during pregnancy. *Hypertension* **1,** 410–415.

Black, B. E., Holaska, J. M., Rastinejad, F., and Paschal, B. M. (2001). DNA binding domains in diverse nuclear receptors function as nuclear export signals. *Curr. Biol.* **11,** 1749–1758.

Bourget, W., Germain, P., and Gronemeyer, H. (2000). Nuclear receptor ligand-binding domains: Three-dimensional structures, molecular interactions and pharmacological implications. *Trends Pharmacol. Sci.* **21,** 381–388.

Bourget, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995). Cristal structure of the ligand-binding domain of the nuclear receptor RXR-alpha. *Nature* **375,** 377–382.

Brzozowski, A. M., Pike, A. C. W., Dauter, Z., Hubbard, R. E., Bonn, T., Engstron, O., Ohman, L., Greene, G. L., Gustaffson, J. A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the estrogen receptor. *Nature* **389,** 753–758.

Bumke-Vogt, C., Bähr, V., Diederich, S., Herrmann, S. M., Anagnostopoulos, I., Oelkers, W., and Quinkler, M. (2002). Expression of the progesterone receptor and progesterone-metabolizing enzymes in the female and male human kidney. *J. Endocrinol.* **175,** 349–364.

Burton, G., Galigniana, M. D., deLavallaz, S., Brachet-Cota, A. L., Sproviero, E. M., Ghini, A. A., Lantos, C. P., and Damasco, M. C. (1995). Sodium-retaining activity of some natural and synthetic 21-deoxysteroids. *Mol. Pharmacol.* **47,** 535–543.

Ceballos-Picot, I., Witko-Sarsat, V., Merad-Boudia, M., Nguyen, A. T., Thevenin, M., Jaudon, M. C., Zingraff, J., Verger, C., Jungers, P., and Descamps-Latscha, B. (1996). Glutathione antioxidant system as a marker of oxidative stress in chronic failure. *Free Rad. Biol. Med.* **21,** 845–853.

Chen, S. Y., Bhargava, A., Mastroberardino, L., Meijer, O. C., Wang, J., Buse, P., Firestone, G. L., Verrey, F., and Pearce, D. (1999). Epithelial sodium channel regulated by aldosterone-induced protein sgk. *Proc. Natl. Acad. Sci. USA* **96,** 2514–2519.

Cheung, J., and Smith, D. F. (2000). Molecular chaperone interactions with steroid receptors: An update. *Mol. Endocrinol.* **14,** 939–946.

Chubb, J. R., and Bickmore, W. A. (2003). Considering nuclear compartmentalization in the light of nuclear dynamics. *Cell* **112,** 403–406.

Clouse, S. D. (2002). Brassinosteroids, plant counterparts to animal steroid receptors? *Vitam. Horm.* **65,** 195–223.

Colombe, L., Fostier, A., Bury, N., Pakdel, F., and Guiguen, Y. (2000). A mineralocorticoid-like receptor in the rainbow trout, *Oncorhynchus mykiss*: Cloning and characterization of its steroid binding domain. *Steroids* **65,** 319–328.

DeFranco, D. (2002). Navigating steroid hormone receptors through the nuclear compartment. *Mol. Endocrinol.* **16,** 1449–1455.

Dluhy, R. G. (2001). Glucocorticoid-remediable aldosteronism. *Endocrinologist* **11,** 2263–2268.

Duax, W. L., Cody, V., Griffin, J. F., Rohrer, D. C., and Weeks, C. M. (1978). Molecular conformation and protein binding affinity of progestins. *J. Toxicol. Environ. Health* **4,** 205–227.

Duax, W. L., Griffin, J. F., Strong, P. D., Funder, J. W., and Ulick, S. (1982). Molecular structure of 18-deoxyaldosterone and its relationship to receptor binding and activity. *J. Am. Chem. Soc.* **104,** 7291–7293.

Edwards, C. R. W., Stewart, P. M., Burt, D., Bret, L., McIntyre, M. A., Sutanto, W. S., de Kloet, E. R., and Monder, C. L. (1988). Localization of 11β-hydroxysteroid dehydrogenase-tissue specific protector of the mineralocorticoid receptor. *Lancet* **2,** 986–989.

Ehrlich, E. N., and Lindheimer, M. D. (1972). Effect of administered mineralocorticoids on ACTH in pregnant women. *J. Clin. Invest.* **51,** 1301–1309.

Escriva, H., Safi, R., Hanni, C., Langlois, M. C., Saumitou-Laprade, P., Stehelin, D., Capron, A., Pierce, R., and Laudet, V. (1997). Ligand binding was acquired during evolution of nuclear receptors. *Proc. Natl. Acad. Sci. USA* **94,** 6803–6808.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240,** 889–895.

Fagart, J., Wurtz, J. M., Souque, A., Hellal-Levy, C., Moras, D., and Rafestin-Oblin, M. E. (1998). Antagonism in the human mineralocorticoid receptor. *EMBO J.* **17,** 3317–3325.

Falkenstein, E., Christ, M., Feuring, M., and Wehling, M. (2000). Specific non-genomic actions of aldosterone. *Kidney Int.* **57,** 1390–1394.

Fejes-Tóth, G., Pearce, D., and Náray-Fejes-Tóth, A. (1998). Subcellular localization of mineralocorticoid receptors in living cells: Effects of receptor agonists and antagonists. *Proc. Natl. Acad. Sci. USA* **95,** 2973–2978.

Forman, H. J., Torres, M., and Fukuto, J. (2002). Redox signaling. *Mol. Cell. Biol.* **234/235,** 49–62.

Funder, J. W. (2003). Aldosterone resurgens—Letter from EPHESUS. *J. Clin. Endocrinol. Metab.* **88,** 2373–2375.

Funder, J. W., and Miles, K. (1996). Exclusion of corticosterone from epithelial mineralocorticoid receptors is insufficient for selectivity of aldosterone action: *In vivo* binding studies. *Endocrinology* **137,** 5264–5268.

Funder, J. W., Pearce, P., Smith, R., and Smith, A. I. (1988). Mineralocorticoid action: Target tissue specificity is enzyme, not receptor, mediated. *Science* **242,** 583–585.

Galigniana, M. D. (1996). Stability study on renal type I mineralocorticoid receptor. *Life Sci.* **59,** 511–521.

Galigniana, M. D. (1998). Native rat kidney mineralocorticoid receptor is a phosphoprotein whose transformation to a DNA-binding form is induced by phosphatases. *Biochem. J.* **333,** 555–563.

Galigniana, M. D. (2000). Functional regulation of corticosteroid receptors by phosphorylation and redox potential. *Curr. Top. Steroid Res.* **3,** 1–22.

Galigniana, M. D., Harrell, J. M., Murphy, P. J. M., Chinkers, M., Radanyi, C., Renoir, J. M., Zhang, M., and Pratt, W. B. (2002). Binding of hsp90-associated immunophilins to

cytoplasmic dynein: Direct binding and *in vivo* evidence that the peptidylprolyl isomerase domain is a dynein interaction domain. *Biochemistry* **41,** 13602–13610.

Galigniana, M. D., and Piwien-Pilipuk, G. (1999). Comparative inhibition by hard and soft metal ions of steroid-binding capacity of renal mineralocorticoid receptor cross-linked to the 90-kDa heat-shock protein heterocomplex. *Biochem. J.* **341,** 585–592.

Galigniana, M. D., Piwien-Pilipuk, G., and Assreuy, J. (1999). Inhibition of glucocorticoid receptor by nitric oxide. *Mol. Pharmacol.* **55,** 317–323.

Galigniana, M. D., Radanyi, C., Renoir, J. M., Housley, P. R., and Pratt, W. B. (2001). Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. *J. Biol. Chem.* **276,** 14884–14889.

Galigniana, M. D., Vicent, G. P., Lantos, C. P., and Burton, G. (1993). Ligand properties of 11, 19-xidoprogesterone: A preliminary report. *An. Asoc. Quím. Argent.* **81,** 333–340.

Galigniana, M. D., Vicent, G. P., Lantos, C. P., and Burton, G. (1994). 11,19-Oxidoprogesterone: A potent Na$^+$-retaining steroid binding to an alternative site in rat kidney type I receptor. *Eur. J. Endocrinol.* **130,** 260.

Galigniana, M. D., Vicent, G. P., Lantos, C. P., and Burton, G. (1997). Features of the shuttle pair 11β-hydroxyprogesterone/11-ketoprogesteron. *Steroids* **62,** 358–364.

Galigniana, M. D., Vicent, G. P., Piwien-Pilipuk, G., Burton, G., and Lantos, C. P. (2000). Mechanism of action of the potent sodium-retaining steroid 11,19-oxidoprogesterone. *Mol. Pharmacol.* **58,** 58–70.

Gasser, S. M. (2002). Visualizing chromatin dynamics in interphase nuclei. *Science* **296,** 1412–1416.

Geller, D. S., Farhi, A., Pinkerton, N., Fradley, M., Moritz, M., Spitzer, A., Meinke, M., Tsai, F. T. F., Sigler, P. B., and Lifton, R. P. (2000). Activating mineralocorticoid receptor mutation in hypertension exacerbated by pregnancy. *Science* **289,** 119–123.

Geuns, J. M. C. (1978). Steroid hormones and plant growth and development. *Phytochemistry* **17,** 1–14.

Gómez-Sánchez, E. P., Venkataraman, M. T., Thwaites, D., and Fort, C. (1990). ICV infusion of corticosterone antagonizes ICV-aldosterone hypertension. *Am. J. Physiol.* **258,** E649–E653.

Grassi, G., Fagart, J., Calas, B., Adenot, M., Rafestin-Oblin, M. E., and Auzou, G. (1997). Structure-activity relationships of steroids with mineralocorticoid activity. *Eur. J. Med. Chem.* **32,** 869–879.

Harrell, J. M., Kurek, I., Breiman, A., Radanyi, C., Renoir, J. M., Pratt, W. B., and Galigniana, M. D. (2002). All of the protein interactions that link steroid receptor-hsp90-immunophilin heterocomplexes to cytoplasmic dynein are common to plant and animal cells. *Biochemistry* **41,** 5581–5587.

Heap, R. B., Holzbauer, M., and Newport, H. M. (1966). Adrenal secretion rates of C19 and C21 steroids after hypophysectomy in the pig and the dog. *J. Endocrinol.* **36,** 159–176.

Heun, P., Laroche, T., Shimada, K., Ferrer, P., and Passer, S. M. (2001). Chromosome dynamics in the yeast interphase nucleus. *Science* **294,** 2181–2186.

Hutter, H., Vogel, B. E., Plenefisch, J. D., Norris, C. R., Proenca, R. B., Spieth, J., Guo, C., Mastwal, S., Zhu, X., Scheel, J., and Hedgecock, E. M. (2000). Conservation and novelty in the evolution of cell adhesion and extracellular matrix genes. *Science* **287,** 989–994.

Iida, T., Furutani, M., Nishida, F., and Maruyama, T. (1998). FKBP-type peptidyl-proplyl *cis-trans* isomerase from a sulfur-dependent hyperthermophilic archaeon, *Thermococcus sp.* KS-1. *Gene* **222,** 249–255.

Ikeda, U., Kanbe, T., Nakayama, I., Kawahara, Y., Yokoyama, M., and Shimada, K. (1995). Aldosterone inhibits nitric oxide synthesis in rat vascular smooth muscle cells induced by interleukin-1b. *Eur. J. Pharmacol.* **290,** 69–73.

Johansson, E. D., and Johansson, L. E. (1971). Progesterone levels in amniotic fluid and plasma from women. Levels during normal pregnancy. *Acta Obstet. Gynecol. Scand.* **50,** 339–343.

Kitagawa, H., Yanagisawa, J., Fuse, H., Ogawa, S., Yogiashi, Y., Okuno, A., Nagasawa, H., Nakajima, T., Matsumoto, T., and Kato, S. (2002). Ligand-selective potentiation of rat mineralocorticoid receptor activation function 1 by a CBP-containing histone acethyl-transferase complex. *Mol. Cell. Biol.* **22,** 3698–3706.

Klug, A., and Schwabe, J. W. R. (1995). Protein motifs 5. Zinc fingers. *FASEB J.* **9,** 597–604.

Kotelevtsev, Y., Brown, R. W., Fleming, S., Kenyon, C., Edwards, C. W. R., Seckl, J. R., and Mullins, A. J. (1999). Hypertension in mice lacking 11β-hydroxysteroid dehydrogenase type 2. *J. Clin. Invest.* **103,** 683–689.

Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H., and Yahara, I. (1986). *Proc. Natl. Acad. Sci. USA* **83,** 8054–8058.

Kraulis, I., Traikov, H., Li, M., and Birmingham, M. K. (1973). The effects of corticosterone, 18-OH-DOC, DOC and 11-hydroxyprogesterone on the adrenal pituitary axis of the stressed rat. *J. Steroid Biochem.* **4,** 129–137.

Lantos, C. P., Burton, G., Roldán, A., Damasco, M. C., and Aragonés, A. (1981). Structure-activity relationship in certain glucocorticoids and mineralocorticoids. *In* "Physiopathology of Endocrine Diseases and Mechanisms of Hormone Action" (R. J. Soto, A. de Nicola, and J. Blaquier, Eds.), pp. 477–494. Alan R. Liss, New York.

Latif, S. A., Sheff, M. F., Ribeiro, C. A., and Morris, D. J. (1997). Selective inhibition of sheep kidney 11β-hydroxysteroid dehydrogenase isoforms 2 activity by 5α-reduced (but not 5β) derivatives of adrenocorticosteroids. *Steroids* **62,** 230–237.

Laudet, V. (1997). Evolution of the nuclear receptor superfamily: Early diversification from an ancestral orphan receptor. *J. Mol. Endocrinol.* **19,** 207–226.

Leid, M., Kastner, P., and Chambon, P. (1992). Multiplicity generates diversity in the retinoic acid signaling pathways. *Trends Biochem. Sci.* **17,** 427–433.

Liang, X., Lu, B., Scoot, G. K., Chang, C. H., Baldwin, M. A., and Benz, C. C. (1988). Oxidant stress impaired DNA-binding of estrogen receptor from human breast cancer. *Mol. Cell. Endocrinol.* **146,** 151–161.

Lim-Tio, S. S., and Fuller, P. J. (1998). Intacellular signaling pathways confer specificity of trans-activation by mineralocorticoid and glucocorticoid receptors. *Endocrinology* **139,** 1653–1661.

Liu, W., Wang, J., Sauter, N. K., and Pearce, D. (1995). Steroid receptor heterodimerization demonstrated *in vitro* and *in vivo. Proc. Natl. Acad. Sci. USA* **92,** 12480–12484.

Lombès, M., Binart, N., Delahaye, F., Baulieu, E. E., and Rafestin-Oblin, M. E. (1994). Differential intracellular localization of human mineralocorticoid receptor on binding of agonists and antagonists. *Biochem. J.* **302,** 191–197.

López-Bernal, A., Flint, A. F. P., Anderson, A. B. M., and Turnbull, A. C. (1979). 11β-hydroxysteroid dehydrogenase activity (E.C.1.1.1.146) in human placenta and deciduas. *J. Steroid Biochem.* **13,** 1081–1087.

Losel, R. M., Feuring, M., Falkenstein, E., and Wehling, M. (2002). Nongenomic effects of aldosterone: Cellular aspects and clinical implications. *Steroids* **67,** 493–498.

Losel, R. M., and Wehling, M. (2002). *Nature Rev. Mol. Cell. Biol.* **4,** 46–56.

Lupo, B., Mesnier, G., and Auzou, G. (1998). Cysteines 849 and 942 of human mineralocorticoid receptor are crucial for steroid binding. *Biochemistry* **37,** 12153–12159.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schultz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995). The nuclear receptor superfamily: The second decade. *Cell* **83,** 835–839.

Mannick, J. B., and Schonoff, C. M. (2002). Nitrosylation: The next phosphorylation? *Arch. Biochem. Biophys.* **408,** 1–6.

Marks, A. R. (1996). Cellular functions of immunophilins. *Physiol. Rev.* **76,** 631–649.

Marshall, H. E., Merchant, K., and Stamler, J. S. (2000). Nitrosation and oxidation in the regulation of gene expression. *FASEB J.* **14,** 1889–1900.

Matias, P. M., Donner, P., Coelho, R., Thomaz, M., Peixoto, C., Macedo, S., Otto, N., Joschko, S., Scholz, P., Wegg, A., Bäsler, S., Schäfer, M., Egner, U., and Carrondo, M. A. (2000). Structural evidence for ligand specificity in the binding domain of the human androgen receptor. *J. Biol. Chem.* **275,** 26164–26171.

McDonnell, D. P., Shabaz, M. M., Vegeto, E., and Goldman, M. E. (1994). Progesterone receptor A-form functions as a transcriptional modulator of mineralocorticoid receptor transcriptional activity. *J. Steroid Biochem. Mol. Biol.* **48,** 425–432.

Montano, M. M., Muller, V., Trobaugh, A., and Katzenellenbogen, B. S. (1995). The carboxy-terminal F domain of the human estrogen receptor: Role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Mol. Endocrinol.* **9,** 814–825.

Morimoto, R. I. (2002). Dynamic remodeling of transcription complexes by molecular chaperones. *Cell* **110,** 281–284.

Murphy, B. (1981). Demonstrations of novel compounds in human fetal tissues and a consideration of their possible role in parturition. *Am. J. Obstet. Gynecol.* **139,** 353–358.

Náray-Fejes-Tóth, A., Canessa, C., Cleaveland, E. S., Aldrich, G., and Fejes-Tóth, G. (1999). *sgk* is an aldosterone-induced kinase in the renal collecting duct. *J. Biol. Chem.* **274,** 16973–16978.

Nath, K. A., and Salahudeen, A. K. (1990). Induction of renal growth and injury in the intact rat kidney by dietary deficiency of antioxidants. *J. Clin. Invest.* **86,** 1179–1192.

Nishi, M., Ogawa, H., Ito, T., Matsuda, K.-I., and Kawata, M. (2001). Dynamic changes in subcellular localization on mineralocorticoid receptor in living cells: In comparison with glucocorticoid receptor using dual-color labeling with green fluorescent protein spectral variants. *Mol. Endocrinol.* **15,** 1077–1092.

Nolten, W. E., Lindheimer, M. D., Oparil, S., and Ehrlich, E. N. (1978). Desoxycorticosterone in normal pregnancy. Sequential studies of the secretory patterns of desoxycorticosterone, aldosterone, and cortisol. *Am. J. Obstet. Gynecol.* **132,** 414–420.

Owen, G. I., and Zelent, A. (2000). Origins and evolutionary diversification of the nuclear receptor superfamily. *Cell. Mol. Life Sci.* **57,** 809–827.

Picard, D. (2002). Heat-shock protein 90, a chaperone for folding and regulation. *Cell. Mol. Life Sci.* **10,** 1640–1648.

Piwien-Pilipuk, G., Ayala, A., Machado, A., and Galigniana, M. D. (2002a). Impairment of mineralocorticoid receptor (MR)-dependent biological response by oxidative stress and aging. Correlation with post-translational modification of MR and decreased ADP-ribosylatable level of elongation factor 2 in kidney cells. *J. Biol. Chem.* **277,** 11896–11903.

Piwien-Pilipuk, G., and Galigniana, M. D. (1998). Tautomycin inhibits phosphatase-dependent transformation of the rat kidney mineralocorticoid receptor. *Mol. Cell. Endocrinol.* **144,** 119–130.

Piwien-Pilipuk, G., and Galigniana, M. D. (2000). Oxidative stress induced by L-buthionine-(S,R)-sulfoximine, a selective inhibitor of glutathione metabolism, abrogates mouse kidney mineralocorticoid receptor function. *Biochem. Biophys. Acta* **1495,** 263–280.

Piwien-Pilipuk, G., Kanelakis, K. C., Ghini, A. A., Lantos, C. P., Litwack, G., Burton, G., and Galigniana, M. D. (2000). Modification of an essential amino group in the mineralocorticoid receptor evidences a differential conformational change of the receptor protein upon binding of antagonists, natural agonists and the synthetic agonist 11,19-oxidoprogesterone. *Biochem. Biophys. Acta* **1589,** 31–48.

Piwien-Pilipuk, G., Kanelakis, K. C., and Galigniana, M. D. (2002b). Correlation between pregnanesteroid conformation, receptor affinity, and anti-natriuretic effect. *Eur. J. Pharmacol.* **454,** 131–143.

Pratt, W. B. (1993). The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *J. Biol. Chem.* **268,** 21455–21458.

Pratt, W. B., and Toft, D. O. (1997). Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.* **18,** 306–360.

Quinkler, M., Bumke-Vogt, C., Meyer, B., Bähr, V., Oelkers, W., and Diederich, S. (2003). The human kidney is a progesterone-metabolizing and androgen-producing organ. *J. Clin. Endocrinol. Metab.* **88,** 2803–2809.

Quinkler, M., Johanssen, S., Bumke-Vogt, C., Oelkers, W., Bähr, V., and Diederich, S. (2001). Enzyme-mediated protection of the mineralocorticoid receptor against progesterone in the human kidney. *Mol. Cell. Endocrinol.* **171,** 21–24.

Quinkler, M., Meyer, B., Bumke-Vogt, C., Grossmann, C., Gruber, U., Oelkers, W., Diederich, S., and Bähr, V. (2002). Agonistic and antagonistic properties of progesterone metabolites at the human mineralocorticoid receptor. *Eur. J. Endocrinol.* **146,** 789–800.

Rafestin-Oblin, M. E., Fagart, J., Souque, A., Seguin, C., Bens, M., and Vandewalle, A. (2002). 11β-hydroxyprogesterone acts as a mineralocorticoid agonist in stimulating Na^+ absorption in mammalian principal cortical collecting duct cells. *Mol. Pharmacol.* **62,** 1306–1313.

Rees, D. D. (1995). Role of nitric oxide in the vascular dysfunction of septic shock. *Biochem. Soc. Trans.* **23,** 1025–1029.

Renaud, J. P., Rochel, N., Ruff, M., Vivat, P., Chambon, P., Gronemeyer, H., and Moras, D. (1995). Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans reinoic acid. *Nature* **378,** 681–689.

Robertson, N. M., Schulman, G., Karnik, S., Alnemri, E., and Litwack, G. (1993). Demonstration of nuclear translocation of the mineralocorticoid receptor (MR) using anti-MR antibody and confocal scanning microscopy. *Mol. Endocrinol.* **7,** 1226–1239.

Rusnak, F., and Reiter, T. (2000). Sensing electrons: Protein phosphatase redox regulation. *Trends Biochem. Sci.* **25,** 527–529.

Sato, A., Sheppard, K. E., Fullerton, M. J., Sviridov, D. D., and Funder, J. W. (1995). Glucocorticoid receptor expression is down-regulated by Lp(a) lipoprotein in vascular smooth muscle cells. *Endocrinology* **136,** 3707–3713.

Selye, H. (1946). The general adaptation syndrome and the diseases of adaptation. *J. Clin. Endocrinol.* **6,** 117–230.

Sen, C. K. (1998). Redox signaling and the emerging therapeutic potential of thiol antioxidants. *Biochem. Pharmacol.* **55,** 1747–1758.

Shimkets, R. A., Lifton, R., and Canessa, C. M. (1998). *In vivo* phosphorylation of the epithelial sodium channel. *Proc. Natl. Acad. Sci. USA* **95,** 3301–3305.

Silverstein, A. M., Galigniana, M. D., Kanelakis, K. C., Radanyi, C., Renoir, J. M., and Pratt, W. B. (1999). Different regions of the immunophilin FKBP52 determine its association with the glucocorticoid receptor, hsp90, and cytoplasmic dynein. *J. Biol. Chem.* **274,** 36980–36986.

Souness, G., Latif, S., Laurenzo, J., and Morris, D. J. (1995). 11α- and 11β-hydroxyprogesterone, potent inhibitors of 11β-hydroxysteroid dehydrogenase (isoforms 1 and 2), confer marked mineralocorticoid activity on corticosterone in the ADX rats. *Endocrinology* **136,** 1809–1812.

Spach, C., and Streeten, D. H. (1964). Retardation of sodium exchange in dog erythrocytes by physiological concentrations of aldosterone. *J. Clin. Invest.* **43,** 217–227.

Spittle, M. A., Hoenich, N. A., Handelman, G. J., Adhikarla, R., Homel, P., and Levin, N. W. (2001). Oxidative stress and inflammation in hemodialysis patients. *Am. J. Kidney Dis.* **38,** 1408–1413.

Trapp, T., Rupprecht, R., Castren, M., Reul, M., and Holsboer, F. (1994). Heterodimerization between mineralocorticoid and glucocorticoid receptor: A new principle of glucocorticoid action in the CNS. *Neuron* **13,** 1457–1462.

Ullian, M. (1999). The role of corticosteroids in the regulation of vascular tone. *Cardiovasc. Res.* **41,** 55–64.

Van Steensel, B., Van Binnendjik, E. P., Hornsby, C. D., Van der Voort, H. T. M., Krozowski, Z. S., de Kloet, E. R., and Van Driel, R. (1996). Partial colocalization of glucocorticoid and mineralocorticoid receptors in discrete compartments in nuclei of rat hippocampus neurons. *J. Cell Sci.* **109,** 787–792.

Vander, A. J., Malvin, R. L., Wilde, W. S., Lapides, J., Sullivan, L. P., and McMurray, V. M. (1958). Effects of adrenalectomy and aldosterone on proximal and distal tubular sodium reabsorption. *Proc. Soc. Exp. Biol. Med.* **99,** 323–325.

Vicent, G. P., Pecci, A., Ghini, A. A., Piwien-Pilipuk, G., and Galigniana, M. D. (2002). Differences in nuclear retention characteristics of agonist-activated glucocorticoid receptor may determine specific biological responses. *Exp. Cell Res.* **276,** 142–154.

Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B. L., Baxter, J. D., and Fletterick, R. J. (1995). A structural role for the hormone in the thyroid hormone receptor. *Nature* **378,** 690–697.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* **77,** 591–625.

Williams, S. P., and Sigler, P. B. (1998). Atomic structure of progesterone complexed with its receptor. *Nature* **393,** 392–396.

Yamakawa, M., Ezumi, K., Shiro, M., Nakai, H., Kamata, S., Matsui, T., and Haga, N. (1986). Relationships of the molecular structure of aldosterone derivatives with their binding affinity for mineralocorticoid receptor. *Mol. Pharmacol.* **30,** 585–589.

Yoshika, T., Bills, T., Moore-Jarret, T., Greene, H. L., Burr, I. M., and Ichikawa, I. (1990). Role of intrinsic antioxidant enzymes in renal oxidant injury. *Kidney Int.* **38,** 282–288.

Young, D. B. (1988). Quantitative analysis of aldosterone's role in potassium regulation. *Am. J. Physiol.* **255,** F811–F822.

Young, M., Fullerton, M., Dilley, R., and Funder, J. (1994). Mineralocorticoids, hypertension, and cardiac fibrosis. *J. Clin. Invest.* **93,** 2578–2583.

Zilliacus, J., Carlstedt-Duke, J., Gustaffson, J. A., and Wright, A. P. (1994). Evolution of distinct DNA-binding specificities within the nuclear receptor family of transcription factors. *Proc. Natl. Acad. Sci. USA* **91,** 4175–4179.