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Mechanism of Action of the Potent Sodium-Retaining Steroid 11,19-Oxidoprogesterone

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ABSTRACT

We have demonstrated previously that a planar conformation of the molecular frame is required for steroids to acquire optimal sodium-retaining activity and binding properties to the mineralocorticoid receptor (MR). One of the most active sodium-retaining compounds tested in those studies was 11,19-oxidoprogesterone. Despite its biological potency, the relative affinity of 11,19-oxidoprogesterone for the MR is 5-fold lower than that of 21-deoxycorticosterone and 10-fold lower than aldosterone. Such a discrepancy may be assigned to uncommon biopharmacological properties of this synthetic steroid or an unusual molecular mechanism of action. In this work, we studied the biopharmacological and mechanistic features of 11,19-oxidoprogesterone. We show that both the pharmacokinetic properties of 11,19-oxidoprogesterone and its ability to transform and translocate the MR into the nucleus are undistinguishable from aldosterone. However, the capability of the serine/threonine phosphatase inhibitor tautomycin to impair nuclear translocation of the aldosterone-MR complex is not observed for the 11,19-oxidoprogesterone-MR complex. In addition, the binding properties of both steroids are differentially affected by modification of crucial lysyl residues of the MR. Kinetic studies performed on the aldosterone-MR complex in the presence of low concentrations of oxidopregnane suggest that 11,19-oxidoprogesterone may bind to the MR in a different binding site from the aldosterone binding pocket. Consistent with this postulate, a biologically inactive dose of 0.6 ng of oxidopregnane is able to potentiate the mineralocorticoid effect of a suboptimal dose of aldosterone.

In contrast to glucocorticoids, which exhibit a slightly torsioned steroid nucleus at the A/B-ring junction, mineralocorticoids seem to require a flat conformation for optimal activity (Duax et al., 1978; Lantos et al., 1981; Yamakawa et al., 1986; Burton et al., 1995). Consistent with this structural requirement, we have reported previously (Burton et al., 1995) that the highly planar synthetic steroid 11,19-oxidoprogesterone (11-OP; see structure in Fig. 1) exhibits potent sodium-retaining activity. In this sense, 11-OP is equivalent to the biological activity measured with 21-deoxycorticosterone (DOC) and, at some doses, even approaches the effects of aldosterone (ALDO). On the other hand, 11-OP differs from endogenous mineralocorticoids in its relatively lower affinity for mineralocorticoid receptor (MR), which is ~50 nM as compared with 4 nM for ALDO and 10 nM for DOC.

In contrast to the natural mineralocorticoids ALDO and DOC, the synthetic steroid 11-OP lacks a C_{21}-hydroxyl group. Also, 11-OP does not possess a functional C_{18}-methyl group as found on ALDO and the strong synthetic mineralocorticoid 18-vinylprogesterone. Nevertheless, the overall flat 11-OP is a strong sodium-retaining steroid. In contrast, its highly bent isomer 6,19-oxidoprogesterone (6-OP) exhibits negligible, if any, mineralocorticoid activity. These interesting features suggest that a planar steroid conformation confers per se mineralocorticoid properties to pregnane steroids rather than a specific functional group. Consistent with this hypothesis, similar properties as those described for 11-OP and 6-OP were also demonstrated for 5α-dihydroprogesterone and its bent isomer 5β-dihydroprogesterone.

When a relationship between the structure of the steroids and the biological effect was investigated, we demonstrated that the orientation of the C_3═O group with respect to ring D rather than the angle of the A/B junction correlates with MR affinity as a semilogarithmic function (Burton et al., 1995). That linear function was highly significant if 11-OP is excluded from that series. In effect, this steroid exhibits a lower relative binding affinity (RBA) for MR than what is

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ABBREVIATIONS: 11-OP, 11,19-oxidoprogesterone; MR, mineralocorticoid receptor; GR, glucocorticoid receptor; hsp90, 90-kDa heat shock protein; RBA, relative binding affinity; ADX, adrenalectomized; CBG, corticosterone-binding protein; ALDO, aldosterone; CORT, corticosterone; PROG, progesterone; DOC, 21-deoxycorticosterone; SPO, SC9420-spironolactone; 6-OP, 6,19-oxidoprogesterone.

expected, according not only to its planar conformation but also to its significant biological effect.

As a consequence of these studies, several questions arise in relation to such paradoxical behavior of 11-OP, and many hypotheses were considered to interpret its biological potency. One reasonable explanation may be that 11-OP possesses an exceptionally long plasma half-life. Because 11-OP exhibits an extremely rigid molecular frame owing to the presence of an additional ring on the β face of the steroid, the idea of a long half-life is entirely possible because this structural feature may affect its recognition by plasma proteins or inactivating enzymes, which in turn may increase its bioavailability in target organs. Another possibility envisions that 11-OP may interact with a different binding site on the MR or with a receptor different from the known "type I" MR. Even if 11-OP binds to the classical type I MR, it should be considered that the resultant steroid-receptor complex may not follow classical transformation or transactivational features. As it has been suggested for other steroids (Vicent et al., 1999), one may speculate that even if the biological effect is receptor-mediated, this mechanism may justify only in partial form the complete biological action. It may also be possible that the steroid triggers "nonclassical" activation pathways (Kato et al., 1995; Migliaccio et al., 1996), or the effects may be nongenomic so that the steroid receptor is not involved in the molecular mechanism of action (Nemere et al., 1993). All of these hypotheses are not mutually exclusive and could even integrate a series of events that mediate the biological effect.

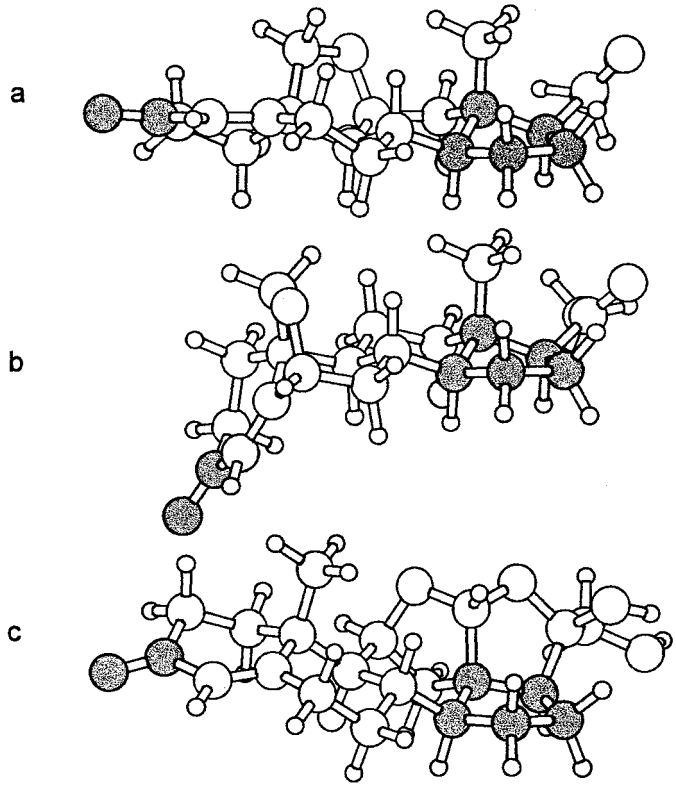

Fig. 1. Structures of the most stable conformers of 11-OP, 6-OP, and ALDO. Geometry optimizations were carried out with AMPAC 5.1 (Semichem) using the AM1 semiempirical method. The C_3═O/ring D angles (gray atoms) are +8.9° for 11-OP (structure a), −55.2° for the bent isomer 6-OP (structure b), and −15.5° for the most potent physiological mineralocorticoid ALDO (structure c).

In this work, we focus on the elucidation of the mechanism of action of 11-OP, and most of the speculations described previously are tested. We investigate the pharmacokinetic properties of 11-OP and its capability to trigger biochemical events related to MR activation such as the dissociation of the 90-kDa heat shock protein (hsp90) heterocomplex and nuclear translocation. We clearly demonstrate that 11-OP binds to the renal MR, dissociates the hsp90 heterocomplex, and translocates the receptor into the nucleus. However, our results also provide evidence that the binding of 11-OP to the MR is not equivalent to the binding of the natural steroid, most likely because 11-OP may bind to an alternative site that is different from the ALDO-binding site.

Materials and Methods

Chemicals. [1,2-^{3}H]ALDO (specific activity = 59.0 Ci/mmol), [1,2,6,7-^{3}H]corticosterone (CORT; specific activity = 87 Ci/mmol), [4-^{14}C]DOC (specific activity = 52 Ci/mmol), [methoxy-^{3}H]ZK-91587 (specific activity = 86.5 Ci/mmol), and ^{125}I-conjugated counterantibodies were purchased from NEN Life Science Products (Boston, MA). Unlabeled steroids [ALDO, DOC, CORT, progesterone (PROG), and SC9420-spironolactone (SPO)], phenylmethylsulfonyl fluoride, trypsin-chymotrypsin inhibitor from soybean, aprotinin from bovine lung, tautomycin, sodium borohydride, pyridoxal 5′-phosphate, dimethyl pimelimidate, and protein A-Sepharose were purchased from Sigma Chemical Co. (St. Louis, MO). Benzon nuclease was from Merck (Darmstad, Germany). RU-28362 was a kind gift from Roussel-Uclaf (Romainville, France). 11-OP and 6-OP were synthesized as described (Brachet-Cota and Burton, 1990). Concentrated rabbit reticulocyte lysate was purchased from Green Hectares (Oregon, WI). The rabbit pAbhMR polyclonal antibody against MR was a kind gift of Drs. G. Litwack and N. Robertson (Jefferson Cancer Institute, Philadelphia, PA). The AC88 monoclonal antibody against hsp90 was from StressGen (Victoria, BC, Canada). Horseradish peroxidase-conjugated counterantibodies were from Pierce (Rockford, IL).

Bioassays. Male Sprague-Dawley rats weighing 250 g underwent adrenalectomy 48 h before the experiments and were maintained on Purina chow and 0.9% NaCl and fresh water ad libitum. Food was removed the previous night, and liquids were removed 4 h before the experiment. Steroids were dissolved in ethanol/propylene glycol/0.9% NaCl (3:3:34) and injected in thighs. Rats were simultaneously given s.c. injections of 3 ml of 0.9% NaCl. Three hours later, blood and urine samples were taken, and excretion rates for Na^+, K^+, and creatinine were measured. The control group was given the vehicle mixture injection.

Liver glycogen deposition was measured as described (Vicent et al., 1997). Briefly, the steroids were dissolved in vehicle, and 100 μg of steroid/100-g body weight were injected (i.m.) to adrenalectomized (ADX) male Sprague-Dawley rats on the evening before the study. On the morning of the experiment, we repeated that dose (i.p.). After 3 h, the rats were sacrificed by cervical dislocation, and liver glycogen was purified and quantified.

Tyrosine amino-transferase activity was measured on isolated hepatocytes following a standard protocol (Vicent et al., 1998). Cell viability (>95%) was determined by trypan blue exclusion.

Binding Assays. ADX rats were sacrificed by cervical dislocation and bled by heart puncture. Ice-cold saline solution was perfused through the aorta until organs were completely blanched. Thymus cytosol was used as a source of glucocorticoid receptor (GR) and kidney cytosol as a source of MR. Tissue was homogenized with 2 volumes of cold TEGI buffer (0.1 M Tris, 10 mM EDTA, 25% glycerol, 10 mM β-mercaptoethanol, 20 mM Na_2MoO_4, 0.1 mM phenylmethylsulfonyl fluoride, 2 IU/ml aprotinin, 30 μg/ml trypsin-chymotrypsin inhibitor, pH 7.4). Homogenates were centrifuged at 67,000*g* for 30 min at 0°C, and the resultant supernatant was referred to as

cytosol. Binding assays were performed as reported previously (Galigniana, 1996; Galigniana et al., 1997) with 5.0 nM [^{3}H]CORT for the GR or 5.0 nM [^{3}H]ALDO in the presence of 1.0 μM RU-28362 for the MR. The RBA for steroid receptors was determined by competition between the appropriate ^{3}H-labeled tracer and increasing concentrations of unlabeled steroids. The nonspecific binding was determined with a 1000-fold excess of unlabeled steroid and subtracted from the total binding. Scatchard plots were performed using increasing concentrations of radiolabeled ALDO as described (Galigniana and Piwien-Pilipuk, 1999).

Rat plasma was used to evaluate the steroid binding to corticosterone-binding protein (CBG). Blood from ADX rats was collected in 5 mM EDTA and centrifuged at 2000 rpm for 5 min, and the plasma was fractionated with solid ammonium sulfate at 30% to 60% of saturation. The resultant albumin-free fraction was dissolved in TEGI buffer and used as the source of partially purified CBG. The RBA for the carrier protein was determined by competition of unlabeled steroid with 5 nM [^{3}H]CORT as described (Vicent et al., 1997).

When binding assays were performed in vivo, rats were given i.p. injections of 10 μCi of [^{3}H]ALDO in saline solution and increasing amounts of radioinert steroids. After 20 min, blood samples were taken for the determination of plasma [^{3}H]ALDO levels. Rats were sacrificed immediately, and kidneys were excised, perfused, and processed as described previously.

In Vivo Half-Lives of Steroids. ADX rats were anesthetized with 6 mg of sodium barbiturate per 100 g of body weight. Steroids in saline solution were then injected as an i.v. bolus. Two micrograms of unlabeled steroid and 5 μCi of ^{3}H-labeled steroid (expressed per 100 g of body weight) were used for ALDO and CORT, and the biological half-lives were measured according to Morris et al. (1976). For 11-OP and 6-OP, 100 μg of oxidopregnane steroid/100 g were injected, and plasma samples were analyzed by HPLC using a liquid chromatography system (Isochrom LC pump and Spectraseries UV1 detector with a 1.2-μl cell; Spectra-Physics Analytical, Mountain View, CA), an injector with a 1-μl internal loop (Rheodyne Inc., Cotati, CA), and C_{18} 5-μm column (1 $\times$ 250 mm; Ultremex, Phenomenex Co., Torrance, CA). The solvent used for isocratic elution (50 μl/min) was methanol/acetonitrile/H_2O (35:28:37, v/v/v).

Sucrose Gradient Ultracentrifugation. Kidney cytosol was incubated with either radioinert ALDO or 11-OP for 3 h at 0°C in TEGI buffer. Free steroid was then removed with 1% charcoal, 0.1% dextran, and the supernatant was incubated for 10 min at 30°C to allow hsp90 to dissociate from the MR. Samples were centrifuged on a 5-to-20% sucrose gradient in TEGI buffer supplemented with 20 mM Na_2MoO_4. The gradient was centrifuged at 80,000 rpm (463,000g) to bring about a cumulative centrifugal effect (w^2t) of 3.0×10^{11} rad^2/s in a Beckman vTi65.2 vertical rotor for 90 min at 0°C. [^{14}C]Ovalbumin (3.5 S) and ^{14}C-labeled rabbit muscle aldolase (7.9 S) were used as internal standards (Galigniana, 1998). Fractions collected by gravity flow were pooled and concentrated by ultrafiltration. The hsp90-based chaperone heterocomplex was reconstituted by incubation of cytosol with 1 volume of concentrated rabbit reticulocyte lysate and an ATP-regenerating system for 30 min at 30°C (Scherrer et al., 1990). After reconstitution, the MR-hsp90 heterocomplex was incubated overnight on ice in the presence of 100 nM [^{3}H]ZK-91587. One sample was processed as described above, but the final incubation with radioactive steroid was performed in the presence of 10 μM radioinert ZK-91587 to estimate the nonspecific binding.

Specific Nuclear Uptake of Steroids. Rat kidneys were sliced, and the medulla region was removed. Cortices and medulla-cortex interphases were pooled and finely minced with scissors. The minced tissue was suspended in 1 volume of Ham's F-12 medium containing steroid and incubated for 30 min at 37°C under a 95% O_2/5% CO_2 atmosphere with continuous shaking. After this incubation, the tissue was washed with Earle's balanced saline and homogenized in buffer containing 0.3 M sucrose, 3 mM $MgCl_2$, and 10 mM phosphate at pH 6.5. Nuclear fractions were separated from cytosol (Piwien-Pilipuk and Galigniana, 1998) and resuspended in lysis buffer (10 mM Tris at pH 7.4, 2 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol, 0.05% Nonidet P-40 and inhibitors of proteases as detailed for TEGI buffer). Nuclei were first disrupted by two cycles of freeze-thawing. Then, 300 U/ml benzon nuclease and 0.3 M NaCl were added to the medium and incubated on ice for 30 min. Samples were centrifuged at 10,000g for 5 min, and salt was washed out from the supernatants using ultrafiltration. The samples were simultaneously concentrated by this procedure. The resulting concentrated nuclear fraction was reconstituted with rabbit reticulocyte lysate as described above and subsequently labeled with 100 nM [^{3}H]ZK-91587 (in the presence or absence of 10 μM unlabeled ZK-91587).

Immunoprecipitation of the MR. Kidney slices were incubated with 1 μM steroid for 30 min at 37°C. The tissue was homogenized in TEGI buffer supplemented with 0.05% Nonidet P-40, 0.1% Triton X-100, and 500 U/ml benzon nuclease. After two cycles of freeze-thawing, the homogenate was centrifuged at 67,000g for 30 min at 0°C. The supernatant of this centrifugation was used for immunoprecipitation of MR as described (Piwien-Pilipuk and Galigniana, 2000). Briefly, precleared renal cytosol was immunoadsorbed by duplicate with pAbhMR antibody (or nonimmune rabbit serum) bound to protein A-sepharose for 3 h at 4°C, and washed five times with 1 ml of ice-cold buffer containing 10 mM Tris, 1 mM EDTA, 10% glycerol, 0.02% Nonidet P-40, 150 mM NaCl, at pH 7.5. Proteins from one immunopellet were resolved in a 9% polyacrylamide-SDS gel, electrotransferred to an Immobilon-P membrane, and probed with a solution of pAbhMR for MR and AC88 antibody for hsp90. Donkey anti-rabbit antibody labeled with either iodine or horseradish peroxidase was used to visualize immunoreactive bands. The second immunopellet was resuspended with 100 μl of TEGI buffer and incubated overnight on ice with 10 μM [^{3}H]ALDO ($\pm$10 μM unlabeled ALDO). After this incubation period, the immunopellet was washed three times and the specific radioactivity bound to MR was quantified.

Inhibitory Effect of Tautomycin on Receptor Translocation. Renal tissue was minced as described above and preincubated for 45 min at 37°C with 100 nM tautomycin. Controls were preincubated with vehicle only (0.02% dimethyl sulfoxide). Then, either 1 μM ALDO or 1 μM 11-OP was added to the medium, and the incubation continued for 30 min. After this time period, the tissue was washed three times with saline solution and fractionated into nuclei and cytosols as described above. The MR associated to the nuclear fraction was extracted with nuclease and high ionic strength, and both cytosolic and nuclear MRs were reconstituted with the reticulocyte lysate system. Finally, a steroid binding assay with 100 nM [^{3}H]ZK-91587 was performed.

Modification of Lysyl Residues in the MR-hsp90 Cross-Linked Heterocomplex. Covalent cross-linking of MR and hsp90 was performed by using the cross-linking reagent dimethyl pimelimidate, exactly as described in a previous work (Galigniana and Piwien-Pilipuk, 1999). To modify lysine residues, the MR-hsp90 cross-linked heterocomplex (12 mg of protein/ml) in PEG buffer (25 mM sodium phosphate at pH 7.4, 10 mM EDTA, 10% glycerol, protease inhibitors as detailed for TEGI buffer) was incubated in the darkness with 10 mM pyridoxal 5′-phosphate for 20 min at 8°C. The Schiff base formed after this incubation was immediately stabilized by reduction with 10 mM sodium borohydride. When the reaction was completed, the samples were placed on ice and an additional treatment with 10 mM sodium borohydride was performed. The excess of reagents was removed by centrifugation in columns of Sephadex G-50 equilibrated in PEG buffer containing 2 mM β-mercaptoethanol. Proteins were finally concentrated by ultrafiltration, and a steroid binding assay was achieved. Control samples underwent exactly the same treatment described above, but pyridoxal 5′-phosphate was omitted in the incubation medium.

The reversion of the chemical modification of lysine residues was accomplished by addition of an excess of amino groups to the medium. Thus, when the reaction of MR with pyridoxal 5′-phosphate

was completed after 20 min, 60 mM L-lysine was added to the medium and incubated at 8°C for 30 min. After this time period, the reaction with sodium borohydride was performed and the treatment continued as detailed above. In experiments in which either ALDO or 11-OP was prebound to the MR before the treatment with the lysyl modifying reagent, bound unlabeled steroid was first dissociated from the cross-linked receptor by incubation at 30°C for 30 min and cleared from the medium by adsorption with charcoal- dextran. Then a steroid binding assay with [^{3}H]ALDO was performed.

Statistical Tests. Data were analyzed by one-way nonparametric ANOVA followed by the Kruskal-Wallis test.

Results

Structures of the Most Stable Conformers. Figure 1 depicts the most stable conformers of the flat pregnanesteroid 11-OP (Fig. 1a) and its bent isomer 6-OP (Fig. 1b). The structure of ALDO (Fig. 1c) is also included for comparative purposes. Despite the similarity between 11-OP and 6-OP with respect to chemical functions, the overall conformations are very different. In this sense, 11-OP shares similar overall flatness with ALDO, whereas 6-OP is dramatically bent toward the α-face. As a common feature, the three steroids exhibit an extra ring on the β-face, a property that transforms these steroids in highly rigid structures. It should be noted that the extra rings present in ALDO can open reversibly under physiological conditions; however, this does not occur with 11-OP and 6-OP.

Biological Effects. Figure 2 depicts the mineralocorticoid properties of both oxidopregnanes as compared with the natural steroids PROG, CORT, DOC, and ALDO. 11-OP elicits significant sodium retention (Fig. 2A) as well as kaliuresis (Fig. 2B). These effects are quite similar to those measured with the endogenous mineralocorticoids DOC and ALDO. However, the natriuretic effect of ALDO is significantly more potent ($P < .005$) than the effect exhibited by DOC and 11-OP if doses lower than 10 μg/100 g are analyzed. In turn, both natriuresis and kaliuresis are indistinguishable between 11-OP and DOC. On the other hand, PROG, CORT, and 6-OP did not significantly affect the electrolyte excretion under this experimental condition. Inasmuch as the creatinine clearance rate remained unaffected for all the treatments (2.62 ± 0.06 ml/min), any quantitative difference cannot be ascribed to variations in the glomerular filtration. Plasma electrolyte levels remained unchanged for all treatments (data not shown).

The RBA for renal MR was measured by competition of unlabeled steroid with [^{3}H]ALDO in kidney cytosol. Figure 2C shows that the relative potency is ALDO > DOC > PROG = 11-OP > CORT > 6-OP. The cytosol used in this experiment is a crude preparation; therefore, it contains CBG from both plasma and renal origin. This, in turn, could be a reason for underestimating the RBAs because of competition of the carrier protein with the MR for the steroids. Therefore, we first eliminated such contamination by adsorption of the MR on hydroxylapatite gel, and the competition curves were then measured in the CBG-free medium. As can be seen in Fig. 2D, the efficiency of 11-OP and 6-OP to compete with the tracer was not affected, suggesting that there is not significant binding of these steroids to the carrier protein. On the other hand, CORT and DOC show efficiency similar to that of ALDO to compete with the tracer. PROG also improved its ability to compete with [^{3}H]ALDO, although less efficiently.

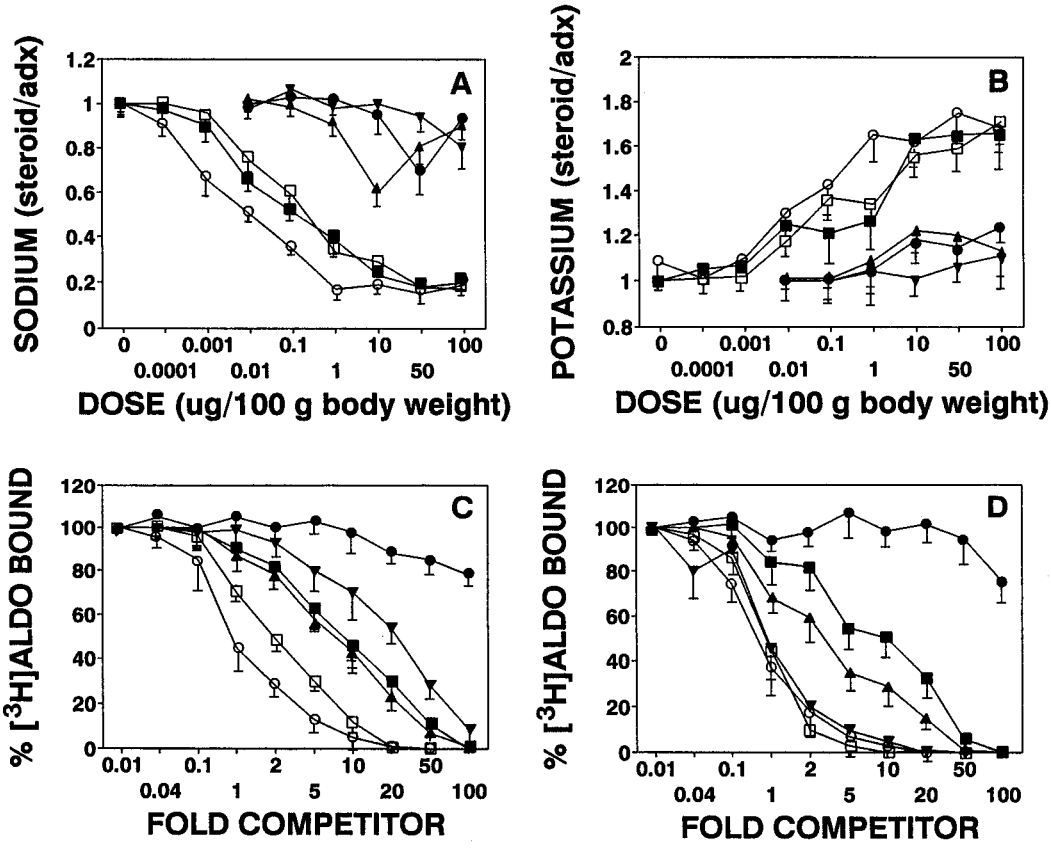

Fig. 2. Mineralocorticoid properties. ADX male rats were injected with several doses of steroid, urine was collected by bladder puncture after 3 h, and the elimination rates for sodium (A) and potassium (B) were quantified. Results represent the mean ± S.E. for 8 rats/dose/group. The RBAs for the MR were measured in crude kidney cytosol (C) or CBG-free kidney cytosol (D) incubated with [^{3}H]ALDO and increasing concentrations of unlabeled steroids. Incubations were performed at 0°C for 12 h in the presence of RU-28362 to prevent cross-reaction with the GR. Nonspecific binding was determined with a 1000-fold excess of unlabeled ALDO and subtracted from the total. Values are expressed as a percentage of the maximum binding measured in the absence of radioinert steroid (mean ± S.E. for five experiments performed in triplicate). Symbols used in all panels represent: ALDO (○); DOC (□); PROG (▲); CORT (▼); 11-OP (■); and 6-OP (●).

The RBA for each steroid was calculated from the competition curves and is summarized in Table 1.

Despite the fact that 11-OP is as potent a sodium retainer as DOC and slightly less potent than ALDO, 11-OP shows at least 5- and 10-fold less affinity for MR ($P < .001$), respectively. Based on the RBAs, 11-OP resembles the weak mineralocorticoid PROG rather than the potent physiological mineralocorticoids DOC and ALDO. The results depicted in Fig. 2 confirm our previous findings for 11-OP (Burton et al., 1995) and extend the mineralocorticoid features to its kaliuretic action.

Table 2 summarizes the biological and biochemical glucocorticoid properties. CORT, the most important glucocorticoid in rodents, stimulates the deposition of liver glycogen 5-fold, and the induction of tyrosine amino-transferase activity is stimulated 7-fold in isolated rat hepatocytes. DOC, a mineralocorticoid that also exhibits partial glucocorticoid effects, shows moderate induction of tyrosine amino-transferase activity and a medium capacity to stimulate glycogen storage in liver. Therefore, neither 11-OP nor 6-OP exhibits glucocorticoid effects in vivo or in vitro. Consistent with this lack of biological effect, both synthetic steroids also fail to bind to the GR. Table 2 provides direct evidence that both synthetic steroids do not bind to CBG. This is an important observation for 11-OP because it implies that its RBA for MR (and the subsequent biological effect) cannot be affected by competition with contaminating CBG when kidney cytosol is used as a source of MR. These results rule out one of the hypotheses outlined in the Introduction.

Taken together, the experiments shown in Fig. 2 and Table 2 demonstrate that 11-OP is a specific and selective agonist for MR, which is devoid of glucocorticoid properties.

Plasma Clearance. To ascertain whether the biological half-life of the steroid influences its biological potency, we measured the half-lives of ALDO, CORT, 6-OP, and 11-OP. Radioactive ALDO or CORT was injected as an i.v. bolus, and both total and dichloromethane extractable radioactivity were measured. Figure 3A shows the plasma clearance for the natural steroids. The nonextractable fraction represents polar metabolites that do not exhibit significant binding to MR (Morris, 1981). As can be seen, the total radioactivity in plasma decreases rapidly for both steroids following first-order kinetics. The calculated half-lives for total radioactivity are 17.3 ± 0.7 min for ALDO and 46.2 ± 0.7 min for CORT. After extraction with Cl_2CH_2, the radioactivity exhibits similar first-order kinetics and half-lives of 12.1 ± 0.3 min for ALDO and 22.9 ± 1.4 min for CORT. These half-lives agree with findings from previous reports (Colby and Kitay, 1972; Morris et al., 1976). Urine samples collected at the end of the experiment (data not shown) showed insignificant amounts of excreted radioactivity (<5% of the total).

Plasma levels of 11-OP and 6-OP were measured using HPLC, and the results are depicted in Fig. 3B. The clearance of both steroids also follows first-order kinetics. The calculated half-lives are 13.0 ± 0.7 min for 11-OP and 20.4 ± 1.3 min for 6-OP. Because ALDO and 11-OP exhibit similar half-lives, the differences observed for the mineralocorticoid effect cannot be assigned to different plasma clearance rates.

RBA for MR in Vivo. To ascertain whether the RBA for MR measured in a cell-free system is representative of the actual RBA in vivo, ADX rats were coinjected with [^{3}H]ALDO and increasing amounts of unlabeled ALDO, 6-OP, or 11-OP. The nonspecific binding measured by coinjection of the tracer with 1000-fold unlabeled ALDO was subtracted from each sample. Figure 4A shows that the total radioactivity reached a maximum in kidneys after 20 min. Therefore, kidneys were excised at this time, and the specific binding was measured in cytosol after adsorption of free tracer with charcoal-dextran. Figure 4B depicts the curves obtained by competition in vivo. The RBA order is similar to that observed in vitro: ALDO (16.9 ± 3.3 nM) > 11-OP (52.7 ± 6.1 nM) > 6-OP (>2000 nM). Both synthetic steroids show identical values of RBA as those measured in vitro. Even so, 11-OP is still significantly less efficient than ALDO to compete for the tracer ($P < .001$).

The distribution volume of 11-OP was calculated to be 21.6 ml. This value represents ~10% of body weight, equaling the rat plasma volume (Waynforth, 1980). In other words, the biological availability of 11-OP is the same as that calculated for the natural steroid (19.6–22.2 ml).

Transformation and Nuclear Translocation of the MR. The aforementioned experiments indicate that neither the pharmacokinetic nor pharmacodynamic properties of 11-OP are responsible for the biological activity of this synthetic mineralocorticoid. In turn, the common factor in the mechanism of action of ALDO and 11-OP seems to be the

TABLE 1
RBAs for MR

The RBA for each steroid was calculated from the competition curves shown in Fig. 2 by using a four-parameter logistic function. Values in the first column (−HAP) represent assays performed in crude cytosol, whereas those in the second column (+HAP) represent RBAs obtained in cytosol previously adsorbed on hydroxylapatite. Data are presented as the mean ± S.E. of five independent curves, each one performed in triplicate. Differences of RBAs are significant ($P < .001$), with the exception of: −HAP, 11-OP versus PROG; +HAP, ALDO versus DOC, ALDO versus CORT, CORT versus DOC.

Steroid	RBA	
	−HAP	+HAP
	nM	
ALDO	4.0 ± 0.1	2.3 ± 0.3
DOC	10.9 ± 0.8	3.6 ± 0.4
PROG	46.9 ± 5.0	26.6 ± 6.2
11-OP	52.1 ± 5.2	56.0 ± 6.0
CORT	111.3 ± 6.7	4.1 ± 0.9
6-OP	>2000	>2000

TABLE 2
Glucocorticoid properties

	TAT[a]	Glycogen[b]	GR[c]	CBG[c]
ALDO	1.1 ± 0.1	1.5 ± 0.2	74.6 ± 7.6	905.5 ± 50.1
DOC	2.8 ± 0.3	3.1 ± 0.6	6.9 ± 2.1	14.8 ± 3.5
CORT	7.4 ± 0.6	5.7 ± 0.5	1.0 ± 0.2	1.0 ± 0.1
PROG	2.0 ± 0.5	0.9 ± 0.1	6.2 ± 0.5	289.1 ± 9.9
11-OP	1.0 ± 0.1	1.4 ± 0.2	>2000	>2000
6-OP	1.0 ± 0.1	0.8 ± 0.3	>2000	>2000

[a] Isolated hepatocytes from ADX rats were incubated with 100 nM steroid, and the induction of tyrosine amino transferase (TAT) activity was measured. Results represent fold induction (mean ± S.E. of three experiments performed in duplicate) with respect to the control incubated with vehicle alone (4.0 ± 1.0 μmol of tyrosine × 10^{-6} cells × min^{-1}).

[b] Glycogen was obtained from the liver of ADX rats after acute treatment with the indicated steroid. Values are expressed as a fold increase (mean ± S.E. for eight rats per group) with respect to controls injected with vehicle (9.1 ± 1.2 mg of glycogen/g of liver).

[c] RBAs were calculated from competition curves between the steroid and 5.0 nM [^{3}H]CORT. Thymocyte cytosol homogenized in TEGI buffer was used as a source of GR. Rat plasma was fractionated with solid ammonium sulfate at 30 to 60% of saturation, dissolved in TEGI buffer afterward, dialyzed, and used as a source of albumin-free CBG. Values are expressed in nanomolar and represent the mean ± S.E. of three independent experiments, each one performed in quintuplicate.

interaction with the MR. Hence, we performed studies on the ability of 11-OP to transform MR as compared to ALDO.

A conventional technique used to detect transformation of steroid receptors is to label the protein by incubation with radioactive ligand at low temperature. Then, the preformed radioligand-receptor complex is heated to allow the dissociation of the hsp90 heterocomplex. Thus, the transformed receptor can be further separated from untransformed receptor (still associated to the hsp90 heterocomplex) by ultracentrifugation. Because the receptor requires association with hsp90 to be in the steroid binding conformation (for a recent review, see Pratt and Toft, 1997), the transformed receptor will not be detected after disassembly. Therefore, additional incubation of gradient fractions with a radioactive tracer does not detect the peak of transformed MR. Because 11-OP is not available in radioactive form, it is not possible to detect the presence of the 11-OP-transformed MR by reincubation with, for example, [^{3}H]ALDO or [^{3}H]ZK-91587, unless the heterocomplex with the hsp90 chaperone system can be previously refolded with MR to recover the steroid binding capacity. We attempted to reconstitute the transformed 11-OP·MR complex by incubating it with concentrated reticulocyte lysate and an ATP-regenerating system by adapting the reassembly system first described by Scherrer et al. (1990). We further performed a steroid binding assay with [^{3}H]ZK-91587 as tracer to detect the presence of refolded MR. The reticulocyte lysate functions as a donor source of all the proteins that are required to reconstitute the MR heterocomplex, and the refolded heterocomplex is indistinguishable from the native untransformed receptor.

Figure 5A depicts a control experiment that validates its additional use for detecting transformed receptor. We knew from preliminary approaches that the [^{3}H]ALDO·MR complex dissociates during the incubation period required for the reconstitution with reticulocyte lysate at 30°C. Inasmuch as the mass of tritiated tracer released to the medium during such incubation is insignificant, the putative rebinding of the same molecules of ^{3}H-labeled steroid to the refolded MR is not quantitatively important. This is clearly demonstrated by the incubation shown in column 3 of Fig. 5A as compared with the untreated (column 1) and reticulocyte lysate-treated (column 2) controls.

Figure 5A also depicts the effect of ALDO- and 11-OP-induced transformation of the MR. Cytosolic MR was preloaded at 0°C with radioinert ALDO (columns 4 and 5) or radioinert 11-OP (columns 6 and 7). Temperature was then raised to 30°C for 10 min (columns 4 and 6) to allow MR transformation, and the resulting stripped MR was incubated overnight on ice with 100 nM [^{3}H]ZK-91587. Negligible specific binding could be measured, demonstrating that both steroids transformed the MR to the nonbinding form. However, when the stripped receptor was reconstituted with the reticulocyte lysate system in the presence of an ATP-regenerating system (columns 5 and 7), approximately 50% of the binding capacity exhibited by controls could be recovered. As can be seen in column 8, reticulocyte lysate itself lacks detectable steroid binding capacity. The experiment depicted in Fig. 5A demonstrates that transformed MR by either ALDO or 11-OP is detectable in a similar manner, even if the transforming ligand is not available in radioactive form.

To provide direct evidence for 11-OP-dependent transformation, we next used the reconstitution system tested in the previous experiment to refold the transformed MR resolved by sucrose gradient ultracentrifugation (Fig. 5B). Kidney cytosol was first incubated with unlabeled steroid, cleared of free steroid with charcoal, heated to 30°C, and subjected to a 5 to 20% sucrose gradient. Samples collected from the gradient were reconstituted with rabbit reticulocyte lysate, and a binding assay with 100 nM [^{3}H]ZK-91587 was performed. Figure 5B depicts the hydrodynamic profile of the receptor. The control gradient shows that molybdate-stabilized MR sediments as untransformed 9.2 S species, whereas preincubations with 10 nM ALDO or 500 nM 11-OP (both radioinert) followed by heating of the steroid-MR complex results in a large increase of the 5.2 S transformed receptor with a concomitant loss of the 9.2 S untransformed species. According to these hydrodynamic properties, we conclude that 11-OP does transform the renal MR, and this transformation is indistinguishable from that observed with ALDO.

Despite the fact that 11-OP dramatically affects the hydrodynamic properties of the MR, this transformation achieved in vitro might not be in agreement with the nuclear uptake of the steroid-receptor complex in intact cells, for instance, due to ineffective interactions of a conformationally anomalous

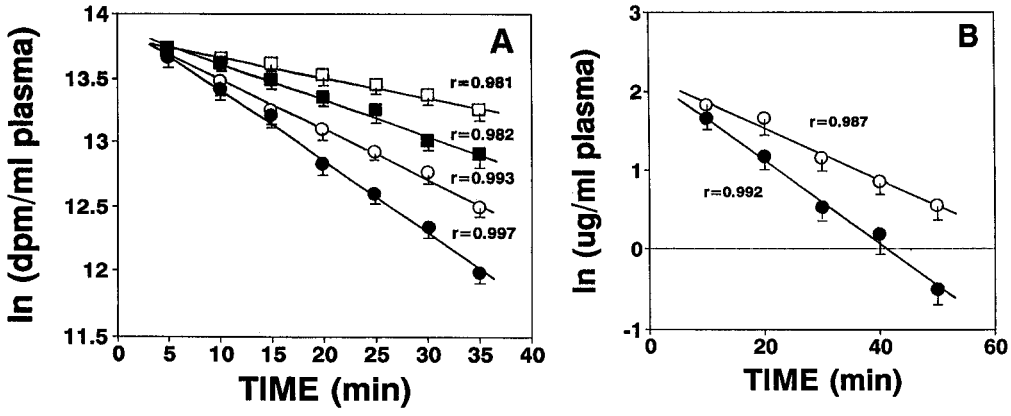

Fig. 3. Biological half-lives. A, ADX male rats (~200 g) were injected with 10 μCi of either [^{3}H]ALDO (circles) or [^{3}H]CORT (squares) and 2 μg of the respective unlabeled steroid. After i.v. injection, blood samples were taken and the plasma clearance of total radioactivity (open symbols) and Cl_2CH_2-extractable fraction (closed symbols) was measured. Results are the mean ± S.E. (n = 6) for each time-interval. B, 100 μg of 11-OP (●) or 6-OP (○) were injected and processed as indicated in A. Quantification was carried out by HPLC using standard solutions of 11-OP and 6-OP made in ADX rat plasma. Results are the mean ± S.E. of three independent experiments. The total plasma level at zero time ranged from 6.1 to 8.5 nM for ALDO and CORT, and from 32.1 to 35.7 nM for the synthetic oxidopregnanes.

11-OP·MR complex with the trafficking machinery and/or nuclear import receptors such as α-importin. To evaluate the nuclear import of the MR, we incubated minced cortex-medulla kidney interphases with 10 nM ALDO or 500 nM 11-OP. Then, the tissue was washed and homogenized, and cytosolic and nuclear fractions were obtained. The MR was extracted from the nuclear fractions by treatment with nuclease and high ionic strength and afterwards reconstituted with reticulocyte lysate to allow the binding of [^{3}H]ZK-91587. As has been demonstrated previously by other laboratories (Alnemri et al., 1991; Binart et al., 1991), the experiment depicted in Fig. 5C shows that the MR is localized primarily in the cytoplasm in the absence of hormone. Incubations with either ALDO or 11-OP translocate the MR into the nucleus with equal efficiency, evident from steroid binding assays performed after extraction and reconstitution of translocated nuclear MR. Therefore, 11-OP not only transforms but also efficiently translocates the MR into the nucleus, suggesting there is normal interaction of the steroid-receptor complex with both trafficking and translocating machinery.

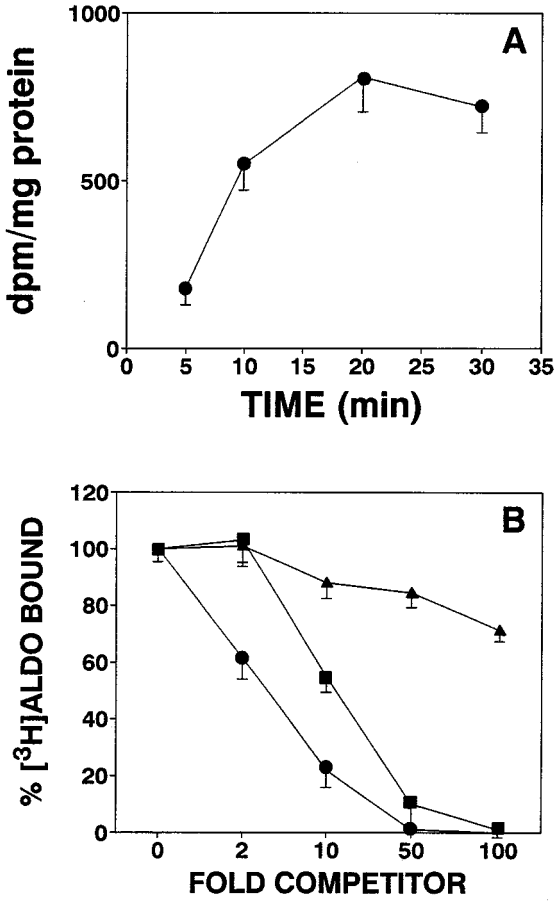

Fig. 4. In vivo steroid binding to kidney MR. A, ADX rats were given i.p. injections of a solution of 10 μCi of [^{3}H]ALDO in saline solution and a 1000-fold excess of radioinert ALDO. Kidneys were excised at different times, perfused, and the specific binding of radioactivity by the organs was measured after charcoal-dextran adsorption of renal cytosol. Results are the mean ± S.E. of three experiments, each one performed with four rats per condition. B, specific binding of [^{3}H]ALDO was measured in kidney cytosol 20 min after the coinjection of the tracer and increasing amounts of radioinert steroids. Results are the mean ± S.E. of five independent experiments, each one performed with four rats per condition: ALDO (●); 11-OP (■); 6-OP (▲).

Tautomycin Does Not Inhibit the Translocation of 11-OP/MR into the Nucleus. In a recent study (Piwien-Pilipuk and Galigniana, 1998), we reported that tautomycin, a polyketide which is a selective inhibitor of PP1/PP2A/PP5 serine/threonine phosphatases, inhibits the transformation and translocation of the MR into the nucleus. This process is triggered physiologically by ALDO, and tautomycin abrogates such an event.

We examined whether the translocation into the nucleus of 11-OP-activated MR is inhibited by tautomycin as demonstrated for the ALDO-activated MR complex. These results are shown in Table 3. Consistent with the results obtained in Fig. 5, most of the MR is localized in the cytosolic fraction in the absence of steroid (70% of the total), whereas 75% of the MR is recovered in the nuclear fraction after the addition of either ALDO or 11-OP. The presence of 100 nM tautomycin does not affect the normal subcellular distribution of MR in the absence of ligand. However, if the samples are pretreated with tautomycin and then incubated with ALDO, the translocation of the ALDO·MR complex is inhibited significantly and only 40% of the MR is recovered in the nucleus. On the other hand, more than 75% of the MR was recovered in the nucleus of samples treated with tautomycin and 11-OP. In other words, the activation of the MR on binding of 11-OP differs from the activation achieved by binding of ALDO because the former induces a translocation process that is resistant to the inhibitory effect of tautomycin. This experiment demonstrates that the binding of the oxidopregnane steroid affects the relative sensitivity of the MR to tautomycin with respect to the physiological activation triggered by the natural ligand ALDO. Sucrose gradient experiments (data not shown) demonstrated that the steroid-dependent dissociation of the hsp90 chaperone system from MR is not affected by the treatment with tautomycin in any case.

Chemical Modification of Lysyl Residues in the MR Inhibits the Binding of ALDO, but Not the Binding of 11-OP. To assess the effect of chemical modification of lysine residues on the steroid binding capacity of MR, we first cross-linked the receptor with the hsp90 heterocomplex to prevent its eventual dissociation under the conditions required for that reaction. Thus, kidney cytosol was first treated with the cross-linker reagent dimethyl pimelimidate. We have reported previously (Galigniana and Piwien-Pilipuk, 1999) that hsp90 can be recovered associated to the MR in a covalent form after this treatment, and that the cross-

TABLE 3

Inhibitory effect of tautomycin on the nuclear translocation of the MR

Minced renal tissue was preincubated with 100 nM tautomycin for 45 min at 37°C. Nuclear translocation was induced with 1 μM ALDO or 1 μM 11-OP for 30 min. The tissue was homogenized and fractionated into nuclear and cytosolic fractions, and the MR was extracted from the nuclei by treatment with nuclease and high ionic strength. Both cytosolic and nuclear fractions were then reconstituted with the reticulocyte lysate system, and a steroid binding assay with 100 nM [^{3}H]ZK-91587 was performed. Results are given as the mean ± S.E. of five experiments.

Treatment	Cytosol	Nucleus
	dpm / mg of protein	
None	380 ± 35	153 ± 28
ALDO	88 ± 28[a]	417 ± 16[a]
11-OP	111 ± 19[a]	399 ± 24[a]
Tautomycin	367 ± 50	141 ± 34
ALDO + tautomycin	276 ± 31[b,c]	212 ± 11[b,c]
11-OP + tautomycin	122 ± 13[a]	431 ± 33[a]

Different from the respective control at [a] $P < .001$ and [b] $P < .005$.
[c] Different from treatment with ALDO alone at $P < .007$.

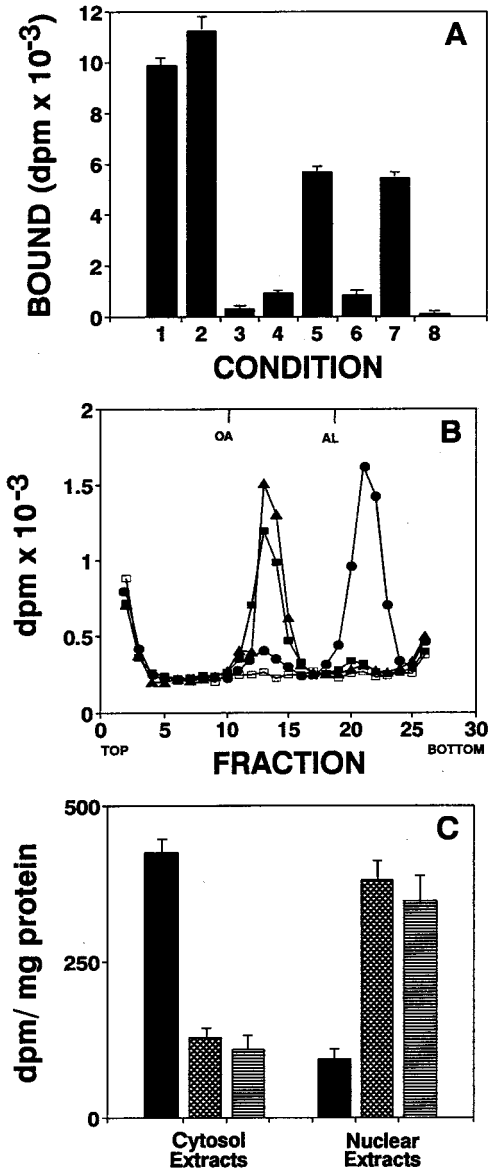

Fig. 5. 11-OP transforms MR and promotes its nuclear translocation. A, transformed MR can be refolded to the steroid binding form by reconstitution with reticulocyte lysate and ATP. Cytosolic MR in TEGI buffer was obtained from renal medulla-cortex interphases of ADX rats. 300 μl of cytosol was preincubated for 3 h at 0°C with either vehicle (control bars 1 and 2), 5 nM [^{3}H]ALDO (bar 3), 5 nM unlabeled ALDO (bars 4 and 5), or 500 nM 11-OP (bars 6 and 7). Steroid-MR complexes were cleared of free steroid with 50 μl of 7% charcoal, 0.7% dextran. One volume of concentrated rabbit reticulocyte lysate (bars 2, 3, 5, and 7) or buffer (bars 1, 4, and 6) was added to the medium and incubated for 30 min at 30°C in the presence of the ATP-generating system. The MR was then labeled overnight at 0°C with 100 nM [^{3}H]ZK-91587 ($\pm$10 μM unlabeled ZK-91587) and adsorbed with charcoal-dextran, and the radioactivity was measured. Incubation in bar 8 shows the steroid binding capacity of 300 μl of reticulocyte lysate (no cytosol was added) incubated overnight with 100 nM [^{3}H]ZK-91587. Bars depict the specific binding (mean $\pm$ S.E.) for triplicate samples per condition. Numbers below each bar represent the following treatments previous to the overnight incubation at 0°C with 100 nM [^{3}H]ZK-91587 (this incubation was omitted in condition 3): 1, control of native receptor: no treatment; 2, control of native receptor for the reconstitution treatment: untreated cytosol was incubated for 30 min at 30°C with reticulocyte lysate and an ATP-generating system; 3, control of residual [^{3}H]ALDO binding: cytosol was labeled with 5 nM [^{3}H]ALDO for 3 h at 0°C (no transformation takes place), free steroid was adsorbed with charcoal, and the resultant [^{3}H]ALDO·MR complex was incubated with reticulocyte lysate for 30 min at 30°C (bound [^{3}H]ALDO is dissociated) followed by an overnight incubation at 0°C without [^{3}H]ZK-91587; 4, control of lack of binding capacity for the ALDO-transformed receptor: cytosol was incubated with 5 nM radioinert ALDO for 3 h at 0°C, adsorbed with charcoal, and incubated for 30 min at 30°C to allow receptor transformation; 5, test of reconstitution for the ALDO-transformed receptor: same conditions as in bar 4, but the incubation for 30 min at 30°C was performed in the presence of reticulocyte lysate and an ATP-generating system; 6, control of lack of binding for 11-OP-transformed receptor: cytosol was incubated with 100 nM 11-OP for 3 h at 0°C, adsorbed with charcoal, and heated for 30 min at 30°C to allow receptor transformation; 7, test of reconstitution for 11-OP-transformed receptor: same conditions as in bar 6, but the incubation for 30 min at 30°C was performed in the presence of reticulocyte lysate and ATP-generating system; 8, control of lack of binding by the reticulocyte lysate: 300 μl of reticulocyte lysate was incubated overnight with [^{3}H]ZK-91587. B, hydrodynamic profile of 11-OP-transformed MR. Renal cytosol was preincubated with 10 nM ALDO (▲) or 500 nM 11-OP (■) for 3 h at 0°C in TEGI buffer without molybdate. The control was not preincubated with steroid and the buffer contained 20 mM molybdate (●). Steroid-receptor complexes were incubated at 30°C for 10 min, after clearing free steroid with charcoal, and centrifuged in a 5% to 20% sucrose gradient. Fractions were collected using gravity flow and reconstituted with rabbit reticulocyte lysate for 30 min at 30°C. The samples were labeled overnight with [^{3}H]ZK-91587 as described for A. [^{14}C]Aldolase (AL) or [^{14}C]ovalbumin (OA) were used as internal standards. The radioactivity measured in fractions 10 to 17 (5.2-S peak) was corrected by the efficiency of the reconstitution. The gradient obtained from samples incubated with [^{3}H]ZK-91587 and a 100-fold excess of radioinert ZK-91587 (□) represents the nonspecific binding. C, 11-OP triggers MR translocation into the nucleus. Minced kidney cortex and medulla-cortex interphases were incubated with vehicle (black bars), 1 μM ALDO (crosshatched bars), or 1 μM 11-OP (hatched bars), washed, homogenized, and fractionated into nuclear and cytoplasmic fractions. Receptors from nuclear fractions were extracted, and the MR·hsp90 heterocomplex was reconstituted with reticulocyte lysate as described in A. The specific binding was then measured with 100 nM [^{3}H]ZK-91587 ($\pm$10 μM radioinert ligand), and the values were adjusted according to the reconstitution efficiency. Results show the mean $\pm$ S.E. of three experiments, each one performed in duplicate.

linked heterocomplex preserves steroid binding capacity even under denaturing conditions (i.e., 4 M urea or high ionic strength). Therefore, the cross-linked MR·hsp90 heterocomplex was incubated with the lysyl modifying reagent pyridoxal 5′-phosphate (Lilley and Engel, 1992). The unstable Schiff base formed between the pyridoxal group and the ϵ-amino group of lysines was rapidly stabilized by reduction with sodium borohydride, and the steroid binding capacity was measured. Controls without pyridoxal 5′-phosphate underwent the same procedure. Results are listed in Table 4.

When the unoccupied MR was treated with pyridoxal 5′-phosphate, the steroid binding was reduced 80% as compared to controls, indicating that the modification of lysyl groups inhibits the steroid binding capacity of the MR. To test the specificity of the reaction, the pyridoxal-modified MR was then incubated with an excess of lysine previous to the treatment with borohydride, and a complete recovery of the steroid binding capacity was obtained. On the other hand, when the MR was first preincubated with ALDO followed by treatment with the modifying reagent, only 30% of inhibition in the steroid binding capacity was obtained. In contrast, when 11-OP was prebound to the receptor, no protection against the treatment with lysyl modifying reagent was observed. Similar results were obtained with the bent stereoisomer 6-OP, a steroid that does not bind to MR. These contrasting features between the natural ligand ALDO and the synthetic agonist 11-OP suggest that both steroids may differ in the binding sites on the MR or may induce different receptor conformation as to protect lysine residues present in or next to the steroid binding pocket. However, it is possible that there are no lysines in the steroid binding pocket of the MR as judged from the crystal structures described for other

members of the nuclear receptor superfamily, i.e., the RAR-γ ligand-binding domain (Renaud et al., 1995) and the PROG receptor (Williams and Sigler, 1998). Therefore, if these models also apply for the MR, the critical lysyl groups modified by pyridoxal 5′-phosphate may be localized on the receptor surface.

Antagonistic Effect of Spironolactone. Inasmuch as the mineralocorticoid activity of 11-OP is MR-mediated (Fig. 5), antagonists that bind to the MR, such as SPO, should inhibit those effects. Table 5 shows that a dose of 1 μg of ALDO yields 20% of the Na^+ excretion measured in ADX rats. This value equals the sodium-retention measured when 100 μg of 11-OP was injected alone or coinjected with ALDO. When 100 μg of SPO was coinjected with ALDO, 11-OP, or both agonists, sodium excretion was recovered to the values measured for the ADX group. Kaliuresis behaved in a similar fashion. Because both ALDO (1 μg) and 11-OP (100 μg) represent saturating doses for the mineralocorticoid response, it is not surprising to observe that there is no addition in the effect obtained by cotreatment. Nevertheless, these results suggest that ALDO and 11-OP may share a common pathway of activation to trigger the mineralocorticoid response. If another mechanism would take place (e.g., altered plasma membrane permeability for ions), a biological response stronger than the maximum effect observed at saturating doses could be possible.

Interestingly, a dose of 0.6 ng of 11-OP, which is ineffective per se to trigger a biological response (Fig. 2), amplifies the mineralocorticoid effect of a suboptimal dose of 60 ng of ALDO. Consistent with an MR-dependent mechanism, this effect is competed by SPO. It should be noted that these combined treatments did not affect the creatinine clearance rate (data not shown).

Dissociation Rate of the Steroid-Receptor Complex. The previous experiment demonstrates that 11-OP is also able to amplify the ALDO biological effect. Inasmuch as 11-OP functions through the activation of MR, we studied whether 11-OP exerts any effect of addition or potentiation of the ALDO binding to the MR. Figure 6A shows the competition curve between 5 nM [^{3}H]ALDO and increasing concentrations of unlabeled ALDO (●) by the unoccupied receptor. The RBA for ALDO measured under these conditions is 4.8 ± 0.4 nM. If 11-OP and ALDO operate through the same mechanism, it would be expected that the oxidopregnane adds its competitive effect to the competition of unlabeled ALDO by the tracer. As a consequence, a shift toward the left in the competition curve with ALDO would be expected. On the other hand, if 11-OP is ineffective in modifying the competitive profile of ALDO, a similar curve as that observed by competition with radioinert ALDO alone should be observed. Surprisingly, the simultaneous presence of an equimolar concentration of 11-OP (▲) with respect to the tracer shows that ALDO is now less effective to compete. A significantly higher ($P < .003$) RBA equal to 8.4 ± 0.2 nM is measured under this condition. This shift is dependent on the concentration of 11-OP. Thus, in the presence of 30 nM 11-OP (■), a level of RBA equal to 14.6 ± 3.1 nM was measured (different from control at $P < .001$).

A detailed analysis of the competition curves shows that a 10-fold excess of radioinert ALDO (50 nM) totally competes with the radioactive steroid specifically bound to the MR, but this displacement decreases, respectively, to 22% and 35% if 5 nM 11-OP or 30 nM 11-OP is also present in the incubation medium ($P < .001$ for both concentrations).

To confirm such a peculiar behavior of 11-OP, we repeated the displacement curve shown in Fig. 6A, but [^{14}C]DOC was used as tracer. Figure 6B shows similar results to those described above. As expected, radioinert ALDO was more efficient than DOC in competing the tracer (open symbols) yielding RBAs equal to 3.2 ± 0.3 nM and 9.2 ± 0.7 nM, respectively. As observed previously with [^{3}H]ALDO, the simultaneous presence of 30 nM 11-OP also abrogates the efficiency of unlabeled steroid to displace [^{14}C]DOC (solid symbols). Thus, the RBAs increase to 17.0 ± 2.1 for ALDO and 35.2 ± 3.6 nM for DOC. Consistent with results shown in A, a 10-fold excess of either ALDO or DOC fully competes with the tracer, whereas more than 30% of residual binding can be measured if 11-OP is also present in the medium.

Two main reasons may explain the data obtained in the competition curves: 1) because 11-OP exhibits 10-fold less affinity for MR than ALDO, it may be envisioned that the presence of 11-OP affects the binding of ALDO and DOC in a similar fashion as potent glucocorticoids such as cortisol or dexamethasone are inhibited by weaker agonists (i.e., cortexolone or RU-28362). Two previous experiments argue against this hypothesis: the biological activity of ALDO is not inhibited (but amplified) by 11-OP (Table 5), and a concentration as low as 5 nM 11-OP does not affect per se the [^{3}H]ALDO binding in a significant manner (Fig. 2); 2) it is entirely possible that the binding of 11-OP to MR stabilizes an "active" receptor conformation, allowing 11-OP to trigger an ALDO-like biological response in ADX rats. This "active" receptor conformation may abrogate unlabeled ALDO to compete with [^{3}H]ALDO already bound to the agonist binding site, e.g., due to restricted access to the ALDO-binding pocket.

If the latter hypothesis is correct, 11-OP should affect the dissociation rate of the ALDO·MR complex. Figure 6C depicts the dissociation rates of the [^{3}H]ALDO·MR complex measured by isotope dilution in a medium precleared of free tracer. The dissociation rate constant, k_{-1}, measured at 20°C in the presence of 100-fold excess of unlabeled ALDO (●) is 37.7 ± 2.9 $min^{-1} \times 10^{-3}$, and equaled 11.5 ± 0.9 $min^{-1} \times 10^{-3}$ in the presence of 5 nM 11-OP (▲) (equimolar with tracer). It should be pointed out that this concentration of 11-OP fails to compete with ALDO, as can be also seen in Fig. 2. The simultaneous presence of 500 nM ALDO and 5 nM 11-OP (■) yields a k_{-1} equal to 11.5 ± 0.1 $min^{-1} \times 10^{-3}$, indicating a deceleration of the dissociation rate caused by the presence of the synthetic steroid. These results suggest

TABLE 4
Protective effect of ALDO and 11-OP on the inhibition of the steroid binding capacity of MR by pyridoxal 5′-phosphate

Treatment	Binding of [^{3}H]ALDO
	cpm/incubation
Control	8180 ± 435
10 mM Pyridoxal 5′-phosphate + 60 mM L-Lys	7295 ± 785
10 mM Pyridoxal 5′-phosphate	1488 ± 381[a]
Preincubation with 20 nM ALDO	5988 ± 880[b,c]
Preincubation with 300 nM 11-OP	1377 ± 279[a]
Preincubation with 1 μM 6-OP	1607 ± 556[a]

Different from control at [a] $P < .001$ and [b] $P < .05$.
[c] Different from treatment of unoccupied MR at $P < .001$.

that the binding of 11-OP to MR may take place on a site different from that of ALDO.

When the kinetic study was performed in the presence of 2.5 nM DOC (Fig. 6D), the k_{-1} remains unaffected ($29.7 \pm 3.0 \times 10^{-3}$ min^{-1} for incubation with both steroids as compared with $34.3 \pm 2.1 \times 10^{-3}$ min^{-1} for ALDO alone). The k_{-1} measured when only DOC is added to the medium is $9.5 \pm 0.9 \times 10^{-3}$ min^{-1}. The same type of kinetic experiments was also performed with the bent isomer 6-OP (data not shown), and no effect of this steroid on the dissociation rate of ALDO was obtained. The lack of effect of DOC and 6-OP under equivalent experimental conditions confirms the specificity of the observations performed with 11-OP.

11-OP Enhances ALDO Binding at Low Concentration and Competes with the Natural Ligand at High Concentration. The experiments depicted in Fig. 7 were designed to confirm the identity of the protein receptor and to evaluate the type of competition between ALDO and 11-OP for binding to the MR. Kidney slices were first incubated with 1 μM steroid, and then the MR was immunoprecipitated. As can be seen in Fig. 7A, immunopellet B exhibits specific ALDO binding capacity as compared with the nonimmunopellet A. Accordingly, the Western blot analysis shows hsp90 coimmunoprecipitated with the MR. On the other hand, both ALDO (condition C) and 11-OP (condition D) dissociated hsp90 from the MR, and no steroid binding can be consequently measured in these immunopellets unless the MR·hsp90 heterocomplex is reconstituted with rabbit reticulocyte lysate (conditions E and F). These results obtained by using a purified system confirm that the oxidopregnane is certainly capable to transform the MR.

The experiments shown in Figs. 2 (in vitro) and 4 (in vivo) indicate that the binding of 11-OP competes with ALDO for the MR, whereas the experiments described in Fig. 6 and Tables 3 and 4 demonstrate that the MR binding is not equivalent for both steroids. To confirm whether or not 11-OP is capable of competing for the ALDO binding site, we immunopurified renal MR from renal cytosol and performed Scatchard plots by saturation with [^{3}H]ALDO in the presence of increasing concentrations of 11-OP. The results are depicted in Fig. 7B and clearly demonstrate a dual behavior for 11-OP. At a concentration equal to 1 nM, which is incapable of competing with ALDO, 11-OP significantly increased the affinity of MR for ALDO by approximately three times (from 0.99 ± 0.10 nM to 0.28 ± 0.03 nM, $P < .005$). In contrast, higher concentrations of 11-OP, which had exhibited competitive binding properties for MR, showed a typical competitive inhibitory pattern on ALDO binding to MR. In effect, the MR apparent dissociation constant (K_{app}) for ALDO increases twice (1.95 ± 0.25 nM) and more than 3-fold (3.54 ± 0.42 nM) in the presence of 30 and 100 nM 11-OP, whereas the number of total binding sites remained constant. A Dixon plot shows a straight line ($r = 0.99$) if 1 nM 11-OP is omitted from the regression, and an apparent inhibitory constant equal to 34 nM was calculated for 11-OP. Importantly, such a K_i value is in the same order of magnitude as the RBA values measured previously for 11-OP in the competition curves.

These results confirm that the binding of 11-OP to the MR enhances the affinity of ALDO for the receptor and, consequently, may explain the potentiation effect observed when the mineralocorticoid biological response was measured. In turn, this experiment shows that high doses of 11-OP may exert mineralocorticoid effects per se.

Discussion

It is well known that the intrinsic sodium-retaining specificity of mineralocorticoids is not exclusively receptor-dependent but also depends on the competitive effects of sequestering proteins (Krozowski and Funder, 1983) and/or enzymes able to transform active hormones into inactive metabolites (Funder et al., 1988) or even more active derivatives (Galigniana et al., 1997). The experiments performed in this work clearly exclude the first possibility. Moreover, the putative metabolic effects of a key enzyme for the mineralocorticoid action such as 11β-hydroxysteroid dehydrogenase is unlikely because 11-OP engages the functional group in C_{11} in an oxido bridge with C_{19}.

To prove that 11-OP is able to transform the MR, we used an original approach to reconstitute the oligomeric structure of MR and detect changes in its hydrodynamic properties when unlabeled steroid is not available. The analysis of the sucrose gradient ultracentrifugation profile shows that the binding of 11-OP to renal MR shifts the 9.6 S untransformed form to the 5.2 S transformed form, which proves that 11-OP transforms MR as efficiently as ALDO. As shown in Fig. 5C, those properties also agree with the ability of MR to translocate into the nucleus on steroid binding.

The results obtained in this work provide evidence that many of the potential mechanisms by which 11-OP elicits mineralocorticoid effects are indistinguishable from those of ALDO. Although it is clearly demonstrated that the effects of

TABLE 5

Spironolactone inhibits the mineralocorticoid effect of 11-OP

ADX male rats were injected with the indicated doses (expressed per 100 g of body weight) of ALDO, 11-OP, or both steroids together. To inhibit the mineralocorticoid effect, 100 μg of SPO was also coinjected with the agonist. After 3 h, urine samples were collected by bladder puncture and both natriuresis and kaliuresis were measured. Results represent the mean ± S.E. (9 rats/group). The inhibitory effect observed for SPO is statistically different for all treatments at $P < .001$.

Steroid	+SPO (100 μg)	Urinary Electrolyte (Steroid/ADX Ratio)		
		Na^+	K^+	Na^+/K^+
1 μg of ALDO	No	0.195 ± 0.024	1.595 ± 0.061	0.132 ± 0.046
1 μg of ALDO	Yes	0.958 ± 0.088	1.014 ± 0.114	0.957 ± 0.094
100 μg of 11-OP	No	0.231 ± 0.017	1.449 ± 0.152	0.161 ± 0.089
100 μg of 11-OP	Yes	0.924 ± 0.091	1.081 ± 0.047	0.857 ± 0.060
1 μg of ALDO + 100 μg of 11-OP	No	0.178 ± 0.024	1.634 ± 0.551	0.110 ± 0.046
1 μg of ALDO + 100 μg of 11-OP	Yes	0.935 ± 0.132	1.154 ± 0.077	0.819 ± 0.110
0.06 μg of ALDO	No	0.568 ± 0.055	1.200 ± 0.209	0.479 ± 0.122
0.6 ng of 11-OP	No	0.944 ± 0.072	0.973 ± 0.098	1.040 ± 0.147
0.06 μg of ALDO + 0.6 ng of 11-OP	No	0.171 ± 0.075	1.560 ± 0.124	0.249 ± 0.150
0.06 μg of ALDO + 0.6 ng of 11-OP	Yes	0.860 ± 0.132	1.020 ± 0.091	0.870 ± 0.126

11-OP are MR-mediated, several evidences suggest that the binding of 11-OP to the MR may be attributed to a second (regulatory?) binding site on the MR, which may be different from the ALDO-binding pocket. One reasonable argument to explain the biopotency of 11-OP despite its relatively low MR affinity is that the extra ring present in 11-OP can interfere with its binding to plasma proteins. This speculation is supported by the experiments performed with hydroxylapatite-adsorbed MR (Fig. 2) and the lack of binding capacity to CBG (Table 2). However, this speculation does not explain either the significant potentiation effect exhibited by a dose as low as 0.6 ng of 11-OP or the unexpected results obtained in experiments performed in vitro. Furthermore, the effect of tautomycin on the nuclear translocation of 11-OP·MR (Table 3), the differential sensitivity of the 11-OP·MR complex to pyridoxal 5′-phosphate (Table 4), the kinetic experiments shown in Fig. 6, and the Scatchard plot performed in the presence of 1 nM 11-OP (Fig. 7B) provide support for the notion that the binding of 11-OP and ALDO to the MR is not identical. Similar kinetic studies as those depicted in Fig. 6 have been used previously as a tool to demonstrate the presence of a second binding site on the GR (Jones and Bell, 1980; Svec et al., 1989).

The sodium-retaining activity of 11-OP is inhibited by the antimineralocorticoid spironolactone, a steroid with proven binding capacity to MR. Similarly, kaliuresis is also inhibited. No potentiation of the ALDO effect was observed when a saturating dose of 1 μg of ALDO was assayed, which suggests that, if the activation pathway was already saturated by ALDO, no improvement of the biological response can be

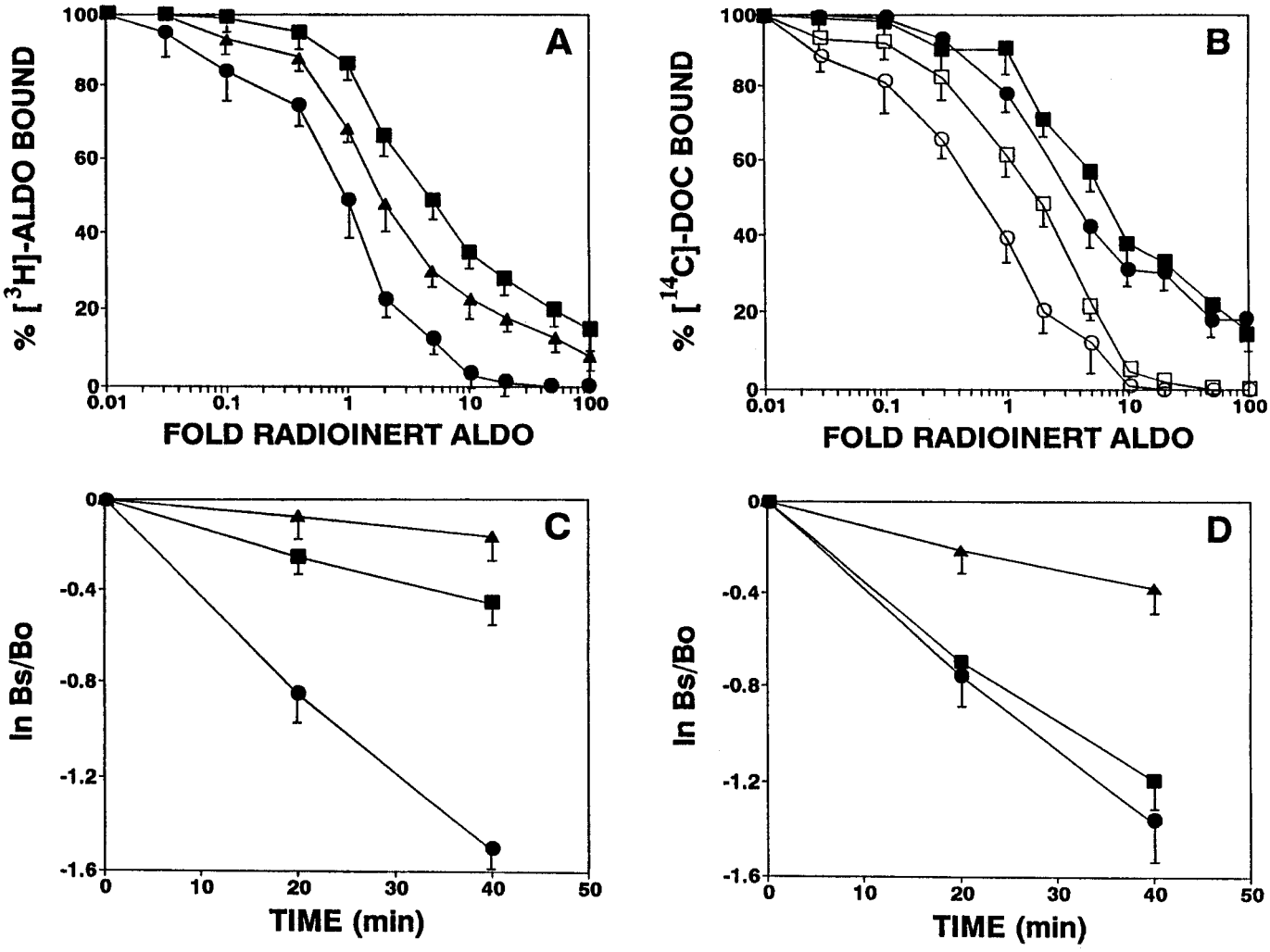

Fig. 6. The dissociation of the complex [^{3}H]ALDO·MR by competition with unlabeled ALDO is decelerated by the simultaneous presence of 11-OP. A, kidney cytosol in TEGI buffer was incubated for 12 h at 0°C with 5.0 nM [^{3}H]ALDO and increased amounts of radioinert ALDO, in the absence (●) or presence of a constant concentration of 11-OP (▲ = 5 nM 11-OP; ■ = 30 nM 11-OP). To compare different treatments, results were normalized as a percentage of the maximum binding, being the respective 100% absolute values for 0, 5, and 30 nM 11-OP: 10456 ± 674, 9902 ± 339, and 6808 ± 594 dpm. B, the same experiment as described for A, but the tracer was replaced by 5 nM [^{14}C]DOC and competed with ALDO (circles) or DOC (squares), either in the absence (open symbols, maximum binding = 11277 ± 332 dpm) or presence (solid symbols, maximum binding = 10143 ± 422 dpm) of an equimolar concentration of 11-OP. C, kidney cytosol in TEGI buffer was incubated with 5.0 nM [^{3}H]ALDO for 3 h at 0°C. Free steroid was removed with 1% charcoal, 0.1% dextran, and the supernatant was incubated at 20°C in the absence (Bo) or presence (Bs) of the indicated unlabeled steroid. Samples were taken at different time intervals (0, 20, and 40 min), and the bound steroid was measured and expressed as a Bs/Bo ratio. Incubations with unlabeled steroid are symbolized as: ▲, +5 nM 11-OP; ●, +0.5 μM ALDO; ■, +5 nM 11-OP + 0.5 μM ALDO. D, this panel depicts the same experiment as described for C, but competition of unlabeled steroid with tracer was performed as follows: ▲, +2.5 nM DOC; ●, +0.5 μM ALDO; ■, +2.5 nM DOC + 0.5 μM ALDO. All panels depict results as the mean ± S.E. of three independent experiments, each one performed in quadruplicate.

reached in the presence of 11-OP. Therefore, both steroids may share the MR activation pathway to trigger such a response. These data argue against the possibility that nongenomic effects may be relevant for the mechanism of action of 11-OP. Despite the fact that kaliuretic and natriuretic components of the physiological response to ALDO in kidney are separable, 11-OP also exhibits similar properties to ALDO and DOC. In addition, the effect of 0.06 μg of ALDO, a dose able to generate 50% of the maximum biological effect, is amplified to a maximum sodium retention level by a dose as low as 0.6 ng of 11-OP, which is, in turn, ineffective in generating per se a detectable mineralocorticoid effect. This important finding also argues in favor of the notion that 11-OP may exert its biological action by binding to a regulatory site on the MR. It is possible that such a site may function as a stabilizer of the activated form of the receptor. The low concentrations of 11-OP required to observe both an effect on ALDO binding (nanomolar) and on the mineralocorticoid action (nanogram), as well as the fact that its bent isomer 6-OP is devoid of activity, makes an artifactual observation unlikely. Moreover, our interpretation of the kinetic experiments agrees with the hypothesis that the off-rate of the steroid from the MR may be critical in determining the ligand potency (Galigniana et al., 1994; Souque et al., 1996).

We have recently reported (Piwien-Pilipuk and Galigniana, 1998) that tautomycin inhibits the transforming capacity of endogenous phosphatases on renal MR. This effect of tautomycin correlates with the inhibition of the nuclear translocation of the ALDO·MR complex. Similar findings were also reported for the GR, and a potential role for the hsp90 heterocomplex in the receptor translocation has been postulated (Czar et al., 1997; Galigniana et al., 1998, 1999). Importantly, the rat renal MR undergoes phosphorylation in a physiological milieu (Galigniana, 1998), a process which is more evident if renal tissue is treated with okadaic acid, another serine/threonine protein phosphatase inhibitor that is chemically and functionally related to tautomycin. Interestingly, tautomycin does not inhibit the translocation of the 11-OP·MR complex into the nucleus, whereas the ALDO·MR complex is retained in the cytoplasm in the presence of this protein phosphatase inhibitor. One possible explanation for this experimental evidence may be found in the possible induction of different conformations of the steroid-receptor complex, which in turn may affect the capability of tautomycin to disrupt the MR-signaling pathway, i.e., by inhibition of a protein phosphatase activity required for receptor trafficking and/or translocation. Beyond these recent intriguing findings about the molecular mechanism of action of both corticosteroid receptors, the evidence shown in the functional study performed with tautomycin in Table 3 also argues in favor of the hypothesis that a differential interaction between 11-OP and ALDO may certainly take place with the MR.

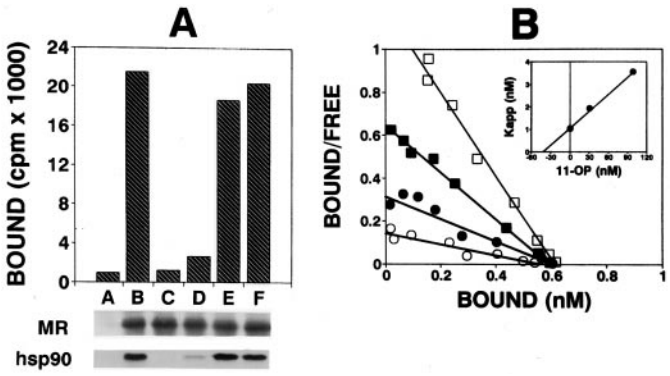

Fig. 7. Concentration-dependent competing effect of 11-OP on [^{3}H]ALDO binding to immunopurified renal MR. A, kidney slices were incubated for 30 min at 37°C with vehicle (conditions A and B), 1 μM ALDO (conditions C and E), or 1 μM 11-OP (conditions D and F). The MR was immunoadsorbed with rabbit pAbhMR antibody anti-MR (conditions B to F) or rabbit nonimmune serum (condition A). After the immunoadsorption, immunopellets E and F were incubated with rabbit reticulocyte lysate and ATP-generating system to reconstitute the MR·hsp90 complex. The steroid binding capacity of immunopellets was measured by incubation with [^{3}H]ALDO (bar graph), whereas the MR transformation was analyzed by Western blots for MR and hsp90 (lower panel). B, the MR was immunoprecipitated from kidney cytosol obtained in TEGI buffer, and Scatchard plots were performed by using increasing concentrations of [^{3}H]ALDO in the absence (■) or presence of 11-OP (□, 1 nM; ●, 30 nM; ○, 100 nM). The insert depicts a Dixon plot as a function of the 11-OP concentration (0, 30, and 100 nM).

Our studies do not provide direct evidence about the localization of the putative second binding site on MR. However, because Scatchard plots performed with [^{3}H]ALDO exhibit a single slope (if the GR is excluded from competition), we envision the possibility that this "11-OP site" fails to recognize ALDO as a ligand. In contrast, 11-OP shows binding properties to compete with ALDO for the primary binding site (Fig. 2), but the insertion of 11-OP in the ALDO-binding site differs from the natural steroid as evidenced by the lack of capacity to protect lysine groups (Table 4). Nevertheless, the experiments shown in Fig. 7B demonstrate that 11-OP does compete with ALDO at higher concentrations. Perhaps the capability of 11-OP to compete with ALDO is thermodynamically favored at high concentrations, and 11-OP can occupy a steroid binding pocket that is permissive for flat molecules (Burton et al., 1995). As a consequence, 11-OP behaves like the endogenous agonist. However, 11-OP not only fails to exhibit competitive properties with ALDO at low concentrations but also stabilizes the binding of the natural agonist as evidenced in the experiments shown in Figs. 6 and 7. Such a dual behavior is difficult to explain in view of the experimental evidence presented in this work. However, we can certainly affirm that 11-OP does exhibit both biological and biochemical mineralocorticoid properties, which may be assigned to a differential ligand positioning in the receptor protein and/or to a differential induction of receptor conformation. A rigid lock model between steroid and protein would certainly be sufficient to account for the differential binding of ligands, as stated by the classical theory, which considers that the receptor switches from an inactive to active form on ligand binding. However, there is no reason why 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 more subtle repositioning, taking into account several factors in addition to the functional groups, e.g., length of the molecule, conformational rigidity, and overall flatness (Burton et al., 1995; Teutsch et al., 1995). In this sense, note that 11-OP lacks a C_{21}-hydroxyl, a group that was always thought to be essential for the biological activity.

Therefore, an "all or nothing" event is unlikely in view that the ligand binding is an adaptive process in which the overall structure of the steroid-receptor complex can be influenced by the nature of the ligand. Consistent with this speculation, the analysis of the mechanism of action of sex steroids has

provided insights into how the cell recognizes and responds to ligand-activated receptor. Thus, agonists and antagonists appear to interact with distinct, although overlapping regions within the human PROG receptor, resulting in the conformation of structurally different complexes (Benhamou et al., 1992; Vegeto et al., 1992). Similarly, estrogen ligands were classified into four classes according to the agonist activity, which, in turn, reflects differential alterations in the estrogen receptor structure (McDonnell et al., 1995).

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References

Alnemri ES, Maksymowych AB, Robertson NM and Litwack G (1991) Overexpression and characterization of the human mineralocorticoid receptor. *J Biol Chem* **266:**3925–3936.

Benhamou B, Garcia T, Lerouge T, Vergezac A, Gofflo D, Bigogne C, Chambon P and Gronemeyer H (1992) A single amino acid that determines the sensitivity of progesterone receptors. *Science* **255:**206–209.

Binart N, Lombes M, Rafestin-Oblin ME and Baulieu EE (1991) Characterization of human mineralocorticoid receptor expressed in the baculovirus system. *Proc Natl Acad Sci USA* **88:**10681–10685.

Brachet-Cota AL and Burton G (1990) An improved preparation of 11,19-oxidopregn-4-ene-3,20-dione and 6,19-oxidopregn-4-ene-3,11,20-trione. *Z Naturforsch Teil [B]* **45:**711–715.

Burton G, Galigniana MD, de Lavallaz S, Brachet-Cota AL, Sproviero EM, Ghini AA, Lantos CP and Damasco MC (1995) Sodium-retaining activity of some natural and synthetic 21-deoxysteroids. *Mol Pharmacol* **47:**535–543.

Colby H and Kitay J (1972) Sex and substrate effects on hepatic corticosteroid metabolism in rat. *Endocrinology* **90:**473–478.

Czar MJ, Galigniana MD, Silverstein AM and Pratt WB (1997) Geldanamycin, a heat shock protein 90-binding benzoquinone ansamycin, inhibits steroid-dependent translocation of the glucocorticoid receptor from cytoplasm to the nucleus. *Biochemistry* **36:**7776–7785.

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

Funder JW, Pearce P, Smith R and Smith AI (1988) Mineralocorticoid action: Target tissue specificity is enzyme, not receptor, mediated. *Science (Wash DC)* **242:**583–585.

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

Galigniana MD (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 MD, Housley PR, DeFranco DB and Pratt WB (1999) Inhibition of glucocorticoid receptor nucleocytoplasmic shuttling by okadaic acid requires intact cytoskeleton. *J Biol Chem* **274:**16222–16227.

Galigniana MD 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 MD, Vicent GP, Lantos CP and Burton G (1994) 11,19-Oxidoprogesterone: A potent sodium retaining steroid binding to an alternative site in kidney type I receptor. *Eur J Endocrinol* **130 (Suppl 2):**260.

Galigniana MD, Vicent GP, Burton G and Lantos CP (1997) Features of the shuttle pair 11β-hydroxyprogesterone/11-ketoprogesterone. *Steroids* **62:**358–364.

Galigniana MD, Scruggs JL, Herrington J, Welsh MJ, Carter-Su C, Housley PR and Pratt WB (1998) Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton. *Mol Endocrinol* **12:**1903–1913.

Jones TR and Bell PA (1980) Glucocorticoid-receptor interactions: Studies of the negative cooperativity induced by steroid interactions with a secondary, hydrophobic, binding site. *Biochem J* **188:**237–245.

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D and Chambon P (1995) Activation of estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science (Wash DC)* **270:**1491–1494.

Krozowski ZS and Funder JW (1983) Renal mineralocorticoid receptors and hippocampal corticosterone binding species have identical intrinsic steroid specificity. *Proc Natl Acad Sci USA* **80:**6056–6060.

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

Lilley KS and Engel PC (1992) The essential active-site lysines of clostridial glutamate dehydrogenase: A study with pyridoxal-5′-phosphate. *Eur J Biochem* **207:**533–540.

McDonnell D, Clemm D, Herman T, Goldman ME and Pike JW (1995) Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. *Mol Endocrinol* **9:**659–669.

Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E and Auricchio F (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J* **15:**1292–1300.

Morris D (1981) The metabolism and mechanism of action of aldosterone. *Endocr Rev* **2:**234–247.

Morris DJ, Tsai R and De Conti G (1976) Regulation of plasma levels of aldosterone and its metabolites during the latent period of aldosterone. *J Steroid Biochem* **7:**971–978.

Nemere I, Zhou L and Norman A (1993) Nontranscriptional effects of steroid hormones. *Receptor* **3:**277–291.

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

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

Pratt WB and Toft DO (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev* **18:**306–360.

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

Scherrer LC, Dalman FC, Massa E, Meshinchi S and Pratt WB (1990) Structural and functional reconstitution of the glucocorticoid receptor-hsp90 heterocomplex. *J Biol Chem* **267:**21397–21400.

Souque A, Fagart J, Couette B and Rafestin-Oblin ME (1996) Sulfhydryl groups are involved in the binding of agonists and antagonists to the human mineralocorticoid receptor. *J Steroid Biochem Mol Biol* **57:**315–321.

Svec F, Teubner V and Tate D (1989) Location of the second steroid-binding site on the glucocorticoid receptor. *Endocrinology* **125:**3103–3108.

Teutsch G, Nique F, Lemoine G, Bouchoux F, Cérède, Gofflo D and Philibert D (1995) General structure-activity correlations of antihormones. *Ann N Y Acad Sci* **761:**5–28.

Vegeto E, Allan GF, Schrader WT, Tsai MJ, McDonnell D and O'Malley BW (1992) The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. *Cell* **69:**703–713.

Vicent GP, Burton G, Ghini A, Lantos CP and Galigniana MD (1998) Influence of calf serum on glucocorticoid-responses of certain progesterone derivatives. *J Steroid Biochem Mol Biol* **66:**211–217.

Vicent GP, Monteserín MC, Veleiro AS, Burton G, Lantos CP and Galigniana MD (1997) 21-Hydroxy-6,19-oxidoprogesterone: A novel synthetic steroid with specific antiglucocorticoid properties in the rat. *Mol Pharmacol* **52:**749–753.

Vicent GP, Pecci A, Ghini A, Piwien-Pilipuk G, Burton G, Lantos CP and Galigniana MD (1999) The glucocorticoid properties of the synthetic steroid pregna-1,4-diene-11β-ol-3,20-dione (ΔHOP) are not entirely correlated with the steroid binding to the glucocorticoid receptor. *Mol Cell Endocrinol* **149:**207–219.

Waynforth HB (1980) *Experimental and Surgical Technique in the Rat*. Academic Press, London.

Williams SP and Sigler PB (1998) Atomic structure of progesterone complexed with its receptor. *Nature (Lond)* **393:**392–396.

Yamakawa Y, 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.

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