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The Synthetic Androgen Methyltrienolone (R1881) Acts as a Potent Antagonist of the Mineralocorticoid Receptor

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ABSTRACT

Aldosterone binds to the mineralocorticoid receptor (MR) and exerts fine control over Na^+ absorption in renal collecting duct cells (CCDs). Many natural and synthetic steroids can also bind to the MR to produce agonist or antagonist effects. Here, we investigate whether androgenic hormones act as MR agonist or antagonist ligands in CCDs. Testosterone (T), dihydrotestosterone (DHT), and methyltrienolone (R1881), a synthetic androgen agonist, all bind to the MR. R1881 displayed the same affinity for MR as aldosterone. Androgens did not activate the MR transiently expressed in human embryonic kidney 293T cells but did antagonize aldosterone-induced MR *trans*-activation activity (R1881>DHT>T). Short-circuit current (I_{sc}) experiments, used to measure transepithelial Na^+ transport, revealed that 10^{-5} M T and DHT or R1881 prevented the increase in the amiloride-sensitive component of I_{sc} caused by aldosterone in mouse mpkCCD$_{cl4}$ collecting duct cells partially and totally, respectively. In contrast, androgens had no effect on stimulated I_{sc} elicited by the specific glucocorticoid agonist 11β,17β-dihydroxy-17α-(1-propynyl)androst-1,4,6-trien-3-one (RU26988). Docking of steroids within the crystal structure of the ligand-binding domain of MR, together with *trans*-activation studies, revealed that the contacts between the 17β-hydroxyl group of androgens and the Asn770, Cys942, and Thr945 residues of the ligand-binding cavity stabilize ligand binding complexes but are not strong enough to keep the receptor in its active state. Altogether, these findings indicate that androgen ligands, particularly R1881, act as MR antagonists in aldosterone target cells and provide new insights into the requirements for MR activation to occur and for the designing of new selective MR antagonists.

Aldosterone, the major mineralocorticoid hormone, is involved in the control of water and sodium homeostasis and regulation of blood pressure. The effects of aldosterone are mediated by the mineralocorticoid receptor (MR), a transcription factor present in sodium-transporting epithelia, such as those of the distal renal tubules, distal colon, and sweat and salivary glands (Pearce et al., 2003). In the kidney, the fine regulation of Na^+ reabsorption takes place in the distal nephron. In the principal cortical collecting duct (CCD) cells, Na^+ is reabsorbed via the apical ENaC, which constitutes the rate-limiting step for Na^+ entry, and is extruded from the cells by the basolaterally located Na^+,K^+-ATPase (Rossier and Palmer, 1992).

The human MR is a member of the superfamily of nuclear receptors that is organized into three functional domains (Arriza et al., 1987). The N-terminal domain harbors a ligand-independent activation function. This domain is important for interactions with transcriptional coactivators (Kitagawa et al., 2002; Pascual-Le Tallec et al., 2003) and with the ligand binding domain (LBD) (Rogerson and Fuller, 2003). The centrally located DNA binding domain is involved in DNA binding and in receptor homo- and heterodimerization.

A.-N.T., G.M.P., M.-E.R.-O., and A.V. made equal contributions to the study.

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ABBREVIATIONS: MR, mineralocorticoid receptor; CCD, cortical collecting duct; Aldo, aldosterone; I_{sc}, short-circuit current; Ams I_{sc}, amiloride-sensitive short-circuit current; AR, androgen receptor; DHT, dihydrotestosterone; T, testosterone; DM, defined medium; DMEM, Dulbecco's minimal essential medium; ENaC, epithelial sodium channel; FCS, fetal calf serum; GR, glucocorticoid receptor; HF, hydroxyflutamide; HFM, hormone-free defined medium; LBD, ligand binding domain; MMTV, mouse mammary tumor virus; 18OVP, 18-oxo-18-vinylprogesterone; 19-nor T, 19-nor-testosterone; 17-met T, 17α-methyl testosterone; hsp90, heat shock protein 90; HEK, human embryonic kidney; Spiro, spironolactone; R1881, 17β-hydroxy-17α-methyl-19-norandrost-4,9,11-trien-3-one; RU486, 11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(1-propynyl)-19-norandrost-4,9-dien-3-one; RU26752, 7α-(propyl)-17α-hydroxy-3-oxopregn-4-en-21-carboxylic acid γ-lactone; RU26988, 11β,17β-dihydroxy-17α-(1-propynyl)androst-1,4,6-trien-3-one.

The LBD is located in the C-terminal region and contributes to nuclear localization. The LBD is involved in homo- and/or heterodimerization and in the interaction with heat-shock protein 90 (hsp90). It is also characterized by a ligand-induced *trans*-activation function (Arriza et al., 1988; Mangelsdorf et al., 1995; Ribeiro et al., 1995). The crystal structures of the LBD of human MRs carrying the S810L mutation (Fagart et al., 2005) and/or the C808S mutation (Bledsoe et al., 2005; Li et al., 2005) and forming a complex with an agonist ligand have been solved. Consistent with sequence homologies between MR and the glucocorticoid receptor (GR), the progesterone receptor, and the androgen receptor (AR), the structure of the MR closely resembles the agonist-bound structures of these oxosteroid receptors (Williams and Sigler, 1998; Matias et al., 2000; Bledsoe et al., 2002). The structure is composed of 11 α-helices and 4 small β-strands that are folded to form a three-layered structure surrounding the ligand cavity.

The aldosterone-dependent activation of gene transcription is believed to be a multistep process. In its ligand-free state, MR is found predominantly in the cell cytoplasm as a hetero-oligomeric complex in association with the heat shock protein hsp90 (Rafestin-Oblin et al., 1989). The MR-hsp90 interaction seems to be required to maintain the receptor in a nonfunctional state and to fold the MR-LBD in a ligand binding-competent state (Couette et al., 1998). Aldosterone binding induces a change in receptor conformation that allows the dissociation of the associated proteins, transfer into the nucleus, and the subsequent recruitment of transcriptional coactivators (Trapp and Holsboer, 1995; Couette et al., 1996; Fejes-Toth et al., 1998; Hellal-Levy et al., 2000; Hultman et al., 2005). The aldosterone-associated receptor binds as a dimer to hormone response elements in the promoter region of target genes and initiates hormone-mediated transcription by specific interactions with the transcriptional machinery. Site-directed mutagenesis experiments based on three-dimensional homology models have shown that the contact between the Asn770 residue of the MR-LBD and the 21-hydroxyl function, present in all natural steroids with agonist properties, plays a key role in maintaining MR in its active conformation (Fagart et al., 1998).

Many natural and synthetic steroids bind to MR and, depending on the nature of their substituents, may exhibit MR-antagonist properties. This is the case, for instance, of progesterone, the antagonist activity of which has been linked to its inability to establish contact with the Asn770 residue of MR-LBD (Fagart et al., 1998). Two progesterone derivatives, 17α- and 20α-hydroxyprogesterone (but not 11β-hydroxyprogesterone) act as potent MR antagonists and inhibit the aldosterone-stimulated absorption of Na^+ in cultured mouse mpkCCD$_{cl4}$ collecting duct principal cells (Rafestin-Oblin et al., 2002). Rossier et al. (1980) first reported that testosterone (T) specifically displaces [^{3}H]aldosterone from its nuclear and cytoplasmic binding sites and antagonizes the stimulating action of aldosterone on Na^+ transport in the toad bladder. However, the ability of natural and synthetic androgens to activate or inactivate MR and to stimulate or inhibit Na^+ absorption has not been investigated yet in appropriate models of mammalian collecting duct cells.

In this study, the ability of the two main natural androgens, T and its active metabolite dihydrotestosterone (DHT), and the potent synthetic steroidal AR agonist methyltrienolone (R1881) to modulate Na^+ absorption was analyzed using the short-circuit current (I_{sc}) method in cultured mpkCCD$_{cl4}$ collecting duct cells, which had retained the main features of intact CCDs, including a Na^+ transport mechanism that was stimulated by aldosterone (Bens et al., 1999; Rafestin-Oblin et al., 2002). The ability of T, DHT, and R1881 to activate or inactivate MR was investigated in *cis-trans* cotransfection assays in HEK 293T and COS-7 cells. The antagonist properties of androgens are discussed in the light of docking experiments performed using the structure of the MR-LBD (Bledsoe et al., 2005).

Materials and Methods

Chemicals. Aldosterone (4-pregnen-11β,21-diol-18-al-3,20-dione; Aldo), testosterone (17β-hydroxy-4-androsten-3-one; T), 19-nortestosterone (17β-hydroxy-19-norandrost-4-en-3-one; 19-nor T), 17α-methyl testosterone (17β-hydroxy-17α-methyl-4-androsten-3-one; 17-met T), and dihydrotestosterone (5α-androstan-17β-ol-3-one; DHT) were from Sigma (St. Louis, MO). 18-Oxo-18-vinyl progesterone (18-vinyl-4-pregnen-3,18,20-trione; 18OVP) was a gift from A. Marquet (Paris, France). Spironolactone [7α-(acetylthio)-17β-hydroxy-3-oxopregn-4-en-21-carboxylic acid γ-lactone; Spiro] was a gift from Pharmacia/Pfizer Laboratories (Chicago, IL). R1881 was purchased from PerkinElmer (Courtaboeuf, France). Mifepristone (RU486), RU26752, and RU26988 were kindly provided by Sanofi-Aventis (Paris, France). Hydroxyflutamide [2-hydroxy-2-methyl-n-(4-nitro-3-(trifluoromethyl) phenyl)propanamide; HF] was from Merck, Theramex (Monaco, France). [1,2-^{3}H]Aldosterone (40–60 Ci/mmol) was purchased from GE Healthcare (Orsay, France). The ligands used in this study are depicted in Fig. 1. All other hormones and reagents were from Sigma.

Expression Vectors and Reporter Constructs. The expression plasmids pchMR, pchMR$_{Ala770}$, and pchMR$_{Ala945}$ contain the entire coding sequence for the wild-type human MR and of mutant MRs in which alanine is substituted for asparagine at position 770 (MR$_{Ala770}$) or for threonine at position 945 (MR$_{Ala945}$) (Fagart et al., 1998). The C942A mutation was created on the recombinant pchMR using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotide sense primer, 5′-CTGCTGGAATTCGCCTTCTAC-3′, was used together with the corresponding antisense oligonucleotide. The desired mutation was identified by direct sequencing (MWG Biotech, Roissy CDG, France). After identifying the mutated clones, MR fragments were excised with BpU1102i/AflII and subcloned into a new pchMR vector. The expression plasmid pchGR contains the entire coding sequence for the wild-type human GR (Hellal-Levy et al., 1999). The plasmid pFC31Luc, which contains the mouse mammary tumor virus (MMTV) promoter driving the luciferase gene, was kindly provided by H. Richard-Foy (LBME, Toulouse, France). The plasmid pcβgal encodes β-galactosidase.

Coupled Cell-Free Transcription and Translation. The plasmid pchMR (1 μg), containing cDNA encoding the wild-type MR, was transcribed for 90 min at 30°C using T7 RNA polymerase and translated into the rabbit reticulocyte lysate system purchased from Promega according to the manufacturer's instructions (Promega, Charbonnières, France).

Steroid Binding Experiments. After translation of the wild-type MR, the lysate was diluted (1:3) with ice-cold buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 20 mM sodium tungstate, 1 mM β-mercaptoethanol, and 10% glycerol). The lysate was then incubated for 4 h at 4°C with 1 nM [^{3}H]aldosterone with or without various concentrations of unlabeled competitors (10^{-11} to 10^{-5} M). Bound and unbound steroids were separated by the charcoal-dextran method (Fagart et al., 1998). The radioactivity was determined in an LKB liquid scintillation spectrometer (LKB Instruments, Mt. Waverley, Victoria, Australia) after adding 5 ml of Optiphase Hisafe

(PerkinElmer Wallac, Turku, Finland). The apparent dissociation constant (K_{Dapp}) was calculated according to the equation $K_{DappX} = K_{Daldo} \times [x]/[Aldo]$, where K_{Daldo} is the dissociation constant at equilibrium of aldosterone measured by Scatchard plot analysis (Fagart et al., 1998), and [x] and [Aldo] are the concentrations of a compound x and of aldosterone, respectively, required to inhibit 50% of the [^{3}H]aldosterone binding.

Cultured Cells. Experiments were performed using human embryonic renal HEK 293T cells or COS-7 cells (American Type Culture Collection, Manassas, VA), and the mouse renal mpkCCD$_{cl4}$ collecting duct principal cells established in our laboratory previously (Bens et al., 1999). HEK 293T cells and COS-7 cells were cultured in T75 flasks with high-glucose Dulbecco's modified essential medium (DMEM; Invitrogen NV, Leek, The Netherlands) supplemented with 2× nonessential amino acids, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, and 10% charcoal-stripped and heat-inactivated fetal calf serum (FCS) in a 5% CO_2/95% air atmosphere. mpkCCD$_{cl4}$ cells were grown until confluent in a modified defined medium [DM; DMEM/Ham's F-12 medium [1:1 (v/v)], 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 2% FCS, and 20 mM HEPES, pH 7.4] at 37°C in a 5% CO_2/95% air atmosphere. The medium was changed every 2 days, and all experiments were carried out between the 25th and 36th passages.

Transfection Procedures. HEK 293T cells were cultured and transfected in high-glucose-containing DMEM supplemented with 10% charcoal-stripped and heat-inactivated FCS, 25 mM HEPES, 2× nonessential amino acids, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2/95% air atmosphere. Transfection was carried out using the phosphate calcium precipitation method. For one 75-cm^2 flask, the phosphate solution contained 2 μg of one of the receptor expression vectors (pchMR, pchMR$_{Ala770}$, pchMR$_{Ala942}$, pchMR$_{Ala945}$, or pchGR), 7 μg of pFC31Luc construct, and 1 μg of pcβgal. Twelve hours after transfection, the cells were rinsed with phosphate-buffered saline, trypsinized, and replated in 12-well trays. The steroids to be tested were added to the cells 24 h after seeding, and incubation of the steroids with the transfected cells was performed for 24 h at 37°C. Cell extracts were then assayed for luciferase (De Wet et al., 1987) and β-galactosidase (Herbomel et al., 1984). To standardize the transfection efficiency, the relative light units obtained in the luciferase assay were divided by the optical density obtained in the β-galactosidase assay. The transfection protocol used for COS-7 cells was similar to that for HEK 293T cells but used 4 μg of receptor expression vector, 14 μg of pFC31Luc construct, and 2 μg of pcβgal.

Short-Circuit Current Studies. mpkCCD$_{cl4}$ cells were seeded on Snapwell filters (insert growth area, 1 cm^2; 0.4 μm pore size; Corning Costar Corporation, Cambridge, MA) and grown until confluent in the same modified DM as described above. After 5 days, confluent cells were placed in hormone-free, epidermal growth factor-free DM, referred to as HFM, and supplemented with charcoal-treated steroid-free FCS for 24 h and then in FCS-free HFM (containing 29 mM $NaHCO_3$) for a further 18 h (Bens et al., 1999; Rafestin-Oblin et al., 2002). Cells were mounted in a modified Ussing-type chamber (Diffusion Chamber System, Costar Corporation) connected to a voltage-clamp apparatus via glass-barrel Micro-Reference Ag/AgCl electrodes. Experiments were always performed using sets of steroid-treated and untreated cells from the same passages to avoid interpassage variations. Cell layers were bathed on both sides (8 ml) with HFM warmed to 37°C and continuously gassed with 95% O_2/5% CO_2 with the pH kept at 7.4 The various steroids and compounds to be tested were added to both the apical and basal sides of the filters. I_{sc} (microamperes per square centimeter) was measured by clamping the open-circuit transepithelial voltage (P_D) to 0 mV for 1 s. By convention, a positive I_{sc} value corresponded to a flow of positive charges from the apical to the basal compartment.

Ligand Docking within the Human MR Ligand-Binding Domain. Aldosterone, DHT, and R1881 were docked within the crystal structure of the MR-LBD associated with aldosterone (Bledsoe et al., 2005; Protein Data Base identification number 2AA2) using the O package (Jones et al., 1991). Androgen ligands were manually positioned within the binding pocket in an orientation similar to that of aldosterone. The steroids positioning was refined using the VOIDOO-generated probe-accessible software and van der Waals volumes as guides (Kleywegt and Jones, 1994).

Statistical Analysis. Results are expressed as means ± S.E.M. from n separate experiments. Significant differences between groups were analyzed by Student's t test. A p value <0.05 was considered significant.

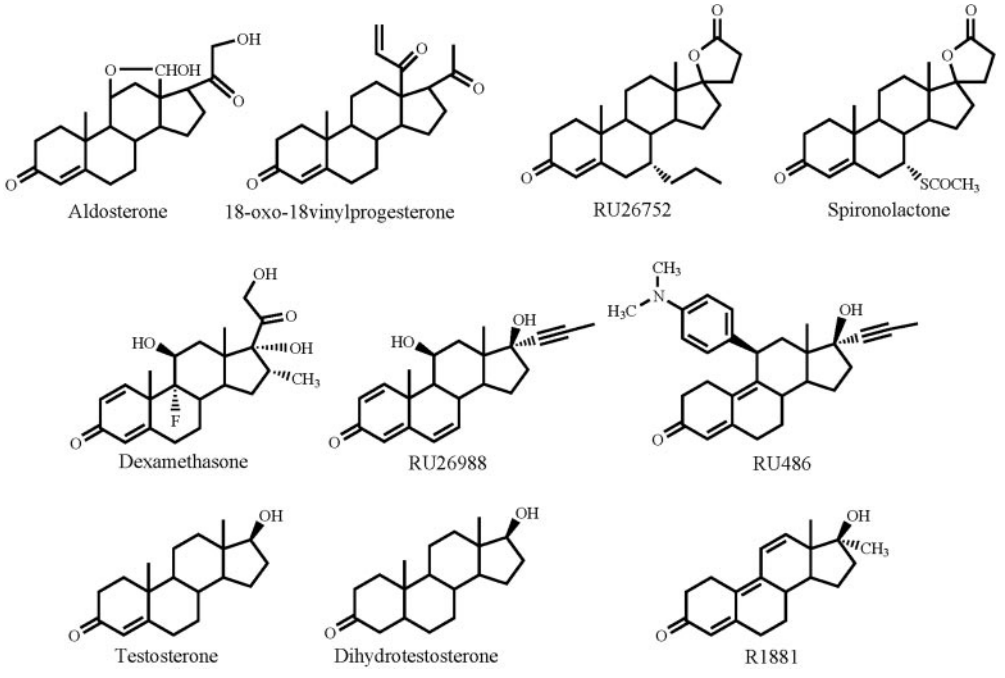

Fig. 1. Structural formulae of the natural and synthetic steroids.

Results

Androgen Ligands Inhibit Aldosterone-Stimulated Na^+ Absorption. The effects of androgen ligands on Na^+ transport were investigated in $mpkCCD_{cl4}$ cells using the short-circuit current (I_{sc}) method. Confluent cells grown on filters maintained the typical features of a tight epithelium as assessed by high electrical transepithelial resistance (R_T, 3033 ± 246 Ω · cm^2, n = 17) and negative transepithelial potential (P_D, −65.6 ± 4.7 mV, n = 17). I_{sc} recordings were performed on confluent $mpkCCD_{cl4}$ cells grown on filters and incubated for 3 h without or with steroids added to both the apical and basal side of the filters. Amiloride (10^{-5} M), a potent inhibitor of ENaC, was then added to the apical side of the cells to determine the amiloride-sensitive (Ams) component of I_{sc}, which reflects the Na^+ absorption mediated by ENaC (Bens et al., 1999). As reported previously, 5×10^{-7} M aldosterone, a concentration shown to induce maximal Na^+ absorption in $mpkCCD_{cl4}$ cells (Bens et al., 1999), significantly increased the total and Ams component of I_{sc} by almost 2.4-fold (Fig. 2A and inset). T (5×10^{-7} M) did not have any effect on the total and Ams components of I_{sc} measured in untreated cells (Fig. 2B and inset). Likewise, DHT and R1881 (5×10^{-7} M) did not alter total or Ams I_{sc} (data not shown). Compared with basal I_{sc} values measured in untreated cells, an excess of spironolactone (10^{-5} M), a classic antagonist of MR, inhibited the increase in total I_{sc} (72%) and Ams I_{sc} (65%) caused by aldosterone (Fig. 2C and inset). An excess of hydroxyflutamide (10^{-5} M), a nonsteroidal AR antagonist (Singh et al., 2000), had no effect on the increase in total and Ams components of I_{sc} stimulated by aldosterone (Fig. 2D and inset). In contrast, T and DHT partially inhibited the increase in total and Ams I_{sc} induced by aldosterone by 56 and 51%, respectively (Fig. 2, E and F, and insets), and R1881 almost completely inhibited both total and Ams component of I_{sc} stimulated by aldosterone (Fig. 2G and inset). These results suggest that androgens act as MR antagonists in inhibiting the increase in Na^+ transport caused by aldosterone in cultured $mpkCCD_{cl4}$ collecting duct principal cells.

Androgen Ligands Do Not Alter Na^+ Absorption Stimulated by the Selective GR Agonist RU26988. Aldosterone is known to act on both MR and GR to stimulate sodium absorption. We therefore recorded I_{sc} to find out whether the inhibitory action of androgen ligands on aldosterone-stimulated Na^+ absorption is mediated via MR and/or GR. We used the synthetic steroid RU26988, a selective GR agonist (Moguilewsky and Raynaud, 1980; Gomez-Sanchez and Gomez-Sanchez, 1983; Rafestin-Oblin et al., 1986). RU26988 (5×10^{-7} M) significantly increased the total I_{sc} by a factor of 2 in confluent $mpkCCD_{cl4}$ cells grown on filters, and RU486, a potent GR antagonist, almost completely inhibited this effect (Fig. 3A). As a result, RU486 significantly inhibited the Ams I_{sc} caused by RU26988 (Fig. 3A, inset). In contrast, 10^{-5} M DHT or R1881 did not impair the increase in total or Ams I_{sc} elicited by RU26988 in $mpkCCD_{cl4}$ cells (Fig. 3, B and C, and insets). T (10^{-5} M) also had no inhibitory effect on the RU26988-stimulated I_{sc} (data not shown). These findings indicate that androgen ligands do not inhibit the Na^+ absorption stimulated by the GR agonist RU26988 in cultured $mpkCCD_{cl4}$ collecting duct cells.

Androgens Do Not Activate GR, and Do Not Inhibit the GR Activity Induced by the Specific GR Ligand RU26988. To confirm that the inhibitory effect of T, DHT, and R1881 on aldosterone-stimulated Na^+ transport does not occur via GR, *trans*-activation experiments were performed on HEK 293T cells transiently transfected with GR. RU26988 produced a dose-dependent stimulation (ED_{50}, 10^{-9} M) of the GR *trans*-activation activity, as did dexamethasone (Fig. 4A). High concentrations of T, DHT, and R1881 did not *trans*-activate GR, although R1881 did induce a slight stimulation of the GR *trans*-activation at 10^{-5} M (Fig. 4A).

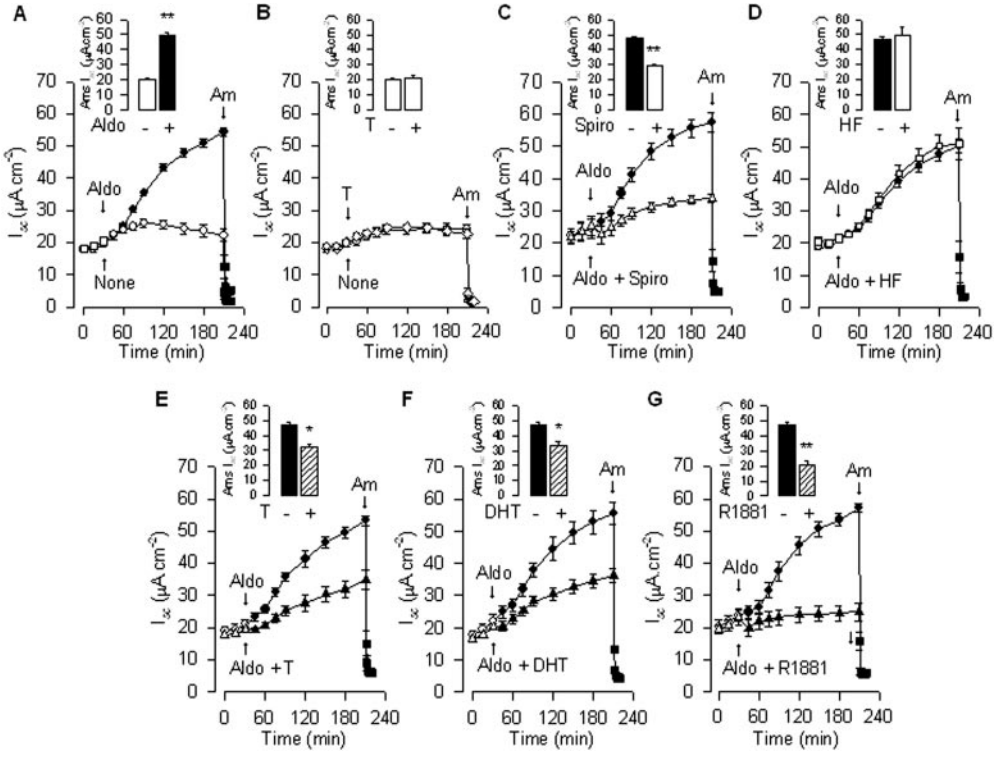

Fig. 2. Effects of androgens on Na^+ absorption stimulated by aldosterone. A, I_{sc} was measured on sets of confluent $mpkCCD_{cl4}$ cells grown on filters and incubated for 48 h in steroid-free and hormone-free medium as described under *Materials and Methods*. A–G, after the traces has stabilized (open symbols), cells were incubated for 3 h without (○) or with 5×10^{-7} M aldosterone (●), testosterone (◇), or with aldosterone 5×10^{-7} M plus 10^{-5} M Spiro (△), 10^{-5} M HF (□), Spiro (△), T, DHT, or R1881 (▲). All steroids were added to both the apical and basal side of the cells for 3 h, and 10^{-5} M amiloride (Am, ■) was added for 10 min to the apical side of the cells. A–G, insets, the bars represent the amiloride-sensitive component of I_{sc} (Ams I_{sc}) measured in cells incubated for 3 h without (−, □) or with aldosterone alone (−, ■) or T alone (+, □) and with aldosterone plus T, Spiro, HF, T, DHT, or R1881 (+, ▨). Values are means ± S.E.M. from five to six individual recordings for each steroid tested. *, $p < 0.05$; **, $p < 0.01$ between groups.

The GR antagonist activities of androgens were then tested by incubating the transfected HEK 293T cells with 10^{-8} M RU26988 alone (100%) or with increasing concentrations of T, DHT, R1881, or RU486 (Fig. 4B). RU486 induced a dose-dependent inhibition of the RU26988-induced *trans*-activation activity of the GR, whereas T, DHT, and R1881 had only weak inhibitory action on GR activity (Fig. 4B).

Androgen Ligands Bind to MR. To check that the effect of androgen ligands on sodium transport does occur as a result of their binding to MR, we investigated the ability of androgen ligands to bind to MR by competition experiments. MR was expressed in vitro using the rabbit reticulocyte lysate expression system and was incubated with 5×10^{-9} M [^{3}H]aldosterone in the absence or presence of unlabeled ligands. A 100-fold excess of unlabeled aldosterone inhibited the binding of [^{3}H]aldosterone to MR by 90 to 95% (Fig. 5A). As expected, a 100-fold excess of the two MR antagonists, spironolactone and RU26752 (Rafestin-Oblin et al., 1992), have a high efficiency to inhibit [^{3}H]aldosterone to MR (Fig. 5A). A 100-fold excess of R1881 also displaced 90% of the [^{3}H]aldosterone bound to the MR, whereas a 100-fold excess of T or DHT inhibited the binding of [^{3}H]aldosterone to MR by 36 and 52%, respectively (Fig. 5A). As also shown in Fig. 5B, increasing concentrations of T, DHT, and R1881 (10^{-10} to 10^{-6} M) induced dose-dependent displacements of the [^{3}H]aldosterone bound to MR. The apparent dissociation constant of R1881 for MR (K_{Dapp} ~0.8×10^{-9} M) is very similar to that of aldosterone (K_{Dapp} ~0.5×10^{-9} M). The apparent dissociation constants of T (K_{Dapp} ~4×10^{-8} M) and DHT (K_{Dapp} ~2.2×10^{-8} M) for the MR indicated that their affinities for this receptor were lower than that for R1881. These studies indicate that androgen ligands have the ability to bind to MR expressed in vitro and indicate that R1881 displays a high affinity for MR.

Androgens Inhibit the Aldosterone-Induced Activity of MR. To further investigate the properties of the androgen ligands when bound to MR, *cis-trans* cotransfection assays were performed in HEK 293T cells with pchMR and a reporter plasmid containing the luciferase gene under the control of the MMTV promoter. Incubating transfected HEK 293T cells with various concentrations of aldosterone for 24 h led to a dose-dependent increase in MR *trans*-activation activity, with maximum induction at 10^{-10} M aldosterone and an ED_{50} value of ~5×10^{-11} M (Fig. 6A), a value that is in accordance with previous studies (Arriza et al., 1987, 1988; Hellal-Levy et al., 1999). T and DHT had no effect on the *trans*-activation activity of the MR (Fig. 6A). At a concentration of 10^{-5} M, R1881 increased the *trans*-activation activity by only 20%. The antagonist activities of androgens were then tested by incubating the transfected HEK 293T cells with 10^{-9} M aldosterone alone (100%) or with increasing concentrations (10^{-9} to 10^{-5} M) of T, DHT, or R1881 or with one of the two MR antagonists, spironolactone or RU26752 (Fig. 6B). T, DHT, and R1881 all induced dose-dependent inhibition of the aldosterone-induced MR activity (Fig. 6B, left). The antagonist potencies of T (IC_{50} ~5×10^{-7} M) and DHT (~10^{-6} M) were lower than those of RU26752 (IC_{50} ~3×10^{-8} M) and spironolactone (IC_{50} ~8×10^{-8} M) (Fig. 6B, left). It is interesting that R1881 (IC_{50} ~7×10^{-8} M) seemed to be as potent as spironolactone in inhibiting aldosterone-induced MR activity (Fig. 6B, left). Transient transfections were also performed using COS-7 cells with no receptor expression: R1881 (IC_{50} ~5×10^{-8} M), T (IC_{50} ~4×10^{-6} M), and DHT (IC_{50} ~6×10^{-6} M) also induced dose-dependent inhibition of the aldosterone-induced MR activity (data not shown).

R1881 is a 19-nor steroid, whereas T and DHT have a C19-methyl group. R1881 is characterized by having a 17α-methyl group and by three double bonds (C4–C5, C9–C10, and C11–C12), T has a C4–C5 double bond, and DHT has no double bond. The question arises as to whether the presence of the 17α-methyl group and/or the lack of the 19-methyl group could be responsible for the potent antagonist activity of R1881. To test this hypothesis, the ability of 17-met T and 19-nor T to inactivate MR was analyzed. The efficiency of these two molecules in inhibiting aldosterone-induced activity was equivalent to that of testosterone (~5×10^{-7} M) (Fig. 6B, right). This suggests that the efficiency with which R1881 inhibits aldosterone-induced MR activity is not due to the presence of the methyl group at the C17α position or to the absence of methyl group at the C19 position.

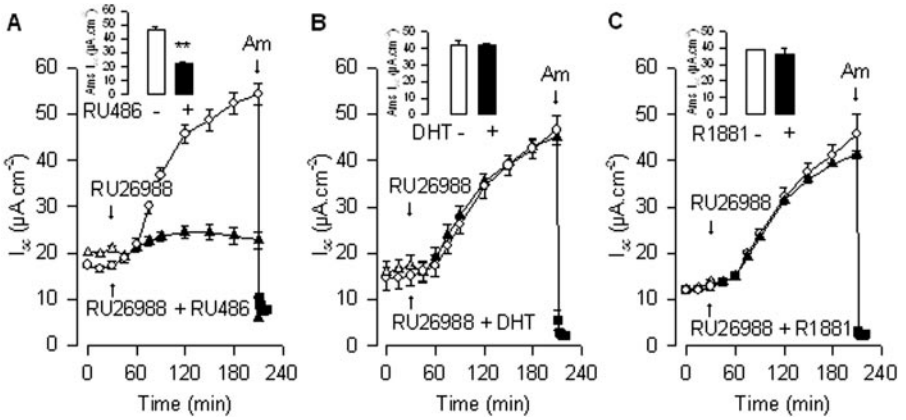

Fig. 3. Effects of androgens on Na^+ absorption stimulated by RU26988. A–C, I_{sc} was measured on sets of confluent mpkCCD$_{cl4}$ cells grown on filters as described in the legend to Fig. 2. After the traces has stabilized (open symbols), cells were incubated for 3 h with 5×10^{-7} M RU26988 alone (○) or with RU26988 plus 10^{-5} M RU486, DHT, or R1881 (▲). All steroids were added to both the apical and basal side of the cells for 3 h, and 10^{-5} M amiloride (Am, ■) was added for 10 min to the apical side of the cells. A–C, insets, the bars represent the amiloride-sensitive component of I_{sc} (Ams I_{sc}) measured in cells incubated for 3 h with RU26988 alone (−, □) or with RU26988 plus RU486, DHT, or R1881 (+, ■). Values are means ± S.E.M. from three to five individual recordings for each steroid tested. $p < 0.01$ versus RU486 values.

Docking of Androgen Ligands within the Ligand-Binding Cavity of MR. Some of us have shown previously that the contact between the Asn770 residue of the MR-LBD and the hydroxyl function at the 21 position, common to all MR agonist ligands, is essential to stabilize MR in its active state (Fagart et al., 1998). Conversely, the MR antagonist character of progesterone and spironolactone seems to be attributable to their inability to establish stabilizing contacts with the Asn770 residue (Fagart et al., 1998). To explain the antagonist property of androgen ligands when bound to MR, we examined the positioning of the androgen ligands within the ligand binding cavity of the crystal structure of the MR-LBD associated with aldosterone (Bledsoe et al., 2005). In this structure, the C3 ketone of aldosterone is hydrogen-bound to Gln776 (H3 helix) and Arg817 (H5 helix) (Fig. 7A). The C18 hydroxyl forms a hydrogen bond with the side chain carbonyl of Asn770 and is in position to interact with the Cys942 (Bledsoe et al., 2005). As shown in Fig. 7A, aldosterone is also anchored by two hydrogen bonds between Thr945 and C21 hydroxyl and C20 ketone groups. Docking of R1881 in the MR structure revealed that R1881 adopts the same orientation as aldosterone within the ligand binding cavity (Fig. 7, compare A and B). R1881 is favorably positioned to establish hydrogen bonds with Gln776 and Arg817 (Fig. 7B). The C17-hydroxyl of R1881 is at a distance of 3.6 Å from Asn770 and is 4.05 Å from Cys942 and Thr945 (Fig. 7B). These distances are too large for hydrogen bonds to be established, but are compatible with van der Waals interactions (Fig. 7, B and C). Moreover, the Δ4, Δ 9, and Δ 11 double bonds of R1881 are in the vicinity of the aromatic rings of Trp806 and Phe829, allowing interactions to occur (Fig. 7C). The two other androgen ligands, T and DHT, are positioned in a similar way to R1881 within the ligand binding cavity (data not shown).

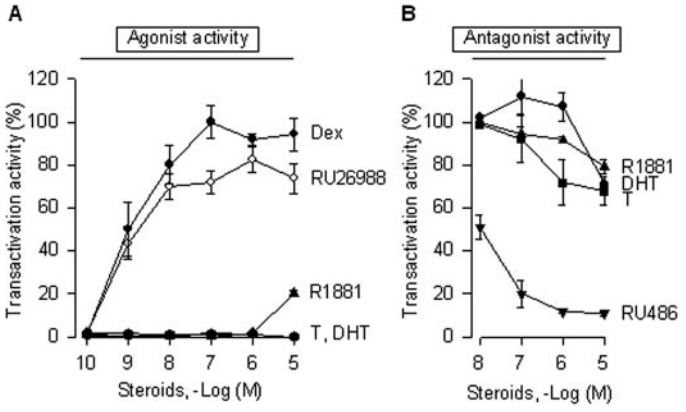

Fig. 4. Effects of androgens on the transactivation properties of wild-type GR. HEK 293T cells were transfected with the wild-type GR expression vector, the pFC31Luc reporter plasmid, and the pcβgal vector containing the gene coding for the β-galactosidase enzyme. A, the steroid agonist activity was tested by incubating cells with increasing concentrations (10^{-10} to 10^{-5} M) of dexamethasone (Dex), RU26988, T, DHT, or R1881 for 24 h. The GR *trans*-activation activity was determined by luciferase activity, normalized versus the internal β-galactosidase control, and expressed as a percentage of the maximum aldosterone-induced MR activity (data not shown on the graph). Values are means ± S.E.M. from three separate experiments. B, the steroid antagonist activity was tested by incubating cells with 10^{-8} M RU26988 alone and with RU26988 plus increasing concentrations (10^{-8} to 10^{-5} M) of T, DHT, R1881, or RU486 for 24 h. The GR *trans*-activation activity was determined as described above and is expressed as the percentage of the GR activity measured in the presence of RU26988 alone (100%). Values are means ± S.E.M. from three separate experiments.

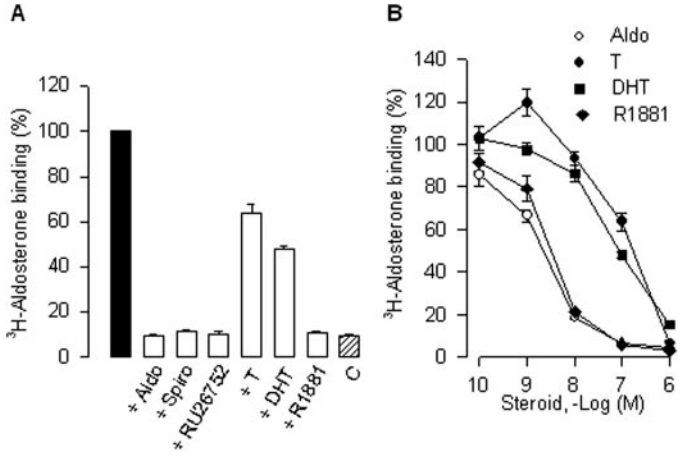

Fig. 5. Steroid binding to the wild-type MR synthesized in vitro in rabbit reticulocyte lysate. A, the lysate was diluted 1:3 in buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 20 mM sodium tungstate, 1 mM β-mercaptoethanol, and 10% glycerol and incubated with 5×10^{-9} M [^{3}H]aldosterone alone or with a 100-fold excess of unlabeled Aldo, Spiro, RU26752, T, DHT, or R1881. The hatched bar represents the binding of aldosterone to the nonrelevant protein β-galactosidase. B, the diluted lysate was incubated with 5×10^{-9} M [^{3}H]aldosterone alone or with increasing concentrations (10^{-10} to 10^{-6} M) of unlabeled Aldo, T, DHT, or R1881. In all cases, bound and unbound steroids were separated by the charcoal-dextran method. The results are expressed as the percentage of [^{3}H]aldosterone binding measured in the absence of any competitors (100%). Values are means ± S.E.M. from three to six separate experiments.

Anchoring of Androgen Ligands within the Ligand Binding Cavity of MR. To find out whether the Asn770, Thr945, and Cys942 residues are involved in the stability of the ligand receptor complex, we used three mutant MRs, MR_{Ala770}, MR_{Ala942} and MR_{Ala945}, in which alanine was substituted for Asn770, Cys942, and Thr945, respectively. Aldosterone is unable to bind to MR after N770A mutation, so we used the synthetic steroid 18OVP as the agonist ligand (Souque et al., 1995; Fagart et al., 1998). T, DHT, and R1881 all inhibited the 18OVP-induced *trans*-activation activity of MR_{WT} and MR_{Ala770}, although their inhibitory efficiencies were lower for MR_{Ala770} than for MR_{WT} (Fig. 8, A and B). A 10-fold excess of T, DHT, or R1881 inhibited the 18OVP-induced *trans*-activation activity of MR_{Ala770} by only 20 to 50% compared with 50 to 85% for MR_{WT}. These results suggest that the contact between Asn770 and the C17β-hydroxyl of the androgen ligands contributes to stabilizing the androgen-MR complex but is not strong enough to maintain MR in its activate state.

Aldosterone is still able to *trans*-activate MR after the C942A and T945A mutations. However, the efficiency with which it *trans*-activates the two mutant receptors (MR_{Ala942}, ED_{50} ~5×10^{-7} M; MR_{Ala945}, ED_{50} ~ 5×10^{-8} M) is lower than that observed with MR_{WT} (ED_{50} ~5×10^{-11} M). This indicates that the hydrogen bonds between aldosterone and Cys942 and Thr945 residues stabilize the aldosterone-MR complex but do not seem to be essential for aldosterone-induced MR activation. T, DHT, and R1881 still act as MR antagonists after the C942A and T945A mutations (Fig. 8, C and D). However, T and DHT produced less inhibition of the aldosterone-induced activity of MR_{Ala942} and MR_{Ala945} than of that of MR_{WT} (compare Fig. 8, C and D, with 6B). These

findings suggest that the interaction between T or DHT and the Thr945 and Cys942 residues contribute to the stability of the complex. In contrast to T and DHT, R1881 displays the same efficiency in inhibiting the *trans*-activation activity of MR_{WT}, MR_{Ala942}, and MR_{Ala945} (compare Fig. 8, C and D, with 6B). In addition, the partial agonist activity of R1881 (10^{-5}M) with MR_{WT} (Fig. 6B) was no longer observed with the mutant MRs in which the Cys942 or Thr945 residues had been replaced by an alanine (Fig. 8, C and D). These results indicate that the contacts between the C17β-hydroxyl of R1881 and the residues Thr945 and Cys942 contribute to its partial agonist activity.

Discussion

This study shows that the androgen ligands T, DHT, and R1881 display antagonist properties when bound to the human MR and inhibit Na^+ absorption stimulated by aldosterone in renal collecting duct cells. The MR antagonist properties of androgen ligands were demonstrated by testing their ability to inhibit the aldosterone-induced *trans*-activation activity of the wild-type MR on the MMTV-luciferase reporter gene in HEK 293T cells expressing low levels of endogenous MR or in COS-7 cells, which have no steroid receptors. T, DHT, and R1881 did not exert any agonist activity in either cell line, although R1881 displayed a slight capacity to activate MR at high concentrations. In contrast, the androgen ligands T, DHT, and R1881 all inhibited the aldosterone-induced activity of the transiently expressed MR. *Trans*-activation experiments performed in HEK 293T cells transiently expressing GR revealed that androgen ligands have no effect on GR activity. They also show that androgen ligands exert almost no inhibitory action on the GR activity induced by RU26988. Thus, our MR and GR *trans*-activity studies have provided evidence that androgen ligands act as MR antagonists.

The functional consequence of the MR antagonist properties of androgen ligands was further investigated in renal collecting duct cells by means of short-circuit current measurements. We used immortalized $mpkCCD_{cl4}$ cells that have retained the main functions of the cortical collecting duct cells from which they have been derived, particularly that of possessing Na^+ absorption, which is stimulated by aldosterone (Bens et al., 1999). The fact that spironolactone produced 72% inhibition in the increase in Na^+ absorption caused by 5×10^{-7} M aldosterone indicates that the increase in Na^+ absorption caused by aldosterone is in part attributable to MR occupancy (Rafestin-Oblin et al., 2002). Consistent with their MR antagonist features, T, DHT, and R1881 also prevented the increase in Na^+ absorption elicited by aldosterone in $mpkCCD_{cl4}$ cells. This effect seems to be dependent on MR, because androgens failed to inhibit the increase in Na^+ absorption caused by the specific GR agonist RU26988. It is interesting that the I_{sc} recordings also show that spironolactone exerted only a weak inhibitory effect on RU26988- stimulated Na^+ absorption, which contrasted with its potent inhibitory effect on the aldosterone-stimulated Na^+ current (Fig. 7). Thus, the results from I_{sc} experiments indicate that aldosterone stimulates Na^+ absorption via the occupancy of both MR and GR but that androgen ligands have almost no impact on GR-mediated Na^+ transport. In accordance with the data from transactivation studies, I_{sc} recordings show that T, DHT, and R1881 all inhibited both aldosterone-stimulated MR activity and aldosterone-stimulated Na^+ absorption.

R1881 has an affinity for MR (0.75 nM) that is similar to that of aldosterone and much higher than the affinities of T and DHT (40 and 22 nM, respectively). In accordance with these findings, R1881 seems to exert a more potent antagonist activity than T and DHT. These differences could be explained if more contacts are involved in the interaction between the MR and R1881 than in those with T or DHT. It is unlikely that the presence of the C17α-methyl group and the absence of the C19-methyl group that characterize R1881

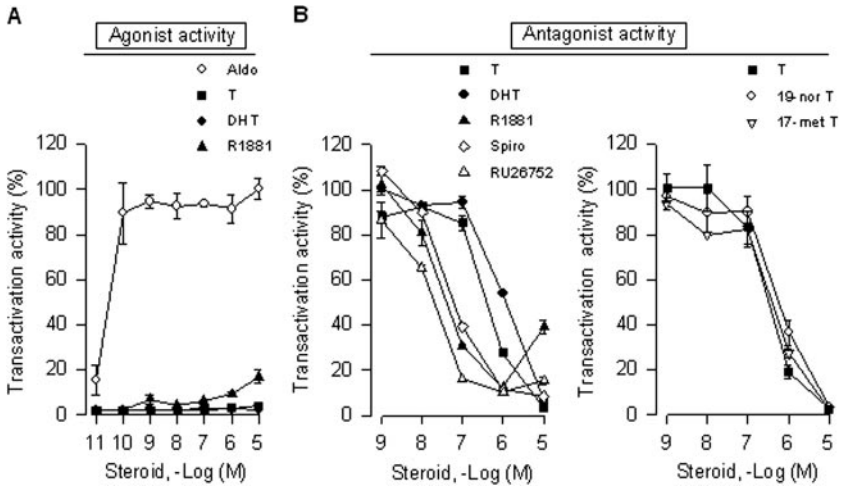

Fig. 6. Effects of androgens on the *trans*-activation properties of wild-type MR. HEK 293T cells were transfected with the wild-type MR expression vector, the pFC31Luc reporter plasmid, and the pcβgal vector. A, the steroid agonist activity was tested by incubating the transfected cells with increasing concentrations (10^{-11} to 10^{-5} M) of Aldo, T, DHT, or R1881 for 24 h. The MR *trans*-activation activity was determined by luciferase activity, normalized in terms of internal β-galactosidase control, and expressed as the percentage of the maximum aldosterone-induced MR activity. Values are means ± S.E.M. from three separate experiments. B, the steroid antagonist activity was tested by incubating the transfected cells with 10^{-9} M aldosterone alone (100%) or with aldosterone plus various concentrations (10^{-9} to 10^{-5} M) of T, DHT, R1881, RU26752, Spiro, 19-nor T, or 17-met T for 24 h. The MR activity was determined as described above and expressed as the percentage of the MR activity found in the presence of aldosterone alone (100%). Values are means ± S.E.M. from three separate experiments.

is responsible for its high activity. Indeed, 17α-methyl testosterone and 19-nortestosterone (which lacks the C19-methyl group) are as effective as testosterone in inhibiting aldosterone-induced MR activity. However, the interactions between the aromatic rings of Trp806 and Phe829 and the Δ4, Δ9, and Δ11 unsaturations of R1881 are sufficient to stabilize R1881 in the binding pocket of MR and thereby to improve its binding affinity compared with that of T or DHT, which lack Δ9 and Δ11 or Δ4, Δ19, and Δ11 insaturations, respectively.

Crystallographic and in vitro studies of numerous nuclear receptors have suggested that, in the absence of ligand, the LBD that surrounds the ligand binding cavity is rather dynamic and exhibits some of the properties of a molten globule (Nagy and Schwabe, 2004). Binding a ligand compacts the LBD by establishing many polar and hydrophobic contacts. Some of these are involved only in the stability of the ligand receptor complex, but some others are required to stabilize the ligand binding domain in its active conformation. The active conformation can be stabilized in two ways. First, the ligand itself may make direct contact with residues in the helix 12, thereby promoting its active position. Second, the ligand may stabilize the lower part of the LBD so that helix H12 tends to adopt its active position (Nagy and Schwabe, 2004). The crystal structure of the MR-LBD associated with aldosterone has revealed that the ligand D-ring substituents are in position to make hydrogen bonds with Asn770, a residue of helix 3, and with Cys942 and Thr945, two residues of the helix 11 (Bledsoe et al., 2005). The mutagenesis results presented in this study, together with previous analyses (Fagart et al., 1998), show that aldosterone retains its ability to activate mutant receptors carrying T945A and C942A mutations. However, the aldosterone efficiency reflected by the ED_{50} values is much lower for the two mutant receptors than for the wild-type MR. Taken together, these findings suggest that the hydrogen bonds between aldosterone and the Cys942 and Thr945 residues stabilize the aldosterone-MR complex. They probably contribute to maintaining the receptor in its active conformation after aldosterone binding but seem not to be essential in the activation process. In sharp contrast, the accommodation of aldosterone within the ligand binding cavity and the subsequent MR activation requires Asn770, a residue of helix 3 that simultaneously acts as a hydrogen bond acceptor from the ligand and a hydrogen bond donor to the backbone carbonyl of Glu955 residue located in the loop H11–H12 (Fagart et al., 1998; Hellal-Levy et al., 2000; Bledsoe et al., 2005).

The androgen ligands T, DHT, and R1881, which all have

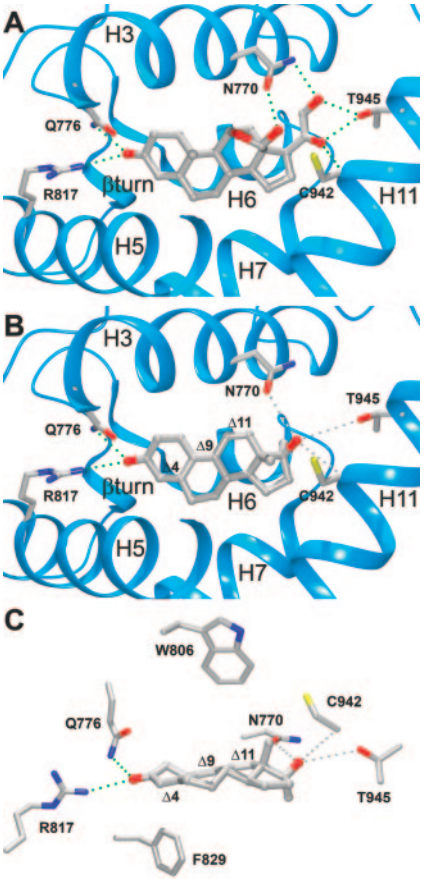

Fig. 7. Anchoring of aldosterone and R1881 within the ligand binding cavity of MR. A, crystallographic structure of the ligand binding domain of MR complexed with aldosterone according to Bledsoe et al. (2005). B and C, positioning of R1881 within the structure of the ligand binding domain of MR. The networks of hydrogen and van der Waals contacts involved in the anchoring of the ligands are depicted as green and gray dots, respectively.

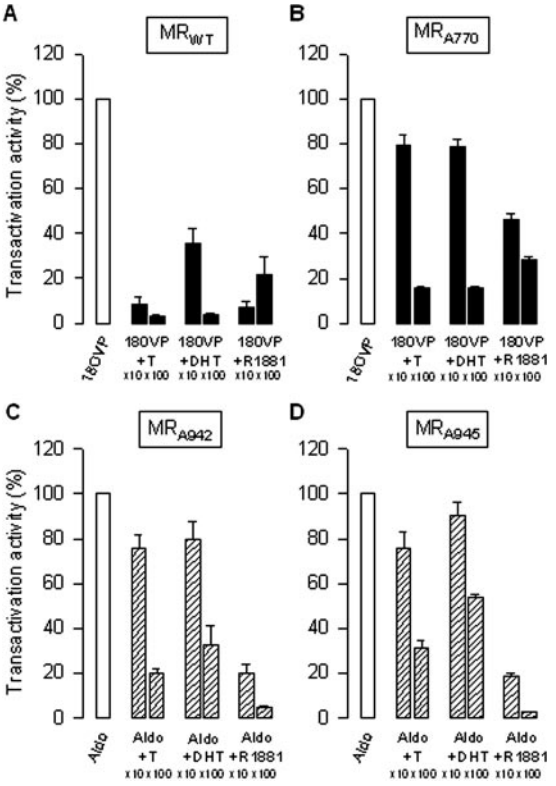

Fig. 8. Effects of androgens on the *trans*-activation properties of the mutant MRs. HEK 293T cells were transfected with an expression vector of MR_{WT} (A) or MR_{Ala770} (B), MR_{Ala942} (C), and MR_{Ala945} (D), together with the pFC31Luc reporter plasmid and the pcβgal. Before harvesting, cells were incubated with 10^{-7} M 18OVP (A and B) or aldosterone (C and D) in the absence or presence of 10^{-6} M ($\times$10) or 10^{-5} M ($\times$100) T, DHT, or R1881 for 24 h. The *trans*-activation activity of the wild type and mutant MRs was determined by luciferase activity, normalized versus the internal β-galactosidase control, and expressed as a percentage of the maximum aldosterone or 18OVP-induced MR activity. Values are means $\pm$ S.E.M. from three separate experiments.

a 17β-hydroxyl, display either no MR agonist activity (T and DHT) or only very weak activity (R1881). The fact that the agonist activity displayed by MR_{WT} and MR_{Ala770} is no longer observed in MR_{Ala942} and MR_{Ala945} strongly suggests that the contacts between R1881 and Cys942 and Thr945 residues are required for MR agonist activity. Stabilization of MR in its active state independently of Asn770 has been already reported for the synthetic compound 18OVP (Fagart et al., 1998). In this case, MR activation is believed to be due to an interaction between Cys942 and the C18 enone oxygen that provides a stabilizing interaction between the ligand and helix 11 (Bledsoe et al., 2005).

The results from the docking experiments and *trans*-activation studies led us to propose that the antagonist character of the androgen ligands T, DHT, and R1881 is due to the inability of their 17β-hydroxyl groups to form strong hydrogen bonds with the Asn770, Cys942, and Thr945 residues of the MR ligand binding cavity. The MR antagonist character of T, DHT, and R1881 contrasts with the ability of these ligands to activate the AR (Kemppainen et al., 1992). The crystal structure of the AR-LBD in association with R1881 revealed that the 17β-hydroxyl group of R1881 forms two strong hydrogen bonds with Asn705, corresponding to Asn770 in MR, and with Thr877, corresponding to Cys942 in MR, and establishes a van der Waals contact with Leu880, corresponding to Thr945 in MR (Matias et al., 2000). Mutation of AR-Thr877, and to a greater extent mutation of AR-Asn705, interferes with ligand recognition and reduces the transactivation efficiency (Poujol et al., 2000). Nevertheless, the two corresponding mutant receptors, AR_{Cys877} and AR_{Ala705}, retain their capacity to be activated by R1881, suggesting that either one of the two contacts is sufficient and strong enough to maintain the receptor in its active state. The bulky side chain of the leucine residue at position 880 in AR reduces the volume of the cavity compared with that of the MR cavity, and along with the presence of a threonine at position 877 in AR, this allows the formation of the strong hydrogen bonds that stabilize R1881-AR in its active state. The inability of R1881 to form strong hydrogen bonds with the corresponding residues in the MR ligand binding cavity could explain, at least in part, its antagonist properties.

References

Arriza JL, Simerly RB, Swanson LW, and Evans RM (1988) The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. *Neuron* **1:**887–900.

Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, and Evans RM (1987) Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science (Wash DC)* **237:**268–275.

Bens M, Vallet V, Cluzeaud F, Pascual-LeTallec L, Kahn A, Rafestin-Oblin ME, Rossier BC, and Vandewalle A (1999) Corticosteroid-dependent sodium transport in a novel immortalized mouse collecting duct principal cell line. *J Am Soc Nephrol* **10:**923–934.

Bledsoe RK, Madauss KP, Holt JA, Apolito CJ, Lambert MH, Pearce KH, Stanley TB, Stewart EL, Trump RP, Wilson TM, et al. (2005) A ligand-mediated hydrogen bond network required for the activation of the mineralocorticoid receptor. *J Biol Chem* **280:**31283–31293.

Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, et al. (2002) Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell* **110:**93–105.

Couette B, Fagart J, Jalaguier S, Lombes M, Souque A, and Rafestin-Oblin ME (1996) Ligand-induced conformational change in the human mineralocorticoid receptor occurs within its hetero-oligomeric structure. *Biochem J* **315:**421–427.

Couette B, Jalaguier S, Hellal-Levy C, Lupo B, Fagart J, Auzou G, and Rafestin-Oblin ME (1998) Folding requirements of the ligand-binding domain of the human mineralocorticoid receptor. *Mol Endocrinol* **12:**855–863.

De Wet JR, Wood KV, Deluca M, Helinski DR, and Subramani S (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol* **7:**725–737.

Fagart J, Huyet J, Pinon GM, Rochel M, Mayer C, and Rafestin-Oblin ME (2005) Crystal structure of a mutant mineralocorticoid receptor responsible for hypertension. *Nat Struct Mol Biol* **12:**554–555.

Fagart J, Wurtz JM, Souque A, Hellal-Levy C, Moras D, and Rafestin-Oblin ME (1998) Antagonism in the human mineralocorticoid receptor. *EMBO (Eur Mol Biol Organ) J* **17:**3317–3325.

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

Gomez-Sanchez CE and Gomez-Sanchez EP (1983) RU-26988: a new tool for the study of the mineralocorticoid receptor. *Endocrinology* **113:**1004–1009.

Hellal-Levy C, Couette B, Fagart J, Souque A, Gomez-Sanchez C, and Rafestin-Oblin ME (1999) Specific hydroxylations determine selective corticosteroid recognition by human glucocorticoid and mineralocorticoid receptor. *FEBS Lett* **464:**9–13.

Hellal-Levy C, Fagart J, Souque A, Wurtz JM, Moras D, and Rafestin-Oblin ME (2000) Crucial role of the H11–H12 loop in stabilizing the active conformation of the human mineralocorticoid receptor. *Mol Endocrinol* **14:**1210–1221.

Herbomel P, Bourachot B, and Yaniv M (1984) Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell* **39:**653–662.

Hultman ML, Krasnoperova NV, Li S, Du S, Xia C, Dietz JD, Lala DS, Welsch DJ, and Hu X (2005) The ligand-dependent interaction of mineralocorticoid receptor with coactivator and corepressor peptides suggests multiple activation mechanisms. *Endocrinology* **19:**1460–1473.

Jones TA, Zou JY, Cowan SW, and Kjeldgaard A (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A* **47:**110–119.

Kemppainen JA, Lane MV, Sar M, and Wilson EM (1992) Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones. *J Biol Chem* **15:**968–974.

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 acetyltransferase complex. *Mol Cell Biol* **22:**3698–3706.

Kleywegt GJ and Jones TA (1994) Detection, delineation, measurement and display of cavities in macromolecular structures. *Acta Crystallogr D Biol Crystallogr* **50:**178–185.

Li Y, Choi M, Suino K, Kovach A, Daugherty J, Kliewer SA, and Xu HE (2005) Structural and biochemical mechanisms for the specificity of hormone binding and coactivator assembly by mineralocorticoid receptor. *Proc Natl Acad Sci USA* **102:**9505–9510.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, et al. (1995) The nuclear receptor superfamily: the second decade. *Cell* **83:**835–839.

Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, Otto N, Joschko S, Scholz P, Wegg A, et al. (2000) Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. *J Biol Chem* **275:**26164–26171.

Moguilewsky M and Raynaud JP (1980) Evidence for a specific mineralocorticoid receptor in rat pituitary and brain. *J Steroid Biochem* **12:**309–314.

Nagy L and Schwabe JWR (2004) Mechanism of the nuclear receptor molecular switch. *Trends Biochem Sci* **29:**317–324.

Pascual-Le Tallec L, Kirsh O, Lecomte MC, Viengchareun S, Zennaro MC, Dejean A, and Lombès M (2003) Protein inhibitor of activated signal transducer and activator of transcription 1 interacts with the N-terminal domain of mineralocorticoid receptor and represses its transcriptional activity: implication of small ubiquitin-related modifier 1 modification. *Mol Endocrinol* **17:**2529–2542.

Pearce D, Bhargava A, and Cole TJ (2003) Aldosterone: its receptor, target genes and actions. *Vitam Horm* **66:**29–76.

Poujol N, Wurtz JM, Tahiri B, Lombroso S, Nicolas JC, Moras D, and Sultan C (2000) Specific recognition of androgens by their nuclear receptor. A structural study. *J Biol Chem* **275:**24022–24031.

Rafestin-Oblin ME, Couette B, Radanyi C, Lombès M, and Baulieu EE (1989) Mineralocorticoid receptor of the chick intestine, oligomeric structure and transformation. *J Biol Chem* **254:**9304–9309.

Rafestin-Oblin ME, 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.

Rafestin-Oblin ME, Lombès M, Couette B, and Baulieu EE (1992) Differences between aldosterone and its antagonists in binding kinetics and ligand-induced hsp90 release from mineralocorticosteroid receptor. *J Steroid Biochem Mol Biol* **41:**815–821.

Rafestin-Oblin ME, Lombès M, Lustenberger P, Blanchardie P, Michaud A, Cornu G, and Claire M (1986) Affinity of corticosteroids for mineralocorticoid and glucocorticoid receptors of the rabbit kidney: effect of steroid substitution. *J Steroid Biochem* **25:**527–534.

Ribeiro RC, Kushner PJ, and Baxter JD (1995) The nuclear hormone receptor gene superfamily. *Annu Rev Med* **46:**443–453.

Rogerson FM and Fuller PJ (2003) Interdomain interactions in the mineralocorticoid receptor. *Mol Cell Endocrinol* **200:**45–55.

Rossier BC, Geering K, Gaggeler HP, Claire M, and Corvol P (1980) Testosterone: a specific competitive antagonist of aldosterone in the toad bladder. *Am J Physiol* **239:**F433–F439.

Rossier BC and Palmer LG (1992) Mechanisms of aldosterone action on sodium and potassium transport, in *The Kidney: Physiology and Physiopathology* (Seldin DW and Giebisch G eds) pp 1373–1409, Raven, New York.

Singh SM, Gauthier S, and Labrie F (2000) Androgen receptor antagonists (antiandrogens): structure-activity relationships. *Curr Med Chem* **7:**211–247.
Souque A, Fagart J, Couette B, Davioud E, Sobrio F, Marquet A, and Rafestin-Oblin ME (1995) The mineralocorticoid activity of progesterone derivatives depends on the nature of the C18 substituent. *Endocrinology* **136:**5651–5658.
Trapp T and Holsboer F (1995) Ligand-induced conformational changes in the mineralocorticoid receptor analyzed by protease mapping. *Biochem Biophys Res Commun* **215:**286–291.
Williams SP and Sigler PB (1998) Atomic structure of progesterone complexed with its receptor. *Nature (Lond)* **393:**392–396.

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