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11β-Hydroxyprogesterone Acts as a Mineralocorticoid Agonist in Stimulating Na^+ Absorption in Mammalian Principal Cortical Collecting Duct Cells

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ABSTRACT

The binding of mineralocorticoid hormones to the mineralocorticoid receptor is the first step in a cascade of events leading to the stimulation of Na^+ reabsorption by renal cortical collecting duct (CCD) principal cells. The agonist properties of mineralocorticoid hormones are linked to contacts between their 21-hydroxyl group and Asn770, a residue of the ligand-binding domain of the human mineralocorticoid receptor (hMR). Here, we investigate whether the presence of a hydroxyl group at position 11, 17, or 20 could also alter the activity of progesterone (P), a mineralocorticoid antagonist without the 21-hydroxyl group. Both 17α-hydroxyprogesterone (17OHP) and 20α-hydroxyprogesterone (20OHP) antagonized the aldosterone-induced *trans*-activation activity (IC_{50}: 17OHP, 10^{-7} M; 20OHP, 10^{-8} M) of the hMR transiently expressed in COS-7 cells lacking steroid receptors. In cultured mouse mpkCCD_{cl4} principal cells, 17OHP and 20OHP also prevented the aldosterone-stimulated amiloride-sensitive component of the short-circuit current (Ams I_{sc}), reflecting Na^+ absorption mediated by the epithelial Na^+ channel (ENaC). In contrast, 11β-hydroxyprogesterone (11OHP) activated the transiently expressed hMR in COS-7 cells in a dose-dependent manner (ED_{50}: 10^{-8} M) and, like aldosterone, stimulated Ams I_{sc} in mpkCCD_{cl4} cells. Docking 11OHP within the hMR-ligand-binding domain homology model revealed that the agonist activity of 11OHP is caused by contacts between its 11β-hydroxyl group and Asn770. Furthermore, 11OHP was unable to activate the mutant hMR/N770A, in which Ala is substituted for Asn at position 770. These findings demonstrate that in the absence of the 21-hydroxyl group, the 11β-hydroxyl group can produce the contact with the hMR-Asn770 required for the hMR activation leading to stimulated Na^+ absorption.

In the kidney, the collecting duct is the main site of Na^+ reabsorption and is subjected to a fine hormonal control by aldosterone and vasopressin (Rossier and Palmer, 1992). In the principal cells of the cortical collecting duct (CCD), sodium enters via the amiloride-sensitive epithelial sodium channel (ENaC) and exits via the basolaterally located Na,K-ATPase pump. The regulation of sodium reabsorption by aldosterone requires it to be bound to the mineralocorticoid receptor (MR), a member of the nuclear receptor superfamily (Arriza et al., 1987). These receptors share a common modular structure with three major functional domains. The N-terminal region contains a constitutive *trans*-activation function. The central DNA-binding domain consists of two zinc fingers that are involved in DNA binding and receptor dimerization. The ligand-binding domain (LBD) lies in the C-terminal region and is involved in several functions, including nuclear localization, interaction with the 90-kDa heat-shock protein, homo- and/or hetero-dimerization, and a ligand-dependent activation function (Evans, 1988; Tsai and O'Malley, 1994; Mangelsdorf et al., 1995; Ribeiro et al., 1995).

The crystal structure of the MR-LBD has not yet been elucidated. However, three-dimensional homology models have been generated using crystallographic data for the human retinoic acid receptor (Fagart et al., 1998) and progesterone receptor (Auzou et al., 2000) as templates. These models have been used to predict the three-dimensional organization of the hMR-LBD and the docking of aldosterone within the ligand-binding cavity (Fagart et al., 1998). Several amino acid residues involved in the interaction with aldosterone have been identified by site-directed mutagenesis. It has been shown that Gln776 and Arg817 residues interact with

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ABBREVIATIONS: CCD, cortical collecting duct; EnaC, epithelial Na^+ channel; LBD, ligand-binding domain; MR, mineralocorticoid receptor; hMR, human mineralocorticoid receptor; P, progesterone; MMTV, mouse mammary tumor virus; 18OVP, 18-oxo-18-vinylprogesterone; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; DM, defined medium; HFM, hormone-free, epidermal growth factor-free defined medium; SFM, steroid-free, hormone-free medium; I_{sc}, short-circuit current; BAm, benzamyl amiloride; BAms, benzamyl amiloride-sensitive.

the 3-ketone function, and the Asn770 residue forms a hydrogen bond with the 21-hydroxyl group of aldosterone (Fagart et al., 1998). Contact between Asn770 of the hMR and the 21-hydroxyl group, common to all natural mineralocorticoid agonists, is crucial for the active conformation state of hMR. Other steroid substituents can alter the steroid accommodation of the steroid within the ligand-binding pocket, and modulate receptor *trans*-activation activity. For example, deoxycorticosterone, which has no substituent at the C11 and C17 positions, is a potent mineralocorticoid agonist, nearly as potent as aldosterone. In contrast, corticosterone, cortexolone, and cortisol, each of which has a hydroxyl group at the C11, C17, or C11/C17 position, respectively, are less efficient mineralocorticoid agonists than deoxycorticosterone (Hellal-Levy et al., 1999).

Progesterone binds to hMR with the same affinity as aldosterone and displays antagonist properties under in vitro conditions (Rupprecht et al., 1993; Souque et al., 1995; Myles and Funder, 1996). The antagonist activity of progesterone has been linked to its inability to establish contact with the Asn770 residue of hMR (Fagart et al., 1998). The question therefore arises as to whether hydroxylation at the C11, C17, or C20 positions of progesterone can alter its activity. To answer this question, the *trans*-activation activity of the hMR in response to 11β-hydroxyprogesterone (11OHP), 17α-hydroxyprogesterone (17OHP), and 20α-hydroxyprogesterone (20OHP) were examined in *cis-trans* cotransfection assays in COS-7 cells, using mouse mammary tumor virus (MMTV)-luciferase as a reporter gene. The ability of progesterone derivatives to regulate Na^+ absorption, assessed by the short-circuit current (I_{sc}) method, was also investigated in immortalized mouse mpkCCD$_{cl4}$ collecting duct principal cells which have kept the main features of the intact CCD from which they were derived (Robert-Nicoud et al., 2001; Hasler et al., 2002), including Na^+ absorption that is stimulated by aldosterone (Bens et al., 1999).

The present study shows that 17OHP and 20OHP, like progesterone, are able to antagonize the aldosterone-induced *trans*-activation activity of the transiently expressed hMR. I_{sc} recordings on mpkCCD$_{cl4}$ cells revealed that 17OHP and 20OHP also prevent the aldosterone-induced increase in amiloride-sensitive (Ams) I_{sc}, which reflects Na^+ absorption mediated by the epithelial Na^+ channel (ENaC). In contrast, 11OHP displayed agonist mineralocorticoid activity, because it produces dose-dependent activation of the transiently expressed hMR in COS-7 cells and stimulates the Ams I_{sc} in mpkCCD$_{cl4}$ cells. The mechanism of activation of the hMR caused by 11OHP has been further examined and discussed in the light of a three-dimensional hMR-LBD homology model constructed from the crystallographic data of the human progesterone receptor (Auzou et al., 2000) and in the light of the inability of 11OHP to *trans*-activate the aldosterone-insensitive N770A mutant of hMR (hMR/N770A) (Fagart et al., 1998).

Materials and Methods

Chemicals. [1,2-^{3}H]Aldosterone and [1,2-^{3}H]progesterone (40–60 Ci/mmol) was purchased from Amersham Biosciences (Saclay, France). Aldosterone, progesterone, 11β-hydroxyprogesterone, 17α-hydroxyprogesterone, and 20α-hydroxyprogesterone were purchased from Sigma (St. Louis, MO). 18-oxo-18-Vinylprogesterone (18OVP) was gift from A. Marquet (Paris, France). Spironolactone was kindly provided by Searle Laboratories (Chicago, IL). Dulbecco's minimal essential medium (DMEM) and Ham's F12 medium were from Invitrogen (Cergy Pontoise, France). All other compounds were from Sigma.

Expression and Reporter Constructs. The expression plasmids pchMR and pchMR/N770A contain the entire coding sequence of the wild-type hMR and the mutant hMR/N770A, respectively (Fagart et al., 1998). The plasmid pFC31Luc, which contains the MMTV promoter driving the luciferase gene, was obtained from H. Richard-Foy (LMBE, Toulouse, France). The pSVβ vector was from Promega (Charbonnières, France).

Coupled Cell-Free Transcription and Translation. Plasmids (1 μg) containing cDNA encoding the full-length wild-type hMR or the mutant hMR/N770A were transcribed for 1 h at 30°C using T7 RNA polymerase and translated in rabbit reticulocyte lysate system purchased from Promega according to the manufacturer's instructions (Promega).

Steroid Binding and Competition Studies. After translation of the wild-type hMR or the mutant hMR/N770A, the lysate was diluted (1:2) with ice-cold buffer (20 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 20 mM sodium tungstate, and 10% glycerol) and incubated for 2 h at 4°C with 1 nM [1,2-^{3}H]aldosterone with or without various concentrations of unlabeled competitors (10^{-10} M–10^{-6} M). Bound and unbound steroids were separated by the charcoal-dextran method (Fagart et al., 1998). The radioactivity was determined in a LKB liquid scintillation spectrometer after adding 5 ml of OptiPhase HiSafe (PerkinElmer Wallac, Turku, Finland).

Cultured Cells and Transfection Procedures. Experiments were performed using COS-7 cells and the mouse mpkCCD$_{cl4}$ principal cells (Bens et al., 1999). COS-7 cells were routinely grown in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2/95% air atmosphere. Cells were maintained in the same culture medium supplemented with 10% charcoal-stripped FCS for 4 h before and throughout the transfection procedure. Cells grown on six-well trays were transfected using the phosphate calcium precipitation method (ProFection; Promega) according to the manufacturer's instructions. For a six-well tray, the phosphate solution contained 5 μg of pchMR or pchMR/N770A expression plasmid, 10 μg of the pFC31Luc construct, which contains the MMTV promoter driving the luciferase gene and 5 μg of pSVβ containing the gene coding for the β-galactosidase enzyme. The steroids were added to the cells 12 h after transfection. After incubating for 24 h, cell extracts were assayed for luciferase (De Wet et al., 1987) and β-galactosidase activities (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 mpkCCD$_{cl4}$ cells were seeded on permeable Snapwell filters (insert growth area, 1 cm^2; 0.4-μm pore size; Corning Costar Corp., Cambridge, MA). Cells were grown until confluent in a modified defined medium [DM/DMEM/Ham's F12 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. After 5 days, confluent cells were placed in hormone-free, epidermal growth factor-free DM [HFM medium supplemented with charcoal-treated steroid-free FCS for 24 h, and then in FCS-free HFM medium (containing 29 mM $NaHCO_3$) for an additional 18 h]. Cells were then incubated without or with the various steroids (added to both apical and basal sides of the filters) and the agents to be tested. All experiments were performed on sets of cells from the 16th to 26th passages.

Progesterone Metabolism in Cultured mpkCCD$_{cl4}$ Cells. The ability of collecting duct cells to metabolize [1,2-^{3}H]progesterone was examined in mpkCCD$_{cl4}$ cells seeded in six-well trays and grown

in DM for 5 days and in a steroid-free, hormone-free medium (SFM) for additional 48 h. Cells were then incubated with 2 ml of SFM supplemented with 5×10^{-7} M unlabeled progesterone plus 400,000 cpm/well of [1,2-^{3}H]progesterone for 3 h at 37°C. As controls, the same radiolabeled SFM (2 ml) was also maintained for 3 h at 37°C in the absence of cells. Thereafter, the radiolabeled steroids present in the SFM were extracted twice with 2 ml of ethyl acetate. After removal of the medium, cells were rinsed with 2 ml of ice-cold phosphate-buffered saline and incubated with 2 ml ethanol for 1 h at 4°C. Thereafter, the SFM ethyl acetate phase and ethanol cell extract were evaporated. The dried precipitates were resuspended in 100 μl of ethanol and aliquots (60 μl) plus 10 μl of unlabeled progesterone (10^{-2} M) used as carrier were analyzed by thin-layer chromatography using cyclohexane/ethyl acetate (1:1.5, v/v). The radioactivity was counted by using an Automatic TLC-Linear Analyser (LB 282/LB 283) recorded to the data acquisition system LB 500 (B.A.I. Berthold, Elancourt, France).

Short-Circuit Current Studies. The Na^+ transport capacity of the $mpkCCD_{cl4}$ cells was assessed by the short-circuit current (I_{sc}) method. Confluent cells grown on filters were mounted in a modified Ussing-type chamber (Diffusion Chamber System, Costar Cambridge, MA) connected to a voltage clamp apparatus via glass barrel Micro-Reference Ag/AgCl electrodes. Experiments were always performed on sets of untreated and steroid-treated cells from the same passages to avoid interpassages variations. Cell layers were bathed on both sides (0.6 ml for the apical side and 1.2 ml for the basal side) by HFM medium warmed to 37°C and continuously gassed with 95% O_2/5% CO_2 to keep the pH at 7.4. I_{sc} (μA/cm^2) was measured by clamping the open-circuit transepithelial voltage (V_T) 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 solution.

Ligand Docking within the hMR Ligand-Binding Domain. Aldosterone, 11OHP and 17OHP, were docked in the homology model of the hMR ligand-binding domain generated from the crystal structure of the human progesterone receptor obtained in its active conformation state (Hellal-Levy et al., 2000). Complexes were energy minimized in 2000 steps with Discover-Insight II package (Molecular Simulation Inc., San Diego, CA), using the Newton procedure.

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

Results

Transactivation Activities of the hMR in Response to Progesterone Derivatives. We first investigated whether the addition of a hydroxyl group at positions C11, C17, or C20 of progesterone could modify the ability of the steroid to bind to the hMR. For this purpose, hMR was expressed in vitro using the rabbit reticulocyte lysate expression system and tested for its capacity to bind 11OHP, 17OHP, and 20OHP. As these compounds are available as unlabeled molecules, we measured their efficiency to inhibit [^{3}H]aldosterone binding to the hMR. 11OHP, 17OHP, and 20OHP derivatives bound to the hMR because they all displaced [^{3}H]aldosterone binding (Fig. 1). The order of potency in competing [^{3}H]aldosterone was as follows: aldosterone > progesterone ≥ 11OHP > 17OHP > 20OHP (Fig. 1). To find out whether progesterone derivatives have retained the antagonist properties of progesterone, *cis-trans* cotransfection assays were performed in COS-7 cells with pchMR and a reporter plasmid containing MMTV promoter upstream of the luciferase gene. As previously reported (Arriza et al., 1987, 1988; Rupprecht et al., 1993; Lombes et al., 1994), aldosterone stimulates the hMR *trans*-activation activity in a dose-dependent manner, with maximum induction at 10^{-9} M aldosterone, and an ED_{50} value of 10^{-10} M (Figs. 2, A and B). 10^{-6} M P, 17OHP, and 20OHP stimulated hMR *trans*-activation activity to only 5 to 25% of the maximum aldosterone-induced hMR activity. These findings suggest that P and 17OHP and 20OHP derivatives all act as weak MR agonists (Fig. 2A). In contrast, 10^{-6} M 11OHP stimulated hMR *trans*-activation activity almost as much as aldosterone did (Fig. 2A). The data from the dose-response curve reported in Fig. 2B show that 11OHP activated hMR with an ED_{50} of $\sim 5 \times 10^{-8}$ M. These findings indicate that the 11OHP derivative behaves as a mineralocorticoid agonist.

The antagonist activities of 17OHP and 20OHP were tested by incubating the transfected COS-7 cells with 10^{-9} M aldosterone alone (100%) or with various concentrations (10^{-9} M–10^{-6} M) of P, 17OHP, or 20OHP or with 10^{-6} M 11OHP. Compared with the level of hMR *trans*-activation activity induced by aldosterone (100%), the aldosterone-induced hMR *trans*-activation activity was reduced to 75% in the presence of a 100-fold excess of 11OHP (Fig. 3). P, 17OHP, and 20OHP inhibited the aldosterone-induced hMR *trans*-activation activity in a dose-dependent manner with an IC_{50} of 10^{-8} M for P and 20OHP and with an IC_{50} of 10^{-7} M for 17OHP (Fig. 3). Thus, like P, both 17OHP and 20OHP bind to the hMR and act as antagonist ligands, whereas 11OHP displays a weak antagonist activity at high concentration, because 10^{-6} M 11OHP inhibits the aldosterone-induced hMR *trans*-activation activity by no more than 20%.

11OHP Stimulates Na^+ Absorption in Collecting Duct Cells. Because progesterone can be metabolized, we first checked whether $mpkCCD_{cl4}$ cells were able to metabolize progesterone. The results from thin-layer chromatography studies showed that tritiated progesterone accounted for 67.5 ± 0.3% (n = 3) of the total radioactivity of cell extracts. Tritiated progesterone accounted for 33.8 ± 2.2% (n = 3) of the total radioactivity of the SFM medium extract recovered after incubating cells with [1,2-^{3}H]progesterone for 3 h at

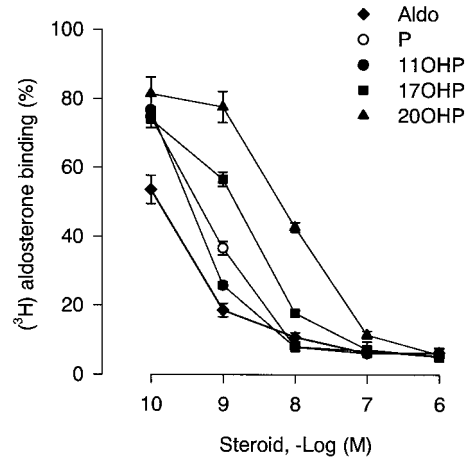

Fig. 1. Steroid binding to the wild-type hMR. The wild-type hMR was synthesized in vitro in rabbit reticulocyte lysate. The lysate was diluted 2-fold with TEGWD buffer and incubated with 10^{-9} M [^{3}H]aldosterone (Aldo), with or without various concentrations (10^{-10} M–10^{-6} M) P, 11OHP, 17OHP, or 20OHP for 2 h at 4°C. Bound and unbound steroids were separated by the charcoal-dextran method. Results are expressed as a percentage of [^{3}H]aldosterone binding measured in the absence of any competitor [100% corresponding to 784 ± 25 dpm (n = 3) for 25 μl rabbit reticulocyte lysate]. Bars are the mean ± S.E.M. from three separate experiments.

37°C compared with a 79.7 ± 0.4% recovery in the SFM medium without cells. These results indicated thus that, despite a significant progesterone metabolism, mpkCCD$_{cl4}$ cells were able to accumulate progesterone in its native form. Unfortunately, the ability of mpkCCD$_{cl4}$ cells to metabolize 11OHP, 17OHP, and 20OHP could not be determined in the absence of the corresponding radiolabeled hydroxylated progesterone derivatives.

We then tested the effects of progesterone and its derivatives on Na^+ transport in cultured mouse mpkCCD$_{cl4}$ cells. I_{sc} recordings were then performed on confluent mpkCCD$_{cl4}$ cells grown on filters and incubated for 3 h with the steroids that were added to both the apical and basal sides of the filters. Benzamyl amiloride (10^{-6} M), a potent inhibitor of ENaC (Kleyman and Cragoe, 1988), was then added to the apical side of the cells to determine the benzamyl amiloride-sensitive (BAms) component of I_{sc} which reflects the Na^+ absorption mediated by ENaC (Bens et al., 1999). As shown in Fig. 4, 5 × 10^{-7} M P and 11OHP significantly increased both total and BAms I_{sc} by 1.7- and 3.7-fold, respectively. In

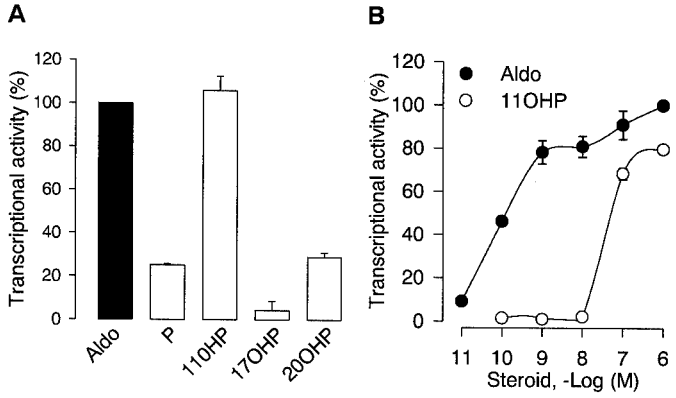

Fig. 2. Transactivation properties of wild-type hMR in response to progesterone and progesterone derivatives. COS-7 cells were transfected with the wild-type hMR expression vector, pFC31luc as reporter plasmid, and a β-galactosidase internal reporter to correct for transfection efficiency. A, before harvesting, cells were treated for 24 h with 10^{-9} M aldosterone (Aldo) or with 10^{-6}M P, 11OHP, 17OHP, or 20OHP. The hMR *trans*-activation activity was determined by luciferase activity, normalized versus the internal β-galactosidase control and expressed as a percentage of the wild-type hMR activity measured in the presence of 10^{-9} M aldosterone. B, before harvesting, cells were also treated for 24 h with rising concentrations of aldosterone or 11OHP. hMR *trans*-activation activity was determined by measuring luciferase activity, normalized versus the internal β-galactosidase control and expressed as a percentage of the maximum aldosterone-induced hMR activity. Each point is the mean ± S.E.M. from three separate experiments.

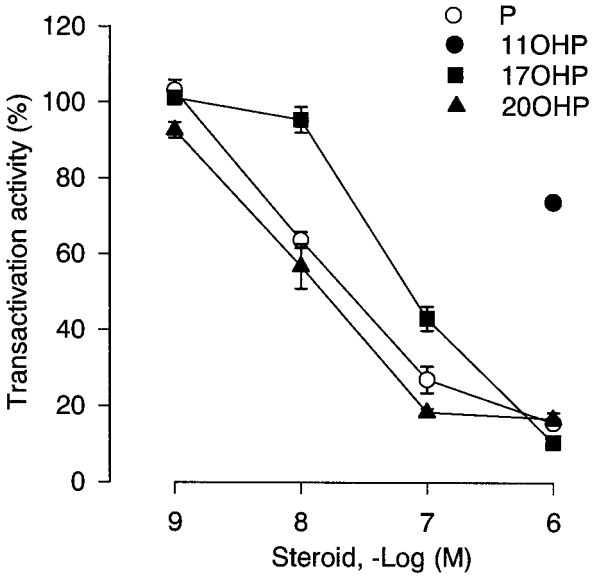

Fig. 3. Effect of progesterone derivatives on aldosterone-induced wild-type hMR *trans*-activation activity. COS-7 cells were transfected with the wild-type hMR expression vector, pFC31luc, and a β-galactosidase internal reporter as described in the legend to Fig. 2. Before harvesting, cells were treated for 24 h with 10^{-9} M aldosterone alone and with 10^{-9} M aldosterone plus 10^{-6} M 11OHP, or various concentrations (10^{-9} M–10^{-6} M) of P, 17OHP, or 20OHP. The hMR *trans*-activation activity was determined by luciferase activity, normalized versus the internal β-galactosidase control and was expressed as a percentage of the hMR activity measured in the presence of aldosterone alone. Each point is the mean ± S.E.M. of three separate experiments.

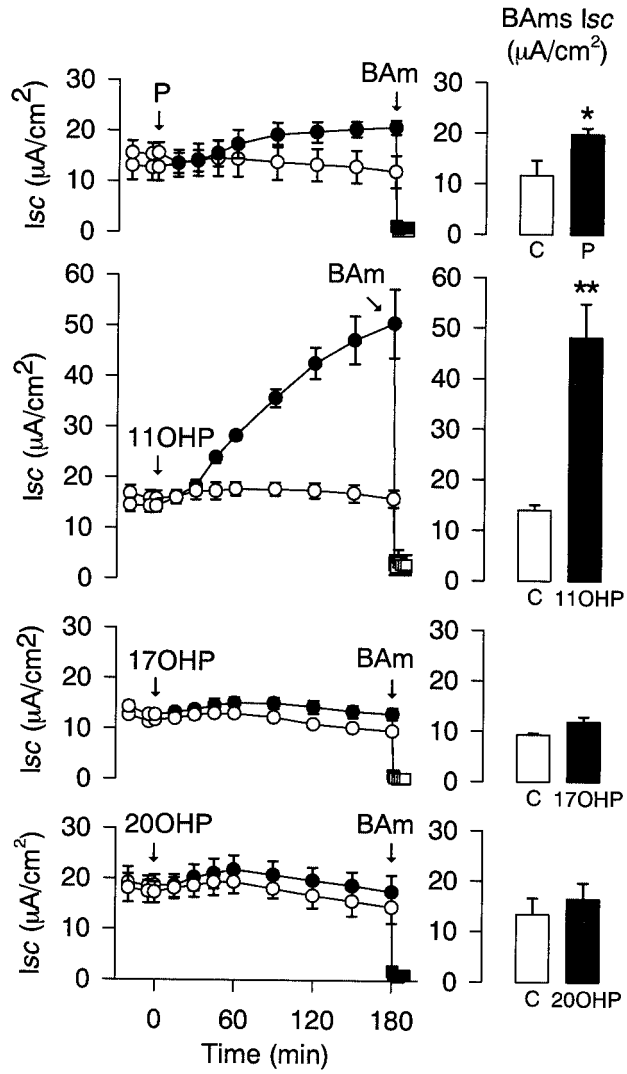

Fig. 4. Effects of progesterone and progesterone derivatives on Na^+ absorption in mpkCCD$_{cl4}$ cells. I_{sc} was measured on sets of confluent mpkCCD$_{cl4}$ cells grown on filters and incubated for 48h in steroid-free and hormone-free medium as described under *Materials and Methods*. Left, after a 1-h equilibration period, cells were incubated without (○) or with (●) 5 × 10^{-7} M P, 11OHP, 17OHP, or 20OHP for 3 h. All steroids were added to both the apical and basal side of the cells. Afterward, 10^{-6} M benzamyl amiloride (BAm) was added for 10 min to the apical side of the cells (■). Right, bars represent the benzamyl amiloride-sensitive I_{sc} (BAms I_{sc}), reflecting Na^+ reabsorption mediated by ENaC, from untreated cells (C, □) and P, 11OHP, 17OHP- and 20OHP-treated cells (■). Values are the mean ± S.E.M. from three to four individual recordings in each condition tested. *, $P < 0.05$; **, $P < 0.01$ versus C values.

contrast, similar concentrations of 17OHP or 20OHP had no effect on the total and BAms component of I_{sc} measured in mpkCCD$_{cl4}$ cells (Fig. 4). To find out whether the increase in Na^+ absorption induced by 11OHP corresponded to a MR-like effect, I_{sc} recordings were performed after adding 5×10^{-7} M aldosterone or 11OHP alone or with the MR antagonist spironolactone (Corvol et al., 1981). 11OHP increased BAms I_{sc} by about 3-fold, which is almost the same extent as aldosterone (Figs. 5, A and B). 10^{-5} M spironolactone almost completely prevented the rise in total (Figs. 5, A and B) and BAms I_{sc} caused by either aldosterone or 11OHP (Fig. 5C). Incubation of the cells with both steroids did not further increase BAms I_{sc} (Fig. 5C). Furthermore, 10^{-5} M carbenoxolone, a potent inhibitor of 11β-HSD (Monder et al., 1989), did not significantly affect the increase in BAms I_{sc} caused by 5×10^{-7} M 11OHP (11OHP: 47.4 ± 3.4 $\mu A/cm^2$, $n = 8$; 11OHP + carbenoxolone: 51.1 ± 5.03 $\mu A/cm^2$, $n = 4$). Overall, these results indicate that, like aldosterone, 11OHP stimulates Na^+ reabsorption mediated by ENaC in renal collecting duct cells.

P, 17OHP, and 20OHP Inhibit the Na^+ Absorption in Collecting Duct Cells That Is Stimulated by Aldosterone. P, 17OHP, and 20OHP were all shown to inhibit the hMR *trans*-activation activity induced by aldosterone (Fig. 3), and so I_{sc} experiments were conducted to find out whether these steroids could also inhibit the rise in I_{sc} caused by aldosterone in mpkCCD$_{cl4}$ cells. Confluent cells grown on filters were incubated for 3 h with 5×10^{-7} M aldosterone alone or with an excess (10^{-5} M) of P, 17OHP or 20OHP. Thereafter, BAms (10^{-6} M) was added to the apical side of the cells to measure the BAms component of I_{sc}. The results are shown in Fig. 6. All three steroids tested prevented the rise in I_{sc} caused by aldosterone. They significantly decreased the BAms I_{sc} to values similar to those for untreated cells (Fig. 6). These findings indicated that P, like 17OHP and

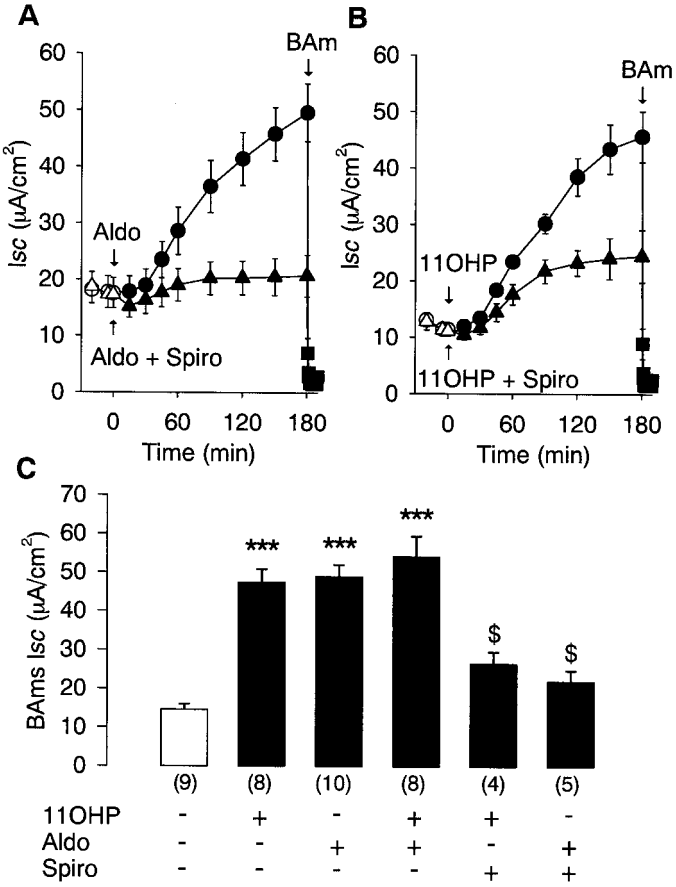

Fig. 5. Effects of spironolactone on the Na^+ reabsorption elicited by aldosterone and 11OHP. A and B, I_{sc} was measured on sets of confluent mpkCCD$_{cl4}$ cells as described in the legend of Fig. 4. After equilibration (○, △), cells were incubated for 3 h with 5×10^{-7} M aldosterone alone (Aldo, ●) or with aldosterone plus 10^{-5} M spironolactone (Aldo + Spiro, ▲) (A) and with 5×10^{-7} M 11OHP alone (●) or with 11OHP plus 10^{-5} M spironolactone (11OHP + Spiro, ▲) (B). All steroids were added to both the apical and basal side of the cells. BAm (10^{-6} M) was added for 10 min to the apical side of the cells (■). Values are the mean ± S.E. from 5 to 6 separate experiments. C, the bars represent the BAms I_{sc} measured in untreated cells (□) and cells incubated with 5×10^{-7} M 11OHP and/or aldosterone alone and in combination with 10^{-5} M spironolactone. Values are the mean ± S.E. from (n) individual recordings performed in each condition tested. ***, $P < 0.001$ versus untreated cells (□), $, $P < 0.05$ versus 11OHP- or aldosterone-treated cells.

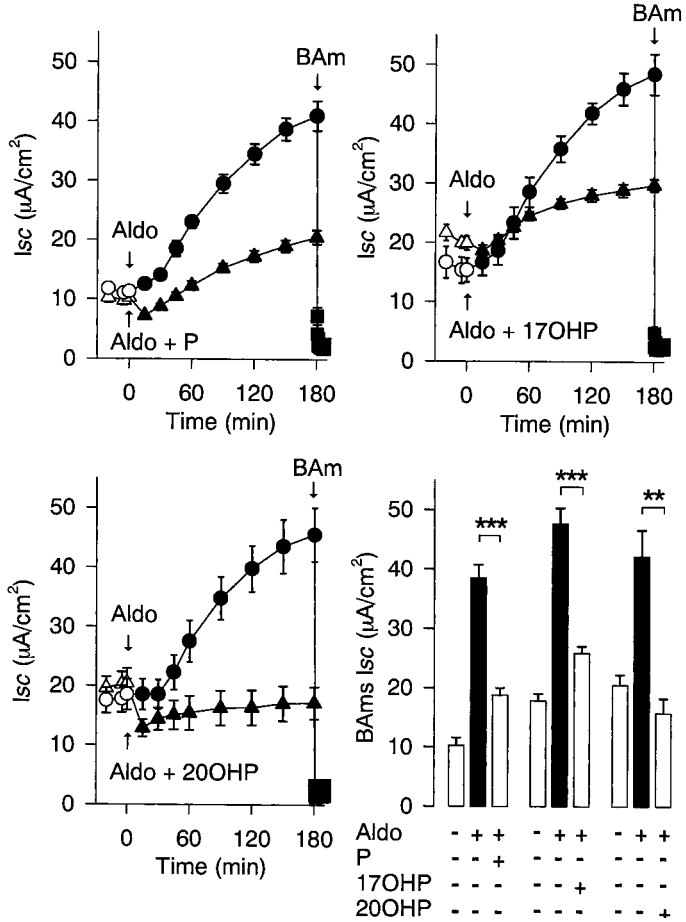

Fig. 6. Inhibitory action of P, 17OHP, and 20OHP on stimulated Na^+ absorption induced by aldosterone. I_{sc} was measured on sets of confluent mpkCCD$_{cl4}$ cells as described in the legend to Fig. 4. After equilibration (○, △), cells were incubated for 3 h with 5×10^{-7} M aldosterone alone (Aldo, [corcf]) or with aldosterone plus 10^{-5} M progesterone (Aldo + P, ▲), 10^{-5} M 17OHP (Aldo + 17OHP, ▲), or 10^{-5} M 20OHP (Aldo + 20OHP, ▲). All steroids were added to both the apical and basal side of the cells. BAm (10^{-6} M) was added for 10 min to the apical side of the cells (■). Values are the mean ± S.E. from five to six separate recordings. The bars represent the benzamyl amiloride-sensitive I_{sc} (BAms I_{sc}) measured in untreated cells and cells incubated with aldosterone alone (■) with an excess of P, 17OHP or 20OHP (□). Values are the mean ± S.E.M. from four to five individual recordings performed in each of the conditions tested. **, $P < 0.01$; ***, $P < 0.001$ between groups.

20OHP, acted as potent MR antagonists and inhibited the increase in Na^+ reabsorption induced by aldosterone.

The Contact between the hMR-Asn770 and the 11β-Hydroxyl Group Determines the Agonist Property of 11OHP. We have previously reported that the antagonist activity of P is related to the lack of contact between P and Asn770 (Fagart et al., 1998). To assess the agonist property of 11OHP, and compared this to the antagonist features of P, 17OHP, and 20OHP, we examined the positioning of the P derivatives within the ligand-binding cavity of a three-dimensional model of the hMR constructed using the crystal structure of the agonist-bound progesterone receptor as a template (Auzou et al., 2000). 11OHP adopts an orientation similar to that of aldosterone (compare Fig. 7, A and B). The 3-ketone function of 11OHP is anchored by Gln776 and Arg817 through hydrogen bonds (Fig. 7B). The 11β-hydroxyl group of 11OHP is in a favorable position to contact Asn770 (Fig. 7B), but this contact does not occur for 17OHP (Fig. 7C).

To check that the contact between the 11β-hydroxyl group of 11OHP and Asn770 is responsible for its agonist property, we examined the ability of 11OHP to activate hMR/N770A, a mutant hMR in which the Ala is substituted for Asn at position 770 (Fagart et al., 1998). Because aldosterone is unable to activate hMR/N770A (Fagart et al., 1998), the synthetic steroid 18OVP (Souque et al., 1995) was used as the agonist ligand. 11OHP was shown to exert slight agonist activity via the mutant hMR/N770A, and as a result, the hMR/N770A *trans*-activation activity induced by 10^{-5} M 11OHP corresponded to only 5% of the maximum *trans*-activation activity (100%) caused by 18OVP (data not shown). We also tested the ability of 11OHP to antagonize the 18OVP-induced hMR/N770A response. The activity of hMR/N770A induced by 10^{-7} M 18OVP was 27 and 4% in the presence of 10^{-6} M 11OHP or 10^{-5} M 11OHP, respectively, and was 3 and 5% in the presence of 10^{-6} M 17OHP and 10^{-5} M 17OHP, respectively, compared with the maximum activity induced by 18OVP alone (100%) (Fig. 8). These findings indicate that 11OHP behaves as an antagonist when bound to hMR/N770A, as does 17OHP. They also demonstrate that the ability of 11OHP to activate the wild-type hMR is caused by the contact between the 11β-hydroxyl group and Asn770. The antagonist property of 17OHP could be attributable to the lack of contact between this steroid and Asn770, as has already been reported for P (Fagart et al., 1998).

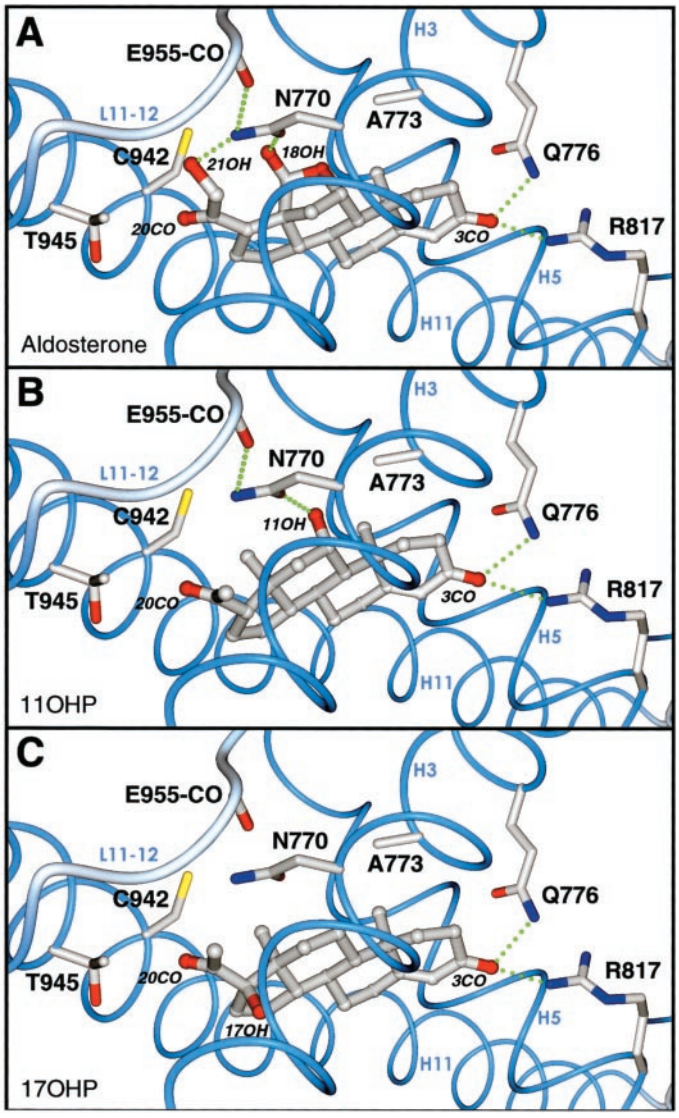

Fig. 7. Anchoring of ligands in the hMR ligand-binding pocket. The hMR-LBD backbone is drawn as blue ribbons and selected residues' side chains in close contact with the ligands are depicted as gray lines. View of the anchoring of aldosterone (A), 11OHP (B), and 17OHP (C) within the LBP. The stabilizing hydrogen bonds between the ligands and the hMR are depicted as green dashed lines. The stabilizing contact between N770 and E955 is depicted as a green dashed line for aldosterone (A) and 11OHP (B). The figure was prepared with Dino (http://www.biozentrum.unibas.ch/~xray/dino/).

Discussion

The present study indicates that 17OHP and 20OHP, like P, exhibit aldosterone antagonist properties in ex vivo conditions. In contrast, 11OHP displays agonist properties related to contacts between the 11β-hydroxyl group of the steroid

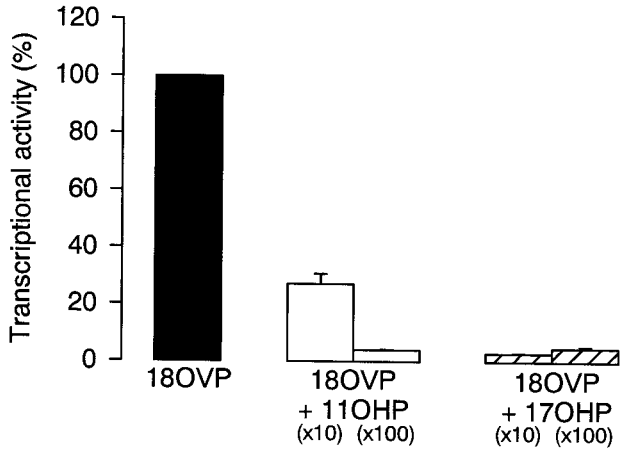

Fig. 8. Effect of progesterone derivatives on the *trans*-activation activity of the 18OVP-induced mutant hMR/N770A. COS-7 cells were transfected with the mutant hMR/N770A expression vector, pFC31luc as the reporter plasmid, and a β-galactosidase internal reporter to correct for transfection efficiency. Before harvesting, cells were treated for 24 h with 10^{-7} M 18OVP alone or with 10^{-7} M 18OVP plus 10^{-6} M or 10^{-5} M 11OHP or 17OHP. The hMR *trans*-activation activity was determined as described in the legend to Fig. 3 and was expressed as a percentage of the hMR/N770A activity measured in the presence of 18OVP alone. Bars are the mean ± S.E.M. of three separate experiments.

molecule and Asn770, a residue of the H3 helix that is crucial for hMR to acquire its active state (Fagart et al., 1998).

In principal cells of the collecting duct, aldosterone-induced sodium reabsorption is thought to be a multistep process in which the MR plays a central role. Aldosterone binds to the MR and induces a change in the receptor conformation of the LBD (Trapp and Holsboer, 1995; Couette et al., 1996). This change is believed to lead to the dissociation of the associated proteins, leaving the receptor in a suitable conformation for recruiting transcriptional coactivators, interacting with the DNA sequences of regulatory regions of target genes and also activating the biosynthesis of the apical epithelial sodium channel ENaC and basolaterally-located Na,K-ATPase pumps. In the present study, the agonist and/or antagonist activities of the progesterone derivatives were investigated using two complementary approaches. First, we investigated the ability of the P derivatives to activate (agonist effect) the *trans*-activation function of the wild-type hMR on a reporter gene (MMTV-luciferase) or to inhibit the aldosterone-induced activity of the hMR (antagonist effect). In this assay, the hMR was transiently transfected in a cellular model, COS-7 cells, which has no steroid receptors. This ensured that any observed effect of the tested steroids was indeed mediated by the hMR, not by the glucocorticoid receptor or progesterone receptor, both of which activate the reporter gene used in this study (Young et al., 1975; Parker, 1988). This strategy enabled us to use the same promoter and cellular context to investigate the response of the mutant hMR/N770A to elucidate the agonist mechanism of the 11OHP. Second, we checked whether P derivatives are able to modulate sodium reabsorption. For this purpose, we used the immortalized mouse mpkCCD$_{cl4}$ cell line that has retained the main properties of cortical collecting duct principal cells (Bens et al., 1999; Vuagniaux et al., 2000; Robert-Nicoud et al., 2001; Summa et al., 2001; Hasler et al., 2002). These cells express the MR and respond to aldosterone by increasing sodium reabsorption in a specific manner (Bens et al., 1999). This was further confirmed by the fact that the MR antagonist spironolactone prevented the rise in I_{sc} caused by aldosterone. The findings obtained by these two approaches are consistent and make it possible to conclude that the antagonist properties of P, 17OHP, and 20OHP and the agonist activity of 11OHP are intrinsic properties of these molecules rather than promoter- and/or cell-dependent effect. The present results also point out that there is no direct relationship between the affinity of P and its derivatives for hMR and their agonist or antagonist activities. For example, 20OHP has the same antagonist activity as P, although 20OHP is less potent than P in displacing tritiated aldosterone binding to hMR (see Figs. 1 and 3). Similar to what was reported for 21-hydroxy steroids (Hellal-Levy et al., 1999), we found that 11OHP is a weaker activator of hMR than aldosterone, although the affinity for hMR of these two steroids is quite similar (see Figs. 1 and 2).

Our understanding of how nuclear receptors are activated has been greatly enhanced by the elucidation of the crystal structures of several nuclear receptors that are now available, in their inactive or active state (Bourguet et al., 1995; Renaud et al., 1995; Wagner et al., 1995; Brzozowski et al., 1997; Williams and Sigler, 1998; Matias et al., 2000). The main difference between the unliganded inactive state and the agonist-associated active state of nuclear receptors seems to be the positioning of the H12 helix that harbors the ligand activated *trans*-activation function (AF-2) (Moras and Gronmeyer, 1998; Bourguet et al., 2000). In the unliganded state, the H12 helix points away from the receptor, whereas in the agonist-associated state, the H12 helix is folded back toward the core of the LBD. This repositioning of H12 after agonist binding, together with other structural changes, such as the bending of H3, brings it into a distinct receptor environment, thus creating an interface suitable for NR-coactivator binding (Moras and Gronmeyer, 1998; Bourguet et al., 2000). The mechanism by which antagonist binding impedes the receptor from activating gene transcription depends upon the antagonist molecule. Antagonists with a bulky side chain, such as raloxifene, cannot be accommodated within the ligand-binding cavity, which prevents the positioning of the H12 helix in its active position (Nichols et al., 1998). Under these conditions, the H12 helix lies over the groove corresponding to the coactivator binding site, thus preventing its interaction with the receptor. An alternative antagonistic mechanism is seen for molecules that can be easily accommodated in the ligand-binding cavity. The antagonism of these molecules is caused by the lack of contact between the molecule and the H12 region of the receptor. Such a mechanism has been proposed to explain the antagonist features of progesterone and spirolactones (Fagart et al., 1998) and can reasonably be extrapolated to the antagonist properties of 17OHP and 20OHP that we report here.

Folding analyses of the hMR-LBD homology model and mutagenesis studies have revealed that several contacts, identified in the region of the H12 helix and also in the loop between the H11 and H12 helices, are involved in stabilizing the active state of the hMR (Fagart et al., 1998; Hellal-Levy et al., 2000). One of them, the oxygen atom of the Glu955 main chain, forms a strong hydrogen bond with Asn770 in helix 3, a residue that is critical for the binding of C21-hydroxylated agonists, such as aldosterone and cortisol (Hellal-Levy et al., 2000). Here we show that 11OHP, 17OHP, and 20OHP all bind to both the mutant hMR/N770A and the wild-type hMR. In contrast, the natural mineralocorticoid hormones, aldosterone and cortisol, are both unable to bind to hMR/N770A (Fagart et al., 1998). It seems likely that the substitution of Asn for Ala at position 770 modifies the ligand-binding cavity, making it impossible to accommodate the 17 side chain when a hydroxyl group is present at position C21 (aldosterone, cortisol), but not if there is no C21-hydroxyl (P, 11OHP, 17OHP, 20OHP). The present findings also provide evidence that the transition from the inactive to the active state of the hMR may be brought about by contact between the 11-hydroxyl group of 11OHP group and Asn770. Because a higher concentration of 11OHP than of aldosterone is required to induce half-maximum activity of the hMR and because high 11OHP concentrations display weak antagonist activity, the contact between the 11-hydroxyl group of 11OHP and Asn770 seems to be less efficient than that between the 21-hydroxyl group and Asn770. An alternative way for the switch from the inactive to the active state of the hMR involves hydrophobic contacts between the hMR and a C18 substituent. Indeed, the synthetic steroid 18OVP is able to activate both the wild-type hMR and the mutant hMR/N770A. It may be concluded that the activation of the hMR can occur independently of Asn770. Interestingly, the 21-hydroxyl group ceases to be required for the receptor activa-

tion when an H3 helix - H5 helix interaction is established by substituting Leu for Ser at the 810 position, leading to a receptor that can be activated by P as well as by aldosterone (Geller et al., 2000). This activated mutation in the hMR causes early onset of hypertension that is markedly exacerbated during pregnancy (Geller et al., 2000).

In conclusion, this study identifies some new determining factors for the hMR activation process and provides new insights relevant to the design of new agonists and antagonists MR molecules.

References

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.

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

Auzou G, Fagart J, Souque A, Hellal-Lévy C, Wurtz JM, Moras D, and Rafestin-Oblin ME (2000) A single amino acid mutation of Ala-773 in the mineralocorticoid receptor confers agonist properties to 11β-substituted spirolactones. *Mol Pharmacol* **58:**684–691.

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.

Bourguet W, Ruff M, Chambon P, Gronmeyer H, and Moras D (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature (Lond)* **375:**377–382.

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

Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, and Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature (Lond)* **389:**753–758.

Corvol P, Claire M, Oblin ME, Geering K, and Rossier BC (1981) The mechanism of the antimineralocorticoid effects of spirolactones. *Kidney Int* **20:**1–6.

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.

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

Evans RM (1988) The steroid and thyroid hormone receptor superfamily. *Science (Wash DC)* **240:**889–895.

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.

Geller DS, Farhi A, Pinkerton N, Fradley M, Moritz M, Spitzer A, Meinke M, Tsai FTF, Sigler PB, and Lifton RP (2000) Activating mineralocorticoid receptor mutation in hypertension exacerbated by pregnancy. *Science (Wash DC)* **289:**119–123.

Hasler U, Mordasini D, Bens M, Bianchi M, Cluzeaud F, Rousselot M, Vandewalle A, Feraille E, and Martin PY (2002) Long-term regulation of aquaporin-2 expression in vasopressin-responsive renal collecting duct principal cells. *J Biol Chem* **277:**10379–10386.

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 receptors. *FEBS Letter* **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.

Kleyman TR and Cragoe EJ (1988) Amiloride and its analogs as tools in the study of ion transport. *J Membr Biol* **105:**1–21.

Lombes M, Kenouch S, Souque A, Farman N, and Rafestin-Oblin ME (1994) The mineralocorticoid receptor discriminates aldosterone from glucocorticoids independently of the 11 beta-hydroxysteroid dehydrogenase. *Endocrinology* **135:**834–840.

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 PD, 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. *J Biol Chem* **275:**26164–26171.

Monder C, Stewart PM, Lakshmi V, Valentino R, Burt D, and Edwards CR (1989) Licorice inhibits 11 β-dehydrogenase of rat kidney and liver: In vivo and in vitro studies. *Endocrinology* **125:**1046–1053.

Moras D and Gronmeyer H (1998) The nuclear receptor ligand-binding domain: Structure and function. *Curr Opin Cell Biol* **10:**384–391.

Myles K and Funder JW (1996) Progesterone binding to the mineralocorticoid receptor: In vitro and in vivo studies. *Am J Physiol* **270:**E601–E607.

Nichols M, Rientjes MJ, and Stewart AF (1998) Different positioning of the ligand-binding domain helix 12 and the F domain of the estrogen receptor accounts for functional differences between agonists and antagonists. *EMBO (Eur Mol Biol Organ) J* **17:**765–773.

Parker M (1988) Characterization of response elements for androgens, glucocorticoids and progestins in mouse mammary tumor virus. *Nucleic Acids Res* **16:**5263–5276.

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

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

Robert-Nicoud M, Flahaut M, Elalouf JM, Nicod M, Salinas M, Bens M, Doucet A, Wincker P, Artiguenave F, Horisberger JD, et al. (2001) Transcriptome of a mouse cortical collecting duct cell line: effects of aldosterone and vasopressin. *Proc Natl Acad Sci USA* **98:**2712–2716.

Rossier BC and Palmer LG (1992) Mechanisms of aldosterone action on sodium transport and potassium transport, in *The Kidney: Physiology and Physiopathology* (Seldin DW and Giebisch G eds) pp 1373–1404, Raven, New York.

Rupprecht R, Reul JMHM, Vansteensel B, Spengler D, Soder M, Berning B, Holsboer F, and Damm K (1993) Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. *Eur J Pharmacol* **247:**145–154.

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 C-18 substituent. *Endocrinology* **136:**5651–5658.

Summa V, Mordasini D, Bens M, Martin PY, Vandewalle A, Verrey F, and Feraille E (2001) Short-term effect of aldosterone on Na,K-ATPase cell-surface expression in kidney collecting duct cells. *J Biol Chem* **276:**47087–47093.

Trapp T and Holsboer F (1995) Ligand-induced conformational changes in the mineralocorticoid receptor analyzed by protease mapping. *Biochem Biophys Res Commun* **215:**286–329.

Tsai MJ and O'Malley BW (1994) Molecular mechanism of action of steroid/thyroid receptor superfamily. *Annu Rev Biochem* **63:**451–486.

Vuagniaux G, Vallet V, Fowler Jeager N, Pfister C, Bens M, Farman N, Courtois-Coutry N, Vandewalle A, Rossier BC, Hummler E, et al. (2000) Activation of the amiloride-sensitive epithelial sodium channel by the mouse serine protease mCAP1 expressed in a mouse cortical collecting duct cell line. *J Am Soc Nephrol* **11:**828–883.

Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD, and Fletterick RJ (1995) A structural role for hormone in the thyroid hormone receptor. *Nature (Lond)* **378:**690–697.

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

Young HA, Scolnick EM, and Parks WP (1975) Glucocorticoid-receptor interaction and induction of MMTV. *J Biol Chem* **250:**3337–3343.

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