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Membrane progestin receptors α and γ in renal epithelium ☆

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ABSTRACT

Sex hormones have broader effects than regulating reproductive functions. Recent identification of membrane progestin receptors expressed in kidney prompted us to investigate their putative involvement in the renal effects of this hormone. We first focused our investigations on mPRα and γ by analyzing three parameters 1/ their distribution along the mouse nephron and their subcellular location in native kidney, 2/ the ability of progesterone to stimulate ERK pathway and/or Ca^{2+} release from internal stores in native kidney structures and 3/ the cellular localization of mPRα and its molecular determinants in heterologous expression system. We observed that 1/ mPRα expression is restricted to proximal tubules of both male and female mice whereas mPRγ exhibits a much broader expression all along the nephron except the glomerulus, 2/ mPRα and γ are not localized at the plasma membrane in native kidney, 3/ this expression does not permit either progesterone-induced ERK phosphorylation or Ca^{2+} release and 4/ in HEK transfected cells, mPRα localizes in the endoplasmic reticulum (ER) due to a C-terminal ER retention motif (−KXX). Therefore, we have characterized mPRs in kidney but their role in renal physiology remains to be elucidated.

1. Introduction

Kidney plays a key role in the maintenance of ionic homeostasis and, *in fine*, blood pressure, and is therefore tightly regulated by many hormones. Among them, sex hormones play a still poorly understood role although strong pieces of evidence support their involvement in such physiological processes [1]. Regarding progesterone, it seems admitted that it acts as a mineralocorticoid antagonist by directly binding to the mineralocorticoid receptor [2]. This would lead to salt waste although clear in vivo demonstration of progesterone-induced natriuesis is lacking. The absence of conventional progesterone receptors in renal epithelium [3,4] contributes to support the idea that progesterone affects renal functions by binding to another steroid receptor. Interestingly, Brunette and Leclerc [5] observed that progesterone increases calcium and decreases sodium uptakes by luminal membrane of distal tubules in the absence of mineralocorticoid, indicating that a mineralocorticoid-independent mechanism may also contribute to the renal effects of progesterone.

Recently, a new type of progesterone receptor has been characterized [6,7]. In vertebrates, there are 3 isoforms of this receptor – α, β, and γ – which are differentially distributed. The α isoform is mainly found in sexual organs (testis and ovaries) and to a lesser extent in epithelial tissues as the kidney. The γ isoform is mainly found in kidney.

As for the β form, its expression seems restricted to the brain. According to Zhu et al. [6], these membrane-bound progesterone receptors (mPR) belong to the seven transmembrane domains receptor family and are associated with G proteins. The group of Peter Thomas has shown several times that their stimulation would lead to a decrease in cAMP and would induce ERK activation. Recently, these results have been directly challenged [8] and the question of mPR location and signaling pathway is still open.

The presence of membrane progesterone receptors in kidney would support the hypothesis of a mineralocorticoid-independent mechanism for progesterone action. To start investigating this hypothesis we focused on the characterization of mPRα and mPRγ, 1/ by analyzing their expression along the nephron and their cellular localization in native tissue, 2/ by testing the ability of progesterone to initiate rapid transduction pathways via ERK kinase activation or Ca^{2+} release and 3/ by identifying the molecular determinants involved in their cellular localization.

2. Materials and methods

2.1. Cell lines

HEK293 cell line (kindly provided by Dr Michael Caplan, Yale University) was grown in a 5%CO2/95% air humidified incubator at 37 °C and in a-MEM (GIBCO BRL) supplemented with 10% FBS, 2 mM L-

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glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. cDNA transfection was performed with Fugene HD (Roche) following manufacturer instructions and using a ratio 5/2 (vol Fugene reactant (μl)/quantity of cDNA (μg)). Experiments were performed 2 days after transfection.

2.2. Membrane fraction preparation and membrane fractionation on iodixanol gradient

After removal, kidneys were rapidly washed in 50 ml of ice-cold PBS, cut into small pieces and placed into ice-cold homogenization buffer (250 mM sucrose, 1 mM EDTA, 10 mM NaOH—Hepes, pH 7.4) containing protease inhibitor (protease inhibitor cocktail, Roche). Minced kidneys were homogenized in a Dounce homogenizer (10 passes). The homogenate was, then, centrifuged at 1000 ×*g* for 10 min, and the supernatant was centrifuged at 200,000 ×*g* for 1 h at 4 °C for direct Western blot analysis or at 100,000 ×*g* for 1 h 30 min at 4 °C for fractionation of membrane compartments. The pellet was resuspended in homogenization buffer and protein contents were determined using the BCA protein assay kit (Pierce).

Fractionation of membrane compartments was performed using separation by buoyant density in pre-formed continuous iodixanol gradients (Optiprep, Axis-shield), mainly as described by the manufacturer with some modifications. Briefly, 0–30% iodixanol gradients were prepared into 8.9 ml tubes by passive diffusion (overnight at 4 °C), top loaded with the 100,000 ×*g* pellet and centrifuged at 200,000 ×*g* for 3 h 30 min at 4 °C. Fractions of 750 μl were collected by bottom puncture and analyzed for density (DO at 340 nm) and protein contents (BCA method).

2.3. Animals and tubule isolation

Animal experiments were carried out according to the French legislation. Before experiments, male and female CD1 mice (8–10 weeks old, Charles Rivers Breeding Laboratories) had free access to food and deionized water ad libitum. Mice were anesthetized by pentobarbital sodium injection (80 μg/g body weight), their left kidney was rinsed (3 ml of Hank's modified medium, Eurobio) and perfused with 120 μg/ml of Liberase blendzyme (Roche) dissolved in Hank's medium. Thin kidney pyramids cut along the corticomedullary axis were incubated at 30 °C for 20 min in aerated Hank's medium containing 40 μg/ml Liberase. Microdissection was performed in ice-cold Hank's medium containing 0.1% BSA. The following structures were microdissected from liberase-treated kidneys according to morphologic and topographic criteria: proximal convoluted tubules (PCT), proximal straight tubules S2 (PST S2) and S3 (PST S3), medullary and cortical thick ascending limb of Henle's loop (mTAL and cTAL), distal convoluted tubules (DCT), connecting tubules (CNT), cortical and outer medullary collecting duct (CCD and OMCD).

2.4. Antibodies

Specific peptide of the mouse mPRα (−V226SPLPAEEDPA236) and mouse mPRγ (−F10RIDQVPQVFHEQGILFGYR) were synthesized (NeoMPS) and coupled to ovalbumin before rabbit immunization (NeoMPS). After the fourth injection, total rabbit sera (SR035 for mPRα, SR036 for mPRγ) were collected and tested for specificity. For cleaner results, 1 ml of SR035 and of SR036 were Ig purified using Ig-Adem kit (Ademtech) according to manufacturer's instructions.

Anti-phosphoERK (phospho-p42/44) and anti-ERK (p42/44) were purchased from Cell Signaling Technology. Anti-Na,K-ATPase α subunit was kindly provided by Dr. Geering, University of Lausanne. Anti-Na,H-exchanger 3 was kindly provided by Dr. Eladari, Centre de recherche des Cordeliers, Paris. Anti-calnexin was purchased from Stressgene and anti-Rab6 from Santa Cruz.

2.4.1. Calcium measurements

Intracellular calcium concentration was determined on single PCT by the fura 2 fluorescence. After rapid isolation, each PCT loaded with acetoxymethyl ester of fura 2 (fura 2-AM, 10 μM, 1 h at room temperature) was transferred to a perfusion chamber, and immobilized by sucking each end within the tip of a holding micropipette. The peritubular fluid (modified Hanks medium, see below) was continuously exchanged at a rate of ~10 ml/min. After 5–10 min equilibration, measurement of fura 2 fluorescence of ~20–30 cells was performed with a standard photometric set up (MSP 21, Zeiss). Tubule autofluorescence was subtracted from the fluorescence intensities of fura 2 at 340 and 380 nm. Values were calculated from the equation of Grynkiewicz et al. [9], using a dissociation constant value of fura 2 for calcium of 224 nM.

2.5. Western blot analysis

For analysis of membrane fractions from mouse kidneys, 20 μg of total protein were denaturated by adding 2× protein sample buffer (4.8% (w/v) SDS, 6.9% (w/v) sucrose, 0.012% (w/v) bromophenol blue, 2.1% (v/v) β-mercaptoethanol) and heated 3 min at 95 °C. The samples were then resolved onto a 10% SDS-polyacrylamide gel. Western blots were performed according the standard procedure using Ig purified-SR035 or -SR036 (1/1000) followed by an incubation with an HRP-conjugated anti-rabbit antibody (1/10,000 Promega). Pre-immune serums were tested at the same dilution as their respective antibodies. As for the blocking peptide experiments, antibodies were incubated 1 h at room temperature with their respective peptides coupled to ovalbumin (20 μg/ml) before incubations with PVDF membranes.

Regarding experiments on nephron segments, 15–20 mm of each microdissected tubule pool (Noesis Visiolog software) or 25 glomeruli were transferred in 20 μl of Hank's medium into 1.5 ml Eppendorf tubes. Protein denaturation was performed by adding 20 μl of 2× protein sample buffer to each sample, incubated 20 min at room temperature before heating 3 min at 95 °C. Samples were resolved by a 10% SDS-polyacrylamide gel-electrophoresis and transferred onto a PVDF membrane. Western blot revelations were performed as described above.

To test the effect of progesterone (Sigma) on ERK activation, mouse kidneys were perfused as described above but using a modified Hanks medium containing 0.5 mM MgCl2, 1 mM sodium acetate, 1 mM pyruvate, 1 mM glutamine, 10 mM glucose and 20 mM HEPES, pH7.4 and microdissection of proximal tubules was carried out in the same medium complemented with protease inhibitors (PMSF 100 μM, leupeptin 5 μg/ml and aprotinin 2.5 μg/ml) and bovine serum albumin 0.1%. Pools of 15–20 mm were transferred into tubes and pre-incubated at 30 °C for 30 min before incubation with either progesterone or EGF for 15 min at 37 °C. Protein sample buffer was then added to stop the incubation and denature the tubules. Western blot analysis was carried out as described above.

For all procedures, revelation was performed using Pierce revelation kit (Pierce).

2.6. RNA extraction, reverse transcription and real-time PCR

RNAs extracted as previously described [10] from pools of 30 microdissected tubules, were reverse-transcribed using the first strand cDNA synthesis kit for RT-PCR (Roche Diagnostics, France) according to the manufacturer's instructions. Real-time PCRs were performed on a LightCycler (Roche Diagnostics) with either the LightCycler FastStart DNA Master SYBR green 1 kit (Roche Diagnostics) or the DyNAmo Capillary SYBR green qPCR kit (Finzymes, Ozyme, St Quentin en Yvelines, France) according to manufacturer's instructions except that the total reaction volume was reduced 2.5-fold. PCRs were performed with cDNA quantity corresponding to 0.1 mm of tubules. No DNA was detectable in samples that did not undergo reverse

transcription or in blank run without cDNA. In each run a standard curve was obtained using serial dilution of stock cDNA prepared from mouse kidney RNA.

2.7. Immunocytochemistry

Transfected cells were washed 2 times in PBS++ (containing 100 μM CaCl2 and 1 mM MgCl2) fixed in 4% paraformaldehyde (stock solution 16%, EMS Hatfield, PA) for 10 min at room temperature and washed again twice in PBS++. For immunolabeling, fixed cells were permeabilized in goat serum dilution buffer (16% goat serum, 20 mM Na3PO4 pH 7.4, 450 mM NaCl, 0.3% Triton X-100) and incubated for 30 min at room temperature with rabbit polyclonal anti-Flag antibodies (1/200, Sigma) and with a mouse monoclonal antibody directed against the protein disulphide-isomerase enzyme (1/200, Affinity Bioreagents) followed by a 30 min incubation with FITC-conjugated anti-rabbit and TRITC anti-mouse IgG (1/200, Sigma). After mounting with Vectashield mounting medium (Vectorlabs), cells were visualized on a conventional fluorescence microscope.

2.8. Cloning and mutants preparation

2.8.1. Constructs

According to the released sequence of the mouse mPRα (GenBank AF313618) we have designed sense 5′ primers covering the first ATG tailed with the coding sequence of the Flag peptide (DYKDDDDK) and containing an EcoR I restriction site and antisense 3′ primers covering the stop codon and containing a Xho I restriction site. Touchdown PCRs (High Fidelity, Roche) were performed using total mouse kidney cDNA (gift from L. Cheval) as a template. cDNA fragment was then digested by EcoR I and Xho I and ligated in pCDNA3.1 Neo expression vector. Sequence was confirmed by full DNA sequencing (Service de séquencage, Institut Cochin).

By analyzing the sequence of mPRα and taking into account its putative tertiary structure as proposed by Zhu et al. [6], we observed that the intracellular C-terminal tail contained two possible ER retention/ER retrieval motifs, namely a double arginine at position 337/338 and a −KXX sequence at the C-terminal extremity that resembles a di-lysine motif. By directed mutagenesis, we created a double mutant NFlag-mPRα RR/AA-K343stop (RR/AA-K343st) in which we have replaced the di-arginine motif by two alanines and the lysine 343 by a stop codon leading to a 3 amino-acid truncation at the C-terminal end. We, also, created single motif mutants, namely NFlag-mPRα RR/AA, NFlag-mPRα K343stop, NFlag-mPRα K343A and NFlag-mPRα K345A. All mutants were confirmed by DNA sequencing (Service de séquencage, Institut Cochin).

3. Results

3.1. Renal distribution and cellular localization of mPRα and mPRγ

The presence of mRNA coding for mPRα and γ isoforms in the kidney was previously shown by Zhu et al. [6] and confirmed by analysis of renal transcriptome [11]. We confirmed these observations by Western blot analysis using our own generated antibodies directed against either mPRα or mPRγ. As shown in Fig. 1A, mPRα antibody (SR035) detected a unique band at an apparent molecular weight of 42 kDa in male (lane 1) and female (lane 2). Pre-immune serum (Fig. 1A, lanes 3–4) and specific blocking peptide (lanes 5–6) confirmed the specificity of our antibody. Regarding mPRγ, SR036 antibody recognized two main bands, one at 39 kDa and the other at 59 kDa, both in males and females (Fig. 1B, lanes 1–2). The 39 kDa-band has the expected size for mPRγ and is not present neither with the pre-immune serum ((Fig. 1B, lanes 3–4) nor after incubation with the blocking peptide (Fig. 1B, lanes 5–6). The band at 59 kDa is more difficult to interpret since it seems present with the pre-immune

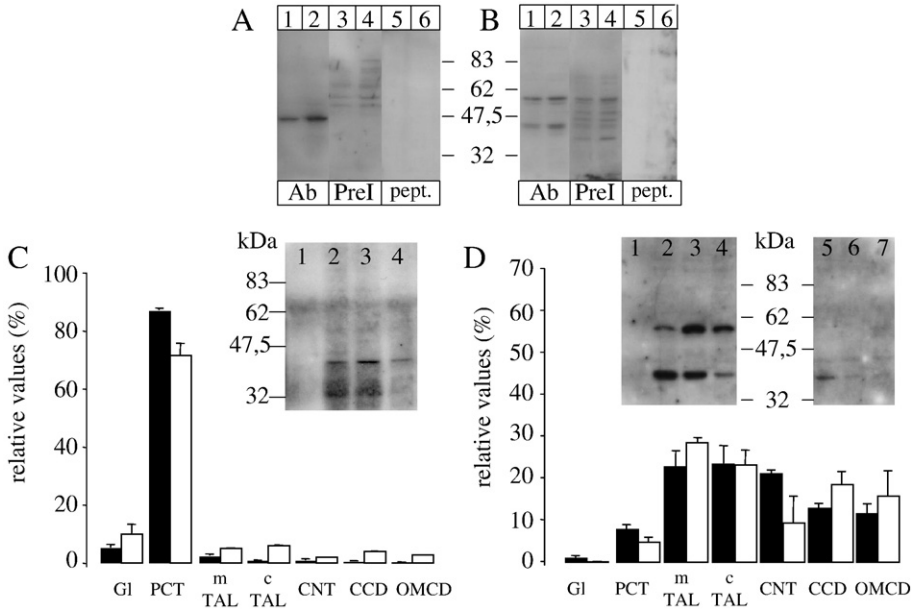

Fig. 1. mPRα and mPRγ expression in mouse kidney and distribution along the nephron. (A) Immunoblots of membrane fraction preprations from male and female kidneys using mPRα antibody (lanes 1 and 2), pre-immune serum (lanes 3 and 4) or mPRα antibody previously incubated with its specific blocking peptide (lanes 5 and 6). (B) Same as (A) but using mPRγ antibody and its respective pre-immune serum and blocking peptide. Pools of 20–30 segments of different types of the nephron structures (or 20 glomeruli) have been manually microdissected, measured and lysed for preparation of RNA from male (black bars) or female (white bars) mouse kidney (Gl.: glomeruli, PCT: proximal convoluted tubules, m and cTAL: medullary and cortical thin ascending limb, CNT: connecting tubule, CCD: cortical collecting duct, OMCD: outer medullary collecting duct). After reverse transcription, cDNAs were diluted in water at 0.25 mm of tubule/μl. Real-time PCR was performed using specific primers for mPRα (C) or mPRγ (D) as described in Materials and methods. Results are mean ± SE of 5 different male mice and 2 female mice, expressed as percent of the total expression in all structures. Pools of 25 glomeruli (C, inset lane 1 and D, inset lane 1) or 20 segments of PCT, PST S2 (cortical proximal straight tubule), PST S3 (medullary proximal straight tubule) (C and D, inset lanes 2–4), CNT, CCD and OMCD (D, inset lanes 5–7) were microdissected and placed into Laemmli buffer before SDS-PAGE analysis and revealed either with mPRα antibody (A, inset) or with mPRγ antibody (B, inset). The results are representative of three independent experiments.

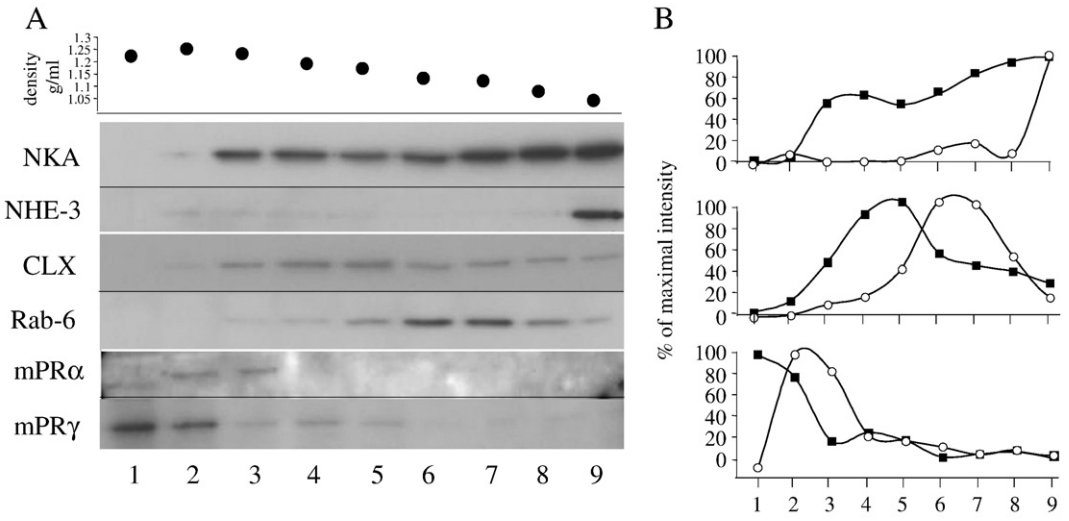

Fig. 2. Cellular localization of mPRα and mPRγ in kidney. (A) Upper panel: iodixanol density of the different fractions collected after centrifugation. Lower panel: 10 μg of proteins from each fraction was resolved by SDS-polyacrylamide electrophoresis and immunoblots using antibodies directed against the Na,K-ATPase α subunit (NKA), the Na,H-exchanger 3 (NHE-3), the calnexin (CLX), Rab-6, the mPRα and the mPRγ were performed. (B) Densitometric analysis of the immunoblots shown in (A). The maximal values obtained is considered as 100%. (B) Upper panel shows the distributions of the Na,K-ATPase α subunit (black squares) and of NHE-3 (white circles). Middle panel: distribution of calnexin (black squares) and of Rab-6 (white circles). Lower panel: distribution of mPRα (white circles) and of mPRγ (black squares). Representative data of 2 independent experiments is shown.

serum but disappear with the blocking peptide. This apparent discrepancy and the fact that the size is not compatible with mPRγ or a putative dimer, led us to consider this band as non-specific. The kidney is a heterogeneous organ containing both epithelial cells forming the nephron, smooth muscles and interstitial cells. The nephron, consists of several structures differing both at a functional and morphological point of view. RNA and protein extractions of nephron structures, after manual microdissection of male or female mouse kidney, allow the analysis of the distribution of particular transcripts or proteins. As shown in Fig. 1C, transcripts for mPRα mainly showed a localization in the proximal convoluted tubule both in male and female mouse. Western Blot analysis on segments from male mice kidney confirmed this distribution, showing that all segments of the proximal tubule (convoluted, straight S2 and S3, Fig. 1C, *inset*, lanes 2–4) expressed mPRα protein. Moreover, protein expression of mPRα cannot be observed in structure expressing no or low level of mRNA like glomeruli (Fig. 1C, *inset*, lane 1). Regarding mPRγ, it is expressed all along the nephron except in the glomerulus at the mRNA level (Fig. 1D) both in male and female mice. This is confirmed by Western blot analysis showing expression of the 39 kDa-band in proximal (PCT, PST S2 and S3, Fig. 1D, *inset*, lanes 2–4) and distal tubules (CNT, CCD and OMCD, lanes 5–7) of male mice. Here again, a mRNA-negative structures like glomeruli did not exhibit a band at 39 kDa. However, structure having similar level of mRNA like PCT and CCD displayed different amount of protein expression. In addition of the 39 kDa protein, we observed, in PCT, PST S2 and PST S3 the non-specific 59 kDa-band. Interestingly, the intensity of these two bands is not correlated. For instance, although PST S3 and CNT (lanes 4 and 5) expressed a similar amount of the 39 kDa-band, the 59 kDa-band is strongly detected in PST S3 but is absent in CNT. These results confirm the nature of the 39 kDa-band as the mPRγ specific band and the 59 kDa-band as the non-specific signal.

To analyze in which cellular compartments the mPRα and γ are localized, we have separated membrane vesicles by buoyant density in pre-formed continuous iodixanol gradients. In addition to the results of immunoblots (Fig. 2A), the intensity of each band was measured and their relative values is depicted in Fig. 2B. For clarity, plasma membrane markers were grouped in the upper panel, the markers of internal compartments are presented in the middle whereas the mPRs are in the lower panel. As shown, in Fig. 2A (upper panel) iodixanol gradients were generated (fractions from a density of 1.06 g/ml to 1.26 g/ml) which allowed to separate plasma membrane vesicles (Fig. 2A, NaK-ATPase and NHE-3 and Fig. 2B, upper panel), endoplasmic reticulum and Golgi apparatus compartments (Fig. 2A, calnexin and

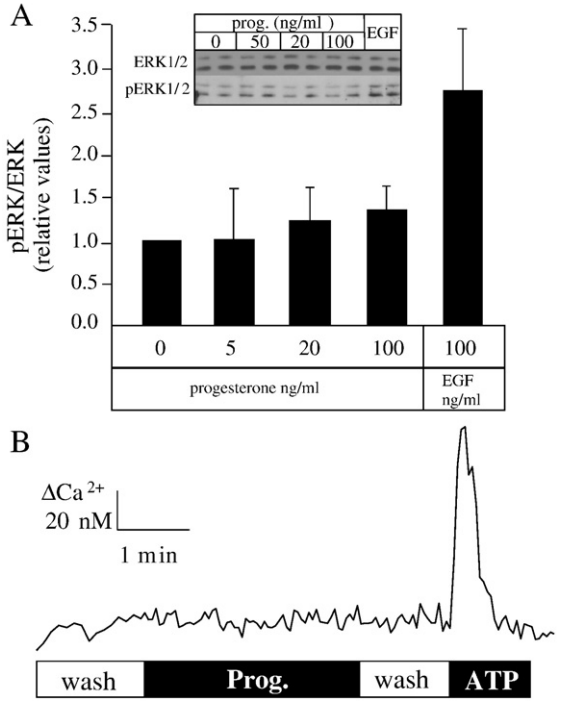

Fig. 3. Functionality of mPRα and mPRγ in proximal tubules. (A) After a pre-incubation of 30 min at 30 °C, pools of PCT were incubated without or with progesterone (5, 20 and 100 ng/ml corresponding to 16, 63 and 318 nM, respectively) or epidermal growth factor (EGF) for 15 min at 37 °C. The incubation is stopped by addition of Laemmli buffer before SDS-PAGE analysis. After p42/44 MAP kinase (ERK) and phospho-p42/44 MAP kinase (pERK) revelation by ECL technology (representative blot is shown in inset), the films were scanned and band intensity measured using ImageJ software (NCBI). Results are means of the ratio pERK/ERK±SE of 4 different male mice done in duplicate. (B) After loading with fura 2, PCT were mounted between two micropipettes and washed with modified Hanks medium until stabilization. Progesterone (1 μg/ml corresponding to 3.2 μM) or ATP (0.1 mM) were then added and fluorescence at both 340 and 380 nm was recorded. Shown is a representative example of 6 tubules from two mice.

rab-6, respectively and Fig. 2B, middle panel). Vesicles containing mPRα and mPRγ are very dense and have migrated at the bottom of the gradients (Fig. 2A mPRα and mPRγ and Fig. 2B, lower panel). There is clearly no overlap with vesicles containing plasma membrane markers or Golgi marker and a slight overlap with calnexin. These data suggest that mPRs are localized in dense vesicles that could have been generated from rough endoplasmic reticulum.

3.2. Signal transduction pathways

Having identified mPRα and γ in proximal tubules of both male and female mice, we tested whether progesterone activates the ERK kinase pathway in this structure. As a positive control, we showed that epidermal growth factor (EGF) increased the amount of phosphorylated ERK (Fig. 3A) after 15 min incubation. However, increasing concentrations of progesterone did not activate the MAP kinase pathway. Five minutes incubation was also tested but failed to induce ERK phosphorylation, either (data not shown). Another possible action of progesterone via mPR is to increase the cytosolic pool of calcium. However, although ATP (100 μM) induced an increase of intracellular calcium on freshly microdissected PCT (Fig. 3B), addition of progesterone (up to 1 μg/ml) did not.

3.3. Cellular location of mPRα and mPRγ in epithelial cells

To investigate in more details the cellular location of mPRα in epithelial cells, we have transiently transfected HEK293 cells with a mouse mPRα tagged with a N-terminus Flag epitope. As shown in Fig. 4Ab mPRα is localized in intracellular compartments. Co-labeling with an antibody directed against an endoplasmic reticulum marker (the protein disulfide isomerase PDI, Fig. 4Ac) and the anti-Flag antibody shows a strong co-localization (Fig. 4Ad, merge) indicating that mPRα is predominantly present in the endoplasmic reticulum. Similar results have been obtained in other cell lines (LLC-PK and MDCK) and using a C-terminal fused mPRα-GFP construct (data not shown).

3.3.1. Presence of ER retention motif in the C-terminal part of mPRα

Taken into account the proposed 7 transmembrane domains topology [6], we identified in the cytoplasmic C-terminus tail a double arginine (−RR-) and a C-terminal lysine motif (−QKTK) which may serve as a retention motif (see Fig. 4B, wild-type). To evaluate the involvement of these motifs in the ER retention of mPRα, we produced different mutants (see Fig. 4B). In a mutant where the double arginine was replaced by a double alanine and the KTK motif was removed, mPRα did not localize in the reticulum anymore but accumulated into large intracellular compartments (Fig. 4C, compare a and b). To discriminate between these two motifs, we mutated them separately. A NFlag-mPRα having only its double arginine motif replaced by two alanine residues showed the same localization as the NFlag-mPRα wild-type (Fig. 4C, compare a and c). On the other hand a truncated form, lacking the 3 last amino-acids (the −KTK motif) displayed the same expression profile than the double mutant (see Fig. 4Cb and d) indicating that this motif plays a role in the ER location of mPRα. To define exactly which amino-acids are important in the KTK motif, we mutated separately the K343 and the K345 into alanines.

As shown in Fig. 4Ce and f, NFlag-mPRα K343A but not K345A displayed localization similar to mPRα K343stop, indicating that lysine 343 is involved in the specific location of mPRα in the reticulum. To confirm this change of intracellular compartment, we performed a co-labeling with anti-Flag and anti-PDI antibodies on cells transfected with the NFlag-mPRα K343A mutant. As shown, in Fig. 4Dc, PDI displayed its classical diffuse labeling whereas K343A mutant is accumulated into big compartments (Fig. 4Db). The overlay (Fig. 4Dd) failed to establish a co-localization of both proteins.

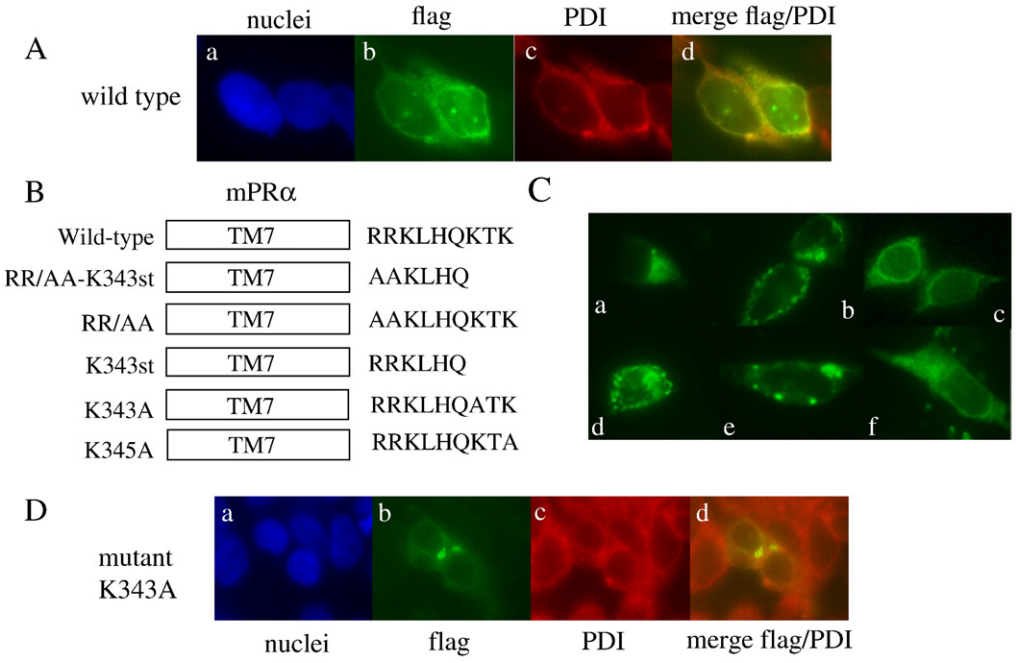

Fig. 4. Cellular localization of mPRα in transfected cells. HEK293 cells were transiently transfected with NFlag-mPRα (Aa, b, c and d)). Two days after transfection, cells were fixed with PFA 4% and subjected to DAPI labeling (Aa) and immunocytochemistry using rabbit anti-Flag (Ab) and anti-PDI antibodies (Ac) and observed under microscope (magnification 63×). Overlay of anti-Flag and anti-PDI labeling is shown (Ad). (B) Schematic representation of the mPRα C-terminus wild-types and mutants. HEK293 cells were transfected with NFlag-mPRα wild-type (Ca), RR/AA-K343stop (Cb), RR/AA (Cc), K343stop (Cd), K343A (Ce) and K345A (Cf) mutants. Two days after transfection, cells were fixed with PFA 4% and subjected to immunocytochemistry using rabbit anti-Flag antibody and observed under microscope (magnification 63×). (D) Cells expressing mutant NFlag-mPRα K343A was also co-labeled with anti-Flag antibody (Db) and anti-PDI (Dc). Nuclei were detected using DAPI (Da) and overlay of Flag and PDI labeling is shown (Dd).

4. Discussion

To characterize mPRα and mPRγ in the kidney, we have generated our own antibodies which can be used for Western blot analysis and detect a band at the expected apparent molecular weight (around 40 kDa) for both proteins. Our mPRα antibody does not revealed any band around 80 kDa as shown by Zhu et al. [6]. On the other hand, our mPRγ antibody revealed a 60 kDa-band that is considered as non-specific (see Results). These tools allowed us to demonstrate that membrane-bound progesterone receptor α and γ isoforms are expressed in kidneys of male and female mice. In addition to potential effect on renal functions during luteal phase or pregnancy in female, the identification of these receptors in male may suggest a more general and gender-independent action of progesterone. In male, circulating progesterone [12] is present and most likely originates from adrenals, since it appears as an intermediate in the biosynthesis of aldosterone and corticosterone [13] but, up to now, no physiological function has been reported for endogenous progesterone produced by male. The major expression of mPRα and mPRγ in proximal tubules may suggest that their activation is important for regulation of functions specific of these segments like protein, glucose, amino-acids or phosphate reabsorption.

In this study, we observed two major discrepancies compared with published observations, mainly by P. Thomas group [6–7,14–18]: 1/ mPRα and mPRγ localized in intracellular compartments, corresponding to endoplasmic reticulum (probably rough ER in native kidney) and 2/ progesterone did not activate ERK phosphorylation and did not increase intracellular Ca^{2+} in native structure expressing mPRα and mPRγ.

The localization of mPRα and mPRγ is based on fractionation of subcellular compartments by separation on density gradients. As expected vesicles formed from plasma membranes (basolateral or apical sides) are more abundant in the light fractions of the gradients, then followed by Golgi and endoplasmic reticulum fractions. It has to be noted that a part of the Na,K-ATPase α subunit co-migrates with calnexine-containing vesicles which may be the newly generated pool of Na,K-ATPase. This observation was done before by Cai et al. [19] using the same technique. Clearly, mPRα and mPRγ do not belong to plasma membrane vesicles since they are found exclusively at the bottom of the gradient in the heaviest fractions. We have not identify unambiguously the compartments in which mPRs are present but these very dense vesicles are likely to be rough endoplasmic reticulum. In transfected cells, using a different technical approach, we confirmed that mPRα is not localized at the plasma membrane but in internal compartments displaying a similar labeling than the protein disulfide isomerase enzyme.

In agreement with the present data, series of published results tend to confirm that mPR are not located at the plasma membrane. Ovine tagged mPRα localized mainly in the ER when expressed in CHO cells and in nuclear membrane of small and large luteal cells [18]. Similarly, Fernandes et al. [20] showed that wild-type or V5- or HA-tagged mPRα, when over-expressed in Cos-7 cells, localized almost exclusively into intracellular compartments. Finally, very recently, Krietsch et al. [8] using the same cell lines (MDA-MB-231) and the same constructs (human mPRα, β and γ) as Zhu et al. [6] have clearly shown that, in their hands, mPRs are present in intracellular compartments but not at the plasma membrane.

In this study, we provide a first explanation for the intracellular location of mPRα. Indeed, we show that a motif (a lysine at position −3 from the C-terminus end) present at the C-terminal extremity is responsible for the ER location of mPRα. This motif (−KXX) is a well established signature for ER retention or ER retrieval of protein specific of the reticulum [21,22] although it is generally composed of a double lysine motif. Zerangue et al. [23] have analyzed the effect of amino-acid residues surrounding the lysine at position −3 for ER retention. Taken into account their results, it turns out that amino-acid sequence of mouse mPRα ($-H_{-5}Q_{-4}K_{-3}T_{-2}K_{-1}$) possesses all requirements for inducing an ER retention since a lysine at position −1 and a glutamine at −4 have a neutral effect whereas a threonine at −2 and an histidine at −5 increase the probability for an ER retention. The C-terminus of mPRα also contains a −RR-motif that is not active as an ER-localization signature (at least in the 3 cell lines we have tested). This may be due to the very close position of this signal to the membrane as predicted by the topology model described by Zhu et al. [6], since RR motif needs at least to be 16 to 46 Å from the membrane bilayer [24]. Mechanisms of retention by C-terminal lysine motifs involve interaction with COPI proteins that promote retrograde transport from the Golgi apparatus back to the ER. Regarding mPRα, the removal of this motif led to accumulation into Golgi-like structure but not to plasma membrane expression indicating that −KXX is, indeed, necessary for ER localization but is not the only motif involved in intracellular expression of this receptor. Exit of intracellular compartments of mPRα, if it should occur, may be strongly regulated and may depend on association with a yet unknown protein that would hide the lysine motif and promote plasma membrane targeting.

Regarding the functionality of these receptors and their ability to transduce a signal via activation of ERK phosphorylation or an increase of intracellular Ca^{2+}, our results are, again, in complete agreement with results obtained by Krietsch et al. [8] who did not observe such activations. Noteworthy, Brunette and Leclerc [5] have tested the rapid response of proximal and distal tubule preparations to progesterone. Although they observed a stimulation of the calcium transport and a decrease of Na uptake by the distal tubules, they failed to observe any change for these parameters with proximal tubules. Therefore, despite the presence of both isoforms of mPR in this structure, progesterone does not modulate cytosolic calcium concentration, ERK kinase pathway and sodium and calcium transport in proximal tubules.

All together, these results showed that potential membrane progesterone receptors are expressed in renal proximal tubules of male and female mice but further experiments will be necessary to assess their physiological role.

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