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The tubulogenic effect of aldosterone is attributed to intact binding and intracellular response of the mineralocorticoid receptor

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Abstract: Little is known about the extra- and intracellular stimuli inducing renal stem/progenitor cells to develop into three-dimensionally structured tubules. To study this specific development in a controlled environment, we used an advanced culture technique. Embryonic tissue derived from neonatal rabbit kidney was placed in a perfusion culture container at the interface of an artificial interstitium made of a polyester fleece. Culture was carried out in chemically defined Iscove's Modified Dulbecco's Medium (IMDM) for 13 days. Development of tubules was histochemically detected on cryosections labeled with Soybean Agglutinin (SBA). The experiments showed that aldosterone exerts a specific tubulogenic effect. Application of aldosterone (1×10^{-7} M) raised numerous SBA-labeled tubules, while in the absence of the steroid hormone the development of tubules was lacking. Specificity of hormone action was analyzed by the use of aldosterone antagonists. Administration of spironolactone (1×10^{-4} M) and canrenoate (1×10^{-5} M) completely inhibited the development of tubules. Finally, disrupting the intracellular molecular complex of the mineralocorticoid receptor (MR) and heat shock proteins by geldanamycin (2 μg/ml) prevented the development of tubules. Our results suggest that the tubulogenic effect induced by aldosterone is attributed to both hormone binding and an undisturbed intracellular response of the MR.

Keywords: Stem/progenitor cells, aldosterone, mineralocorticoid receptor, perfusion culture, artificial interstitium, kidney

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1 Introduction

The development of stem/progenitor cells into structured tubules within the growing kidney is a complex process, since renal parenchyme is derived from two very different kinds of stem cells. On the one hand the nephrogenic mesenchymal stem cells first condensate to form a Comma-shaped and subsequently an S-shaped body [1–3]. The cells located in the capsule-orientated part of the S-shaped body form the nephron tubules, while cells orientated towards the medulla generate the glomerulus. On the other hand, the collecting duct and the connecting tubule are derived from epithelial stem cells located within the tip of each collecting duct ampulla [4, 5].

After determination, the progenitor cells have to form three dimensionally structured tubules. Principally, the development of tubules can happen by wrapping, budding, cavitation, cord or cell hollowing [6–8]. The collecting duct develops by budding [4]. Which of the above processes are involved in the formation of nephron tubules is unknown. The developing tubules show a functional segmentation with individual cell populations of the proximal tubule, the loop of Henle and the distal tubule. Further on, portions of the proximal and distal tubule form convolutes, while the segments of the loop of Henle show a straightforward growth from the cortex towards the medulla. Each segment of the nephron tubule is composed of a single cell type, while the connecting and the collecting duct tubule consist of a heterogenous cell population [9–12]. Finally, tubules derived from all of these processes have a polarized epithelium, a defined lumen and a continuous basal lamina.

Because of the severe shortage of donor organs available for transplantation we are elaborating strategies to help patients suffering from diseased or injured kidneys as proposed earlier [13–17]. In this context, our present experimental work is focused on exploring methods to stimulate the regeneration of renal parenchyme derived from renal stem cells. This broad research field requires intensive investigation of the environmental needs of renal stem/progenitor cells during development.

The present experiments were designed to assess aldosterone-induced mediators involved in the three dimensional development of tubules derived from renal stem/progenitor cells. Previous publications of our group proved the feasibility of generating tubules derived from renal stem/progenitor cells at the interface of an artificial interstitium made of a polyester fleece within a perfusion culture container [18, 19]. We found that the generation of renal tubules depends specifically on the presence of aldosterone. The tubulogenic effect of the steroid hormone is concentration-dependent [20] and can not be mimiked by precursors of the aldosterone synthesis pathway [21]. Applying this innovative technique tubules develop under controlled *in vitro* conditions. The coating of embryonic renal tissue by extracellular matrix proteins is not necessary. A further advantage is that the experiments can be performed in chemically defined medium (IMDM) without serum, protein or protein lysate supplementation.

The experiments presented in this paper were performed to obtain insights into the first steps of tubulogenic signalling induced by aldosterone. The new data shows that the

tubulogenic effect of aldosterone is antagonised to various degrees by spironolactone and canrenoate. Further on, the development of tubules can be blocked by geldanamycin. Our findings suggest that an intact binding of aldosterone to the mineralocorticoid receptor (MR) followed by an intracellular association of the receptor to heat shock proteins is essential for tubulogenic signalling.

2 Material and methods

2.1 Isolation of embryonic tissue containing renal stem/progenitor cells

One day old New Zealand rabbits were anesthetized with ether and killed by cervical dislocation. Both kidneys were removed immediately. Each kidney was dissected in two parts. By stripping off the capsula fibrosa with fine forceps a thin embryonic tissue layer was harvested containing nephrogenic mesenchymal stem cells, numerous collecting duct ampullae and S-shaped bodies [22].

2.2 Perfusion culture of renal tubules at the interface of an artificial interstitium

For a perfusion culture period of 13 days the isolated embryonic tissue was placed in a container with horizontal flow characteristics (Minucells and Minutissue, Bad Abbach, Germany; www.minucells.de) as earlier described [18–21]. To minimize the dead space fluid volume within the container, the tissue was kept between two layers of highly porous biocompatible polyester fleece (Walraf, Grevenbroich, Germany) used as an artificial interstitium. Thus, the embryonic tissue and the polyester material were in close contact. Fresh serum-free Iscove´s Modified Dulbecco´s Medium (IMDM, I) including phenol red (GIBCO/Invitrogen, Karlsruhe, Germany) was provided. An antibiotic-antimycotic solution (1%, GIBCO) was added to the culture media. Up to 50 mmol/l HEPES (H, GIBCO) was used in the medium to maintain a constant pH of 7.4 under atmospheric air containing 0.3% CO_2 during culture for 13 days. The medium was continuously perfused at a rate of 1 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). To maintain a constant temperature of 37°C, the perfusion culture container was placed on a thermoplate (Medax, Kiel, Germany) and covered by a transparent lid. Aldosterone (A) was obtained from Fluka (Taufkirchen, Germany) and was applied in all series at a concentration of 1×10^{-7} M in chemically defined IMDM. Antagonists such as spironolactone and canrenoate were obtained from Sigma (Taufkirchen, Germany). Geldanamycin was ordered from USBiological (Swampscott, Massachusetts, USA) as previously used [23].

2.3 Lectin- and antibody-labeling

For histochemistry cryosections of 20 μm thickness were fixed in ice-cold ethanol. After washing with phosphate buffered saline (PBS) the sections were blocked with PBS con-

taining 1% bovine serum albumin (BSA) and 10% horse serum for 30 minutes. For lectin-labeling the specimens were exposed to fluorescein-isothiocyanate (FITC)-conjugated Soybean Agglutinin (SBA, Vector Laboratories, Burlingame, USA) diluted 1:2000 in blocking solution for 45 minutes as described earlier [18, 19]. Following several washes in PBS, the sections were embedded with Slow Fade Light Antifade Kit (Molecular Probes, Eugene, USA) and then analyzed using an Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany). Fluorescence images were made by a digital camera with a standard exposure time of 1.3 seconds and thereafter processed with Corel DRAW (Corel Corporation, Otawa, Canada). Earlier experiments showed that SBA recognised only matured collecting duct tubules, while isolated embryonic tissue was lacking binding [20, 21]. It was demonstrated that antibodies for markers such as occludin, Na/K-ATPase, cytokeratin 19, TROMA-I and laminin $\gamma 1$ reacted positively on developed tubules indicating a high degree of polar differentiation [20].

2.4 Histological examination

Developed tubules were analyzed as whole mount specimens and as cryosections in longitudinal- or cross-section view. Tubular structures exhibited polarized cells, a clearly visible lumen and a basal lamina bordering the basal aspect as a smooth outer surface.

2.5 Scoring

According to the histochemical profile of SBA-label we used a specific score for evaluation of tubule development. The presence of tubules, intensity of label, lumen formation, the existence of a basal lamina, number and length were registered. Each criterion was scored with one point resulting in a possible maximum of 6 points (Table 1).

2.6 Amount of cultured constructs

A total of 214 embryonic tissues were isolated and generated in culture for the present study. All of the experiments were performed at least in triplicate. The data given in the text are the mean of at least three independent experiments. All experiments are in accordance of the animal ethics committee, University of Regensburg, Regensburg, Germany.

2.7 Detection of the mineralocorticoid receptor

Freshly isolated embryonic renal tissue samples were homogenized in lysis buffer as earlier described and loaded on an acrylamide gel for SDS-electrophoresis [19]. In order to detect the mineralocorticoid receptor separated proteins were electrophoretically transferred to nitrocellulose transfer membranes (Whatman, Schleicher & Schuell, Dassel, Germany)

and probed with primary antibody anti-rMR1-18 1D5 as earlier described [24]. To visualize the antigen-anti-rMR1-18 1D5 complex a secondary antibody goat-anti-mouse IgG (Dianova, Hamburg, Germany) labeled with horseradish peroxidase (HRP) was used. Addition of Western Lightning Chemiluminiscence Reagent (PerkinElmer, Boston, USA) to the blot membrane resulted in light emission, which was captured on a medical X-ray film (Fuji Photo Film, Düsseldorf, Germany). The resulting signals were visualized in a dark room with a negative developer. Films were documented with a Scan Jet 6200 C scanner (Hewlett Packard, Greely, USA). Assessment of apparent molecular weight (kDa) was performed with SDS-PAGE standards (Bio-Rad Laboratories, Hercules, USA and Biolabs, Frankfurt/Main, Germany), which were run in separate lanes of each gel plate.

	ant [c]	tubules	intensity	lumen	bl	amount	length
A		+	+	+	+	+	+
A + Spiro	(1×10^{-7} M)	+	+	+	+	+	+
A + Spiro	(1×10^{-6} M)	+	+	+	+	+	+
A + Spiro	(1×10^{-5} M)	+	+	+	-	-	-
A + Spiro	(1×10^{-4} M)	-	-	-	-	-	-
Spiro	(1×10^{-7} M)	+	-	+	+	-	+
Spiro	(1×10^{-6} M)	+	-	+	+	-	-
Spiro	(1×10^{-5} M)	-	-	-	-	-	-
Spiro	(1×10^{-4} M)	-	-	-	-	-	-
A + Can	(1×10^{-7} M)	+	+	+	+	+	+
A + Can	(1×10^{-6} M)	+	+	+	-	-	-
A + Can	(1×10^{-5} M)	+	-	+	-	-	-
A + Can	(1×10^{-4} M)	-	-	-	-	-	-
Can	(1×10^{-7} M)	-	-	-	-	-	-
Can	(1×10^{-6} M)	-	-	-	-	-	-
Can	(1×10^{-5} M)	-	-	-	-	-	-
Can	(1×10^{-4} M)	-	-	-	-	-	-

Table 1 Scoring the generation of SBA-labeled tubules: A - aldosterone, Spiro - spironolactone, Can - canrenoate, ant - antagonist concentration, bl - basal lamina.

3 Results

To generate renal tubules an innovative culture technique was used. The developing embryonic renal tissue was kept inside a perfusion culture container filled with a polyester fleece as an artificial interstitium (Figure 1a). The standard medium was serum-free IMDM (I) containing HEPES (H) as biological buffer. For quick detection the development of tubules was screened with fluorescent SBA on whole mount specimens. Depending on the treatment we observed numerous tubules at the interface of an artificial interstitium (Figure 1b). The generated tubules had a lumen and a basal lamina (Figure 1c,d).

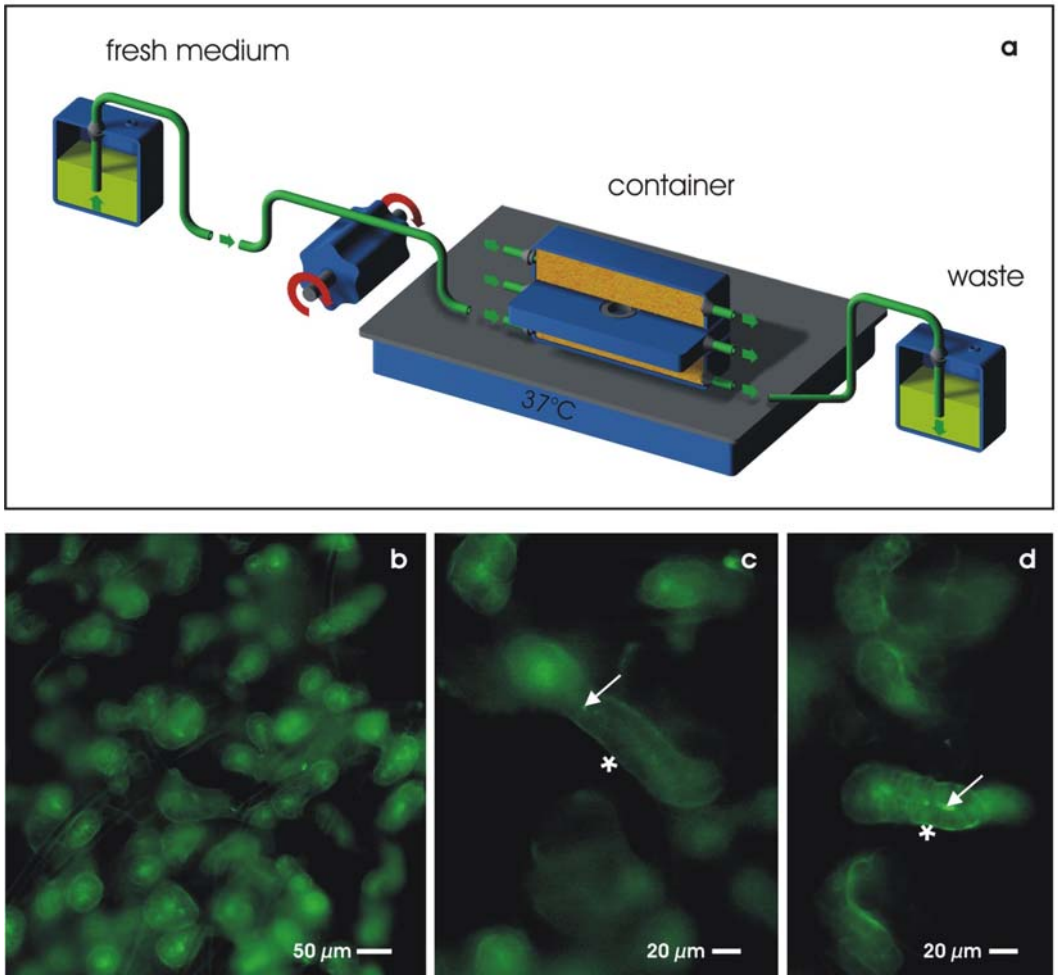

Fig. 1 Schematic illustration of perfusion culture and generation of tubules at the interface of an artificial interstitium. a) Fresh culture medium is constantly transported by a peristaltic pump (1ml/h) into a perfusion culture container on a thermoplate at 37°C. The used medium is collected in a waste bottle. b) SBA-labeled tubules in aldosterone containing medium after 13 days of culture. c) and d) Higher magnification of generated tubules. The asterisk indicates the basal lamina, while the arrow marks a lumen.

3.1 Tubulogenic effect of aldosterone

In a first series of control experiments we could show that culture of renal stem/progenitor cells for 13 days in IMDM (I) with HEPES (H) but without aldosterone resulted in the formation of disintegrated tissue lacking SBA-label [20, 21]. Only thin rows of cells and few cell islets were developed. According to the histological score, no SBA-positive tubules were observed. In contrast, as revealed in cryosections, administration of aldosterone (A) to IH resulted in numerous SBA-labeled tubules (Figure 1b-d, Table 1). In cross and longitudinal cryosections we observed numerous developed tubules with a lumen and a basal lamina (Figure 2a',a", 3a',a", 4a',a"). For control, in the neonatal kidney only the maturing neck and matured shaft of a collecting duct ampulla showed a cellular reaction

with SBA. However, the tip of the ampulla and the surrounding embryonic tissue were negative using the described titer of SBA. This embryonic zone was isolated and used for culture. It was completely negative for the SBA-label after isolation [21]. This was a clear indication that the developed tubules derived from the collecting duct ampulla known as epithelial stem cells of the kidney [18, 22].

3.2 MR and the interference of antagonists

The aim of the present experiments was to get first insights into the tubulogenic signalling induced by aldosterone and to study the role of the MR in this process. To date it is unknown if the tubulogenic effect of aldosterone is exclusively mediated via the mineralocorticoid receptor or if it is an unspecific side effect of the steroid hormone in embryonic renal tissue. Consequently, experiments were performed to interfere with the binding of aldosterone to the MR. For this purpose, MR antagonists were used such as spironolactone (Figure 2) and canrenoate (Figure 3).

Use of low dose spironolactone (1×10^{-7} M, Figure 2b',b"; 1×10^{-6} M, Figure 2c',c") in the presence of aldosterone did not affect the development of SBA-labeled tubules. However, application of a higher concentration of spironolactone (1×10^{-5} M) had an inhibitory effect and resulted in a change in tubule development. The number of structured tubules was reduced. Further on, SBA-labeled structures were found to fuse with each other (Figure 2d') and to form extended cell clusters (Figure 2d"). In contrast, the presence of 1×10^{-4} M spironolactone in aldosterone-containing medium completely prevented the development of SBA-labeled tubules (Figure 2e',e").

Further experiments elucidated whether application of canrenoate (Figure 3) had the same effect on aldosterone-induced tubule development as observed with spironolactone (Figure 2). Again, the control series with aldosterone revealed the development of numerous SBA-positive tubules (Figure 3a',a"). Application of 1×10^{-7} M canrenoate in the aldosterone-containing medium did not affect the development of tubules (Figure 3b',b"). However, administration of 1×10^{-6} M (Figure 3c',c") and 1×10^{-5} M (Figure 3d',d") canrenoate drastically reduced SBA-labeled structures. The use of 1×10^{-4} M canrenoate in aldosterone-containing medium resulted in a complete lack of SBA-labeled tubules (Figure 3e',e").

Comparing both series using aldosterone antagonists, we found that lower concentrations of canrenoate (1×10^{-5} M, Figure 3d',d") compared to spironolactone (1×10^{-4} M) are needed for comparable inhibition of tubulogenic development (Figure 2e',e"). The arise of tubular fusion (Figure 2d') and cell cluster formation (Figure 2d") was only observed in series with spironolactone (1×10^{-5} M) and was not found in experiments performed with canrenoate (Figure 3b',b" - e',e"). There is a different potency of spironolactone and canrenoate, which should be mentioned.

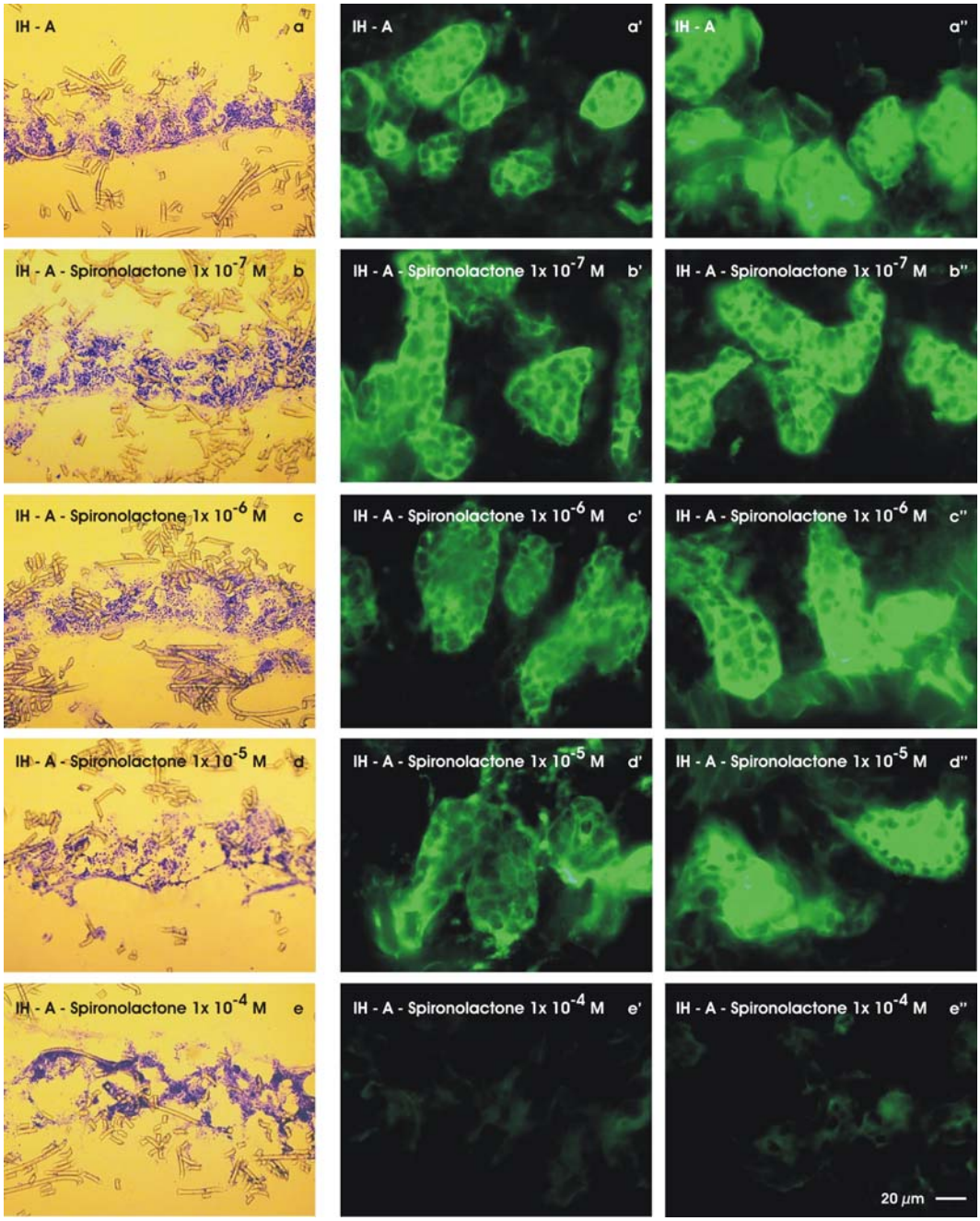

Fig. 2 Effect of aldosterone alone or in combination with spironolactone on the generation of renal tubules. a) - e) Cryosections stained with Toluidine blue, while a') - e') and a") - e") reveal histochemistry of SBA-labeled structures. a,a',a") aldosterone administration, b,b',b") aldosterone + spironolactone (1 x 10-7 M), c,c',c") aldosterone + spironolactone (1×10^{-6} M), d,d',d") aldosterone + spironolactone (1×10^{-5} M) and e,e',e") aldosterone + spironolactone (1×10^{-4} M). The results demonstrate that application of aldosterone shows an intensive tubulogenic effect (a',a"), while it is completely antagonized by spironolactone (1×10^{-4} M) (e',e").

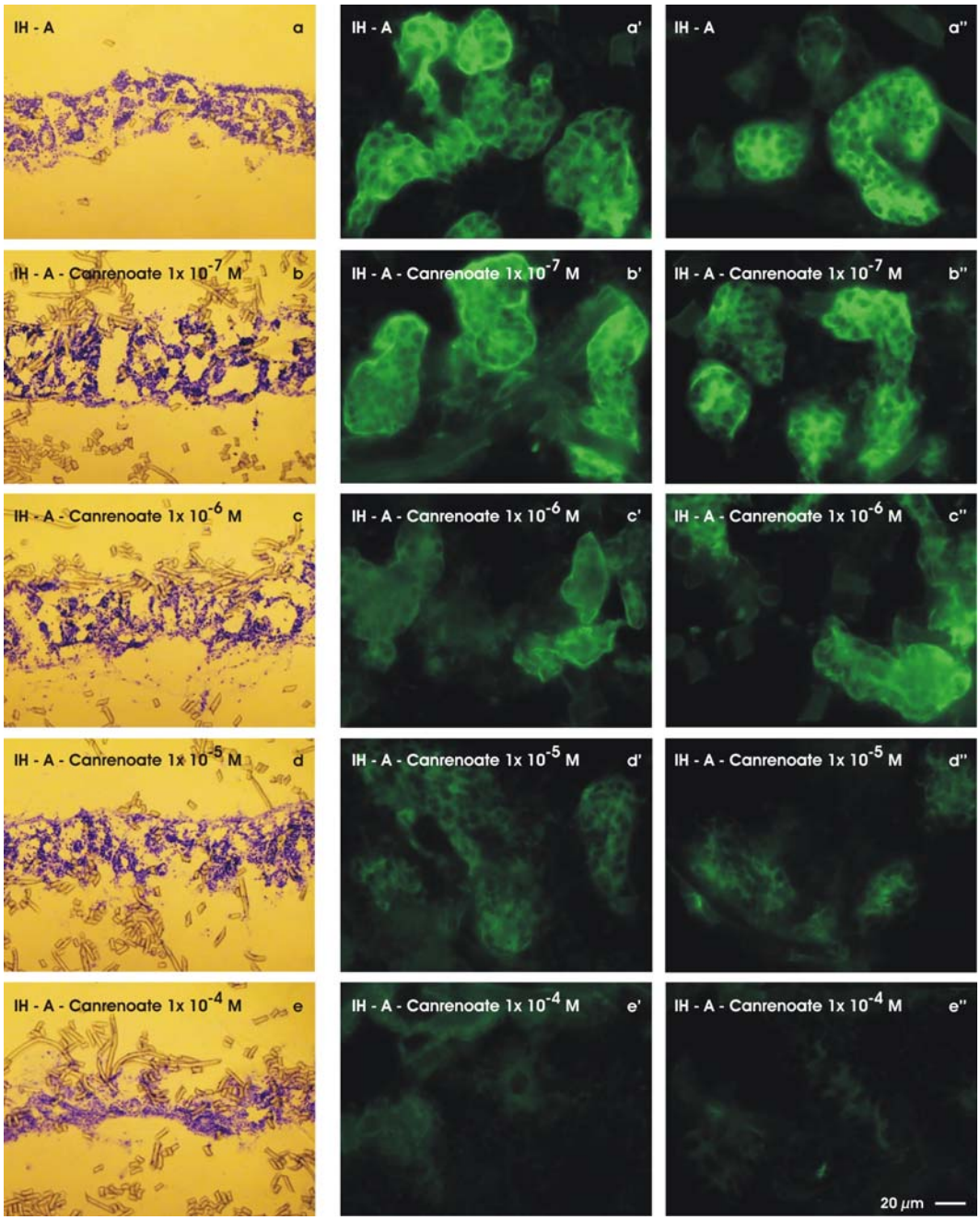

Fig. 3 Effect of canrenoate and aldosterone on the generation of renal tubules. a) - e) Cryosections stained with Toluidine blue, while a') - e') and a") - e") reveal histochemistry of SBA-labeled structures. a,a',a") For control, aldosterone application, b,b',b") aldosterone + canrenoate (1×10^{-7} M), c,c',c") aldosterone + canrenoate (1×10^{-6} M), d,d',d") aldosterone + canrenoate (1×10^{-5} M) and e,e',e") aldosterone + canrenoate (1×10^{-4} M) administration. The results show that application of aldosterone shows an intensive tubulogenic effect (a',a"), while this effect is inhibited (c',d',c",d") and finally, completely antagonized by canrenoate (1×10^{-4} M) (e',e").

In the previous experimental series aldosterone and spironolactone (Figure 2) or canrenoate (Figure 3) were applied for the same period of time. In contrast, in the following series canrenoate was administered first, followed by aldosterone after 1 to 4 hours (Figure 4). In the case that aldosterone exerts MR-independent effects on the development of tubules it should become apparent after pre-blockade of the MR by canrenoate. However, our results clearly demonstrated that blockade of the MR by canrenoate followed by aldosterone application suppressed the development of SBA-labeled tubules. The tubulogenic effect of aldosterone appears to specifically depend on an intact interaction with the MR.

3.3 Possible activation of MR by antagonists

To exclude that MR antagonists may have some unspecific tubulogenic potential, the MR antagonist spironolactone (Figure 5a-d) and canrenoate (Figure 5e-h) were administered during the entire culture period. Use of low doses of spironolactone in the range of 1×10^{-7} M (Figure 5a) and 1×10^{-6} M (Figure 5b) showed the generation of multiple tubules but only few of them showed SBA-label. In contrast, concentrations of 1×10^{-5} M (Figure 5c) and 1×10^{-4} M (Fig. 5d) failed to form tubules as obtained in series with aldosterone application (Figure 1b-d, Fig. 2a',a", 3a',a", 4a',a"). Application of canrenoate in the range of 1×10^{-7} M (Figure 5e), 1×10^{-6} M (Figure 5f), 1×10^{-5} M (Figure 5g) and 1×10^{-4} M (Figure 5h) did not result in the generation of SBA-labeled tubules. These results strongly suggest that MR antagonists are not able to promote a tubulogenic effect as observed after aldosterone treatment (Figure 6).

3.4 MR and the interference with intracellular signalling

To date no information is available regarding the signalling between MR and the intracellular proteins that may mediate the tubulogenic action of aldosterone. Consequently, a further set of experiments was performed to investigate the effect of aldosterone in the presence of geldanamycin (Figure 7). This substance disturbs the binding between the MR and a set of heat shock proteins [23]. Application of geldanamycin (2 μg/ml) alone (Figure 7a) or in combination with aldosterone (Figure 7b) did not result in the development of SBA-labeled tubules. Instead, only some small cell clusters were growing in close vicinity to the polyester fibers of the artificial interstitium. These experiments showed for the first time that the tubulogenic effect of aldosterone can specifically be blocked by interfering with the interaction of the MR and the associated set of heat shock proteins.

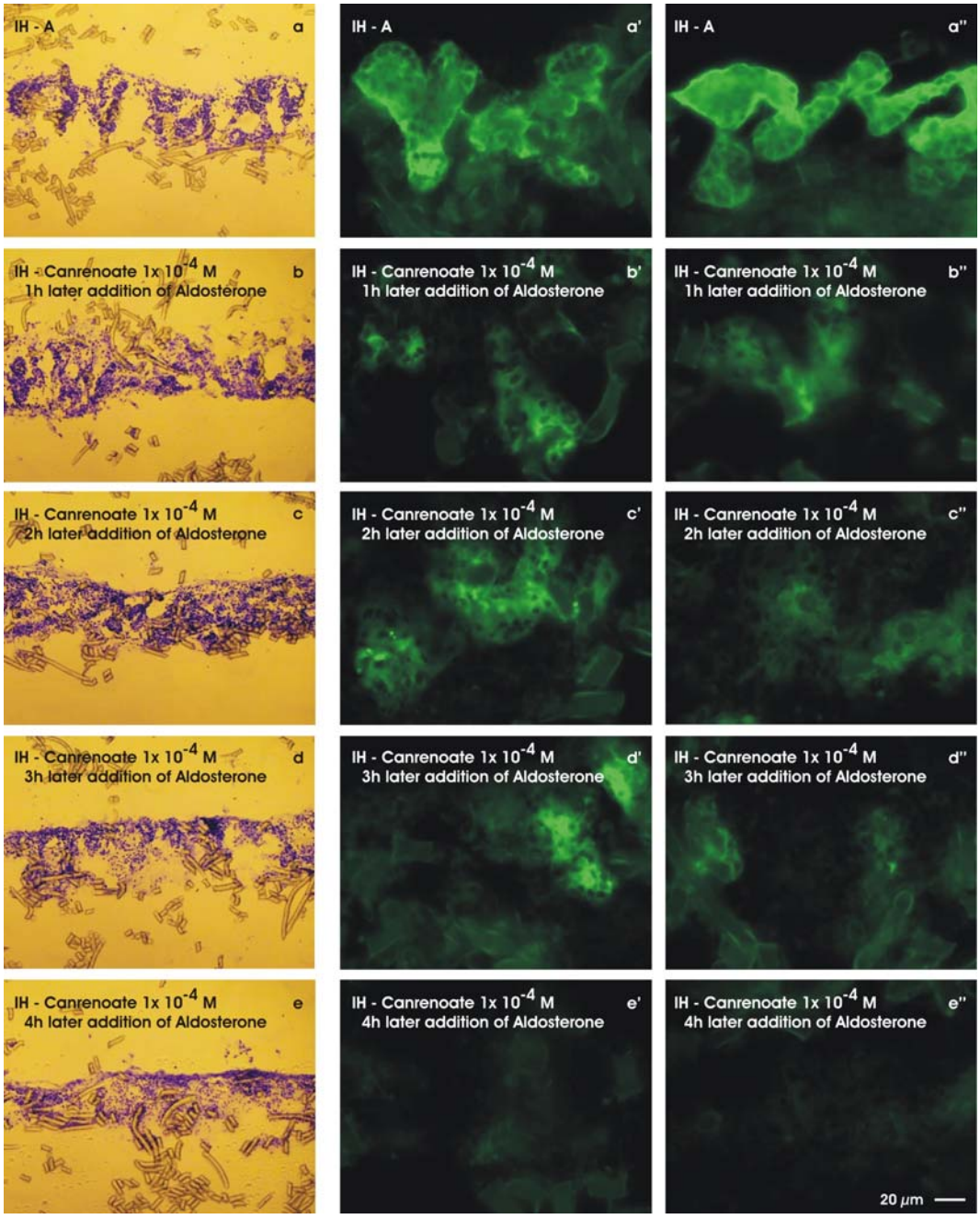

Fig. 4 Effect of canrenoate on the generation of renal tubules followed by aldosterone application after 1 to 4 hours. a) - e) Cryosections stained with Toluidine blue, while a') - e') and a") - e") reveal histochemistry of SBA-labeled structures. a,a',a") For control aldosterone application. Canrenoate (1×10^{-4} M) incubation was followed by aldosterone administration after 1 (b,b',b"), 2 (c,c',c"), 3 (d,d',d") and 4 (e,e',e") hours. While application of aldosterone shows an intensive tubulogenic effect (a',a"), it is completely blocked by canrenoate followed by aldosterone administration (b'-e', b"-e").

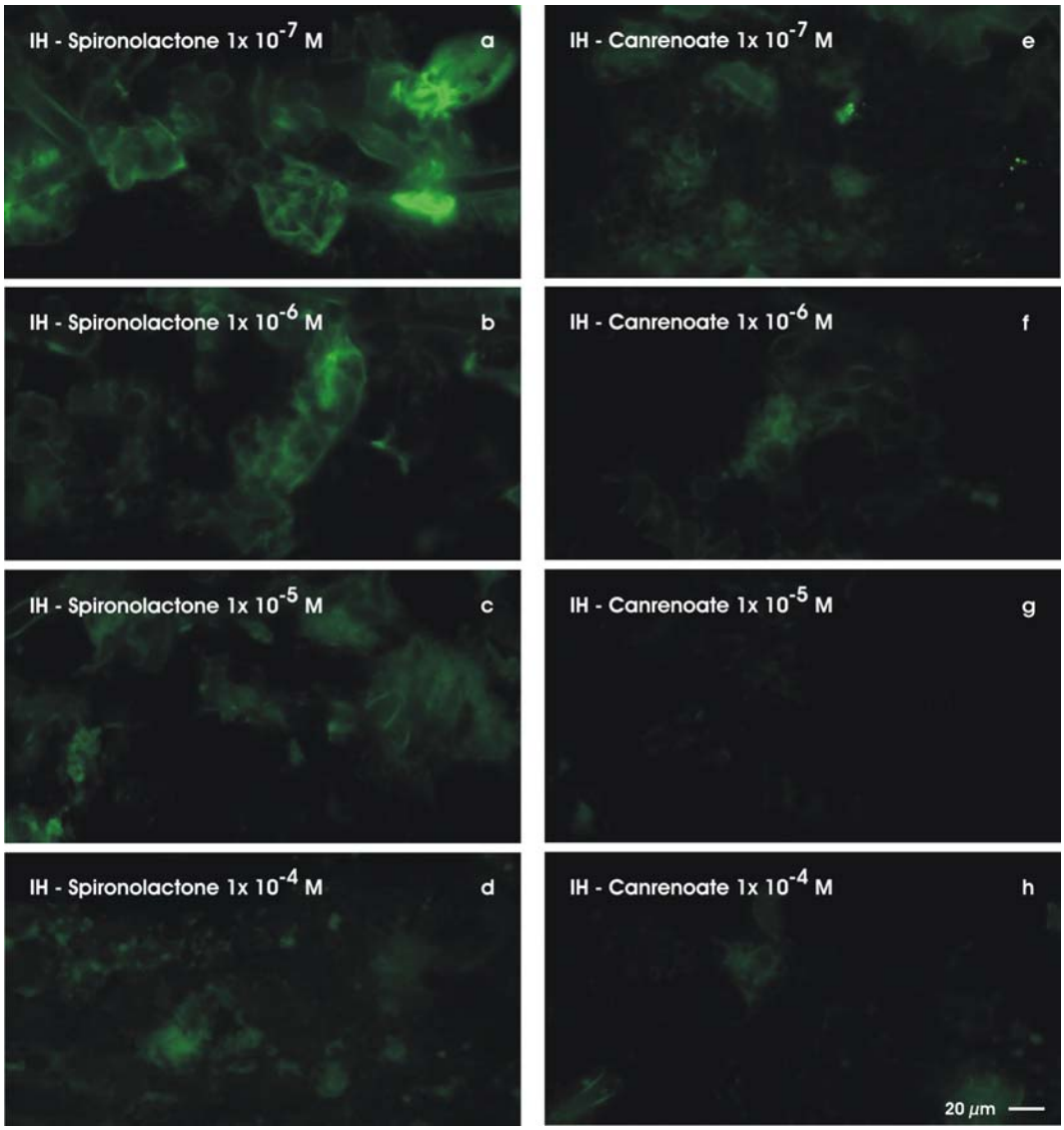

Fig. 5 Effect of spironolactone and canrenoate on the generation of SBA-labeled tubules. Use of a) 1×10^{-7} M and b) 1×10^{-6} M spironolactone resulted in the genereation of few SBA-labeled tubules, while c) 1×10^{-5} M and d) 1×10^{-4} M spironolactone did not reveal SBA-labeled tubules. Application of e) 1×10^{-7} M, f) 1×10^{-6} M, g) 1×10^{-5} M and h) 1×10^{-4} M canrenoate did not show a tubulogenic effect.

3.5 Detection of MR

The last set of experiments was performed to localize MR in embryonic renal tissue by immunohistochemical methods. However, neither the use of various commercially available antibodies (MCR N-17, MCR C-19, anti-idiotypic MCR) nor the application of antibodies provided by Dr. Gomez-Sanchez group such as anti-MRN2 2B7, -rMR79-97 3F10, -rMR1-18 6G1, -rMR365-381 4D6 and -rMR1-18 1D5 allowed to localize immunohistochemically MR in specimens derived from rabbit.

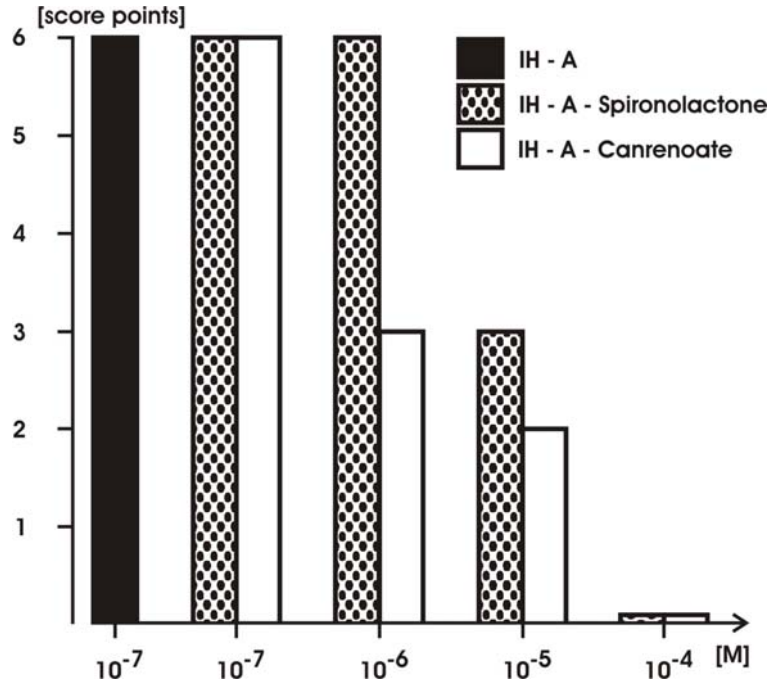

Fig. 6 Action of aldosterone and antagonists on the development of SBA-labeled tubules according to a 6 point score illustration. It is shown that the tubulogenic effect of aldosterone is antagonized by spironolactone and canrenoate. Data are the means of at least three independent experiments.

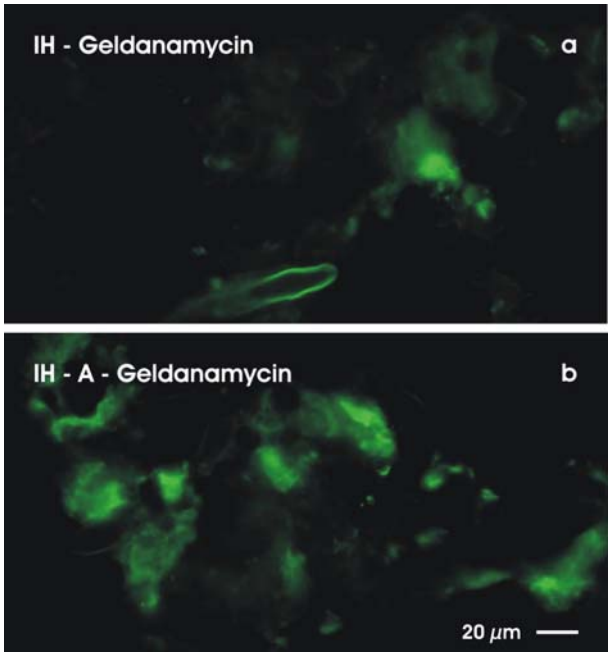

Fig. 7 Effect of geldanamycin on the generation of SBA-labeled tubules. Application of a) geldanamycin (2 μg/ml) or b) aldosterone and geldanamycin did not show any tubulogenic effect.

To gather some information about the molecular features of the MR protein, we performed SDS-electrophoresis followed by western blotting. For control, we probed rat hippocampus protein with antibody anti-rMR1-18 1D5 as earlier described [24]. Comparing it with standard proteins (Figure 8a) we found that the antibody recognised a single protein band revealing an apparent molecular weight of 96 000 (Figure 8b). Reducing the protein content from 3 to 0.6 μg further demonstrated that the antibody consistently labeled a single sharp protein band in a concentration-dependent fashion. In contrast, probing the antibody with embryonic renal tissue (Figure 8c) showed a completely different pattern as compared to rat hippocampus (Figure 8b). Two distinctly separated protein bands were labeled by the antibody anti-rMR1-18 1D5. One band corresponded to a size of 96 kDa, while the second band revealed 88 kDa.

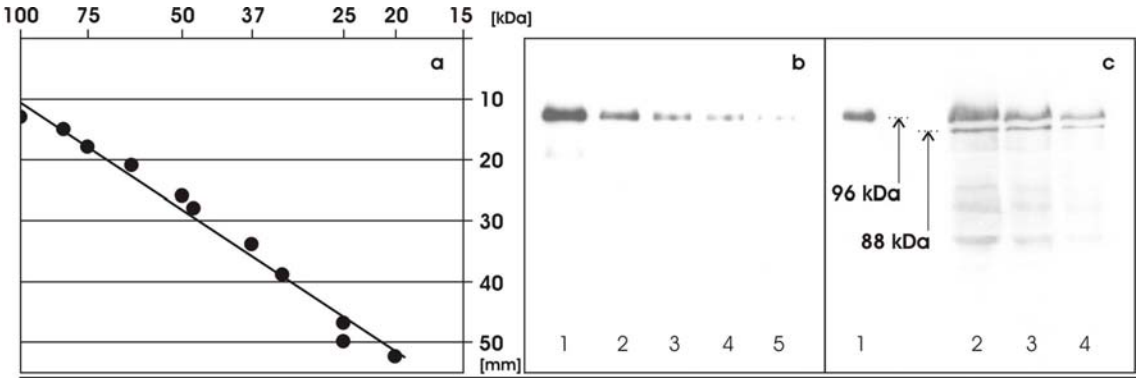

Fig. 8 SDS-electrophoresis and western blotting followed by probing with monoclonal antibody anti-rMR1-18 1D5 on rat hippocampus and freshly isolated rabbit embryonic renal tissue. a) Molecular weight protein standards for equilibration. b) Western blot of rat hippocampus protein probed with antibody anti-rMR1-18 1D5. Lane: 1 (3 μg), 2 (1.5 μg), 3 (1.2 μg), 4 (0.9 μg) and 5 (0.6 μg protein). The label is recognized in a concentration-dependent fashion on a single 96 kDa band. c) Western blot of rat hippocampus (lane 1/1.5 μg protein) and freshly isolated embryonic renal tissue probed with antibody anti-rMR1-18 1D5. Lane: 2 (80 μg), 3 (60 μg) and 4 (40 μg) protein of freshly isolated embryonic renal tissue. Intensive labeling is found of two distinct bands reflecting 96 kDa and 88 kDa.

4 Discussion

For the adult kidney it has been known for many years that aldosterone acts on principal cells of the collecting duct to stimulate Na^+-transport [25, 26]. In addition, we found that embryonic renal tissue is a target for aldosterone, since it promotes the functional maturation of collecting duct epithelium [27]. Furthermore, we detected that aldosterone exerts a tubulogenic effect promoting the development of structured tubules derived from renal stem/progenitor cells [18–20].

A morphogenic effect of aldosterone exists for other tissues. For example, the development of hippocampal neurons was triggered by the stimulation of MR, while activation of the glucocorticoid receptor (GR) suppressed their development [28]. T37i cells differentiated into cells of brown adipose tissue after application of aldosterone [29]. This effect could be inhibited by antagonists such as spironolactone or RU-26752. Other experiments with growth hormone producing cells of the adenohypophysis showed that their development is triggered by MR and is inhibited by spironolactone [30]. During embryogenesis the blockade of MR with ZK 91587 led to a reduction of somite number, to reduced growth and to an alteration of blood vessels in the umbilical cord [31]. Furthermore,, aldosterone increased levels of collagen type I and III synthesis in adult cardiac fibroblasts promoting myocardial fibrosis [32]. It remains a matter of debate whether this is due to a direct effect of aldosterone or due to an increase in endothelin receptor number. Finally, for the adult kidney, it was described that aldosterone promotes the synthesis of fibronectin through a Smad2-dependent TGF-β1 pathway in mesangial cells during the development of glomerular sclerosis [33].

Coming back to the embryonic kidney one may argue that a tubulogenic effect of aldosterone might be related to a more or less nonspecific action on MR in embryonic renal tissue. However, as shown previously, application of dexamethasone led to the formation of numerous huge cell clusters instead of tubules [20]. Furthermore, the use of other steroid hormones such as testosterone or estrogen did not result in the formation of SBA-labeled tubules (Table 2). These results gave the first hints to suggest a specific steroidal action of aldosterone on a cognate receptor in renal stem/progenitor cells.

(+) Generation of tubules	(-) Lack of tubules
	Testosterone Estrogen Cholesterol Pregnenolone Progesterone 11-deoxycorticosterone Corticosterone Dexamethasone
Aldosterone (1×10^{-7} M) / Fluka Aldosterone (1×10^{-7} M) / Sigma Aldosterone concentration-dependent 4 days minimum Aldosterone application	
	Aldosterone + Spironolactone Aldosterone + Canrenoate Aldosterone + Geldanamycin Spironolactone Canrenoate Geldanamycin

Table 2 Steroid hormones, metabolites and antagonists influencing the generation of tubules derived from renal stem/progenitor cells.

A further indicator for a specific tubulogenic effect of aldosterone on MR was obtained by testing precursors of its synthesis pathway [21]. Cholesterol, pregnenolone, progesterone, 11-deoxycorticosterone or corticosterone did not promote the generation of tubules (Table 2). 18-hydroxycorticosterone could not be tested, since the substance was not commercially available. Thus, the lack of a tubulogenic effect of these substances indicated a specific aldosterone-mediated effect on the MR. Despite this clear result, the response of MR-activation in embryonic renal tissue differed from the known physiological effects on MR in the adult kidney. For example, 11-deoxycorticosterone did not show any tubulogenic effects, although the molecule was described to be as potent as aldosterone [34]. Corticosterone was shown to be an MR agonist, albeit about 100 times less potent than aldosterone [35]. However, in our culture experiments corticosterone did not have any tubulogenic effect. Thus, all precursors of the aldosterone synthesis pathway lacked a comparable tubulogenic effect as was observed for aldosterone.

A further important argument for a specific tubulogenic effect of aldosterone on MR was obtained by performing experiments with antagonists (Table 2, Figure 5, 6). The simultaneous administration of aldosterone in combination with spironolactone (Figure 2e',e") or canrenoate (Figure 3d',d",e',e") demonstrated that the tubulogenic effect can be inhibited dose-dependently. These results suggest that the tubulogenic effect of aldosterone is actually mediated via MR. Furthermore, application of higher doses of spironolactone (Figure 5c-d), canrenoate (Figure 5g-h) alone or use of canrenoate followed by administration of aldosterone after 1 to 4 hours (Figure 4b-e) failed to generate SBA-labeled tubules. Thus, binding of an antagonist followed by subsequent application of aldosterone does not activate any tubulogenic effect. However, these results are in contrast with low-dose spironolactone experiments (Figure 5a,b), where numerous unlabeled and few SBA-labeled tubules were found. At the moment we are not able to provide a straightforward explanation for this as finding.

The extracellular signal derived from aldosterone has to be transmitted via MR into an intracellular signal. Previous experiments showed that MR activation is dependent on an intact association with heat shock proteins (hsp) [23, 36, 37]. In consequence, geldanamycin was used to determine if the tubulogenic effect of aldosterone can be prevented by disrupting MR association to the heat shock proteins (Figure 7). We found that the application of geldanamycin alone (Figure 7a) or in the presence of aldosterone (Figure 7b) completely failed to generate SBA-labeled tubules. We consider this an additional clue that the tubulogenic effect of aldosterone is mediated via the cognate MR.

Finally, to obtain information related to molecular features of MR in embryonic renal tissue SDS-electrophoresis was performed followed by western blotting (Figure 8). Probing monoclonal antibody anti-rMR1-18 1D5 on rat hippocampus revealed a single protein band of 96 kDa (Figure 8b). In contrast, in embryonic renal tissue two distinct protein bands of 96 and 88 kDa were detected (Figure 8c). We do not yet know of further molecular features of both these protein bands in embryonic renal tissue. However, the possibility exists that the band with higher molecular weight represents the classical MR, while the other may represent a modification of MR related to the tubulogenic effect of

aldosterone. Protein isolation and sequence analysis shall provide insights into the nature of these two molecules. At the moment it is too early to speculate further about functions of the demonstrated proteins.

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