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Embryonic Renal Collecting Duct Cell Differentiation Is Influenced in a Concentration-Dependent Manner by the Electrolyte Environment

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Key Words

Renal collecting duct · Principal cells · Intercalated cells · Gradient perfusion culture · Electrolyte environment · NaCl load · Differentiation

Abstract

Background: During kidney development, the embryonic collecting duct (CD) epithelium develops into a heterogeneously composed epithelium consisting of principal and intercalated cells. It is unknown by which molecular mechanism the different cell types arise. We have experimental evidence that the electrolyte environment is involved in the process of terminal cell differentiation. ***Methods:*** Embryonic CD epithelia from neonatal rabbit kidneys were microsurgically isolated and maintained in gradient perfusion culture for 13 days under serum-free conditions. Controls were maintained in the same medium (Iscove's modified Dulbecco's medium; IMDM) on basal and luminal sides. Experimental series were performed with IMDM only on the basal side, while on the luminal side IMDM with increasing concentrations of NaCl was used. Finally, the development of principal and intercalated cell features was registered by immunohistochemical labeling with markers specific for adult CD cells. ***Results:*** Immunohistochemical markers show that the differentiation pattern is quite different when the embryonic CD epithelia are cultured in IMDM only as compared with specimens kept in IMDM supplemented with 3–24 mmol/l NaCl on the luminal cell side. First signs of changes in development were seen when low doses of 3–6 mmol/l NaCl were added. ***Conclusions:*** We conclude that facultative protein expression in embryonic CD epithelium is influenced by the electrolyte environment and starts to be upregulated after administration of unexpectedly low doses of 3–6 mmol/l NaCl added to the luminal perfusion culture medium and increases in a concentration-dependent manner.

Introduction

Connecting tubule and collecting duct (CD) are the only portions of the kidney which develop into a heterogeneously composed epithelium consisting of principal (P) and intercalated (IC) cells [1–4]. To date, it is unknown by which molecular mechanism both types of cells develop. It is further unknown by which factors different amounts of P and IC cells appear along the corticomedullary course of the adult rabbit kidney [5–7].

Recent data from in vitro experiments indicate that two very different cell biological mechanisms can influence terminal differentiation of individual CD cell

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populations: the humoral action of aldosterone and changes in the eletrolyte composition are under discussion [7–11]. Culture experiments in the presence of fetal calf serum and in the stagnant environment of a culture dish have shown that aldosterone acts as a differentiation hormone on embryonic CD cells [12]. It increases the transepithelial resistance and potential difference, so that an amiloride-sensitive Na transport comparable to the situation within the adult organ develops [13]. A more paradox effect after aldosterone application is obtained, when CD cells are kept in perfusion culture under serum-free conditions [14]. The epithelium develops from an embryonic into an adult form and upregulates all features of a tight epithelium. However, as compared with controls, between 80 and 90% of the cells develop binding for peanut lectin [10], a marker which reacts with the luminal plasma membrane of β-type IC and not P cells of the adult kidney [15].

Besides the effect of aldosterone on terminal differentiation, embryonic CD cells are sensitive to the electrolyte environment. As demonstrated by nuclear staining with a proliferation marker after perfusion culture under serum-free conditions, the tissue reaches a postmitotic state as observed in the adult rabbit kidney [7]. During this late phase of differentiation, the epithelia were exposed to six different culture media with varying electrolyte contents [9]. According to morphological criteria, each of the media produced perfect CD epithelia.

However, labeling the tissue by immunological markers revealed that each medium produces a very individual differentiation pattern. Epithelia cultured in media with a low Na content showed a completely different developmental pattern as compared with epithelia cultured in media with a high Na content. Evidence for a definitive electrolyte-sensitive reaction during CD development was finally obtained by culturing the embryonic epithelia in the presence of aldosterone and under serum-free conditions in a gradient container [16]. During a culture period of 14 days, epithelia cultured with the same medium on luminal and basal sides develop completely different features as compared with epithelia cultured with different media on both sides [8, 11]. Addition of 12 mmol/l NaCl/17 mmol/l Na gluconate to the luminal culture medium resulted in 90% of cells reacting with CD cell markers, while in controls without additional NaCl only 5% positive cells were found. The stimulating effect of NaCl in the culture medium was found by chance, when the electrolyte concentrations of the culture medium (Iscove's modified Dulbecco's medium; IMDM) were adjusted to serum values.

Since it is unknown, whether the electrolyte-mediated signal for differentiation is able to act only on rather high or also on low doses of NaCl added to the culture medium, we wanted to elaborate whether the embryonic CD epithelia develop individual differentiation patterns dependent on the administered NaCl concentration in the culture medium. The medium on the basal side consisted of standard IMDM, while the tissue was exposed to IMDM with increasing concentrations of NaCl ranging between 3 and 24 mmol/l NaCl on the luminal side. The experiments give new informations that embryonic renal CD react in a very sensitive and concentration-dependent manner to the surrounding electrolyte environment.

Materials and Methods

Tissue Isolation and Generation of an Embryonic CD Epithelium

Cortical explants from the kidneys of newborn New Zealand rabbits were isolated according to methods described earlier [14]. The explants consisted of a piece of capsula fibrosa with adherent CD ampullae, S-shaped bodies, and nephrogenic blastema. The tissue was then mounted in sterile carriers (fig. 1A; Minucells and Minutissue, Bad Abbach, Germany) which were placed in 24-well culture dishes (Greiner, Nürtingen, Germany). During the culture of these explants in IMDM (Gibco BRL-Life Technologies, Eggenstein, Germany) and 10% fetal bovine serum (Boehringer Mannheim, Germany), an outgrowth of cells from the CD ampullae was observed. Within 24 h after the initiation of culture, the entire surface of the explant, 6 mm in diameter, was covered by a polarized CD epithelium. Culture for the 1st day was carried out in a tissue incubator (Heraeus, Hanau, Germany) at 37°C in a humidified atmosphere containing 5% CO_2/95% air.

Gradient Perfusion Culture of Embryonic CD Epithelia

24 h after the initiation of culture, the epithelia on the tissue carriers (fig. 1A) were transferred to a gradient perfusion culture container (fig. 1B; Minucells and Minutissue) to create optimized culture conditions [16]. Fresh medium was continuously superfused on the basal and luminal sides of the epithelia for the following culture period of 13 days at a rate of 1 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany; fig. 1C). To maintain a constant temperature, the culture container was placed on a thermoplate (37°C; Medax, Kiel, Germany) and covered with a transparent lid. 5 isolated CD epithelia were cultured per series of NaCl-loaded media. Therefore, a total of more than 40 epithelia were kept in perfusion culture for the following experiments.

Culture Media

IMDM with phenol red as pH indicator (Gibco BRL-Life Technologies; IMDM No. 21980) served as standard in one compartment of the gradient container. To control full functional tightness of the epithelium during the whole culture period, IMDM without phenol red was applied to the opposite side of the gradient container. Depending on the experimental series, either none or 3–24 mmol/l NaCl was added to the luminal culture medium.

Additives of the Culture Media

None of the perfusion culture media contained serum or growth factors. Aldosterone (1×10^{-7} *M*; Sigma-Aldrich-Chemie, Deisenhofen, Germany) and 1% antibiotic-antimycotic solution (Gibco BRL-Life Technologies) were added to all of the perfusion culture media. Furthermore, up to 50 mmol/l Hepes was added to maintain a constant pH of 7.4 under laboratory air atmosphere (0.3% CO_2).

Physiological Parameters of the Culture Media

Culture parameters such as pH, pCO_2, pO_2, osmolarity, glucose, lactate, and electrolyte contents such as Na^+, K^+, Cl^-, and Ca^{2+} were determined from an undiluted 20-µl sample using a Stat Profile 9 Plus analyzer according to the manufacturer's instructions (Nova Biomedical, Rödermark, Germany). Solutions with defined electrolyte concentrations served as controls.

Histochemical Markers

Development of CD cell features was registered by immunohistochemistry using monoclonal antibodies (mAbs) against CD antigens found in the adult kidney. The following antibodies were used to identify CD cell features: the mAb anticytokeratin 19 recognizes a 40-kD polypeptide in all CD cells of the adult rabbit kidney. The mAb anticytokeratin 19 was produced in the laboratory of Prof. R. Moll (Marburg, Germany) [17] and used diluted 1:10 in blocking solution. The mAb anti-Na/K-ATPase binds to a cytosolic epitope on the α-subunit of the Na/K-ATPase and was used as undiluted supernatant. The mAb anti-Na/K-ATPase α5, developed by Douglas M. Fambrough, was obtained from the Development Studies Hydroma Bank (Department of Biological Sciences, University of Iowa, Iowa City, Iowa, USA; contract No. NO1-HD-7-3263 from the NICHD). MAbs 703 and 503 were generated in the laboratory of Dr. M. Tauc (Nice, France) and diluted in 1:100 blocking solution [18]. MAb 703 detects P cells, while mAb 503 recognizes IC cells in cultured adult renal CD cells.

Immunohistochemistry

For microscopical examination and immunohistochemical detection of CD proteins, exact cross-sections (8 µm thick) of cultured epithelia were prepared with a cryostat HM 500 (Microm, Walldorf, Germany). For histological control, sections were stained with toluidine blue solution. Immunolabeling was started by fixing the cryosections in ice-cold ethanol for 10 min as described earlier [19]. After several rinses with phosphate-buffered saline (PBS; pH 7.2), the sections were incubated with a blocking solution containing PBS, 10% horse serum, and 1% bovine serum albumin for 30 min. The primary antibodies were incubated for 1.5 h. After several rinses with PBS containing 1% bovine serum albumin, the sections were treated for 45 min with a donkey anti-mouse IgG fluorescein isothiocyanate-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, Pa., USA) diluted 1:200 in blocking buffer. After several washes with PBS, the sections were embedded in fluorescein isothiocyanate guard (Testoc, Chicago, Ill., USA) and examined using an Axiovert 35 microscope (Zeiss, Oberkochen, Germany).

To determine the degree of immunopositive cells in the cultured epithelia, a double labeling procedure was applied. The epithelia were first labeled with the nuclear marker propidium iodide (4 µg/ml in PBS; Sigma-Aldrich-Chemie) and then with an immunological marker. By this method, the amount of mAb-labeled and mAb-unlabeled cells within the epithelium could easily be determined. The minimum and maximum (%) of immunopositive cells within the epithelium are given in text.

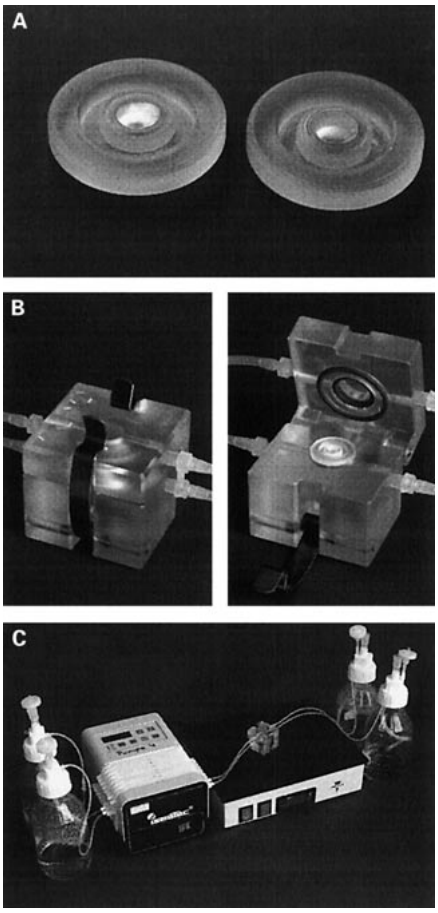


Fig. 1. Gradient perfusion culture of a renal CD epithelium. **A** The renal explant with the epithelium, 6 mm in diameter, is mounted in a tissue carrier. **B** The carriers with the CD epithelia are transferred to a gradient culture container, where the epithelia are superfused on the basal and luminal sides with culture medium. **C** A peristaltic pump transports the media (flow rate 1 ml/h), and a thermoplate ensures a constant temperatures of 37 °C during the 13-day culture period.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting

The cultured CD epithelia were homogenized in a sample buffer containing 2% sodium dodecyl sulfate, 10% glycerin, 125 m*M* Tris-HCl, and 1 m*M* EDTA and centrifuged at 10,000 *g* for 10 min. The supernatants were used in the following experiments. The amount of proteins was measured by a Bio-Rad (Hercules, Calif., USA) DC protein microassay. 10-µg protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% Lämmli minigels according to earlier described methods [20] and then electorphoretically transferred to P-Immobilon membranes (Millipore,

Table 1. Physiological parameters of the culture medium of an individual experiment measured in front, behind, and on the luminal and basal sides of the gradient container

	In front of the container		Behind the container	
	basal	luminal	basal	luminal
pH	7.39	7.42	7.48	7.46
pO_2, mm Hg	195.5	193.7	192.6	191.9
pCO_2, mm Hg	14.2	12.6	6.9	9.5
Na^+, mmol/l	*118.0*	*130.5*	*118.1*	*130.4*
K^+, mmol/l	3.93	3.94	3.94	3.93
Cl^-, mmol/l	*80.8*	*91.2*	*80.8*	*91.6*
Ca^{2+}, mmol/l	1.14	1.11	1.15	1.10
Glucose, mg/dl	476	483	477	475
Lactate, mmol/l	0	0	0.5	0.5
Osmolarity, mosm	*255*	*276*	*255*	*275*

Standard IMDM was superfused on the basal side, while IMDM containing additional 12 mmol/l NaCl was used on the luminal side of the embryonic CD epithelia.

Eschborn, Germany) [21]. In order to detect immunoreactive proteins, the blots were first blocked (PBS, pH 7.2; 0.05% Tween, Sigma; 10% horse serum, Boehringer, Mannheim), and finally mAb 703 (dilution 1:100) and antibody against Na/K-ATPase (1:50) were applied for 3 h. A horseradish peroxidase-conjugated donkey anti-mouse immunoglobulin antiserum (1:2,000; Dianova, Hamburg, Germany) served as detecting antibody applied for 45 min as described in earlier experiments [22]. The staining reaction was started by addition of 0.5 mg/ml diaminobenzidine, 0.02% H_2O_2, and 0.03% cobalt chloride dissolved in citrate buffer (pH 6.3). The reaction was stopped by washing the membrane in tap water. Immunoblots were documented with a Scan Jet 6200 C (Hewlett Packard, Greeley, Colo., USA). Determination of the apparent molecular weight was performed in conjunction with broad-range molecular weight standard proteins (Bio-Rad).

Results

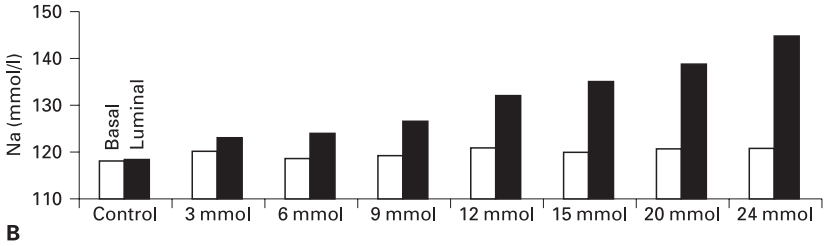
During culture of the epithelia, it has to be assured that the luminal and basal compartments are tightly sealed by the tissue itself and not by the culture tool. Mixing of the media between the luminal and basal compartment has to be prevented. A rough but simple control for the tightness of the epithelia is employed by using IMDM with phenol red on the basal side of the container, while on the luminal side medium without pH indicator is applied. When the epithelium completely separates the luminal from the basal compartment, a purely red and a purely colorless medium can be collected in the waste bottles during the culture period of 13 days. In the case that a mixing of colors between both media is observed, the experiment is stopped immediately. As a consequence, only experiments are described in which a complete functional barrier of epithelia could be observed and maintained over a period of 13 days in gradient perfusion culture.

During differentiation, physiological parameters of the culture media are monitored (table 1). Besides the concentrations of electrolytes and the osmolarity, it is of importance to register pH, pO_2, pCO_2, and lactate and glucose contents during the course of culture to detect deviations from a constant environment. Data from a typical individual experiment with standard medium on the basal side and NaCl load on the luminal side are presented. Measurements are performed before and after the medium reaches the container. As compared with the inlet, a slight increase in pH from 7.33 to 7.48 is found at the outlet of the container. An accumulation of unphysiological concentrations of lactate cannot be observed. All of the other parameters, especially gas, electrolyte, or glucose contents, remain stable. The measured parameters indicate that a constant environment is guaranteed within the culture container over a period of 2 weeks (table 1).

Mimicking an embryonic setup, CD epithelia are exposed to standard medium on luminal and basal sides (fig. 2A). As revealed by the analyzer, in the basal and luminal media, the same concentrations of Na^+, Cl^-, K^+, and Ca^{2+} are found. In contrast, stimulation of CD epithelia is performed by offering standard IMDM on the basal side, while IMDM containing between 3 and 24 mmol/l additional NaCl is applied to the luminal side (fig. 2B). Control measurements with the analyzer show that NaCl concentrations increase continuously step by step, while K and Ca concentrations remain constant (fig. 2C).

	Na^+	CL^-	K^+	Ca^{2+}
Luminal	118.1	76.5	4.07	1.06
Basal	117.6	76.5	3.97	1.04

A



B

Addition of	Na^+	CL^-	K^+	Ca^{2+}
3 mmol/l	3.0	2.9	0.2	0.02
6 mmol/l	4.7	4.1	0.05	0.1
9 mmol/l	7.7	8.3	0.03	0.01
12 mmol/l	11.3	10.9	0.16	0.17
15 mmol/l	15.8	16.5	0.18	0.01
20 mmol/l	20.0	21.9	0.05	0.0
24 mmol/l	24.2	24.9	0.05	0.04

C

Fig. 2. A Control: electrolyte concentrations (mmol/l) of IMDM on basal and luminal sides of the cultured CD epithelia. **B** Concentrations of Na^+ in the culture media after addition of 3–24 mmol/l NaCl to the luminal culture media. **C** Resulting measured differences in the electrolyte concentrations (Δmmol/l) between medium administered on the luminal side and medium administered on the basal side of the gradient container by adding NaCl.

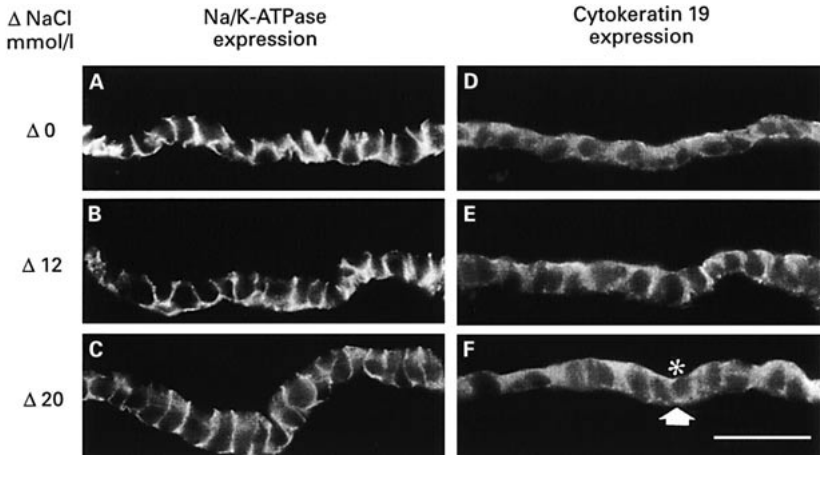


Fig. 3. Expression of Na/K-ATPase and cytokeratin 19 in cultured CD epithelia. Immunohitochemistry reveals that all cells in the epithelia cultured without additional NaCl or with additional 12 and 20 mmol/l NaCl on the luminal side are immunopositive for Na/K-ATPase (**A–C**) and cytokeratin 19 (**D–F**). The bar in **F** represents 20 μm, the asterisk the luminal side, and the arrow the basal aspect of the epithelium.

The degree of cellular differentiation after culture of the embryonic CD epithelia in a gradient container is analyzed by immunohistochemical methods, as shown in earlier investigations [12–14]. In the present experiments, markers for constitutive and facultative protein expression are applied to register the appearance of CD cell features.

Constitutive protein expression is registered by antibodies against Na/K-ATPase and cytokeratin 19. Immunolabeling shows that treatment with the same (fig. 3A,

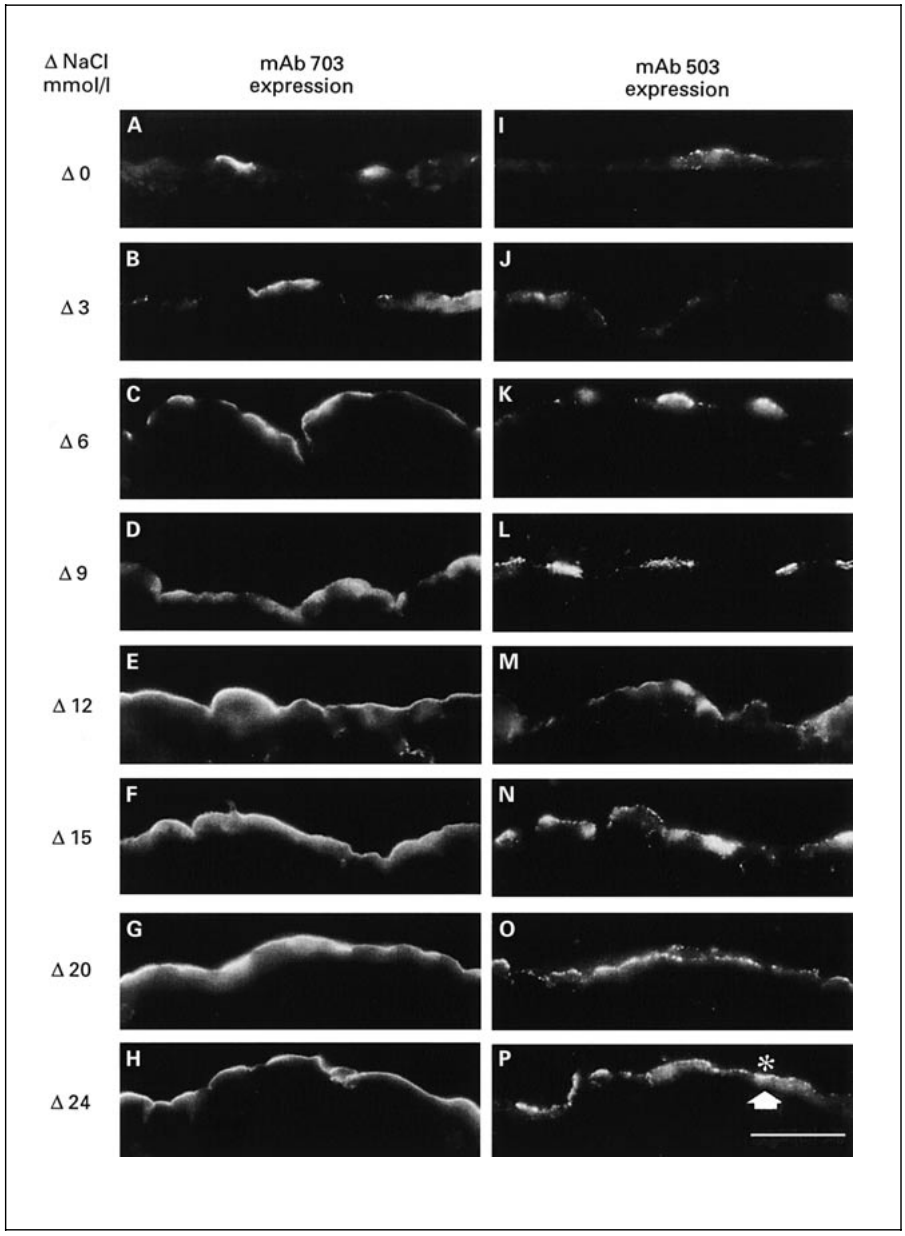


Fig. 4. Immunohistochemical labeling pattern for mAb 703 (**A–H**) and mAb (**I–P**) in CD epithelia exposed on the luminal side to increasing NaCl concentrations. Epithelia cultured in standard IMDM on the luminal side reveal 10% positive cells for mAb 703 (**A**) as well as for mAb 503 (**I**). Addition of 12 mmol/l NaCl to IMDM causes 100% immunopositive cells for mAb 703 (**E**), while 40–60% of the CD cells are positive for mAb 503 (**M**). Addition of 24 mmol/l NaCl shows for both mAb 703 (**H**) and mAb 503 (**P**) 80–100% immunopositive CD cells. The bar in **P** represents 20 μm, the asterisk the luminal side, and the arrow the basal aspect of the epithelium.

D) or with different (fig. 3B–E) media on the luminal and basal sides always produced epithelia in wich all of the cells show a strong positive reaction. This result is seen in series after adding rather high doses of 12 (fig. 3B, E) or 20 (fig. 3C, F) mmol/l NaCl to the luminal IMDM. Astonishingly, the same results are obtained by adding rather low doses of between 3, 6, and 9 mmol/l NaCl (not shown). These data are confirmed by Western blot experiments. Comparing the expression of Na/K-ATPase in epithelia cultured with the same media on both sides (see figure 7, lane a) reveals no difference as compared with epithelia cultured with 12 mmol NaCl on the luminal side (see figure 7, lane b) using the Western blot technique. Thus, administration of NaCl has no recognizable effect on the expression of Na/K-ATPase or cytokeratin, since these typical features are detected in all of the cells within the epithelia treated either with the same media on the luminal and basal sides or with a luminal NaCl load and standard IMDM at the basal aspect.

In contrast, facultatively expressed proteins show a completely different pattern as compared with constitutively appearing proteins. The mAb 703 labels the luminal side of P while mAb 503 labels the luminal side of IC cells kept in culture [18] (fig. 4). Control epithelia with IMDM

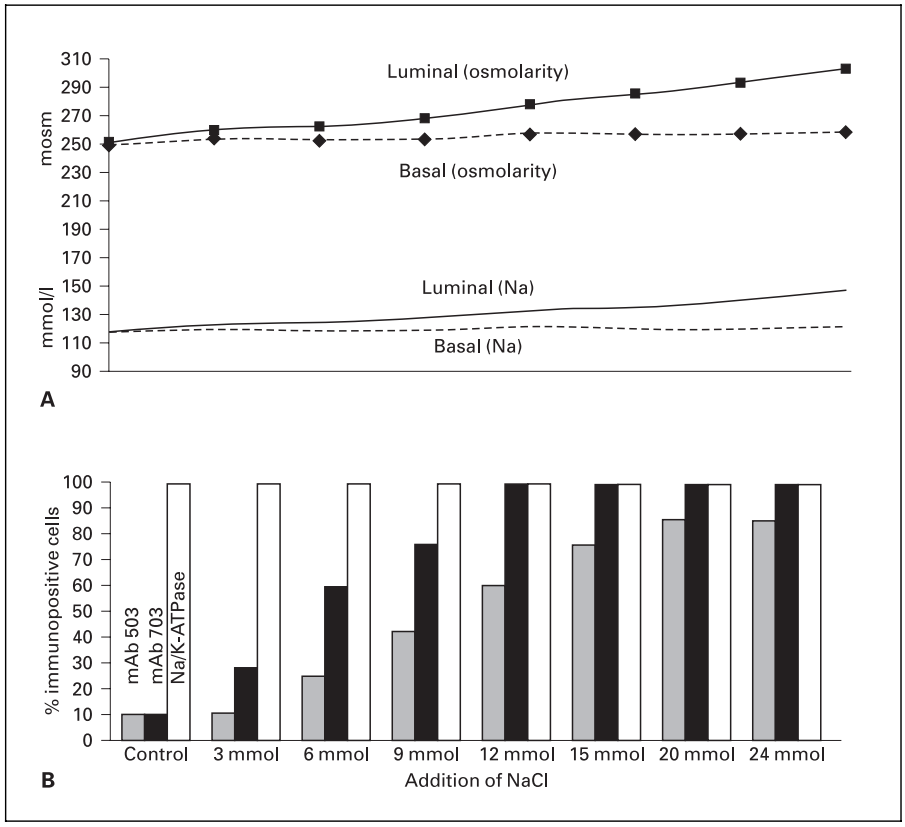


Fig. 5. Dependence of Na^+ load, osmolarity, and protein expression. **A** Alterations of Na^+ content and osmolarity in the culture media (IMDM) used on luminal and basal sides of the epithelia. Increasing concentrations of NaCl were added to the medium used on the luminal side of the gradient container. **B** Amount of immunopositive cells detected by mAb 703, mAb 503, and an antibody directed against Na/K-ATPase. Na/K-ATPase is expressed independently of increasing NaCl concentrations in the tested media. In contrast, mAb 703 and mAb 503 labeling on the CD cells is influenced in a concentration-dependent manner, ranging between 3 and 12 mmol/l NaCl.

on the luminal and basal sides of the epithelia showed only 10% of the cells being positive for mAb 703 (fig. 4A) and mAb 503 (Fig. 4I). However, when increasing concentrations of NaCl were added to the luminal IMDM, the amounts of mAb 703 (fig. 4B–H) and mAb 503 (fig. 4J–P) labeling constantly increase. At NaCl concentrations between 15 and 24 mmol/l (fig. 4F–H, N–P), a minimum of 90 and a maximum of 100% of labeled cells were found within the epithelium. Hence, facultative protein expression can be upregulated by IMDM containing increasing concentrations of NaCl of between 12 and 24 mmol/l (fig. 5B), so that the majority of cells become positive for mAbs 703 and 503. In contrast, low doses of NaCl (3–6 mmol/l) lead only to a moderate increase of mAb 703 (25–50%) and of mAb 503 (10–25%) immunopositive cells.

Western blot analysis should reveal how far an upregulation of synthesized antigens can be identified at the molecular level. In each of the blotting experiments, always the same amount of 10 μg protein was analyzed. In Western blot analysis mAb 703 recognizes protein bands with an apparent molecular weight of 40, 48, 51, 56, 60 and 99 kD after treatment with additional 12 mmol/l NaCl on the luminal side (see figure 7, lane d). In contrast, epithelia cultured with standard IMDM on the luminal and basal sides show only a weak expression of 40-, 48-, 51-, 56-, and 60-kD bands, while the 99-kD band is not found (see figure 7, lane c). Comparing lanes c and d of figure 7, the increased amount of unspecific labeling is due to the increased exposure of luminal NaCl. Na/K-ATPase is always found in the same amount and shows always the same level of expression including the same background staining (see figure 7, lanes a and b).

Addition of NaCl to the culture medium increases the osmolarity. For that reason, it is important to prove whether the change in differentiation is caused by the NaCl ions or by the increase in osmolarity. Consequently, 20 mmol/l glucose (fig. 6C) is added instead of NaCl (fig. 6B) to the luminal culture medium, leading to an osmolarity of 272 mosm (fig. 6D). Administration of glucose reveals only 45–55% of the cells reacting with the antibody (fig. 6D), while epithelia with a luminal load of 10 or 12 mmol/l NaCl show 80–100% mAb-703-positive cells (fig. 6B, C). In contrast, on epithelia with the same

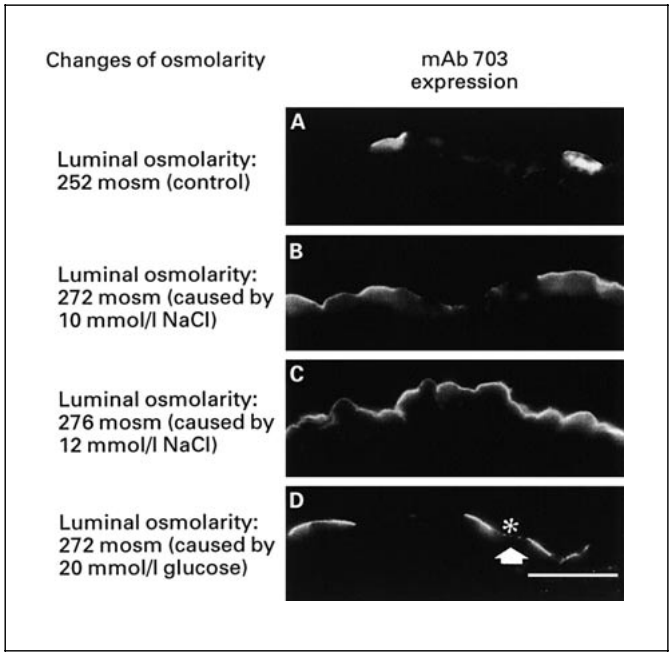


Fig. 6. Labeling pattern for mAb 703 in CD epithelia cultured with different osmolarities on the luminal side of the cultured CD epithelia. **A** The osmolarity of standard IMDM is 252 mosm. Immunohistochemical labeling of these epithelia show only 10% positive cells. An increase of the osmolarity to 272 mosm caused by addition of 10 mmol/l NaCl (**B**) or to 276 mosm caused by addition of 12 mmol/l NaCl (**C**) reveals mAb 703 binding on 80–90% and 100% of the cells, respectively. **D** In contrast, an increase in osmolarity to 272 mosm caused by glucose only shows mAb 703 binding on 45–55% of the cells. The bar in **D** represents 20 μm, the asterisk the luminal side, and the arrow the basal aspect of the epithelium.

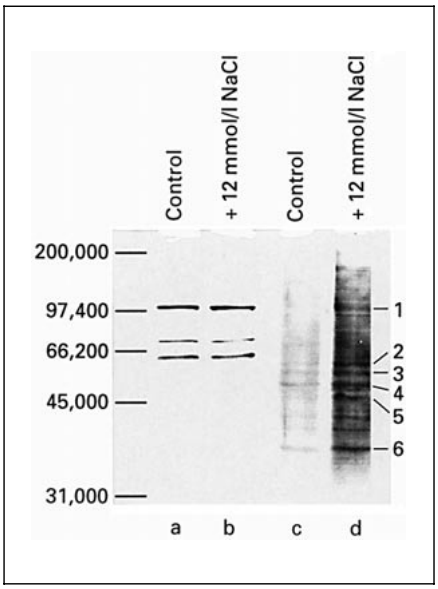


Fig. 7. Immunoblot results using an antibody against the α-subunit of Na/K-ATPase (lanes a and b) and mAb 703 (lanes c and d). 10 μg per lane was separated. Epithelia were cultured with IMDM on the luminal and basal sides (lanes a and c) or with 12 mmol/l NaCl on the luminal side (lanes b and d). The Na/K-ATPase α-subunit is strongly labeled at approximately 100 kD, when the epithelia are cultured with the same media on the luminal and basal sides (lane a) or with a luminal NaCl load (lane b). In addition, weakly labeled 76- and 64-kD protein bands are detected. mAb 703 recognizes intensively labeled protein bands of 99 (1), 60 (2), 56 (3), 51 (4), 48 (5), and 40 (6) kD in epithelia with a luminal NaCl load (lane d), while only weak staining is observed with the same media on the luminal and basal sides (lane c). A 99-kD band (1) is only present after a luminal NaCl load (lane d).

medium on the luminal and basal sides only 10% positive cells could be detected (fig. 6A). This indicates that not the ions alone but also changes in osmolarity have an influence on differentiation.

Discussion

Regarding the developmental potency, morphology, antigenicity, and functionality, the cells of the CD ampulla tip in the neonatal rabbit kidney are completely different as compared with the cells in the matured tubule [23]. The ampullar cells induce the surrounding mesenchyme to form epithelial nephron structures. Factors like FGF, BMP, and GNDF are under discussion triggering this step of development [24, 25]. In the ampullary neck region and further downwards to the midcortical region, the maturing cells develop functional features of P and IC cells [2, 5, 6]. It is unknown by which morphogenic factor and by which molecular mechanism the nephron-inducing ampullar cells change their function and develop into a heterogeneously composed epithelium consisting of P and IC cells. In previous investigations we showed the microsurgical isolation of embryonic ampullar and the generation of a polarized CD epithelium. Developing CD features within the cultured epithelium were registered by morphological [12, 14], immunological [9], and electrophysiological [13] methods, indicating adult CD features and demonstrating the origin of the CD ampulla.

It is assumed that during the initial phase of development a renal epithelium is exposed to the same medium on the luminal and basal sides. In the later process of dif-

ferentiation, it develops a functional barrier, so that different fluid compositions are found at the luminal and basal sides [26, 27]. It is unclear how far a change in the electrolyte environment is able to influence the development of the epithelium. Consequently, we investigated: what happens when a virgin epithelium is exposed for the first time to a luminal-basal medium gradient? Thus, the environment of an embryonic CD epithelium is mimicked in the further experiments by exposing the tissue to the same media on the luminal or basal side. In contrast, a matured situation is simulated by exposing the epithelia to a gradient with increasing concentrations of NaCl on the luminal side.

As shown in the present experiments (fig. 4, 5), the embryonic CD cells can be stimulated by the luminal addition of increasing doses of NaCl to the culture medium. We observe first an acceleration of development (fig. 4, 5, 7) and second after administration of unexpected low doses of 3–6 mmol/l NaCl a qualitative change in differentiation (fig. 4, 5, 7). The changes are dose dependent (fig. 4A–E, I–N), ranging from 3 to 15 mmol/l NaCl, and show in comparison to controls a complete alteration in the immunohistochemical protein expression pattern (fig. 4). Furthermore, the data demonstrate that both changes in NaCl load but also resulting changes in osmolarity may have an influence on cellular differentiation. However, we noticed not the same but different induced profiles of differentiation offering NaCl (fig. 5B, C) or glucose (fig. 5D) to increase osmolarity. Most interestingly, after exposure of 20 mmol/l NaCl to the luminal culture medium, 80–90% of all epithelial cells displayed an intensive labeling for Na/K-ATPase (fig. 3C), mAb 703 (fig. 4G), and mAb 503 (fig. 4O). Thus, the majority of the cells within the cultured epithelia are able to develop both IC cell characteristics as well as P cell features simultaneously. This can only be explained by the fact that hybrid cell characteristics are generated under in vitro conditions. Regarding CD cell development within the kidney, it is indicated that a common precursor cell type for P and IC cells exists in the embryonic ampulla.

In the embryonic CD ampulla, tight junctions with more or less leaky characteristics are found which can be also recognized after outgrowth of the cells on the kidney-specific matrix. It is not known which factors influence the further functional development of the epithelium within the kidney or under in vitro conditions. We assume that embryonic epithelium is exposed to more or less the same fluid compositions on the basal and luminal sides, when the tight junction is upregulating the sealing capacity by inserting finally five to seven anastomizing strands to form a tight barrier [13].

Few data are available which show an influence of NaCl or other ions on the development and differentiation of cells. The results of Yoshim et al. [28] display the upregulation of tissue-unspecific features in pineal body cells into cells with myogenic features by a stepwise increase of the NaCl concentration in the culture medium. To examine whether the change in osmolarity itself is effective for the differentiation or not, a replacement of NaCl by sucrose was done and revealed no effect on the differentiation. Davies and Garrod [29] discovered that lithium is able to trigger epithelial differentiation in early stages of kidney development. Lithium treatment induces the nephrogenic mesenchyme to start a program of differentiation similar to that initiated by classical living inducers like spinal cord [23]. Furthermore, Davies and Garrod [29] reported that mesenchymes induced by lithium show more DNA synthesis than controls and suggested that lithium operates by intervening in a second messenger pathway by altering cyclic nucleotide metabolism or by changing membrane potential. Traebert et al. [30] demonstrated the effect of a phosphate-poor diet on the development and distribution of sodium/phosphate transporters in the brush border membrane of proximal renal tubules. The results show not an up- but a downregulation of the sodium/phosphate transporter during development of the nephron by a phosphate-rich diet.

In adult renal tissues like the inner medullary CD, it was shown that increasing alterations in NaCl load and osmolarity lead to a decreased expression of Na/K-ATPase [31, 32]. In contrast, our data show that the Na/K-ATPase expression at the translational level is not influenced during the differentiation process in the embryonic CD epithelia by the NaCl load (fig. 3). Informations concerning the amount of Na/K-ATPase in the different cell types of the CD are contradictionary in mouse [33] and rat [34, 35]. It is shown that P cells express Na/K-ATPase, at high amounts, while in IC cells the expression is low or lacking [34]. In contrast, other authors [35] described that both P and IC cells express the same level of Na/K-ATPase in rat CD. In the rabbit kidney, a clear basolateral localization of Na/K-ATPase also in IC cells is observed [36]. Our experiments display an expression of Na/K-ATPase in both P and IC cells also in culture of CD epithelium [9], but we could not find a NaCl-dependent expression of Na/K-ATPase at the translational level. Furthermore, multiple data are available describing the influence of pH, osmolarity, or NaCl diet on the expression of adult but not on embryonic CD cell proteins. Met-

abolic acidosis stimulates H^+ secretion [37] and the expression of carbonic anhydrase IV mRNA [38] in the rabbit CD. Changes in osmolarity regulates H^+-ATPase [39], arginine vasopressin V_2 receptors, and aquaporins II and III [40, 41]. Carbohydrates play an important role in withstanding high urea concentrations and changes in extracellular osmolarity [42]. Na^+ channel expression can be maximized by steroid administration in combination with a low-salt diet [43, 44]. Cultured rabbit CD cells respond to aldosterone by increasing γ-rENaC mRNA levels, while a low-NaCl diet increases α-rENaC mRNA in the inner medulla of the kidney [45]. However, to the best of our knowledge, a concentration-dependent action of NaCl on the differentiation profile was shown for the first time in the present experiments.

In summary, our results revealed that electrolytes such as NaCl are able ot influence in an unexpected sensitive manner the development of cell features found in embryonic renal CD cells. The effect of ions is observed in early embryonic development [29] and during late phases of terminal cellular differentiation [11]. The molecular mechanism and the cellular pathway underlying the ion-dependent differentiation are unknown. Consequently, it remains to be elaborated using our system how far changes in NaCl concentration may act on activation of protein kinases A and C and changes in protein phosphorylation, as it was observed, for example, for the action of hypoxia-induced signals [46]. Possibly a similar pathway is activated in embryonic CD cells after the administration of ions. Experiments to clarify the cellular reactions are in progress.

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