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Culture of embryonic renal collecting duct epithelia in a gradient container

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Abstract. During organogenesis the ampullar epithelium of the renal collecting duct acts as an inducer which generates all of the nephron anlagen. As development proceeds, one part of the collecting duct cells in the ampullar tip retain their inducer capability, while others develop into the functional epithelium consisting of principal and intercalated (IC) cells. The events leading from the embryonic inducer to the mature tissue are unknown. We investigated the maturation of embryonic collecting duct epithelium derived from neonatal rabbit kidney under in vitro conditions. To prevent dedifferentiation the epithelia were cultured on kidney-specific support material within a tissue carrier. Apical and basal compartments of the epithelia were simulated in a gradient culture container. The two sides of the epithelium were each constantly perfused with a different medium. During the 14-day incubation the tissue was not subcultured. The development of collecting duct cell features was investigated with morphological and immunohistochemical methods. Both light and electron microscopy revealed morphologically intact epithelia following gradient culture. The polarized cells rested on a uniformly developed basement membrane. The continuous application of aldosterone during the culture modulated the development of collecting duct cell characteristics. Both basal and luminal administration of aldosterone initiated differentiation in the embryonic epithelia. Using the sodium (Na) channel blocker amiloride, it was demonstrated that Na channels are involved in the differentiation of the IC cell phenotype.

Key words: Collecting duct – Development – Perfusion culture – Gradient – Principal and intercalated cells

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Introduction

The epithelia within the kidney develop in three stages. The first step comprises the generation of the nephron anlagen which is triggered by an induction process between the collecting duct ampulla [1] and the surrounding mesenchymal tissue [2, 3]. The budding endothelium is found between these tissues [4]. As a result of the induction process, some of the competent mesenchymal cells are converted into an epithelial form. At this point the first visible stages of the nephron arise, the comma- and S-shaped bodies [5, 6]. In a second step the embryonic epithelia within the S-shaped bodies expand in length and develop into the various types of epithelia [7, 8]. Finally, the development of functional renal features starts. The immature epithelial cells develop into the functional barrier known from the adult organ. It is conceivable that the expansion of the immature nephron epithelia, a process we could liken to the elongation of a tube, is stimulated by a variety of growth factors [9–11]. However, relatively little is known about the mechanisms leading from the immature to the functional epithelia [12].

The collecting duct epithelium changes its function from nephron inducer to regulator of body fluids in the course of kidney development [12, 13]. Within the ampullar shaft, a heterogenous epithelium arises consisting of principal (P) and the different types of intercalated (IC) cells [14–18]. The neonatal rabbit kidney is a readily available model for investigating the transition of an embryonic to a functional epithelium under in vitro conditions. The capsula fibrosa can be stripped off together with collecting duct ampullae [19]. After the explants are mounted in tissue carriers, a collecting duct epithelium develops in the original extracellular matrix environment [20]. To provide improve culture conditions the embryonic epithelia were placed in newly developed gradient culture containers, allowing perfusion of the apical and basal sides of the epithelia with fresh media throughout the culture period. Using this new method we investigated whether the embryonic epithelia could be cultured under continuous perfusion over 13 days

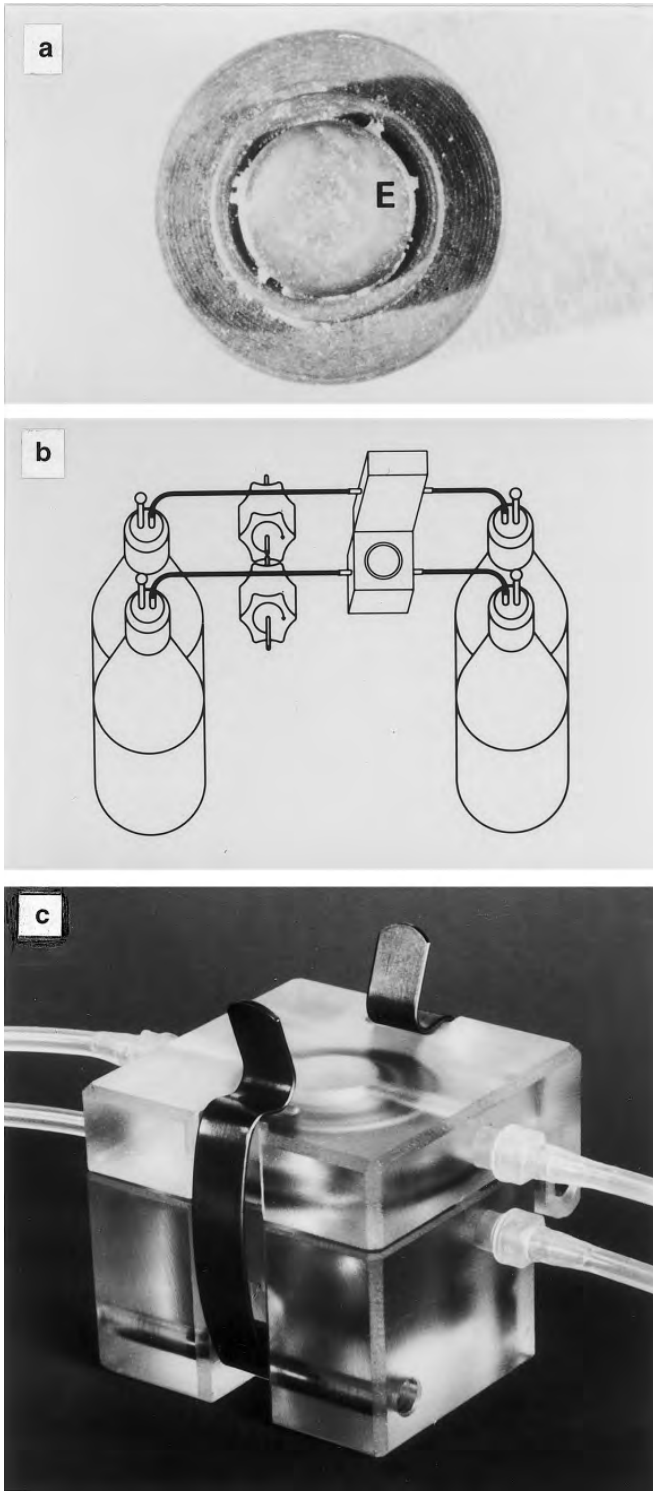


Fig. 1a-c. Renal collecting duct epithelium (*E*) in a tissue carrier for gradient perfusion culture. **a** The renal explant, 6 mm in diameter, is mounted in the carrier. Within 24 h of stationary culture a collecting duct epithelium grew out to completely cover the surface of the explant. **b** The carrier is transferred to a gradient perfusion container, which is connected to a luminal and a basal perfusion line. A peristaltic pump with two channels transports the media. **c** The closed gradient container allows luminal and basal perfusion with two different media

Table 1. Labelling of the different cell types within the adult renal collecting duct by monoclonal antibodies (mAb): mAb $P_{CD}9$ labels all of the collecting duct cells in the neonatal rabbit kidney, while only 70% of the cells are labelled in the adult kidney

Cell type	P	α-IC	β-IC	Reference
mAb 703	+			[24]
mAb 503		+	+	[24]
mAb IVF12		+		[25]
mAb $P_{CD}9$	+	+	+	[26]
PNA			+	[28, 30]

PNA, Peanut lectin; P, principal cells; IC, intercalated cells

and assessed the effects of the continuous application of hormones such as aldosterone.

Materials and methods

Tissue isolation and generation of an embryonic collecting duct epithelium. Cortical explants from the kidneys of newborn New Zealand rabbits were isolated according to methods described earlier [19]. The explants were mounted in sterile tissue carriers (Fig. 1a) (Minucells and Minutissue, Bad Abbach, Germany), which were placed in 24-well culture plates (Greiner, Nürtingen, Germany). The explants consisted of a piece of capsula fibrosa with adherent collecting duct ampullae, S-shaped bodies, and nephrogenic blastema. During the culture of these explants in Iscove's modified Dulbecco's medium (IMDM, Gibco Life Technologies, Eggenstein, Germany) and 10% fetal bovine serum (Boehringer, Mannheim, Germany), an outgrowth of cells from the collecting duct ampullae was observed. Within 24 h of the initiation of culture, the entire surface of the explant, 6 mm in diameter, was covered by a monolayer of collecting duct epithelium cells. Culture was carried out in a tissue incubator (Heraeus, Hanau, Germany) at 37 °C in a humidified atmosphere containing 5% carbon dioxide /95% air.

Culture of epithelia in a gradient container. To create optimized culture conditions the epithelia were exposed 24 h after initiation to continuous perfusion of fresh medium from the luminal and basal sides using a newly developed gradient container (Fig. 1b, c) (Minucells and Minutissue). The container was placed on a 37 °C heating plate (Medax, Kiel, Germany). IMDM without serum was continuously perfused at a rate of 1 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany) for 13 days. IMDM without serum was used as the control medium. Aldosterone (1×10^{-7} M, Ciba Pharma, Wehr, Germany), amiloride (1×10^{-6} M), and spironolactone (1×10^{-5} M) (the last two from Sigma-Aldrich-Chemie, Deisenhofen, Germany) were added in experimental series to the culture medium. Culturing the epithelia in a gradient container made it possible to perfuse hormone-containing medium on either the luminal or the basolateral side, or on both sides of the epithelium at once.

Histology. For light and transmission electron microscopy cultured collecting duct epithelia were fixed in the tissue carriers in IMDM containing 3% glutaraldehyde. Following the initial fixation step, the tissue pieces were postfixed with 1% osmium tetroxide in phosphate-buffered solution (PBS) and dehydrated in a series of alcohols, passed through propylene oxide, and embedded in Epon, according to methods described earlier [21, 22]. The harvested epithelia were embedded horizontally so that precisely oriented cross-sections could be obtained. Semithin sections were stained with Toluidin solution and examined under the light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate. The specimens were examined with an electron microscope 902 (Zeiss, Oberkochen, Germany).

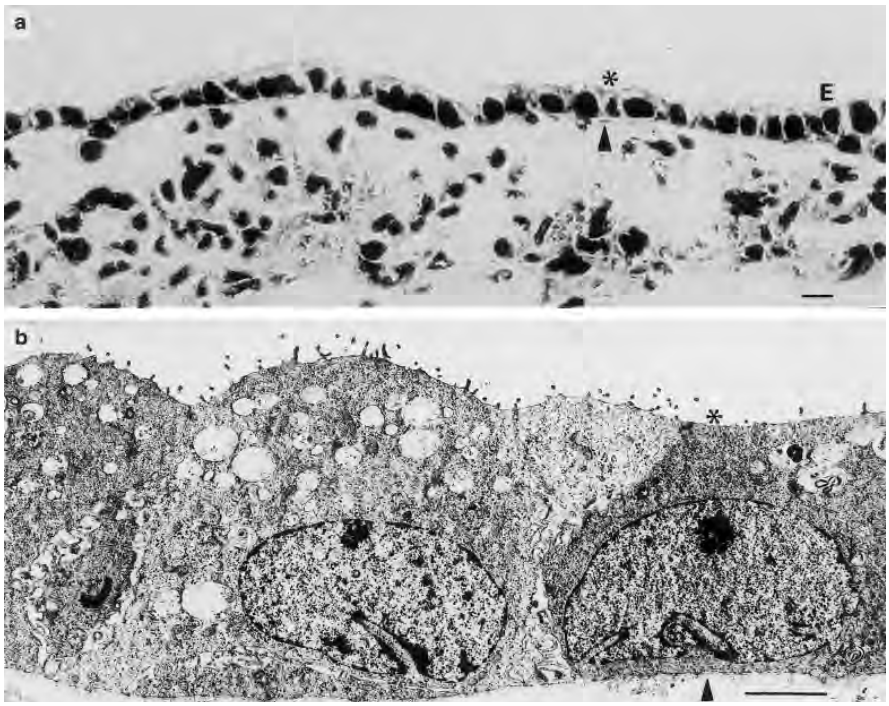


Fig. 2. Light and **b** transmission electron microscopy of renal collecting duct epithelium (E) kept for 13 days in gradient perfusion culture without hormonal additives. **a** The cryosection of a cultured collecting duct epithelium demonstrates that a closed cell monolayer is established at the surface of the explant, which can tolerate the medium flow in the gradient container (bar = 40 μm). **b** Electron microscopy shows the polarized cells with apical microvillar structures (*asterisks*) resting on a uniformly developed basement membrane (*arrowhead*). Deep tight junctions separate the luminal from the basolateral plasma membranes. (bar = 1 μm)

Histochemistry. For the light microscopical monitoring of cell development and the immunohistochemical detection of collecting duct proteins, 7-μm cryosections were prepared with a Cryostat HM 500 (Microm, Walldorf, Germany). The monoclonal antibodies mAb 703, mAb 503, mAb $P_{CD}9$ and mAb IVF12 were used for labelling (Table 1). mAb 703 recognizes a 70-kilodalton kDa protein in P cells, while mAb 503 detects IC cells [23, 24]. mAb IVF12 labels the chloride/bicarbonate (Cl^-/HCO_3^-) exchanger in α-type IC cells [25], while mAb $P_{CD}9$ localizes a 32/39-kDa protein on all of the neonatal collecting duct cells, but on only 70% of adult cells [26]. Cryosections were fixed for 10 min in ice-cold ethanol as described earlier [27]. Following several washing steps with PBS, pH 7.2, the sections were incubated with a blocking solution (PBS) containing 10% horse serum and 1% bovine serum albumin (BSA) for 30 min. The primary antibodies mAb 703, mAb 503, and mAb IVF12 (each diluted 1:100 in blocking buffer) and $P_{CD}9$ (undiluted hybridoma supernatant) were incubated for 1.5 h. Following several washes with PBS containing 1% BSA the sections were treated for 45 min with a fluorescein isothiocyanate (FITC)-labelled donkey anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, USA) diluted 1:200 in blocking buffer. After several rinses in PBS/1% BSA, the specimens were incubated for 40 min with a peanut lectin (PNA)-rhodamine conjugate (Vector, Burlingame, Vermont, USA) diluted 1:2000 in PBS to detect β-type IC cells [28]. Following several washes with PBS, the sections were embedded in FITC guard (Testoc, Chicago, Ill., USA) and examined using an Axiovert 35 microscope (Zeiss, Oberkochen, Germany).

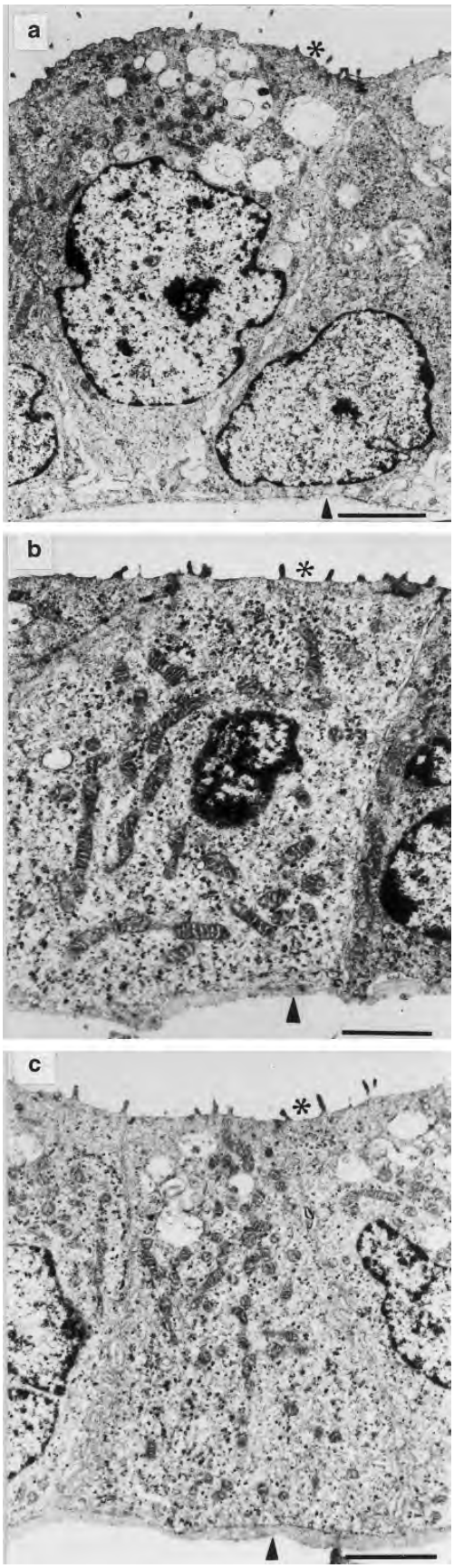
Evaluation. More than 100 epithelia were examined from gradient culture experiments for the present investigation. To obtain an objective result each hormone treatment was repeated at least three times. More than 10 epithelia were analyzed per experimental series. A minimum of 50 vertical cryosections per individual group was examined. The text and figures give the mean numbers of labelled cells.

Results

We investigated factors affecting the transition from an embryonic to a functional collecting duct epithelium. To prevent dedifferentiation the embryonic tissue was maintained under improved culture conditions. The explants were mounted in tissue carriers (Fig. 1a) allowing the epithelium to be be kept on a layer of organospecific extracellular matrix. Within 24 h the cells of the collecting duct ampulla grew outward in serum containing IMDM and formed a polarized epithelium covering the surface of the explant. In order to ensure continuous nutrition, the elimination of harmful metabolic products, and a down-regulation of synthesized paracrine factors, the epithelia were then transferred to gradient containers for the following 13 days (Fig. 1b, c). The epithelia separated the gradient container into an upper and a lower half. On the luminal and basal sides of the epithelia fresh culture medium was perfused by a peristaltic pump through the gradient container at a rate of 1 ml/h for the whole experimental period (Fig. 1c).

Firstly, we examined the appearance of the cultured epithelia under the light microscope after they had been exposed to constant medium flow in the gradient container for a period of 13 days. Over a diameter of 6 mm the surface of the explant in the carrier was completely covered by a polarized epithelium. This was also obtained in the control series where IMDM without addditives was perfused at the apical and basal side (Figs. 2a, 3a). A complete epithelium was also found when medium containing aldosterone was perfused either on the basal (Fig. 3b) or the apical (Fig. 3c) side of the epithelium. Thus, in principle, it is possible to maintain an intact epithelium over a long period of time under gradient perfusion culture.

Electron microscopy revealed that a polarized collecting duct epithelium had been established on the kidney matrix (Fig. 2b). The epithelium consisted of isoprismatic cells and showed distinct polarization. Clearly visible tight junctions separated the luminal from the basolateral plasma membrane. A basement membrane had developed (Fig. 3).



The cytoplasmic staining in the individual cells varied from light to grey to dark, as has also been reported for mature collecting duct of the rabbit [28]. In the control series without hormone, most cells had numerous vacuoles at the apical cell pole (Figs. 2b, 3a); few mitochondria were found. In contrast, aldosterone-treated cells had single vacuoles and abundant mitochondria (Fig. 3b, c). This effect was observed whether aldosterone-containing IMDM was perfused from the basal (Fig. 3b) or from the apical side (Fig. 3c). In the control series without hormone, wide intercellular spaces were obvious (Figs. 2b, 3a). In contrast, aldosterone-treated epithelia showed narrow intercellular spaces (Fig. 3b, c). Furthermore, we found that the administration of aldosterone led to densely interdigitating microplicae at the lateral plasma membranes (Fig. 3b, c), which was not observed in control experiments (Fig. 3a). Conspiciuous differences in the synthesis of the basement membrane were observed between controls (Fig. 3a) and aldosterone-treated epithelia (Fig. 3b, c). While in the controls (Figs. 2b, 3a) a very thin layer of newly synthesized basement membrane was always seen, aldosterone-treated epithelia exhibited a substantially thicker basement membrane (Fig. 3b, c).

From previous experiments we knew that aldosterone can modulate differentiation [22] and increase the number of cells which bind PNA on their luminal plasma membrane, as do β-type IC cells in the adult kidney [16, 29, 30]. It is not known if aldosterone acts on the embryonic epithelia on the basolateral side as it does in the adult organ, or if there is a luminal effect during organ maturation. For this reason aldosterone was added either to the basal (Fig. 4b) or to the luminal (Fig. 4c) perfusion medium for the whole culture period. Regardless of whether the medium containing aldosterone was perfused from the basal (Fig. 4b) or from the luminal (Fig. 4c) side, nearly all of the cells showed an intense reaction with the fluorescent PNA. In contrast, control epithelia without aldosterone showed only 15% strongly positive cells and 15% with a weak reaction at the luminal cell poles (Fig. 4a).

We also investigated the appearance of collecting duct proteins in the cultured epithelia using histochemical markers (Table 1; Fig. 5). Epithelia perfused with aldosterone-containing medium from the basal side showed intense labelling with PNA (Fig. 5a) and mAb P_{CD} 9 on all cells (Fig. 5e). In contrast, there was no labelling with mAb 503 (Fig. 5b), mAb IVF 12 (Fig. 5c), and mAb 703 (Fig. 5d).

Fig. 3 a–c. Electron microscopy of renal collecting duct epithelia after 13 days in a gradient perfusion culture container. **a** During luminal and basal perfusion with Iscove's modified Dulbecco's medium (IMDM) aldosterone was omitted. Numerous vacuoles are observed in the luminal cell poles (bar = 0.5 μm). **b** Aldosterone was added to IMDM perfused at the basal side of the epithelium in the lower part of the container. Numerous mitochondria are visible in the cytoplasm. **c** Aldosterone was added to IMDM perfused at the luminal side of the epithelium in the upper part of the container. Numerous mitochondria are visible in the cytoplasm. A thickened basement membrane (*arrowhead*) has developed (bar = 0.75 μm). *Asterisk* luminal side

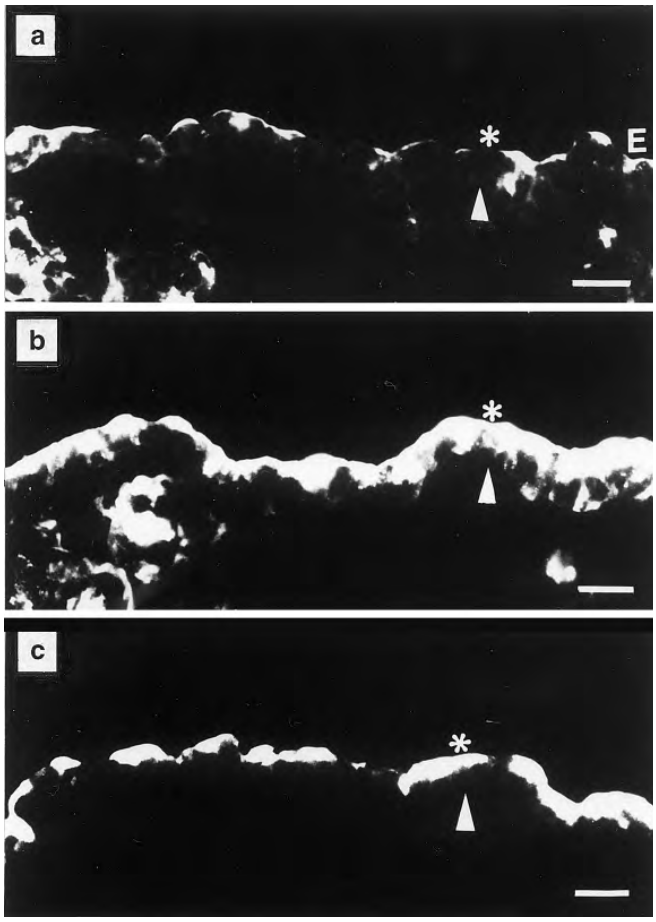


Fig. 4 a–c. Fluorescent peanut lectin (PNA) labelling on collecting duct epithelia (E) cultured for 13 days in a gradient perfusion container. **a** Control epithelium without hormonal treatment; only 30% of the cells are labelled. **b** Aldosterone was added to IMDM on the basal side of the epithelium; all of the cells are labelled. **c** Aldosterone was added to the luminal IMDM. Nearly all of the cells are labelled at the luminal cell poles. The *arrowhead* marks the basement membrane region; bar = 1 μm

Aldosterone stimulates sodium transport in the P cells of the adult collecting duct epithelium. In embryonic cells the hormone influences differentiation via an unknown mechanism. To find out whether sodium channels are involved in this process we added amiloride to the culture medium [31]. While aldosterone-treated epithelia showed 100% PNA-positive cells (Fig. 5a), aldosterone in combination with amiloride reduced this to less than 30% (Fig. 6a) [the same level found in control epithelia without any hormonal

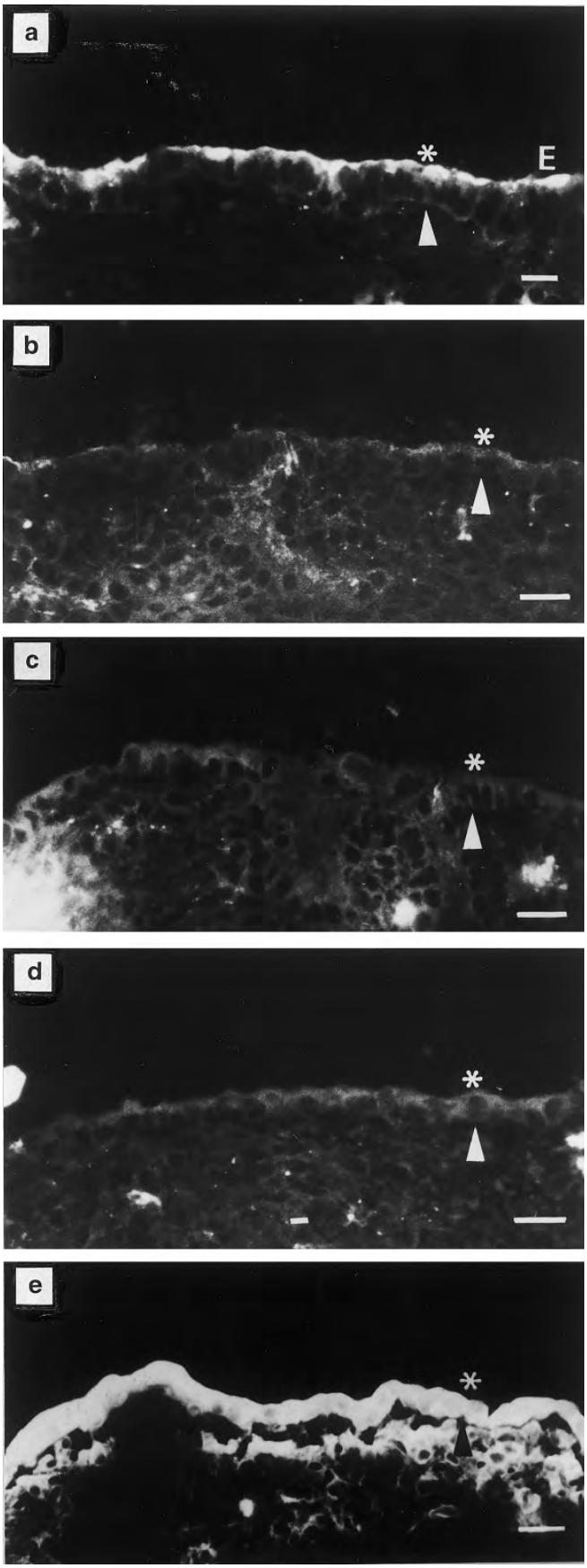


Fig. 5 a–e. Immunohistochemistry of renal collecting duct epithelia (E) after 13 days in a gradient perfusion container. Aldosterone was added to the IMDM perfused at the basal side of the epithelium. **a** PNA labels all of the collecting duct cells. **b** mAb 503 recognizes intercalated (IC) cells in the adult kidney but does not label the cultured collecting duct cells. **c** mAb IVF 12, a marker for α-type IC cells, does not label the cultured collecting duct epithelia. **d** mAb 703 labels principal (P) cells within the kidney, but no reaction is found on the cultured epithelia. **e** mAb $P_{CD}9$ recognizes all of the cultured epithelial cells. The *arrowhead* marks the basement membrane region; bar = 20 μm

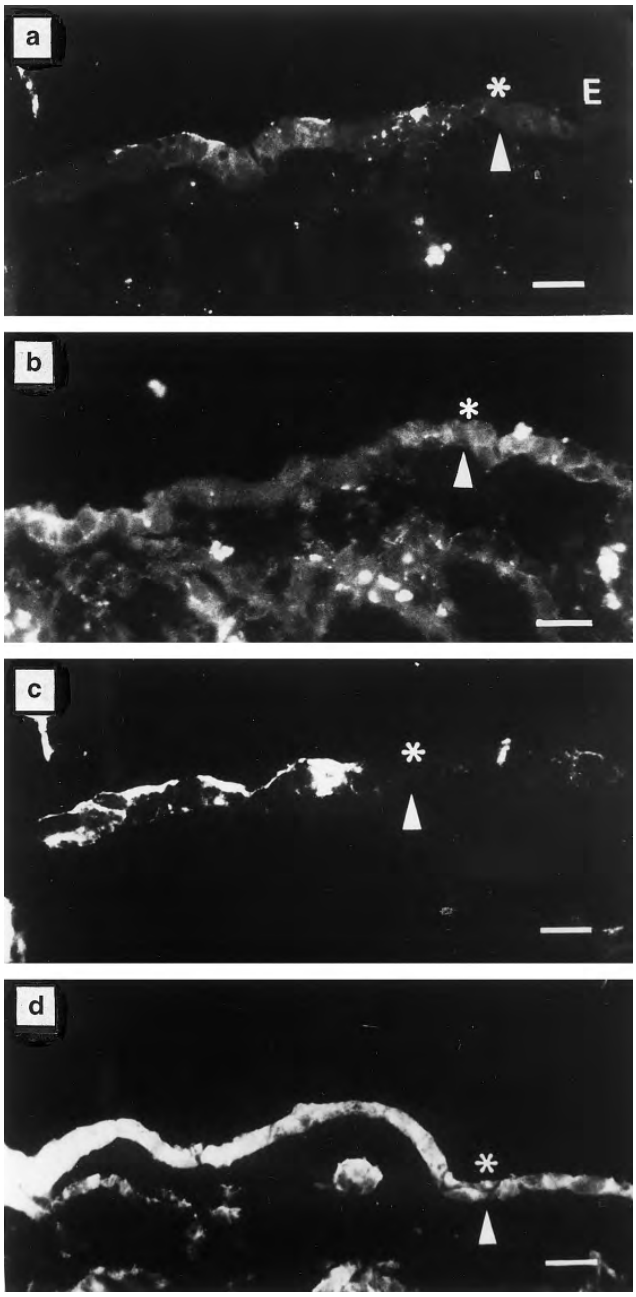


Fig. 6 a–d. Immunolabelling of renal collecting duct epithelia (E) after 13 days in a perfusion culture container. Aldosterone and amiloride were added to IMDM. **a** PNA labelling was found on less than 30% of the cells. **b** mAb 503 did not react with the cells. **c** mAb 703, a marker of P cells, still reacted with more than 50% of the cells. **d** mAb $P_{CD}9$ reacted with all of the cells and was expressed independently of aldosterone or amiloride treatment. The *arrowhead* marks the basement membrane region; bar = 20 μm

treatment (Fig. 4a)]. Furthermore, there was no reaction with mAb 503 (Fig. 6b) and mAb IVF 12 (not shown) on epithelia exposed to IMDM containing aldosterone and amiloride. In contrast, there was a positive reaction with mAb 703 (Fig. 6c). Between 45% and 50% of immunopositive cells were found, indicating the upregulation of a P-cell feature. The appearance of PCD 9 was not dependent on the action of aldosterone (Fig. 5e) and was not downregulated after amiloride application (Fig. 6d). Inhibition of aldosterone at the receptor site was negative. Spironolactone had to be used in rather high concentrations over the whole culture period [32]. We found tissue disintegration in the embryonic collecting duct epithelia.

Discussion

The degree of cellular differentiation in cultured epithelia [33–35] depends not only on the right extracellular matrix [2], but also on a continuous supply of nutrients and growth factors [9–11], the elimination of harmful metabolic products [36], and the prevention of an accumulation of synthesized paracrine factors. Because these demands cannot all be met in the stagnant environment of a culture dish, in vitro conditions were optimized. Collecting duct epithelia were mounted in tissue carriers (Fig. 1a) and placed in newly developed gradient culture containers (Fig. 1c). With this experimental protocol it was possible to superfuse the luminal and basal sides of the epithelia continuously with fresh culture medium (Fig. 1b). We showed that in principle it is possible to harvest tissue fulfilling all the morphological criteria for mature renal collecting duct epithelium after 13 days of such culture (Figs. 2, 3). Regardless of whether a control medium or an aldosterone-supplemented medium was used, a uniformly developed epithelium was observed in all cases.

In contrast to other tubular portions of the kidney, the renal collecting duct epithelium consists of a heterogeneously composed cell population, the P and IC cells [37, 38]. The mechanism by which the different cell types arise is unknown. However, we have long observed indications that aldosterone acts as a differentiation hormone. Causing a physiological sealing in the embryonic collecting duct epithelium and inducing the synthesis of the collecting duct-specific proteins P_{CD} 2 and P_{CD} 3 [39]. These results were not surprising, because high levels of aldosterone receptors are expressed in the developing collecting duct [40]. Data from other groups also showed that aldosterone acts as a differentiation hormone not only in renal but also in other tissues [41–43]. Furthermore, it was shown that aldosterone upregulated PNA binding in embryonic collecting duct epithelia (Fig. 4b, c) [22], a typical feature of β-type IC cells in the adult rabbit kidney [16, 25], but not expected on P cells, which are involved in sodium reabsorption [44, 45].

From adult epithelia it is known that hormone receptors are distributed over the whole cell and are not restricted to the luminal or the basolateral domain [46–48]. For this reason we ran tests to determine the side from which aldosterone is able to act on the embryonic collecting duct epithelia. For these experiments the gradient container was very useful, because it allowed us to perfuse medium containing aldosterone either from the apical or from the basal side of the developing epithelium during the whole culture period (Fig. 1b, c). Aldosterone applied from the basal side was expected to have an effect (Figs. 3b, 4b), because this pathway is known from the adult kidney [45]. However, it was surprising that the embryonic epithelia also responded to the luminal application of aldosterone

(Figs. 3c, 4c). Apparently aldosterone can reach the cytoplasmic receptors either through the apical plasma membrane or via the leaky tight junctions at the beginning of the culture period through the basolateral side. Earlier experiments showed that at least 5 days are needed until the epithelia are sealed with a reasonable transepithelial resistance by aldosterone [39, 49].

The present experiments showed that, according to purely morphological criteria, a mature collecting duct epithelium developed within the gradient container (Figs. 2, 3). However, immunohistochemistry demonstrated that while some of the typical collecting duct features develop under the improved culture conditions, others do not. We now have evidence that aldosterone is able to trigger the development of PNA binding (Fig. 4), while blocking the sodium channels by amiloride-upregulated mAb 703 binding (Fig. 6c). These results showed that both hormone action and sodium channels are involved in the differentiation of the embryonic collecting duct epithelium.

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