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Aldosterone modulates PNA binding cell isoforms within renal collecting duct epithelium

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Aldosterone modulates PNA binding cell isoforms within renal collecting duct epithelium. To investigate the differentiation of the ampullary collecting duct cells into adult principal and intercalated cells, the embryonic cortex of newborn New Zealand rabbit kidney was isolated and brought in culture. With this culture technique the ampullary cells formed a polarized collecting duct epithelium which was kept under permanent exchange of medium and in the presence of aldosterone, argine vasopressin and/or insulin. After 14 days of perfusion culture the epithelia showed light and dark cells resembling the principal and intercalated cells of the adult collecting duct. The differentiation from embryonic into adult collecting duct cells was controlled by applying the monoclonal antibody CD 7. Independent of the hormonal treatment all of the epithelial cells matured in culture and expressed the CD 7 antigen. This corresponded with the situation found within the adult kidney, where the CD 7 antigen was localized in all principal and intercalated (IC) cells, whereas the embryonic ampullary epithelium in the neonatal kidney remained negative. A differentiation feature of the β-type intercalated cell was investigated by labeling the cultured epithelia with peanut agglutinin (PNA). In contrast to the CD 7 antigen the development of PNA binding was highly dependent of time and individual hormone administration. While in control epithelia only 8% of PNA positive cells were found, aldosterone induced epithelia revealed 72% PNA labeled cells. The combination of aldosterone and insulin increased the number of PNA-positive cells to 90%. By scanning electron microscopy it could further be shown that several isoforms of cells were reactive with PNA. Thus, in culture the PNA label is not restricted to the typical β-type IC cells.

During renal organogenesis the collecting duct epithelium undergoes profound functional changes. From the first steps of kidney development until the late neonatal period the ampullary collecting duct (CD) serves as a potent inductor of all nephron generations [1–6] with a hidden microheterogeneity [7]. The embryonic inductor function is obviously lost, while the CD epithelium develops the regulatory capability to control the acid/base status, the Na/K balance and the water content of the urine [8, 9]. These complex physiological functions are triggered by the light principal (P) and at least two types of dark intercalated (IC) cells [10–16]. To date it is not known which are the morphogenetic factors for terminal differentiation influencing the transition from the embryonic homogenously-appearing inductor to the mature and heterogenously composed collecting duct epithelium. It is only known that between the embryonic and mature collecting duct cells marked morphological divergences exist and that the expression of stage-specific proteins occurs [7, 17–20].

The ampullary collecting epithelium of rabbit neonatal kidney can easily be prepared for *in vitro* experiments [21]. The introduction of a newly developed perfusion culture system further allowed the prolonged culture of these cells under very controlled conditions [22, 23]. Combining both methods we investigated the development of embryonic ampullary epithelium after treatment with aldosterone [8], insulin [24] and vasopressin [25], which act on adult collecting duct cells by different cellular mechanisms. Thus, we wanted to investigate if a functional stimulus leads from embryonic to differentiated collecting duct cells.

Methods

Conventional cell culture

Thin cortical explants from the kidney of newborn New Zealand rabbits (Fig. 1a) [21] were mounted on sterile cell holder sets (Fig. 1b) [22, 23] and placed in 24-well tissue culture plates (Falcon, Becton Dickinson, Heidelberg, Germany). The explants consisted of a piece of capsula fibrosa with adherent collecting duct ampulla, S-shaped bodies and nephrogenic blastema. During culture of these explants in Iscove's modified Dulbecco's medium (IMDM/HEPES) (Gibco-BRL Life Technologics, Eggenstein, Germany) containing 10% fetal calf serum we observed an outgrowth of cells from the collecting duct ampulla (Fig. 1c) [21]. Within 24 hours of culture the entire surface of the explant was completely covered by a single-layered collecting duct epithelium (Fig. 1d). Preculture was carried out in a Heraeus tissue incubator (Hanau, Germany) at 37°C in a humidified atmosphere containing 5% CO_2/95% air for 24 hours.

Perfusion cell culture

To mimic the *in vitro* situation comparable to an organ, cell cultures were maintained with permanent superfusion of fresh medium [22, 23]. The perfusion cell culture was started by mounting the working line (Fig. 2). It consisted of two Schott glass bottles (Fig. 2.1) (500 ml), two screw caps (Fig. 2.2) (Nr.47622, Tecnomara, Fernwald, Germany), a cell culture

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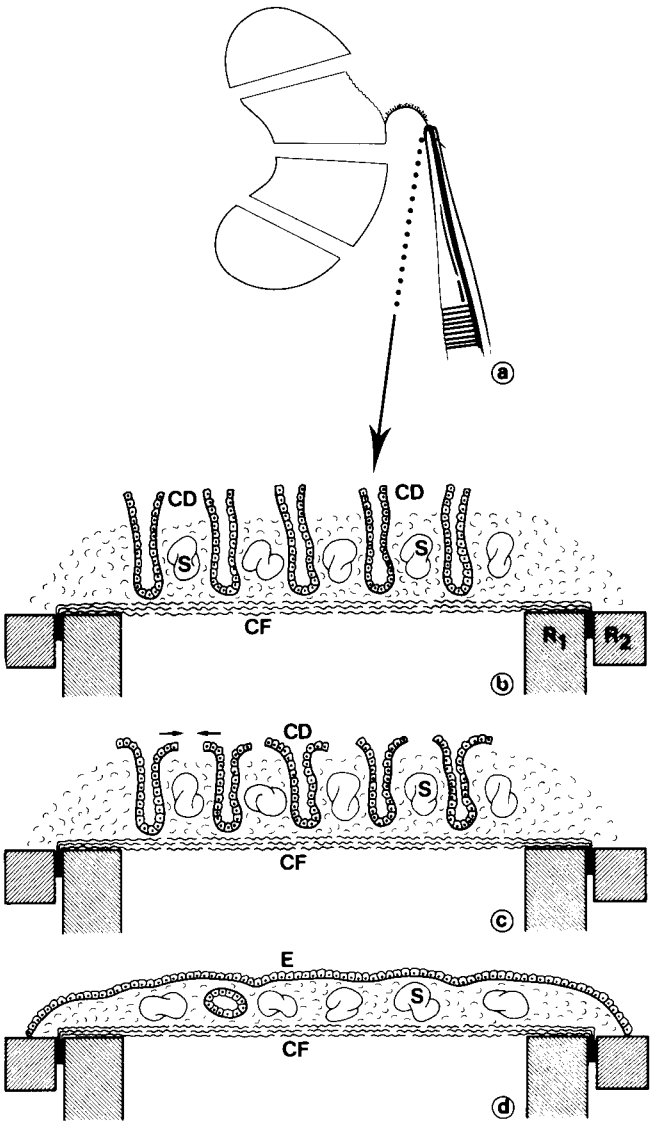


Fig. 1. *Schematic illustration of embryonic collecting duct isolation for cell culture experiments from the outer cortex of neonatal rabbit kidneys.* (**a**) The capsula fibrosa (CF) with adherent collecting duct anlagen or ampulla (CD) is stripped off with jeweler's forceps [21] and (**b**) is placed on a cylinder (R_1) and fixed with an overlapping ring (R_2). (**c**) During culture the ampullary cells spread out (arrows) and (**d**) form a polarized collecting duct epithelium (E). S = S-shaped body.

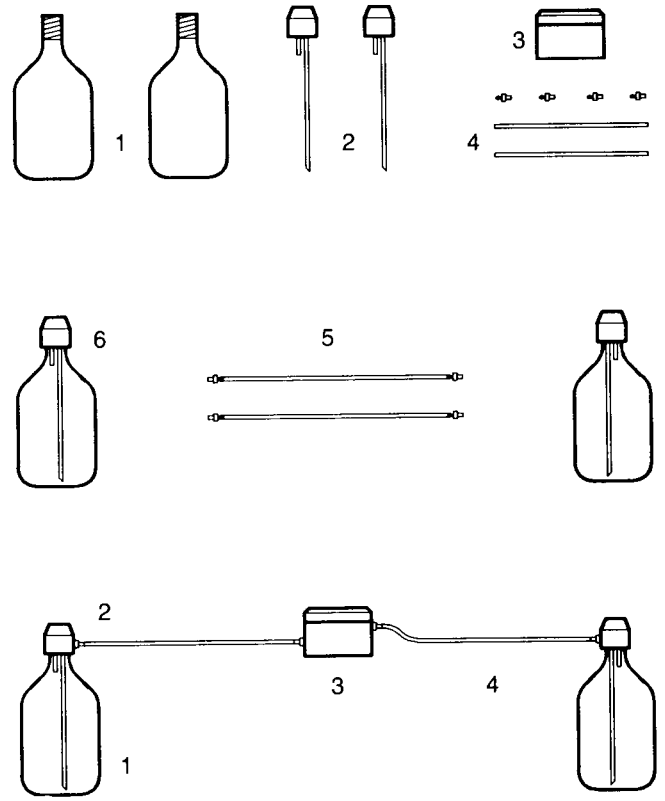


Fig. 2. *Schematic illustration of mounting the complete working line for perfusion cell culture.* (1) glass bottles, (2) screw caps, (3) cell culture container for the epithelia, (4) tubes with fittings.

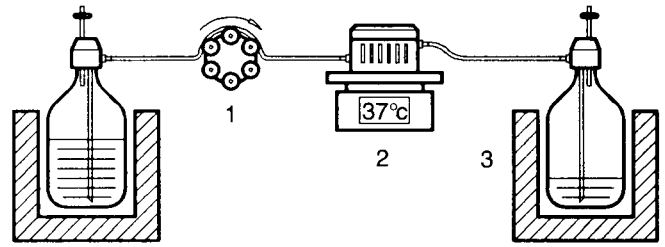


Fig. 3. *Schematic illustration of a complete working line for perfusion cell culture of collecting duct epithelia consisting of a roller pump (1), a heating block (2) and the cooling device (3) for the medium.* The epithelia are kept within the cell culture container on the heating block (2).

container (Fig. 2.3), two silicone tubes with an inner diameter of 1 mm and male luer fittings (Fig. 2.4). The luer fittings were connected with both silicone tubes (Fig. 2.5) and the screw caps were mounted on the glass bottles (Fig. 2.6). The cell culture container (Fig. 2.3) was connected over the silicone tubes with the screw caps (Fig. 2.2) and glass bottles (Fig. 2.1). After sterilization of the complete working line in an autoclave the parts were placed under a sterile laminar air hood for further processing. The screw cap of one glass bottle was opened to fill in the culture medium. After closing the bottles by tightening both screw caps, sterile filters (Nr.6400104, ICN Flow, Meckenheim, Germany) were placed at each top. The cell holder sets were placed in the cell culture container with sterile forceps. The chamber was filled with medium and sterile locked by closing the lid of the cell culture container. The container was placed on a 37°C heating plate and connected to continuous medium supply by a roller pump (Fig. 3). Both medium bottles were placed in a refrigerated circulating water bath.

A constant medium flow rate of 1 ml/hr was used in all experiments. Iscove's Modified Dulbecco's Medium—IMDM/HEPES with or without 10% FCS and 1 ml/100 ml antibiotic-antimycotic mixture was used as the basal control medium. Because the collecting duct cells are the main targets for certain steroidal and nonsteroidal hormones, aldosterone (1×10^{-7} M), arginine vasopressin (1×10^{-6} M) and/or insulin (1×10^{-6} M) were added to the culture medium in the different experimental series [24–26]. The superfusion of cultures with medium and the application of the different hormones were started 24 hours

after preparing the cultures. Total culture time was 14 days (1 day preculture, 13 days perfusion culture).

Culture medium and additives were obtained from Gibco-BRL Life Technologies). Aldosterone, vasopressin and insulin were obtained from Sigma (Deisenhofen, Germany). All parts of the perfusion culture system are available through Minucells and Minutissue (Bad Abbach, Germany).

Fluorescence microscopy

For cryosectioning cultured epithelia were immediately frozen in liquid nitrogen. The epithelia were orientated in the way that exactly vertical cross sections could be obtained. Five micrometer cryosections (Cryostat HM 500, Microm, Heidelberg, Germany) were first fixed in ice cold ethanol, washed several times with phosphate buffered saline (PBS) and incubated with blocking solution (PBS, pH 7.2, 10% horse serum, 1% bovine serum albumin, (Sigma) for one hour. For detection of differentiated CD cells the monoclonal antibody CD 7 was used according an earlier described method [7].

For detection of peanut lectin (PNA) positive cells the sections were incubated for 40 minutes with PNA-rhodamine conjugate (Vector, Burlingame, Vermont, USA) diluted 1:2000 in PBS [7].

For exact determination of the epithelial cell number peanut agglutin labeled tissue was counterstained with 1 μg/ml DAPI (4',6-diamine-2'-phenylindole dihydrochloride), a fluorescent nuclear dye (Boehringer, Mannheim, Germany). The sections were embedded in FITC-guard (Testoc; Chicago, Illinois, USA) and examined using a Zeiss Axiovert 35 microscope (Zeiss, Oberkochen, Germany).

Histology

For transmission electron microscopy (TEM) mounted collecting duct epithelia were fixed after 14 days of long-term perfusion culture for 20 minutes in IMDM containing 3% glutaraldehyde. Following the initial fixation step, the epithelia were removed from the cell holder sets by lifting the span ring with forceps. The epithelia were then postfixed with 1% osmium tetroxide in PBS and dehydrated in a series of alcohols, passed through propylene oxide and embedded in Epon. Semithin sections were stained with Richardson solution and examined under the light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate. The specimens were examined with a Zeiss electron microscope (EM 902).

For conventional scanning electron microscopy (SEM) the fixed specimens were dehydrated in gradual series of ethanol, critical point dried using CO_2 and sputter coated with gold. Examination was carried out in a Zeiss scanning electron microscope DSM 940 A.

PNA binding on the luminal surface of cultured epithelia was visualized by a backscattered electron detector [27, 28]. Cultured epithelia were fixed for 30 minutes with 2% paraformaldehyde and 0.02% glutaraldehyde in phosphate buffered saline (PBS) pH 7.4. The labeling was performed by using biotinylated PNA (Sigma) and a 6 nm colloidal gold coupled antibody against biotin (Aurion/Biotrend, Köln, Germany). These incubation steps were followed by postfixation with 2% glutaraldehyde in PBS and a five minute treatment with silver enhancement (Janssen, Olen, Belgium). For control experiments the PNA/biotin incubation step was omitted. The specimens were dehydrated in an ascending ethanol series and then critical point dried. Then the epithelia were sputter coated with a 3 nm chromium layer. The epithelia were studied with a Hitachi S-800 SEM equipped with an annular single crystal backscattered electron detector. Distribution of colloidal gold label was detected by backscattered electrons at an accelerating voltage of 10 kV. The working distance was 10 nm.

Evaluation

In total more than 200 epithelia were examined from perfusion cell culture experiments for the present investigation. To obtain an objective result each hormonal treatment of epithelia was repeated for three times. Then at least five epithelia were analyzed per experimental series and again 50 cryosections of each individual group were examined. In the text and the figures always the mean of PNA labeled cells is given that was obtained from each individual experiment by counting 1000 cells.

Results

By applying the conventional cell culture technique in conventional Petri dishes we made many attempts to generate intercalated cells from neonatal kidney in a reproducible way. Despite numerous experiments we did not succeed. This was the reason to use a newly developed perfusion cell culture technique [22, 23]. By slow and permanent superfusion of fresh medium on renal collecting epithelium we obtained the following results.

Without any subculturing the renal collecting duct epithelia were kept in perfusion culture for 14 days. As revealed by light and electron microscopy a monolayer of polarity differentiated collecting duct epithelium was established (Fig. 4). Profound morphological differences were observed between epithelia perfused with and without hormonal additions. In transmission electron microscopy control epithelia cultured without any hormonal additives showed only a principal-like cell type within the epithelium (not shown) [23], while treatment with aldosterone caused the expression of light principal cells and mitochondria-rich grey and dark cells (Fig. 4). By morphological criteria these cells resembled the principal (P) and intercalated (IC) cells found within the collecting duct of the adult kidney [10, 12, 13]. Besides the morphological appearance we were interested in elaborating whether the cultured epithelia express any typical proteins of embryonic or differentiated collecting duct cells. Immunoincubation and fluorescence microscopy of the CD 7 antibody revealed that all of the cultured epithelial cells carried this collecting duct specific antigen (Fig. 5). This was independent of the hormonal treatment of cultures. Thus, in this respect all of the cultured ampullary epithelia have differentiated and showed characteristics of adult collecting duct cells [7] as compared to the embryonic ampullary epithelium.

Because both principal and intercalated collecting duct cells express the CD 7 antigen, the PNA lectin binding was used to investigate to which degree this cell characteristic was expressed within the cultured collecting duct epithelium under different hormonal treatment. PNA [29] is a frequently used marker of β-type collecting duct cells, which labels the apical surface of HCO_3^- secreting cells [7, 14, 15, 18, 20]. These cells are further rich in mitochondria, carbonic anhydrase, and diffuse H^+-ATPase, but generally devoid of band 3-like anion exchangers.

To determine the exact amount of PNA-positive cells within

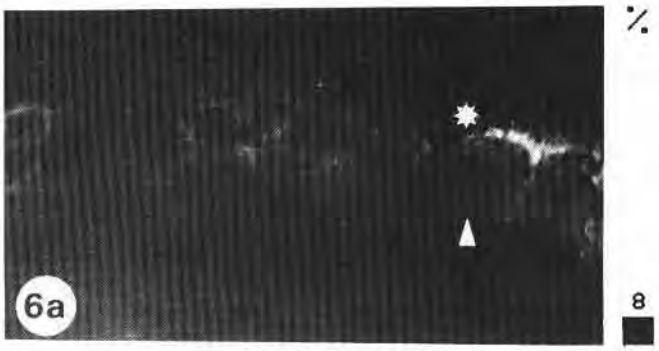
Fig. 5. *Immunofluorescence microscopy using the CD 7 antibody on collecting duct epithelium cultured under permanent perfusion and in the presence of aldosterone for 14 days.* All of the epithelial cells are labeled by the antibody. Arrowhead is the basement membrane; * luminal side. Bar = 30 μm.

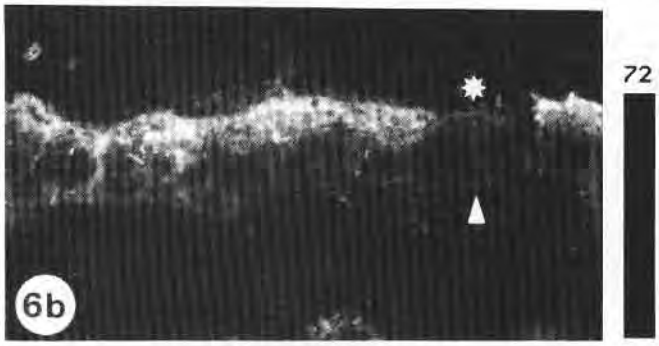
Fig. 4. *Electron microscopic view of collecting duct epithelium cultured under permanent perfusion and in the presence of aldosterone for 14 days.* Various dark IC cells and few light P cells are visible. Arrowhead marks the basement membrane; * is the luminal side. Bar = 4 μm.

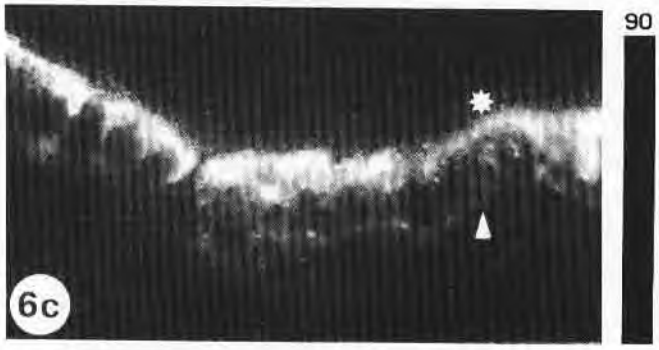
the cultured collecting duct epithelia double labeling experiments with PNA and DAPI were performed. This method allowed to calculate very easily the exact number of nuclei within the epithelium and the amount of labeled or unlabeled luminal cell poles stained by PNA on the same cryosection. In the following text the mean of at least 1000 cells per treated epithelium is given.

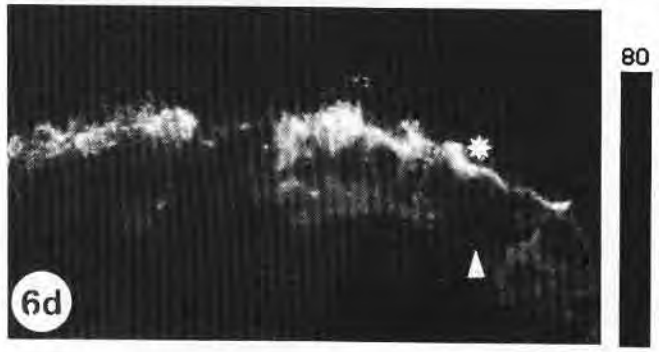
When collecting duct epithelia were cultured for 14 days without any hormones the PNA label was found at the luminal cell poles with a constant low rate of 8% positive between 92% unlabeled cells (Fig. 6a). This relationship dramatically changed if aldosterone was applied for the whole culture period (Fig. 6b). In experimental series including aldosterone a mean of 72% of PNA labeled cells was observed compared to 28% non-reactives. The amount of PNA positive cells still increased to 90% when aldosterone in combination with insulin was used as the hormonal supplement, thus indicating a slight additive effect (Fig. 6c). Addition of AVP to the aldosterone/insulin containing medium did not further increase the number of PNA binding cells (Fig. 6d) as compared to the aldosterone/insulin containing series (Fig. 6c). In contrast, a slight decrease to 80% positive cells was found in the aldosterone/insulin/AVP treated specimens (Fig. 6d). When aldosterone in combination with AVP was used, a number of 44% PNA positive cells could be detected (Fig. 6e), indicating an inhibitory effect of the peptide hormone AVP on the steroidal action of aldosterone.

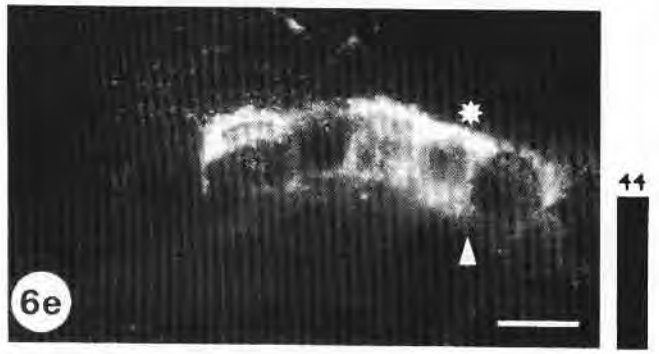
Perfusion culture experiments without aldosterone but with insulin and/or AVP in the medium should illuminate the degree of specific steroidal hormone action. Treatment of collecting duct epithelia with insulin alone gave 18% (Fig. 7a), AVP showed only 10% (Fig. 7b), and insulin in combination with AVP 16% (Fig. 7c) PNA positive cells. On average, the percentage of PNA positive cells in these series is somewhat higher as compared to controls (8%; Fig. 6a); they are, however, extremely lower as compared to aldosterone treated specimens

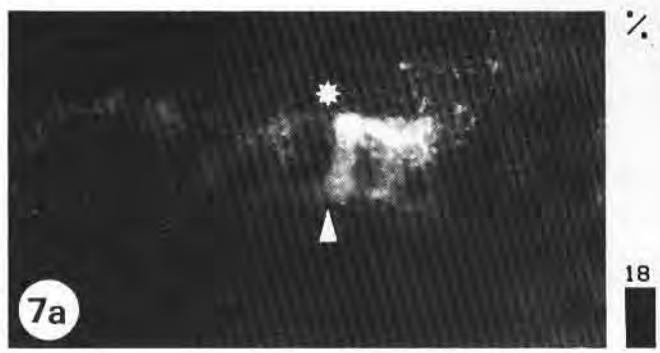


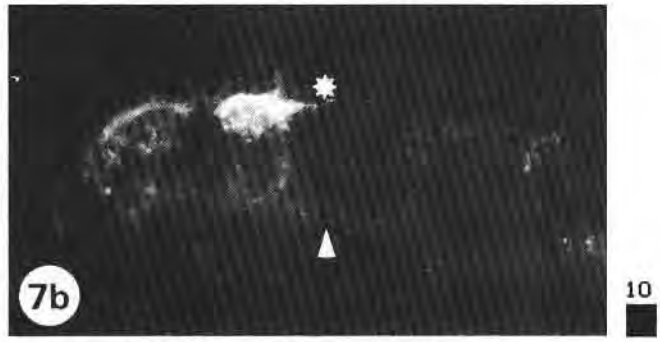












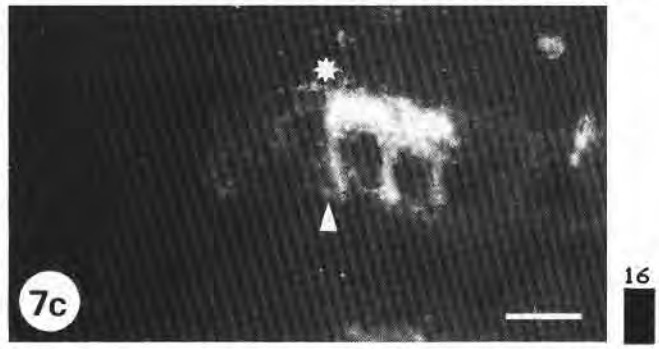


Fig. 7. *Fluorescence microscopy of the PNA label of collecting duct epithelia cultured for 14 days with the perfusion method under various treatment with hormones.* The black bar represents the amount of PNA positive cells within the epithelium. **(a)** Insulin, **(b)** AVP, **(c)** insulin/AVP treatment. Only 10 to 18% of PNA positive cells could be obtained in this series. Bar = 30 μm.

Fig. 6. *Fluorescence microscopy of the PNA label on collecting duct epithelia cultured for 14 days with the perfusion method and under various treatments with hormones.* The black bar at the right side represents the amount of PNA-positive cells within the epithelium. **(a)** Control without hormone; **(b)** aldosterone, **(c)** aldosterone/insulin, **(d)** aldosterone/insulin/AVP, **(e)** aldosterone/AVP series. A total of 90% PNA positive cells were obtained in the aldosterone/insulin treated specimen. Bar = 30 μm.

(72 to 90%; Fig. 6 b–d). In consequence, this result indicates the exclusive ability of aldosterone to induce the development of PNA binding in cultured collecting duct epithelia, while individual treatment with insulin and AVP did not (Fig. 7).

As compared to aldosterone treated specimens (Fig. 6b) a constant down-regulation of PNA binding was obtained by the use of AVP (Fig. 6e). While treatment with aldosterone alone generated 72% PNA-positive cells (Fig. 6b), aldosterone in

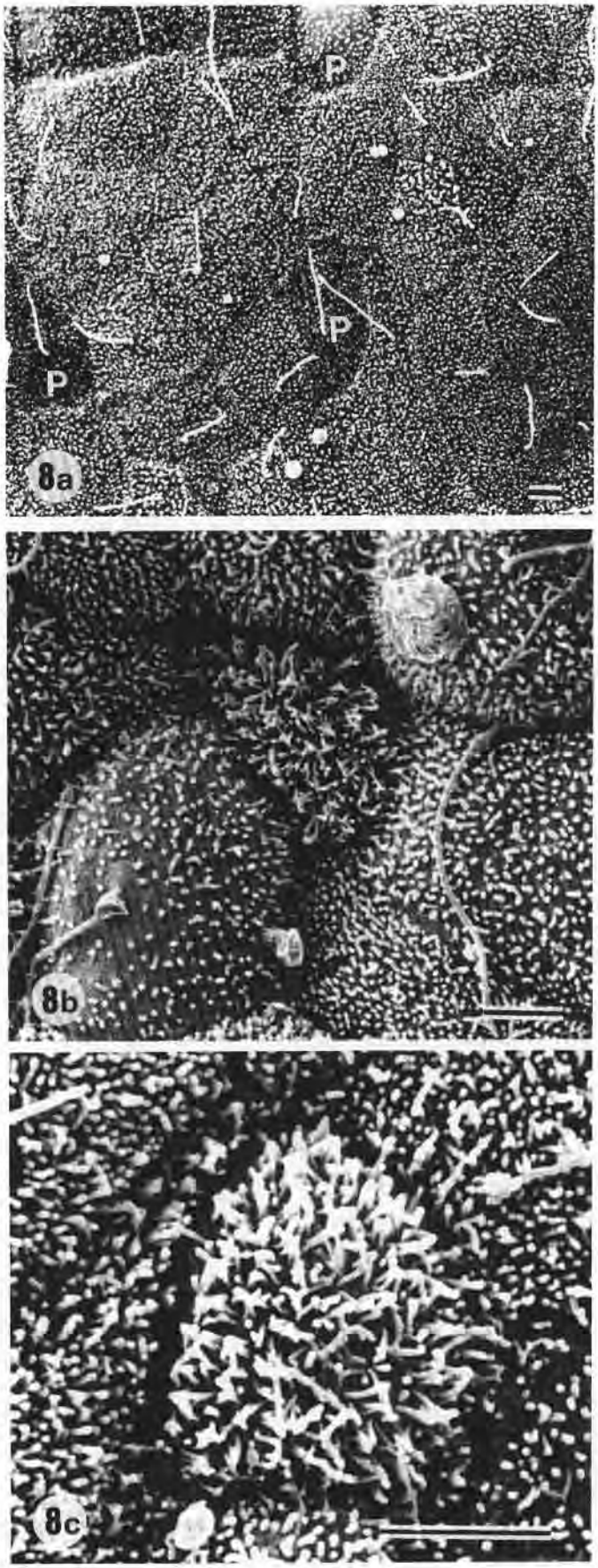


Fig. 8. *Scanning electron microscopy of aldosterone treated epithelia cultured for 14 days with the perfusion method.* (a) The micrograph presents principal (P) like cells surrounded by various intercalated-like cells. (b) and (c) Higher magnification demonstrates a single intercalated like cell surrounded by cells with different densely-packed microvilli each with a cilium. (c) The micrograph shows an intercalated-like cell with very long and densely packed microvilli surrounded by other cells with shorter but even more densely packed microvilli. Bar = 2.5 μm.

combination with AVP gave only 44% (Fig. 6e). Thus, aldosterone and AVP may act as antagonists concerning the expression of PNA binding on IC cells during development of cultured epithelia.

In a further culture series the time-dependent development of PNA binding was investigated. The experiments revealed that the development of PNA binding on aldosterone/insulin treated collecting duct epithelia (Fig. 6 b–d) is a very slow process. After four days in culture the first and very slight appearance of PNA binding was observed on the luminal poles of single cells. Within the next 10 days 90% of the cells became PNA positive and reached a maximum of fluorescence intensity within 14 days.

In a following series of experiments the apical cell surface of aldosterone treated epithelia was investigated by scanning electron microscopy (Fig. 8). The low magnification micrograph of the luminal cell poles of cultured epithelia presented a remarkable heterogeneity (Fig. 8). Principal-like cells with a cilium and short microvilli were in the minority as compared to intercalated-like cells. In contrast, various types of intercalated-cell like features were found [11]. On many of the intercalated-like cells we observed a cilium which is normally not present in the adult rabbit cell type. Such a cell type was described for guinea pig kidney [30]. Because the cultured collecting duct cells derived from ampullary cells where most of the cells have a cilium, we took it as an indication that the terminal differentiation is not completed under the described culture conditions.

In a final experimental series we used the scanning electron microscope to analyze the binding of gold-labeled PNA on the luminal cell surface of aldosterone treated epithelia (Fig. 9). The application of chromium as a coating layer for SEM investigation revealed cell surface morphology and corresponding labeling in one image. Cultured collecting duct cells grown under the hormonal influence of aldosterone/insulin/AVP exhibit a diversity of cell surface morphologies which are characterized by the different equipment with microvilli (Fig. 9).

Figure 9a shows a portion of cultured collecting duct epithelium at a primary magnification of 5000×. Unlabeled cells with a dark appearing surface covered with a relatively low number of short and stubby microvilli surround two cells with a more differentiated surface. The bright appearing cell on the left side is covered with densly packed short microvilli. The decoration of the cell surface with silver-enhanced colloidal gold coupled antibodies against biotinylated PNA results in a bright signal in the material dependent BSE-mode. The neighboring cells on the right side exhibit less densly arranged, longer microvilli. The distinct label of these cells can already be discerned at this low primary magnification as single bright spots located especially on the microvilli. These cell specific labeling properties became more clearly at higher magnification of 12,000×. Figure 9b shows three adjacent cells belonging to different types. They

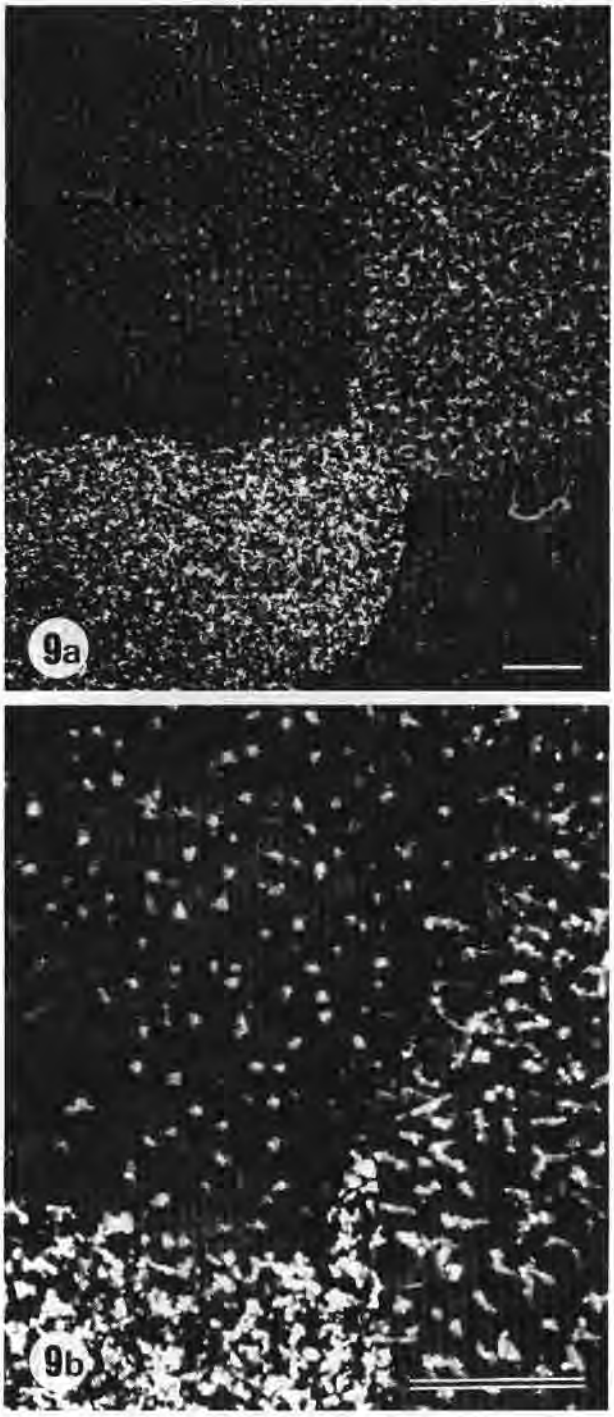


Fig. 9. *SEM of PNA labeled and chromium-coated cultured collecting duct epithelium.* (a) Overview of the luminal surface at a primary magnification of 5000×. Labeled cells are more densely covered with microvilli and appear with a brighter surface. The surrounding cells covered with less microvilli remain unlabeled. (b) Three adjacent cells can be distinguished by their labeling patterns and cell surface morphology. Bar = 2 μm.

can be distinguished by surface morphology and labeling properties as well.

Discussion

One of the most crucial problems in cell biology and especially in cell culture technology is the question of how far cells have developed specific features under *in vitro* conditions. The experiments on renal cell cultures and especially on collecting duct cells are hindered by the lack of such suitable and easily available commercial markers for biological cell identification. Polyclonal antibodies raised in rabbits are in most cases not suitable for cell cultures derived from rabbit tissue, and monoclonal antibodies obtained as a gift from other laboratories differ in quality and availability. In addition, the lectins are frequently used tools for cell recognition. Because PNA is easily available, it became a widly distributed marker to identify the β-type of intercalated cells as well within the mature kidney [14, 15, 20] as in collecting duct cell cultures from adult [26] and neonatal [31] renal tissue. Therefore it has to be mentioned that PNA similarly labels a terminal carbohydrate without knowing the characteristics of the adjacent molecular structures.

We investigated the differentiation process of the embryonic ampullary collecting duct epithelium into differentiated P and IC cells. We knew from our own electron microscopical investigations that IC-like cells were obtained in culture (Fig. 4). Therefore we tested whether embryonic ampullary cells could be stimulated *in vitro* to develop structures which bind PNA like the adult β-type IC cells.

In the present experiments we demonstrated a simple method to isolate the ampullary collecting duct epithelium for long-term culture experiments (Fig. 1). With this model it became possible to get insights initiating and regulating the terminal differentiation from ampullary cells into P and IC cells (Fig. 1). Furthermore, we showed that aldosterone is able to induce within the ampullary epithelium the PNA binding (Fig. 6b) as it is known as a typical differentiation feature of the β-type of IC cells [18, 20]. Comparing the *in vitro* function with the function of aldosterone in the adult kidney this result was unexpected because the hormone acts not on the β-type intercalated cells but on principal cells to stimulate the Na^+-transport [8, 9]. A morphogenetic action of aldosterone on intercalated cells or on embryonic ampullary cells is unknown to our knowledge. On the other side it was found that the embryonic collecting duct showed the highest concentration of aldosterone receptors in the developing kidney as compared to other nephron structures [32]. Thus, the occurrence of aldosterone receptors in the fetal collecting duct in such an unexpected concentration supports a specific role of aldosterone in kidney development as found in our present experiments. However, up to 90% PNA positive cells were found in aldosterone/insulin-treated cell cultures (Fig. 6c). Compared to the renal collecting duct of the adult organ this demonstrates an unnatural overexpression of the PNA binding cells in the cultured epithelia. What makes it again questionable is whether aldosterone acts as a single morphogenetic factor in kidney development. This assumption is supported by the fact that independent of the hormonal treatment, all of the epithelial cells gained the CD 7 antigen (Fig. 5) and AVP acted as an inhibitor to the aldosterone action (Fig. 6e).

In recent literature the β-type of intercalated cells has been found to be the precursor of principal cells [26]. This result was obtained from cell culture experiments in which the amount of PNA positive cells decreased with time. It was not considered that the disappearance of PNA positive cells could be a dedifferentiation process *in vitro*. In contrast, the results presented in our study showed that PNA binding is inducible during prolonged culture of embryonic collecting duct epithelium in the presence of aldosterone.

Under the chosen *in vitro* culture conditions neither completely PNA negative (Fig. 6a) nor 100% positive epithelia (Fig. 6c) were observed, whereas the differentiation marker CD 7 labeled all cultured epithelial cells (Fig. 5). We interpret the failure of a complete 100% induction or a 100% suppression of PNA binding as a hint for two types of precursor cells in the ampulla as shown in a previous investigation [7]. Regarding the PNA binding feature, one cell type seems to express the capability independent of aldosterone, and the other is highly dependent of the hormone.

The scanning electron microcopy demonstrated (Fig. 8) that in aldosterone treated epithelia only few cells with principal-like features but numerous different intercalated-like cells were detected as expected from the transmission electron microscopy (Fig. 4). The micrographs with higher magnification (Fig. 8 b, c) showed that only a minority of the intercalated-like cells revealed the typical appearance known from the β-type IC cells within the adult kidney. In addition, we found that most of the IC-like cells showed a cilium which is normally not present in the adult form. Furthermore, we found that the luminal outfit varied from high and dense to low and less dense microvilli. Despite this fluent pattern up to 90% of the cultured cells showed PNA binding in fluorescence (Fig. 6c) and by scanning electron microscopy (Fig. 9). These results demonstrate that the PNA binding is not restricted only to a specific form of the β-type collecting duct cell. In contrast, various fluent forms of cultured collecting duct cells are labeled by PNA. Not knowing the degree of maturity and the physiological properties of the different PNA binding cell types, it has to be taken into consideration that PNA labels in culture not only a single cell type but a variety of isoforms.

Acknowledgments

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