

Transepithelial Potential in Mesonephric Nephrons of 7-day-old Chick Embryos in Relation to the Histochemically Detected Sodium Pump

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Received January 24, 2001

Accepted June 8, 2001

Summary

In order to obtain basic information on the transport properties of differentiating embryonic nephrons, we examined the 7-day-old chick mesonephros by measuring the transtubular epithelial potential difference (TPD) and by histochemical detection of Na,K-ATPase activity. TPD as an indicator of the electrogenic transport was measured in individual segments of superficial nephrons *in vivo*. Their electric polarity was always lumen-negative. TPD was reduced by addition of 10 mM KCN applied to the mesonephric nephrons from the outside. In the proximal tubules, TPD was significantly lower (mean±SD: -1.0 ± 0.5 mV) than in the distal and collecting tubules (-2.2 ± 1.0 mV, $p\leq 0.05$). Activity of the sodium pump was evaluated histochemically by detection of ouabain-sensitive potassium-dependent p-nitrophenyl phosphatase in cryostat sections of the mesonephros. The enzyme activity was demonstrated only in distal tubules and in the collecting ducts, but not in the proximal tubules. These findings have revealed significant differences between embryonic nephron segments: the distal tubule, in contrast to the proximal one, is supplied by the sodium pump and is able to generate higher TPD. Therefore, we consider that it is only the distal nephron, which possesses the ability of active transport.

Key words

Chick embryonic kidney • Tubular segments • Transepithelial potential difference • Ouabain-sensitive K⁺-NPPase • Active transport

Introduction

Information about the transport functions of embryonic nephrons is rather scarce because nephrons are usually inaccessible to direct examination under *in vivo* conditions. In addition to the difficult position of the embryo or fetus in the mammalian uterus, the organization of the definitive metanephric kidney itself represents an obstacle to direct investigation of

developing nephrons since they are covered by the nephrogenic tissue during the entire prenatal period. The embryonic kidney, mesonephros, on the other hand, offers a unique possibility to investigate individual nephrons of the same population at different stages of development, due to its metameric arrangement of nephrons (Friebová-Zemanová 1981, Friebová-Zemanová and Goncharevskaya 1982). These nephrons can be made permanently accessible to direct experimentation in the

chick embryo after windowing the egg shell and surgical opening of its abdominal cavity at the age of 5 embryonic days (Zemanová *et al.* 1993). For this reason, we consider the chick mesonephros an extraordinarily useful model for functional examinations of embryonic nephrons *in vivo*. The mesonephric nephrons exhibit segmentation analogous to that of the metanephros, with the proximal and distal tubules joining the collecting ducts. Only Henle's loop is lacking. Urine is collected into the allantoic sac instead of into the urinary bladder. We hypothesize that the structural similarity of nephron segments in meso- and metanephros could also imply analogous functional properties. Hitherto, the chick mesonephros ability to handle water and electrolytes has been studied only on the whole kidney. It was assessed that glomerular filtration rate (GFR) and urine flow rates decline in the mature mesonephros during the third quarter of the incubation period (between days 9 - 15) and, in contrast to K^+ , Mg^{2+} and phosphates, the clearance rates of Ca^{2+} , Na^+ and sulphates are low. This demonstrates that the embryonic kidney conserves these ions during this early period (Clark *et al.* 1993). The GFR during mesonephros differentiation between days 6 and 10 increases about three times (from 0.7 to 2.0 $\mu\text{l. min}^{-1}$ per g body mass), and the rate of isotonic NaCl reabsorption in mesonephric proximal tubules concomitantly increases to more than two-fold values between days 8 and 10 (Friebová-Zemanová *et al.* 1982). The observation of a ^{24}Na - labeled salt load shift from amniotic fluid to the embryonic blood and allantoic fluid confirmed that the administered sodium passes through the embryonic kidney into the allantoic fluid. But the Na^+ concentration in the allantoic fluid never exceeds that in blood (Zemanová and Babický 1990). These data provided evidence that the mesonephric kidneys do not yet have a concentrating ability. Single nephron measurements of the basolateral cell membrane potential difference performed *in vitro* on microdissected proximal tubules of the rabbit mesonephros showed an average potential difference of -43 ± 0.5 mV. The cells became depolarized by peritubular exposure to 0.1 mM ouabain, or to 25 and 50 mM potassium or by luminal exposure to 5mM glucose or alanine (Terreros and Tiedemann 1991). The authors consider these results as evidence for the presence of sodium-coupled transepithelial transport mechanisms.

The aim of this report was, for the first, to ascertain if the mesonephric nephrons of the chick

embryo exhibit a measurable transepithelial potential difference, which can be used as an indicator of electrogenic transport. We also questioned whether this parameter differs in individual nephron segments, as it is in the mature metanephric nephron. Our second aim was to supplement the electrophysiological measurements with a demonstration of ouabain-sensitive K^+ -dependent p-nitrophenyl phosphatase (K^+ -NPPase) activity, component of the Na^+ - K^+ -dependent ATPase complex (Ernst 1972), the enzymatic equivalent of the sodium-potassium pump (Katz 1982). The K^+ -NPPase activity was detected using the technique of catalytic enzyme histochemistry. For this study, we chose the superficial, i.e. the oldest population of 7-day mesonephric nephrons, which are already morphologically characterized by the presence of differentiated segments (Friebová-Zemanová 1981, Jirsová and Zemanová 1997).

Methods

Chick embryos of the White Leghorn random bred stock were incubated in an incubator with forced air circulation at a temperature of 38.0 ± 0.2 °C and relative humidity 55-65% for 7 days. After electrophysiological measurements or before freezing of the mesonephros samples, the embryos were killed by decapitation and weighed for assessment of their maturity.

Measurements of the transepithelial potential difference (TPD)

More than 60 5-day-old embryos were prepared by the surgical removal of the right body wall for *in vivo* electrophysiological measurements on the right mesonephros (Zemanová *et al.* 1993). The operated embryos were incubated for the next two days until day 7. The most accessible and healthy-looking ones were selected for the measurements of TPD in the „chick-embryo incubation bath” and kept there at a steady temperature of the incubation medium (modified Krebs-Henseleit-Ringer solution; 37.5 ± 0.5 °C) for 75 to 120 min as described earlier (Zemanová *et al.* 1993). The heart rate of the embryos was monitored visually at 10 - 15 min intervals during their immersion in the bath. This served as an indicator of their physiological status.

The diameters of nephron segments were measured in seven embryos (*in vivo*) at magnification $\times 100$ with an ocular micrometer and the stereomicroscope (Zeiss SMXX).

Transepithelial potential differences across the tubular wall were measured by means of sharpened microelectrodes of 5 – 10 μm tip diameter, filled with 1M KCl. Their resistance was around 1 M Ω . A 3M KCl agar bridge served as the reference electrode. The signal was recorded by a differential D.C. amplifier (Ujec and Dittert 1993), where the measuring electrode was connected to the input (+). The microelectrodes were controlled by a supplementary generator, which repetitively injected brief saw-shaped pulses into the input.

To correlate the relationship between the TPD measured within the tubule and its biological activity, the nephrons in four embryos were poisoned with 10mM KCN in boluses of 30 μl or 50 μl applied by dripping the cyanide solution onto the mesonephros surface at the end of the TPD measurements.

Detection of ouabain-sensitive potassium-dependent p-nitrophenyl phosphatase (K^+ -NPPase) by catalytic enzyme histochemistry

The enzyme reaction was performed with our modification of the method of Kobayashi *et al.* (1987) as described earlier (Zemanová and Gossrau 1994). Samples of 7-day chick mesonephros were frozen in liquid nitrogen, cut in sections at 7-8 μm and stored at -20 °C until use. Cryosections were fixed before the reaction with 0.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, for 1 min at 4 °C. After 30min preincubation with the inhibitor of alkaline phosphatase (8mM levamisole), they were incubated for 15, 30 or 60 min at 37 °C in the reaction medium containing 2mM p-nitrophenyl phosphate (Mg salt), 2mM CeCl_3 , 12mM Mg^{2+} , and 50mM K^+ as activators, and a non-specific alkaline phosphatase inhibitor, levamisole. As controls, the sections were incubated in a substrate-free medium and in a medium with 10mM ouabain. Only ouabain-sensitive enzyme activity was considered as a positive expression of K^+ -NPPase. The enzyme was evaluated in connection with its distribution in the nephron segments and localization within the cells.

Statistical analysis

For statistical evaluation of differences in TPD and tubular diameters, the standard t-test or analysis of variance was employed. For the detection of TPD differences between any two-nephron segments, the Student-Newman-Keuls multiple range test was used. The correlation between body weight and average TPD

values of nephron segments in individual embryos was tested with the Spearman rank order correlation test.

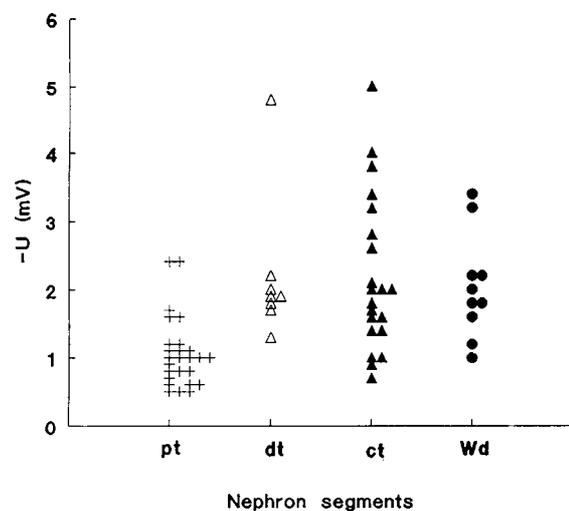


Fig. 1. Scattergram of transepithelial potential difference (U) measured in 7-day chick mesonephric nephron segments. Each point represents the value for one individual measurement; pt - proximal tubule, dt - distal tubule, ct - collecting tubule, Wd - Wolffian duct.

Results

Measurements of transepithelial potential difference (TPD) (Fig. 1)

The TPDs were successfully recorded in 9 embryos. Their heart rates mildly varied around 160 ± 22 beats per minute and their mean body weight was 900 ± 50 mg (mean \pm S.D.).

Altogether 10 - 12 superficial nephrons were accessible through the opening in the body wall of a single embryo.

Nephron segments were further distinguished according to their diameter and topography. The most numerous and best accessible, were the proximal tubules located as a rule on the ventral surface of the kidney. Their external and internal diameters were also the largest: *in vivo* they attained 70 ± 11 μm (mean \pm SD), and 35 ± 10 μm , respectively ($n=23$ and $n=20$, respectively). Less accessible were the collecting tubules arranged transversely to the laterally situated Wolffian duct, and partially hidden beneath the lateral bends of the proximal tubules. Their diameters were close to the size of the distal tubules. The distal tubules running in parallel to the distal part of the proximal tubules, and below them, could very rarely be measured because they were the least

accessible. These distal tubules had external and internal diameters of $56 \pm 8 \mu\text{m}$ ($n=11$) and $30 \pm 9 \mu\text{m}$ ($n=6$) (mean \pm SD), respectively. The differences in the external proximal and distal tubular diameters were highly significant ($P < 0.001$).

In no case was it possible to follow the course of individual nephrons and to measure the TPD along their entire length. We were usually able to measure TPD in two to six proximal tubules, 2 - 3 collecting tubules and to perform one to three measurements in distal tubules and a Wolffian duct in any single embryo. The results of the TPD measurements in segments of the mesonephric nephrons in 7-day embryos are summarized in Fig. 1. The values of TPD were negative in all nephron segments as well as in the urinary passages. In the proximal tubules, the TPD was significantly lower than in the distal and collecting tubules ($p < 0.001$).

Differences in the degree of maturity in embryos of the same age corresponded to differences in their body weight: the more mature embryos were heavier. When testing the correlation between the body weight and the value of TPD in individual nephron segments in order to assess whether the absolute value of TPD increases with advanced state of development, we found a significant relationship only in case of the collecting tubules ($p < 0.01$). This means that their (negative) TPD increased with the embryonic body weight ($r = 0.5975$).

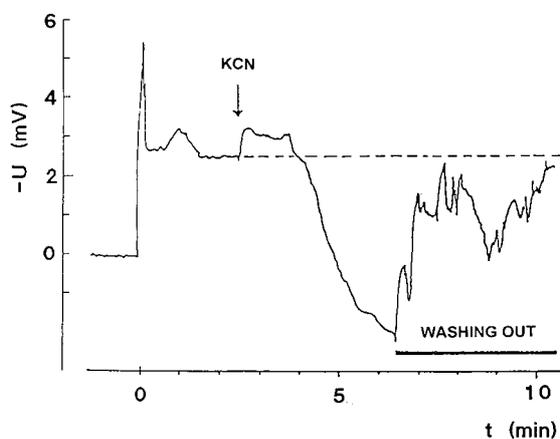


Fig. 2. Record of transepithelial potential (U) in the collecting tubule of 7-day mesonephros beginning with a large potential difference reflecting the penetration of the micropipette through the intracellular space into the tubular lumen. TPD stabilizes during the next two minutes, and after superficial application of $50 \mu\text{l}$ of 10mM KCN it drops by about 4mV to a positive value. After washing out the cyanide solution stained with Evans blue practically complete recovery nearly to original value was observed.

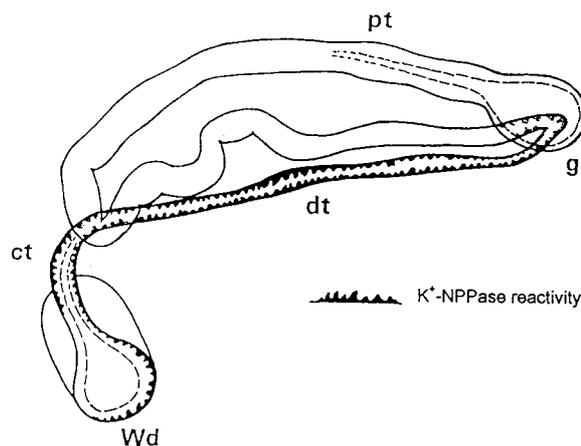


Fig. 3. Diagram of the 7-day mesonephric nephron indicating places with a positive reaction for ouabain-sensitive potassium-dependent *p*-nitrophenylphosphatase, K^+ -NPPase, activity (the black lining of the corresponding tubular segments), ct - collecting tubule, dt - distal tubule, gl - glomerulus, pt - proximal tubule, Wd - Wolffian duct

The effect of cyanide poisoning was striking in the six cases tested. It resulted in a drop of the TPD in proximal, distal and collecting tubules by 50 – 75 % of the original value (Fig. 2). The TPD decline was reversible: after removal of the cyanide by aspiration from the mesonephros surface, the TPD started to return to near-original values.

Ouabain-sensitive potassium-stimulated p-nitrophenyl phosphatase (K^+ -NPPase) (Fig. 3)

The reaction product for K^+ -NPPase in sections of 7-day mesonephros was distributed unequally. Two different product localizations were apparent. The first type was located supranuclearly inside the epithelial cells, but this was not ouabain-sensitive. In the other type, the reaction product lined the basolateral periphery of the tubular cells, but disappeared in the parallel sections treated with ouabain. The latter component was considered to be K^+ -NPPase positive. In order to identify the nephron segments with positive activity in the differentiating mesonephros, it was necessary to be completely familiar with kidney topography: proximal tubules were mostly situated in the ventral and medial part of the kidney, distal tubules could be recognized with certainty in the vicinity of the vascular pole of the glomeruli. Collecting tubules were situated laterally near the Wolffian duct. The positive K^+ -NPPase reaction was

identified in distal and collecting tubules, and in the Wolffian duct wall facing the mesonephros. The proximal tubules were negative to ouabain-sensitive K^+ -NPPase (Fig. 3).

Discussion

TPD is considered an indicator of electrogenic transport. It consists of passive components (solvent drag) proceeding paracellularly and active transport taking place across the cell membranes (Ullrich *et al.* 1979). It is known that higher values of TPD can serve as evidence for the presence of active transport and a tighter epithelium. For example, TPD measured *in vivo* in rat proximal tubules ranges from +0.2 to +1.0 mV and -46.9 mV in distal tubules (see review of Koeppen and Giebisch 1983). The proximal tubular TPD value is always less than that in distal tubules. With respect to polarity, a negative TPD is found only in the most proximal part of the proximal tubules, in proximal segments of the juxtamedullary nephrons and in distal tubules of all types of nephrons. In other segments, the transepithelial voltage is zero or lumen-positive, what is explained as a result corresponding to a chloride diffusion potential (Jacobson and Kokko 1985). In the adult avian metanephric kidney, only proximal tubules are accessible to *in vivo* micropuncture. TPD in these proximal tubules amounts to $+2.24 \pm 1.17$ mV (Lavery and Alberici 1987). As in the rat nephrons, the proximal tubules exhibit positive TPD values there. In the chick embryo mesonephros we could find only negative TPD in any nephron segment. Similarly to the mammalian metanephric nephron, TPD in the proximal tubule of the chick mesonephros was significantly lower than that in the distal tubule. The TPD values for distal and collecting tubules were the same, in contrast to the mammalian metanephric nephron. The experiments with cyanide poisoning of the tubular epithelium, which caused a decrease of TPD, indicated that the transepithelial potential reflected active transport processes.

The differences in maturation that are manifested in our 7-day-embryo group by the relatively wide range of body weight, did not influence the TPD of any particular tubular segments, with the exception of the collecting tubules. TPD in the collecting ducts was

significantly higher in embryos of higher body weight, i.e. presumably the older ones. This might be interpreted as maturation of the ability of the active transport and/or of the tightness of the tubular epithelium accompanying the maturation of collecting tubules.

The distribution of the ouabain-sensitive potassium-stimulated p-nitrophenyl phosphatase, K^+ -NPPase, reaction product in the distal and collecting tubules of the chick embryonic mesonephros corresponded to the findings reported in the differentiated metanephric nephrons of the chick (Zemanová and Gossrau 1994) and rat (Zemanová and Pácha 1996). This enzyme was not found in the proximal tubules of the chick mesonephros. This is in agreement with the findings of Tiedemann and Schlüns (1975) in the mesonephros of sheep foetuses. However, this is in disagreement with the rat metanephros, where the sodium pump was found in the proximal tubules (Laborde *et al.* 1990).

The significantly higher level of TPD and the presence of the K^+ -NPPase activity correlate with their distribution along the nephron. Whereas there is no K^+ -NPPase activity and low TPD in the proximal tubules, this enzyme activity is markedly higher in the distal and collecting tubules and TPD is approximately twice higher than in the proximal tubules. These findings indicate that the distal part of the mesonephric nephron, the collecting tubules, and partially also Wolffian duct could be capable of active transport of solutes. Briefly, a more detailed investigation is required.

Abbreviations

GFR – glomerular filtration rate

K^+ -NPPase - ouabain-sensitive potassium-dependent p-nitrophenyl phosphatase

TPD - transepithelial potential difference

Acknowledgements

We are grateful to Dr J. Pácha for critical reading the manuscript and to Dr P. Hník for revision of the text. The technical assistance of Mrs M. Strnadová is greatly appreciated. This study was supported by grants No.304/95/0626 and No.304/98/0604 of the Grant Agency of the Czech Republic.

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Reprint requests

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