MORPHOLOGICAL CHANGES IN TIGHT JUNCTIONS OF NECTURUS MACULOSUS PROXIMAL TUBULES UNDERGOING SALINE DIURESIS

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ABSTRACT

Tight junctions between epithelial cells are believed to control the paracellular diffusion of substances across epithelia. Epithelia in which tight junctions are poorly developed display a high paracellular electrical conductance, while those with extensive tight junctions show lower conductance values. We describe here a particular epithelium, that of the proximal tubules of the Necturus kidney, in which the development of the tight junctions varies in parallel with a change of paracellular electrical conductance. In control conditions, tight junctions between epithelial cells of the proximal tubules are more developed than in tubules undergoing saline diuresis, a situation which increases the conductance across the paracellular shunt pathway.

Experimental evidence accumulated over the last few years has identified the tight junctions (zonulae occludentes) as the structural elements probably responsible for controlling the paracellular diffusion in epithelia. This conclusion was reached mainly by correlating electrophysiological measurements of transepithelial conductance in a given tissue with morphological evaluation of the tight junctions present between epithelial cells (6, 13). A low electrical conductance was found associated with well-developed tight junctions, and a high conductance with poorly developed junctions. In kidney proximal tubules, the high transepithelial conductance has been ascribed to the existence of a low-resistance paracellular pathway allowing for the ionic currents to bypass the luminal, the lateral, and the basal cell membrane (1, 4, 18). Moreover, variations of paracellular conductance and permeability in proximal tubules were observed according to a given functional state (5, 9). If the correlation between permeability and tight junctions is to hold, local variations in permeability should be accompanied by local changes in the development of tight junctions. The present paper will show that this is the case in the Necturus maculosus kidney undergoing saline diuresis.

MATERIAL AND METHODS

Preparation of the Animals

Nine specimens of N. maculosus, both male and female weighing 80–120 g, were obtained from Connecticut Valley Biological Supply Co. (Southampton, Mass.) in February 1974. During the 4–6 wk preceding the experiments, they were kept in running tap water and fed with living minnows. The anesthesia (Tricaine methane-
sulfate, MS 222, kindly supplied by Sandoz A.G., Basel, Switzerland) and preparation of the right kidney were performed as described previously (5), except that a second catheter (PE 90, outer diameter 1.27 mm, inner diameter 0.86 mm) pulled at its tip was introduced through the caudal portion of the ventral abdominal vein for the fixation of the kidney by perfusion. The retrograde injection into this venous segment allowed the perfusion of the lateral renal veins of both kidneys, and the homogenous distribution of the perfusate to the portal venous capillary system. For this purpose, 20-ml syringes were connected to the PE 90 catheter through a three-way stopcock, allowing the manual control of the infusion rate of fixing fluids.

**Experimental Procedures**

Controls as well as saline loading experiments were carried out exactly as described previously (5, 9).

**CONTROL EXPERIMENTS [FOUR ANIMALS]:** A Necturus Tyrode solution (composition given in reference 5) was infused at low rate (0.1 µl min⁻¹ g⁻¹ body weight) through the first catheter placed in the apical portion of the ventral abdominal vein. A 10 to 30-min equilibration time after surgery was allowed before the fixation of the kidney through the second catheter was initiated (see above, preparation of the animals).

**SALINE LOADING EXPERIMENTS [FIVE ANIMALS]:** Saline loading was induced by infusing the Necturus Tyrode solution at a high rate (1.0 µl min⁻¹ g⁻¹ body weight). The fixation of the kidney was initiated after 2 h of infusion.

**FIXATION OF THE KIDNEY:** The blood of the renal vasculature was washed with a heparinized Necturus Tyrode solution infused for 1 min through the renal portal system. The concentration of heparin USP was 1,000 U/ml. The postcaval vein was cut open immediately after the onset of the washing period, to allow the escape of the solutions from the vascular bed. After the 1-min washing, a glutaraldehyde solution was substituted and infused for the following 10 min. The composition of the fixation solution was 1% glutaraldehyde, buffered with 5.0 mM Na-cacodylate, pH 7.4, equilibrated to 210 mosm/l with sodium chloride, a value approximating the osmolality in Necturus plasma (3). All solutions were perfused at room temperature.

Peritubular, colloid-osmotic and hydrostatic pressures were maintained during the fixation procedure since intrarenal pressures are important in the control of proximal tubular sodium reabsorption (9). The colloid osmotic pressure of the washing solution was adjusted by the addition of polyvinylpyrrolidone (PVP) (mean mol wt, 40,000) (Plasdone K30, Fluka AG, Buchs, Switzerland) at a concentration of 2.0 g% in the control and 0.92% in the saline loading experiments. The PVP concentrations were chosen to parallel the 54% drop in plasma protein concentration observed in volume-expanded animals as compared to controls (plasma protein concentration in control, 2.6 ± 0.2 g%; in saline loaded, 1.2 ± 0.2 g% [9]). The fixing solution contained the same PVP concentrations as the heparinized Tyrode solutions used for washing. Peritubular capillary hydrostatic pressure (P<sub>r</sub>) was maintained constant throughout the whole procedure of fixation in the pressure range of P<sub>r</sub> observed before fixation. P<sub>r</sub> was monitored according to Wiederhielm et al. (17), as described previously (8). The head of the pressure transducer system was a glass micropipette with a tip diameter of 10 µm inserted into a surface peritubular capillary (30-100 µm in diameter), half-way between the medial and the lateral borders of the kidney. The device used allows the detection of pressure changes as low as 1 mm H<sub>2</sub>O, with a response time of 35 ms (8).

**FREEZE-FRACTURE:** Pieces of perfusion-fixed kidney were dissected out of the cortex and further fixed for variable periods of time in a solution of glutaraldehyde similar to that used for perfusion. They were then soaked for 2 h in a 30% glycerol solution in 50 mM cacodylate buffer, pH 7.4, placed on gold disks and quickly frozen in Freon 22 cooled with liquid nitrogen. Fracturing was performed at -100°C according to Moor and Midlethaler (12) in a Balzers BAF 301 unit (Balzers AG, Liechtenstein). Replicas were cleaned in NaClO (Chlorox), washed in distilled water, and recovered on coated 150-mesh copper grids. Replicas were examined in a Philips EM 300 electron microscope.

**MORPHOMETRY:** For each animal (four controls and five saline-loaded), three to five replicas were examined (each replica containing at least one proximal tubule, identified by the presence of a brush border on the epithelial cells). Five micrographs, at least, from each replica (that is, at least 15 micrographs per animal) were taken at a fixed magnification of 51,000, calibrated with a carbon grating replica (Balzers A.G., Liechtenstein cat. no 02005, 2160 lines per millimeter).

Micrographs (negatives) were examined, without knowledge of experimental conditions, in a table projector unit and evaluated with a coherent test system with square unit (16). The number of intersections of the vertical and horizontal test lines with the tight junctional...

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1 Control and volume-expanded kidneys were fixed at different lengths of time after surgery, to avoid changing the experimental conditions in which the permeability and pressure measurements were originally obtained (5, 9). However, on the basis of physiological experiments, showing that the function of the perfused kidney remains stable for several hours (7), there seems to be little ground for suspecting that a long perfusion period induces changes in the fine structure of the junctions.

2 This is approximately the average number of unambiguous tight junctional segments involving proximal tubules which can be found in one replica (A and B faces).
elements (ridges on A faces or grooves on B faces) were recorded and the density of boundary length \((B_A)\) of tight junctional elements was computed according to the following formula:

\[
B_A = \frac{\pi}{2} \frac{l_1 + l_2}{P_T^2 \cdot 2d}
\]

where \(B_A\), density of boundary length on membrane area; \(l_1\), number of intersections of tight junctional elements with horizontal test lines; \(l_2\), number of intersections of tight junctional elements with vertical test lines; \(P_T\), total number of test points in the grid (144); and \(d\), spacing of test points (average = 0.074). This equation is derived from the general relationship \(B_A = (\pi/2)l_1\), where \(l_1\) is the number of intersections per unit length of test lines, using a grid with horizontal test lines only (15). However, since most tight junctions have strands in both horizontal and vertical orientation, we found a grid with a square lattice more adequate for counting.

RESULTS

Fig. 1 shows the peritubular capillary pressure \((P_{pT})\) during the fixation procedure. The baseline of \(P_{pT}\) remained horizontal, except for transient changes in pressure arising from variations of the manual force applied to the syringe. Images of freeze-fracture replicas of proximal tubular cells of control and of saline-loaded animals are shown in Figs. 2-6. The apical part of the lateral face of the proximal tubular cell membrane (identified as proximal due to its characteristic brush border) contains ridges in the A face and grooves in the B face, indicating the presence of tight junctions (11). These junctions contain from one to four ridges (or grooves) which show a variable disposition in the membrane face. In some cases, the ridges (or grooves) are mostly parallel, in others they form a network, the common situation being a mixture of parallel and network patterns. As judged from the rather low number of junctional strands, the presence of focal discontinuities within them, and the looseness of the network, these tight junctions have been ascribed to the "leaky" type (6).

The appearance of these junctions is markedly dependent on the physiological conditions at the time of kidney fixation. Figs. 2 and 4 show examples of tight junctions found commonly in control conditions; Figs. 3, 5, and 6 show examples of tight junctions encountered in saline-loaded animals. The latter junctions differ from control ones in that individual ridges (or grooves) appear more often interrupted for variable lengths and/or that the number of superposed tight junctional elements is reduced. The morphometric evaluation of these qualitative (interruptions) and quantitative (reduced number of junctional strands) changes is shown in Table I. The density of boundary length \((B_A)\) of tight junctions decreases from approximately 4.6 in controls in 3.0 in kidneys fixed after 2 h of isotonic saline infusion. The decreased development of the tight junction network in response to the saline loading is highly significant \((P < 0.005)\). During saline loading, an increase in the conductance of the paracellular shunt pathway has been found (5). This indicates that in a leaky epithelium such as the proximal tubule, a correlation exists between physiological changes in the conductance of the paracellular shunt pathway and the development of the tight junctions.

DISCUSSION

The results presented here point to a decrease in the complexity of the tight junctions in response to saline loading. This important event supervenes in the condition of increased paracellular conductance and sodium back diffusion described by Boulpaep (5) in saline-loaded Necturus. They also complement the work of Bentzel (2) who offered...
FIGURE 2 Control animal. The proximal tubule is recognized by the presence of the brush border (mv). Below the microvilli, the fracture faces (A face on the left, B face on the right side of the picture) contain characteristic ridges or grooves indicating the presence of a tight junction. At the asterisks, one sees interruptions of the junctional strands in both A and B faces. × 45,000

FIGURE 3 Saline-loaded animal. Below the microvilli (mv), junctional strands (ridges) are visible on the membrane face (A face). The individual ridges are less numerous than in the control junction (Fig. 2) and they contain widespread discontinuities (asterisks). The uppermost ridges in the right part of the picture appear nearly erased. × 49,000

semiquantitative evidence in thin-sectioned material for a reduction in the size of tight junctions during volume expansion.

Our freeze-fracture images indicate that the change in tight junctions consists of discontinuities of variable length within individual ridges (or grooves) and/or in the disappearance of entire ridges (or grooves) within a given tight junctional domain. Inasmuch as discontinuities occur in control junctions as well, although in moderate number and extension, it is tempting to interpret the remodeling under saline loading as a progressive enhancement of the “normal” leakiness of the junctions. How a tight junction can modulate the diffusion of solutes in the paracellular space can be imagined if one envisages the single fibril model of the structure of tight junctions (14). In this model, the two apposed plasma membranes are bridged by a structural element, the fibril, shared by the two membranes and sealing the intercellular (paracellular) space. Diffusion would occur only at those points of the junction where the fibril is lacking (see asterisks in Figs. 2–6). Thereby, modifications of the diffusion barrier located at the apical side of
FIGURE 4 Control animal. Tight junction (A face) composed of ridges disposed in a network pattern. Despite the presence of some discontinuities (one labeled with the asterisk) on individual ridges, a complete patency across the entire junction is not evident. × 123,000

FIGURE 5 Saline-loaded animal. Tight junction (A face) composed of a reduced number of ridges. In this case, the interruptions in the horizontal ridges (asterisks) determine open paths across the junction. × 145,000

FIGURE 6 Saline-loaded animal. Tight junction exposed on a B face and showing discontinuities (asterisks) in the individual grooves. × 145,000
TABLE I
Density of Boundary Length of Junctional Elements on Membrane Area

| Animal no. | Control | Saline diuresis |
|------------|---------|----------------|
|            | Mean per animal ± SEM | Mean per animal ± SEM |
| 1          | 4.94 ± 0.65 (18; 2,359) | 2.94 ± 0.51 (42; 3,257) |
| 2          | 4.78 ± 0.39 (33; 4,574) | 2.79 ± 0.46 (32; 2,505) |
| 3          | 3.80 ± 0.25 (15; 1,579) | 2.98 ± 0.21 (33; 2,714) |
| 4          | 4.96 ± 0.37 (17; 2,141) | 2.93 ± 0.30 (22; 1,705) |
| 5          | 3.45 ± 0.79 (30; 2,801) |                |
| Mean for all controls ± SEM | 4.62 ± 0.27 | 3.01 ± 0.11 |

The first number in parentheses indicates the number of micrographs examined per animal, the second the total number of intersections measured in these micrographs.

the paracellular pathway might affect the permeation characteristics of the shunt.

As far as the cause(s) of the remodeling of the junctions is concerned, the possible natriuretic factor(s) responsible for controlling proximal sodium reabsorption should be taken into consideration. For example, physical forces may be involved since the change in peritubular colloid osmotic pressure affects the conductivity of the paracellular shunt path (9).

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