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Tight Junctions are Sensitive to Peptides Eliminated in the Urine

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Abstract. We prepare an extract of dog urine (DLU) that, when applied to monolayers of MDCK cells (epithelial, derived from a normal dog), enhances the transepithelial electrical resistance (TER) in a dose-dependent manner. This increase is not reflected in variations of the linear amount of TJ nor in changes of the pattern of junctional strands as observed in freeze fracture replicas, nor in the distribution of claudin 1 (a membrane protein of the TJ) nor ZO-1 (a TJ-associated protein). A preliminary characterization of the active component of DLU indicates that it weighs 30–50 kDa, bears a net negative electric charge, and is destroyed by type I protease but not by 10-min boiling. DLUs prepared from human, dog, rabbit and cat are effective on MDCK cells. However, dog DLU increases TER in MDCK (dog) as well as $LLCPK_1$ (pig) monolayers, but not in other epithelial cell lines such as $LLCRK_1$ (rabbit), PTK_2 (kangaroo) and MA-104 (monkey), nor in the endothelial cell line CPA_{47} (cow). Given that in its transit from the glomerulus to the urinary bladder the filtrate increases its concentration by more than two orders of magnitude, the substance(s) we report may act at increasingly higher concentrations in each segment, and afford a potential clue to the progressive increase of TER across the walls of the nephron from the proximal to the collecting duct.

Key words: Transepithelial electrical resistance — Tight junctions — Claudin-1 — ZO-1 — MDCK monolayers — Nephron — Urine extracts

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Introduction

As the glomerular filtrate travels from the Bowman capsule to the collecting duct, it is gradually transformed from quasi-plasma to urine, and therefore the epithelia forming the walls of the nephron are exposed to a progressively sharper electrochemical gradient between the lumenal and the interstitial fluid. Exposure to this progressively sharper gradient is accompanied by an increase in the transepithelial electrical resistance (TER), from a mere 5–8 Ω cm^2 at the level of the proximal tube (Hegel, Frömter & Wick, 1967) to 150–600 Ω cm^2 at the distal one (Malnic & Giebisch, 1972; Seely & Boulpaep, 1971) and a further increase to 860–2,000 at the level of the collecting duct (Helman, Grantham & Burg, 1971; Rau & Frömter, 1974). This gradual increase of TER is accounted for by a parallel decrease in the permeability of the paracellular route due to progressively tighter junctions (TJs) (Reuss, 2001), which is in turn matched by an increase in the number of their strands, as observed in freeze fracture replicas (Claude & Goodenough 1973).

In the present article we explore the possibility that the progressive tightening of the different epithelial types constituting the nephron is due to a hypothetic substance (or group of substances) that act on the cells in a concentration-dependent manner. Our search for such substance(s) is based on the following working assumptions: (1) in its course through the nephron, the glomerular filtrate is concentrated some 100- to 200-fold. Therefore, it is expected that a hypothetical substance coming with the glomerular filtrate or produced early in the proximal part of the nephron, will act on the different segments of the nephron with a progressively higher concentration. (2) If such substance increases the tightness

of the TJ in a concentration-dependent manner, the segments would have a higher TER in the distal than in the proximal part of the nephron. (3) Furthermore, if it maintains its activity throughout the nephron and urinary bladder, it may be easily collected and tested in vitro on model systems such as the monolayer of MDCK cells. Taking these assumptions together, we assayed the effect of extracts of dog urine on the TJs of monolayers of MDCK cells, which were also derived from the kidney of this animal species. We also explore the effect of DLUs prepared with urine from different animal species, as well as dog DLU on monolayers prepared with a variety of established cell lines. A preliminary characterization of the active substance in DLU indicates that it may in fact be more than one peptide.

Preliminary observations were presented in poster form (Gallardo et al., 1992).

Materials and Methods

Cell Culture

Starter cultures were obtained from the American Type Culture Collection (MDCK, CCL-34; LLC-PK_1, CRL 1392; PtK2, CCL 56; CPA 47, CRL-1733). Ma104 were a generous gift of Dr. E. Rodriguez-Boulan (Cornell). Upon arrival, cells were cloned and most experiments reported in the present article were performed in MDCK cells of clone 7, chosen because of its intense blistering activity when plated on nonpermeable supports. Cells were grown at 36.5°C in disposable plastic bottles (Costar 3250, Cambridge, MA) with an air-5% CO_2 atmosphere (VIP CO_2 incubator 417, Lab Line Instruments, New Brunswick, N.Y.) and 20 ml of Dulbecco's modified Eagle's medium, DMEM (Grand Island Biological Co., GIBCO 430-1600, Grand Island, N.Y.) with 100 U/ml of penicillin, 100 μg/ml of streptomycin (GIBCO 600-5145), or 0.8 U/ml of insulin (Eli Lilly, México, D.F.), and 10% fetal calf serum (GIBCO 200-6170); in the following text this complete medium is referred to as CDMEM. Cells harvested with trypsin-EDTA (In Vitro, México) were plated on disks of Millipore paper (Bedford, MA, HA pores 0.45 μm in diameter). Cells were usually between the 60–80th passage. Upon allowing 1 hr for cell attachment, medium was discarded and monolayers were switched to fresh media. To assure that incubation media bathe the Millipore filter, the disks were separated from the cell culture dish with a piece of coarse nylon cloth. When one side of the monolayer was exposed to DLU, culture was made on Millipore filters previously glued to the bottom ring of a Lucite cylinder (OD: 19 mm; ID: 15 mm; height 11 mm), and placed in cell culture dish (Contreras et al., 1989).

Transepithelial Electrical Resistance (TER)

The degree of sealing of TJs was assessed by measuring the transepithelial electrical resistance (TER) (Cereijido et al., 1978a; 1978b). After incubation under a given condition, the filter with the monolayers was mounted as a flat sheet between two Lucite chambers with an exposed area of 0.69 cm^2. When the monolayers were prepared in the cylinder described above, the filter with the monolayer was cut out using a scalpel, to permit its mounting as a flat sheet. Current was delivered via Ag/AgCl electrodes placed at 2.0 cm from the monolayer; the voltage deflection elicited was measured with a second set of electrodes placed at 1.0 mm from the membrane. Values of TER reported were obtained by subtracting the contribution of the filter and the bathing solution. A given monolayer was used only for a single determination and discarded to avoid leaks due to edge damage.

Preparation of DLU

Urine was obtained from healthy male mongrel dogs with the help of a sterilized plastic bag. Urine was centrifuged at $1520 \times g$ for 10 min at 2°C in a Beckman centrifuge (TJ-C, Beckman, Palo Alto, CA) and the supernatant was extensively dialyzed overnight against deionized water. It was then lyophilized in a Virtis 10-146 MRBA apparatus (Gardiner, NY). The powder obtained (DLU = dialyzed and lyophilized urine) was stored at −20°C. DLU was dissolved in DMEM or CDMEM.

To obtain DLU fractions of different molecular size, DLU was filtrated through nitrocellulose filters with cut-out values of 30 or 50 kDa in an Amicon Filtration Chamber cooled in ice and under nitrogen gas pressure.

Treatment of DLU with Protease

0.01 units of crude protease Type I from bovine pancreas (Sigma, St. Louis, MO) were incubated with 50 μl of DLU for 12 hr at 37°C. To inactivate the effect of this protease, the mixture was incubated for 10 min at 92°C, followed by 10 min at 4°C.

Ion Exchange Chromatography

A column of DEAE-cellulose as well as DLU were equilibrated with 50 mM TRIS pH 8.0 (10 mg DLU in 1.0 ml). The column was eluted with an increasing stepwise salt concentration 0.2 to 2.0 M NaCl in 50 mM TRIS. Fractions were dialyzed against deionized water before bioassay.

Immunofluorescence

Glass coverslips containing cells cultured under the various experimental conditions described below were rinsed twice with PBS, fixed and permeabilized with methanol at −20°C for 45 seconds, rinsed with PBS, incubated with 3% fetal bovine serum in PBS for 30 minutes, and treated for 1.0 hr with a specific first antibody diluted in PBS. Monolayers were then rinsed 3 times with PBS for 5 minutes each, incubated with a suitable FITC-labeled goat antibody (ZYMED) for 30 minutes, rinsed as above, mounted in Fluorguard (Bio-Rad, Hercules, CA). The sources of antibodies were: rabbit polyclonal anti-claudin 1: Zymed 71-7800; rabbit polyclonal anti-ZO-1: Zymed 61-7300. Observations were made with an MRC-600 BioRad (Hercules, CA) confocal microscope.

Freeze Fracture

Monolayers grown in flasks (Falcon Plastics, Cockeysvillen, MD) were fixed with 2.5% glutaraldehyde in PBS for 30 min at 3°C, washed three times with PBS, and cryoprotected by successive incubations in 10, 20 and 30% glycerol, for 30, 30 and 60 min, respectively. They were then detached from the substratum as a sheet by gently scraping with a rubber policeman, placed on gold specimen holders, and rapidly frozen in the liquid phase of partially solidified Freon 22 cooled with liquid nitrogen. Freeze fractures were performed in a Balzers BAF 400 (Balzers Company, Liech-

tenstein) at −150°C and 5×10^{-9} bar. Fractured faces were shadowed with platinum and carbon at 45 and 90°, respectively. Replicas were cleaned with chromic mixture and washed in distilled water, placed on 300-mesh copper grids and examined in an electron microscope (JEM-2000EX: JEOL, Tokyo, Japan).

Morphometric analysis was performed on micrographs of freeze-fracture replicas, printed at a magnification of 50,000 ×. A line parallel to the main axis of the tight junctions was traced, and a series of perpendicular lines was drawn (one every 300 nm). The number of strands of a given segment of tight junctions was defined as the number of its intersections with the perpendicular line. For further details *see* Balda et al. (2000) and Cereijido, Shoshani & Contreras (2002).

Values are expressed as mean ± standard error, with the number of measurements in parentheses.

Results

The Tightening Effect of Urine Extracts

To facilitate comparisons, all values of transepithelial electrical resistance (TER) reported were normalized as follows:

$$\mathrm{TER} = ter(\mathrm{TER_g}/ter_c)$$

where *ter* is the value actually measured, ter_c is the control value obtained on the same day in monolayers incubated in CDMEM, and $\mathrm{TER_g}$ is the mean value of all recordings in control monolayers 197 ± 8 (419) Ω cm^2.

MDCK cells plated at high density (10^6 cells/ml) form a continuous monolayer in a few minutes, yet TER develops slowly as cells recover from harvesting with trypsin-EDTA and seal the TJs, a process that reaches a maximum in 12–15 hr (Cereijido et al., 1978*a*; 1978*b*). This maximum is often followed by a decrease related to the installation of channels across the strands (Cereijido et al., 1978*b*) and to an increase of the length of intercellular space per unit of monolayer area, due to cell proliferation and packing (Cereijido, González-Mariscal & Borboa, 1983; Rabito 1986). In summary, TER does not reach a constant plateau, but shows some variation (Fig. 1, *open circles*). Nevertheless, DLU produces an increase of TER (Fig. 1, *filled circles*) that starts to be evident and significant in 8 hr and lasts for at least 13 days, albeit 8–12 -day old monolayers are less responsive to this exposure of 24 hr.

Figure 2 shows the effect of 24 hr of treatment with 10% DLU added to monolayers of different ages (*filled circles*). In this particular figure, zero time corresponds to the moment of plating at high density. It may be noticed that the effect that DLU produces in 24 hr starts to be observed on the second day, i.e., in monolayers that had been plated for one day and then treated for a second day with DLU.

Figure 3 depicts the effect of DLU added to the basolateral side (*circles*) or to the apical side of the monolayer (*diamonds*). The increase produced by adding DLU to the apical side is considerably smaller, nevertheless, both of them are dose-dependent.

The effect of DLU is reversible, because its removal after 24-hr exposure (Fig. 4, *open squares*) progressively decreases TER towards control values.

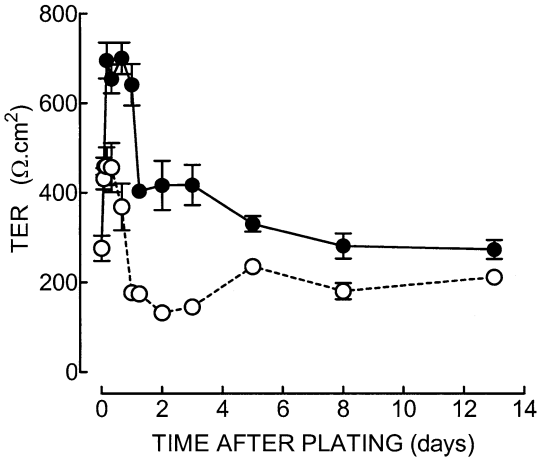

Fig. 1. Effect of DLU on the transepithelial electrical resistance (TER) of MDCK monolayers as a function of time. 10% DLU was added to the basolateral side of 1-day old monolayers (*zero time*) and was continuously present thereafter. Media with DLU were renewed every 3 days. Monolayers were cultured on Millipore filters in multi-well chambers and were mounted as a flat sheet between two Lucite chambers for the measurement of TER. The electrical resistance of the support, chambers, etc. was subtracted, therefore all reported values of TER correspond exclusively to the electrical resistance across the monolayer. In this and the following figures empty symbols and dashed lines represent control conditions and filled ones correspond to values with DLU. Unless otherwise stated, each point is an average of 10–12 individual measurements. When errors are not shown, they are smaller than the size of the symbol.

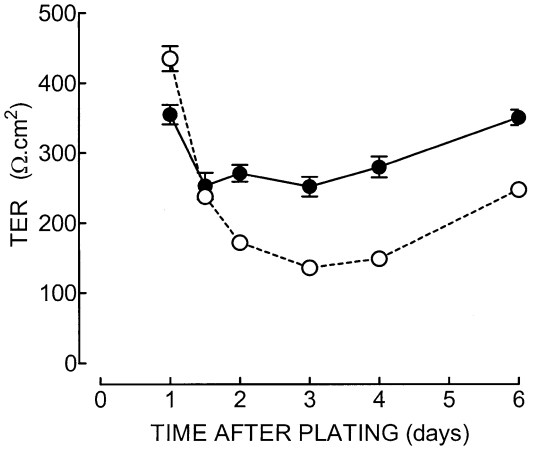

Fig. 2. Effect of DLU as a function of the age of the monolayer. 10% DLU was added for 24 hr to both sides of monolayers whose age at the moment of addition is specified in the abscissa. TER was measured at the end of this exposure period.

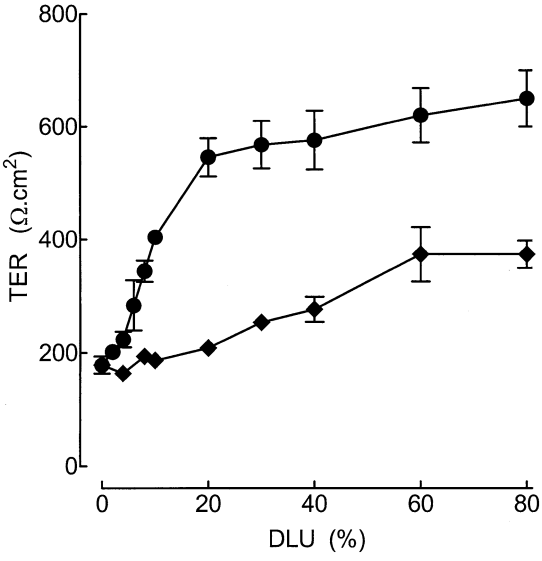

Fig. 3. Effect of DLU as a function of concentration and of the side of the monolayer exposed to the extract. One day old confluent monolayers were treated for another day with CDMEM containing DLU at the percentages specified in the abscissa. DLU was added to the basolateral side (*circles*) or to the apical one (*diamonds*).

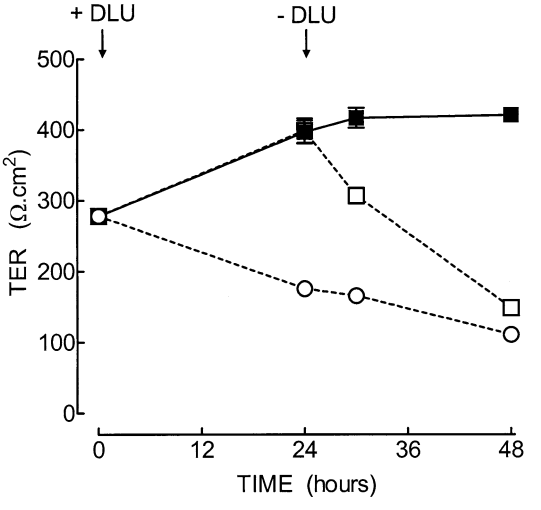

Fig. 4. Effect of removing DLU from monolayers. DLU was added (*zero time*) to two experimental groups of 24 hr-old monolayers (*filled squares*) and was removed from one of them (*empty squares*) one day later. Circles represent untreated controls.

Mechanisms

In principle, the tightness of the paracellular permeation pathway can vary by simply increasing or decreasing the linear amount of TJ. Hence, if DLU acted as an inhibitor of proliferation, cells would be fewer and more extended, the monolayer would contain a smaller linear amount of TJ, and the value of TER would consequently increase. Yet Table 1 shows that DLU increases TER without modifying cell density: 272- *vs* 261-thousand cells per square centimeter. The resistive element of the TJ is assumed to be the strand whose pattern of distribution in a band surrounding the cells as a belt can be observed in freeze-fracture replicas. A morphometric analysis of freeze-fracture replicas (Fig. 5) does not reveal an obvious modification that would account for the increase in TER.

Table 1. Effect of DLU on transepithelial resistance and cell density

Condition	Transepithelial Electrical Resistance Ω cm^2	Cell density (Thousands of cells per cm^2)
Control (CDMEM)	198 ± 26	272 ± 22
DLU 10%	261 ± 31	403 ± 36

The TJ, as it appears in microscopy, is just the membrane extreme of a complex structure involving a dozen molecular species, most of which belong to the cytoplasm and are in intimate association with the cytoskeleton (Cereijido & Anderson 2001; Cereijido, Shoshani & Contreras, 2000). Some of these molecules even shuttle to the nucleus and back (Avila-Flores et al., 2001; Cereijido et al. 2000). To explore whether DLU affects the distribution of junctional molecules, we choose a membrane (claudin-1) and a non-membrane molecule (ZO-1). A study of these molecules performed by phase contrast, indirect immunofluorescence and confocal microscopy (Fig. 6) shows that their pattern of distribution is not perturbed by DLU. Of course, this is only a preliminary exploration, as some of the 20-odd molecular species forming the TJ have a complex pattern of phosphorylation and of association between themselves, which varies in response to a variety of physiological situations and is sensitive to a host of substances. Therefore, the intrinsic mechanism of the DLU effect remains unknown.

Preliminary Characterization of DLU

To gain some insight into the nature of DLU, we explored the effect of a 12-hr treatment with Protease Type I at 37°C. Before using the protease-treated DLU on the monolayer, protease Type I-treated DLU was boiled for 10 min to block the activity of this enzyme. Figure 7 shows that proteolysis completely destroys the TER-enhancing effect of DLU. An assay of DLU filtered through Amicon suggests that active substance(s) are heavier than 30 and lighter than 50 kDa (Fig. 8). Only fractions eluted with 0.2 and 0.4 M NaCl increased TER (Fig. 9). Interestingly, when DLU is fractionated and the fractions are pooled ("all fractions," Fig. 9), the enhancement of TER is significantly higher ($p < 0.001$) than the one elicited by whole DLU. A possible

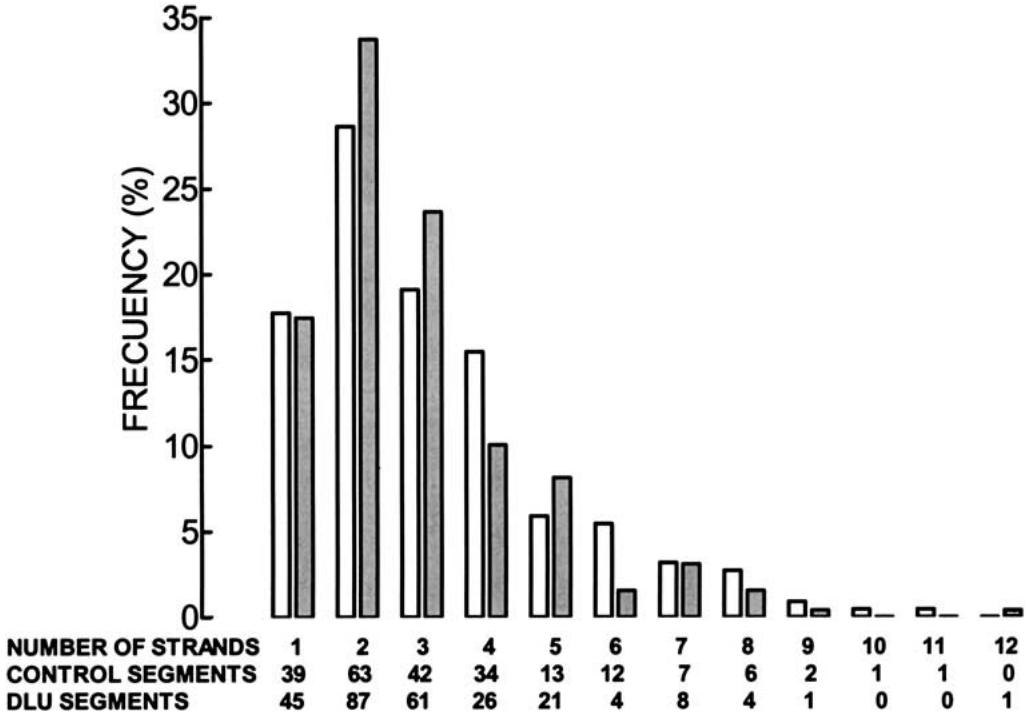

Fig. 5. Morphometric analysis of freeze-fracture replicas of control (*white columns*) and DLU-treated monolayers (*grey columns*). Each pair of columns refers to segments of TJ with 1, 2,. . .*n* strands. "Control segments" refers to the amount of segments found to have 1, 2,. . .*n* strands in control monolayers. "DLU segments" correspond to the same parameter, but in treated monolayers.

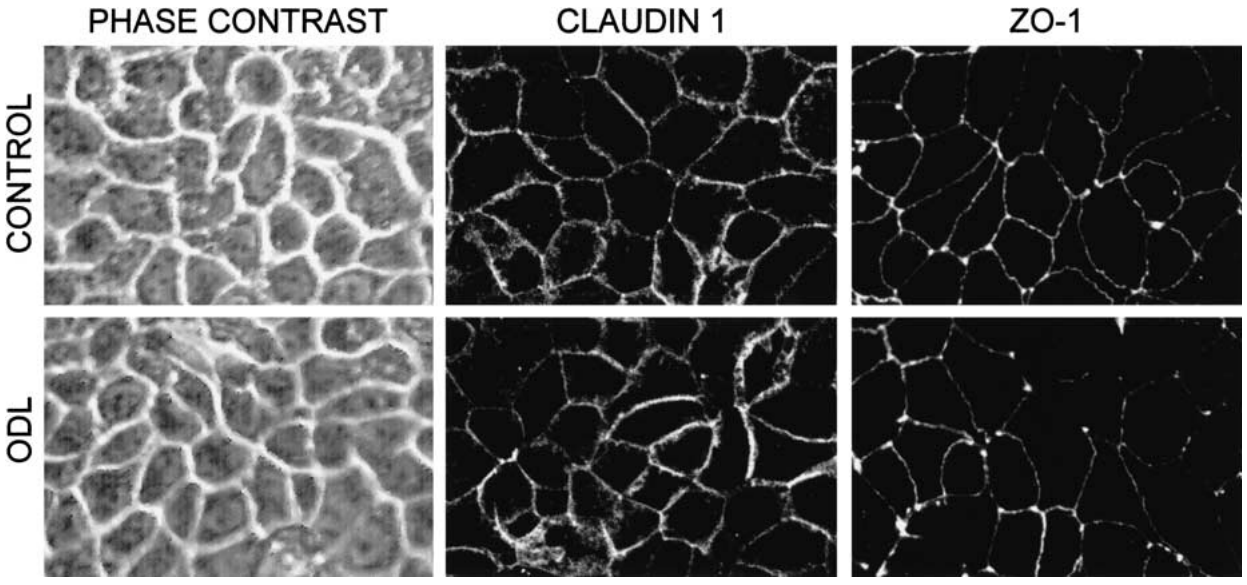

Fig. 6. Confluent monolayers of MDCK cells cultured for one day under control condition, and left for another day as such (*top row*) or treated with 10% DLU for another period of 24 hr (*bottom row*). The smeared appearance of claudin-1 indicates that this molecule is not restricted to the TJ, as it is in the case of ZO-1, but invades also the lateral membrane of the cells.

interpretation of this difference is that DLU consists of a group of active substances, and that one of them, an inhibitory one, does not withstand the fractionation procedure. The ability of DLU to enhance TER is not impaired by heat, as 10 min boiling does not suppress it (Fig. 7). Apparently, boiling does not even destroy the hypothetic TER-inhibitory substance that would account for the difference between columns labeled "DLU" and "All fractions" mentioned in Fig. 9.

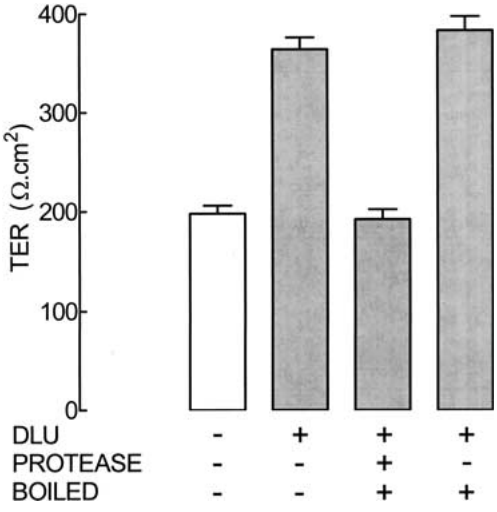

Fig. 7. Influence of protease and heat on the effect of DLU. One day old MDCK monolayers were transferred to medium containing DLU (*gray columns*). The second gray bar (left-to-right) corresponds to DLU that was treated with type I protease for 12 hr at 37°C, then boiled for 10 min to destroy the enzyme. The third gray column corresponds to 10% DLU which was boiled for 10 min.

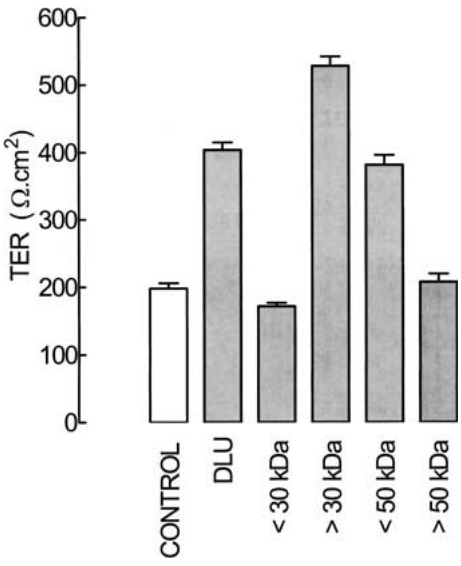

Fig. 8. Effect of urine extracts filtered with Amicon filters of different pore size.

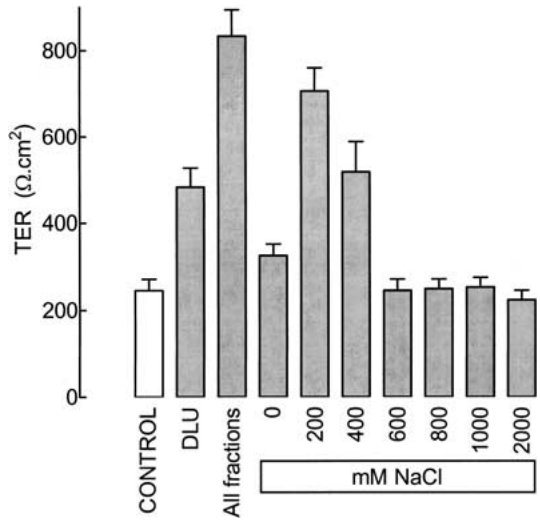

Fig. 9. Values of TER of monolayers exposed for 24 hr to DLU fractions that were preequilibrated, eluted and dialyzed before the assay against various concentrations of NaCl.

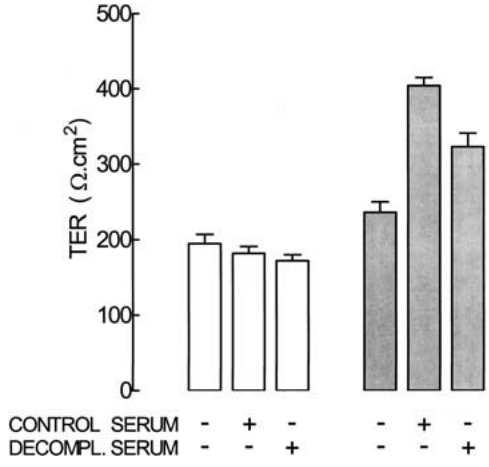

Fig. 10. Influence of serum on the effect of DLU. As in previous figures, white and gray columns correspond to control and DLU-treated monolayers, respectively. In each group the first bar corresponds to monolayers plated and incubated for 1 day without serum, the second to those incubated with 10% bovine serum, and the third to monolayers with 10% bovine serum that was heated for 1 hr at 56°C to destroy the complement.

Taken together, this information suggests that the effect of DLU on TER is elicited by one or more peptides contained in DLU. Yet, since monolayers are routinely cultured with serum-containing medium, the possibility exists that the active substance does not belong to DLU, but is produced by interaction of this extract with proteins of the serum. To explore this alternative we treated monolayers with serum-free medium, or with 10% fetal calf serum either as such or decomplemented, in the absence of DLU (Fig. 10, *white bars*). The group of shaded columns in Fig. 10 corresponds to monolayers that were treated with DLU. This extract does enhance TER in the absence of serum (*first shaded column vs. first white bar*, $p < 0.05$), but this enhancement is clearly larger in monolayers cultured in serum-containing medium (*second shaded columns*). Decomplementation by heating at 57°C for 30 min does make a significant difference, though ($p < 0.02$). This complex relationship between the interaction of DLU and serum proteins is the subject of a systematic study currently in progress.

Table 2. Effect of DLU on different cell types

Cell	Animal species	TER		
		Control Ω cm^2	DLU Ω cm^2	Effect (%)
MDCK	Dog	179 ± 15	404 ± 15	126
LLC-PK$_1$	Pig	201 ± 13	261 ± 26	30
LLC-RK$_1$	Rabbit	24 ± 4	19 ± 4	−28
PtK2	Kangaroo	27 ± 5	24 ± 4	−12
MA-104	Monkey	81 ± 9	90 ± 17	11
CPA 52	Cow	34 ± 1	33 ± 1	−3

All cell types are epithelial, except endothelial CPA 52.

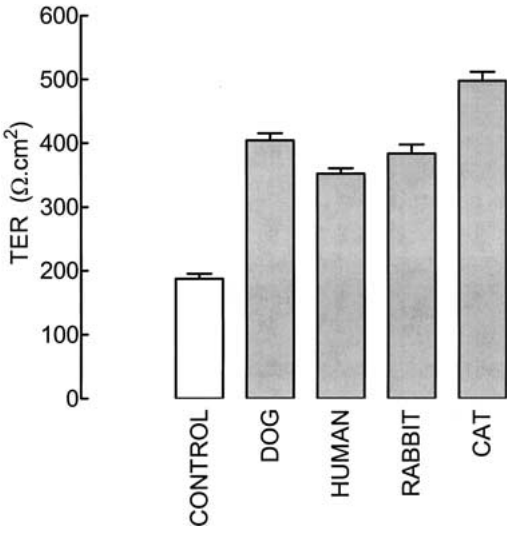

Fig. 11. Effect of DLU prepared with urine obtained from different animal species.

We next explored the specificity of DLU in two different ways. The first was by preparing DLU from urine from different animal species (Fig. 11). Although all extracts tested are able to produce a significant increase of TER, they do it to a different extent. Ironically, even though MDCK cells were derived from a dog kidney, the value of TER they reach when exposed to a cat extract is even higher ($p < 0.001$) than that achieved with extract prepared form dog urine. Again, this might be easily explained by DLU containing more than one active molecular species that elicit different effects, and where a TER-decreasing one would have no affinity for dog cells. The second way of exploring the specificity of DLU was to assay it in cell lines derived from other animal species besides MDCK (dog) (Table 2). Only LLCPK$_1$ cells, derived from the pig kidney, show a comparatively small increase of 23%.

Discussion

During the last century, every feature of the peculiar architecture of the mammalian nephron, from the seemingly "capricious" convolutions of proximal and distal segments, to the "meaningless" detour of fluid through the descending branch of the Henle loop only to come back via a parallel one, came to be recognized as necessary structures that enable the kidney to clear the whole volume of plasma several times a day and eliminate waste products in the urine. One of these intimate structure/function relationships is the progressive tightening of the paracellular pathways that cross the walls of the nephron as the kidney tubule proceeds toward the urinary bladder. The information collected during several decades of work with many animal species and diverse experimental approaches shows that this tightening is due to a reduction of the paracellular permeation route limited by the TJ (Reuss & Finn 1975; Reuss 2001). A mechanism for this gradual progressive sealing of the TJs could be that a substance appearing early in the tubular fluid would increase the degree of sealing of the TJ in a concentration-dependent manner. Because of its gradual increment in concentration as the fluid becomes more and more concentrated on its way along the nephron, it would make the TJs of distal segments more hermetically sealed than those of more proximal segments. In the present work we find a temperature-resistant peptide of 30–50 kDa, with a net negative charge, that enhances TER in proportion to its concentration, and that seems to be continuously required, as its effect is reversed upon withdrawal (Fig. 4). In turn, renal cells seem to be responsive throughout their life, as their doubling time is 20.4 hr and the effect of DLU can be elicited in monolayers up to 6–13 days old (Figs. 2, 3). A brief treatment with 2.0 mM EGTA opens the TJs, and the value of TER drops to practically zero, suggesting that the value of TER is mostly due to the resistance of the TJ itself, and that the contribution of the electrical resistance of the intercellular space is comparatively negligible (Cereijido et al., 1978*a*). This does not discard the possibility that TER could be enhanced by an increase of resistance in the intercellular space, e.g., if DLU were to collapse the intercellular space. Yet this is unlikely, as monolayers treated with DLU do not show any noticeable

structural change, suggesting that the increase of TER elicited by DLU may be attributed to a change in the degree of sealing of the TJ.

The TJ, which was once regarded as an obscure seal at the outermost end of the intercellular space, consists of 20-odd different molecular species that form a cluster spanning from its lips at the plasma membrane to the cytoskeleton (Cereijido et al., 2000). Many of these molecular species have amino-acid sequences that enable them to bind to each other (e.g., the PDZ homology), have nuclear addressing and nuclear exit signals (Islas et al., 2002), bind to gene promoters and undergo phosphorylation/dephosphorylation in response to physiological conditions and pharmacological challenge (Schneeberger & Lynch 1992; Lacaz-Vieira & Jaeger 2001). Furthermore, the TJ assembles in response to Ca^{2+} acting on the extracellular repeats of E-cadherin (Contreras et al., 1992; Gonzalez-Mariscal et al., 1985*a*, 1985*b*), which enables neighboring cells to contact each other, and the signal generated by this contact is transduced to the cytoplasmic side by at least two different G-proteins, a phospholipase C, a PKC and calmodulin (Balda et al., 1991; Contreras et al., 1992). This cascade provokes the phosphorylation of several TJ molecules (Avila-Flores et al., 2001; Balda & Matter, 2000; Perez-Moreno et al., 1998) and the redistribution of actin filaments (Meza et al., 1980). Actually, it comes as no surprise that such complex machinery is sensitive to substances carried in the lumen and eliminated in the urine of the type described in the present work.

We show that urine contains at least one such substance, as DLU is active even in monolayers bathed in serum-free medium. Yet another substance may be produced by interaction of proteins of the serum with those delivered to the tubular fluid by the epithelial cells, because the TER-enhancing effect of DLU is stronger in monolayers incubated in serum-containing medium (Fig. 10). The possibility exists that other substances are produced by the renal cells themselves. Thus Jaeger et al. (Jaeger, Dodane & Kachar, 1994) have found that MDCK cells secrete a substance that enhances TER when applied to a second MDCK monolayer. As long as MDCK monolayers can be taken as a model system for segments of the nephron, the observation of Jaeger et al. suggests that the substance we find in DLU may, in fact, originate in the same kidney tubule.

Conyers et al. (1990) and Marmorstein et al. (1992) have found that human and canine serum have proteins that condense actin and open tight junctions in monolayers of MDCK cells, with peaks of activities of 15, 30, 45, 60, 120 and 240 kDa. It can be speculated that the ones with lower molecular weight may be filtered from plasma to the glomerulus and constitute a TER-depressing component that will, of course, be different from the TER-enhancing one found in the present work. The TER-depressing serum components found by Conyers et al. and Marmorstein et al. act from the basolateral side. This, together with the high molecular weight of some of these molecular species, suggests that they might not be filtered and delivered to urine, but act from the interstitial side. We find that DLU acts from the basolateral as well as from the apical side (Fig. 3), yet the information available does not preclude that it could act with a different molecular species in each case. Canfield et al. (Canfield, Geerdes & Molitoris, 1991) have observed that ATP depletion achieved by antimycin-A causes a rapid and reversible opening of the TJs of LLC-PK_1 monolayers. Ladino et al., (1991) report that inhibition of adenylsuccinate synthetase by hadacidin reduces the cellular levels of ATP and cAMP, and has a marked effect on the TJs of MDCK monolayers, as evidenced by a decrease of TER and the number of strands observed in freeze-fracture replicas. However, in our study the components of serum by themselves do not seem to have an appreciable effect on the value of TER (Fig. 10).

The fact that the value of TER increases from the proximal to the distal nephron by two orders of magnitude suggests the existence of two types of factors: (1) constitutive ones, which would be responsible for the more elaborated meshwork of strands of the TJ in the distal portions (Claude & Goodenough 1973), and (2) regulatory ones, which in a given moment would adapt the permeability of the different segments of the nephron to physiological requirements. The slow reversion of the effect of DLU (Fig. 4) would suggest that DLU belongs to the first group. However, it is premature to reach such a conclusion, or to establish a sharp division between constitutive and regulatory effects. Actually, the pattern of the TJ in freeze-fracture analysis, the amount of junctional cleft, and the distribution of claudin-1 and ZO-1 do not appear to be modified by DLU. Of course, this does not preclude that the extract could act by changing the pattern of phosphorylation of some junctional peptide. In fact, we have observed that DLU modifies the content and distribution of claudins 1 and 2 (manuscript in preparation). Furuse et al. (2001) and Van Itallie et al. (2001) have shown that the expression of some claudins in MDCK cells can actually decrease TER.

As indicated in Fig. 11 and Table 2, the tightening of the TJ elicited by DLU is not a very specific one, suggesting that the active principle(s) may act in TJs of other tissues and animal species, a characteristic that would transform the peptides in DLU into a very useful tool. Thus, the complex molecular structure of the TJ, its consensus with protein-protein binding sequences, with proliferation- and tumor-suppressing activity, as well as the numerous phosphorylated states of its diverse molecules, indicate that the TJ is involved in a variety of important functions other than its role as a barrier to diffusion

along the intercellular permeation route. As mentioned above, currently different laboratories are finding TJ-regulating substances in a variety of luminal fluids (*see* for instance Gorodeski & Goldfarb, 1998; Contreras et al., 1999). Obviously, in a given moment any of those TJ components and TJ-affecting substances can fail, giving rise to pathological conditions. Actually, a number of autoimmune diseases, such as multiple sclerosis, Hashimoto's thyroiditis and some type of diabetes are attributed to failures in the TJ that would allow peptides produced by the intestinal flora gain access to the interstitial side. The antibodies developed by the organism against such peptides would also attack normal cells in the organism, such as those of the nervous system, the thyroid gland and the pancreas. Obviously, substances with the ability to tighten the TJ of the type characterized in the present work may afford a powerful therapeutic tool.

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