Increased urothelial paracellular transport promotes cystitis

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The internal surface of the urinary bladder is lined by the urothelium, a barrier-forming epithelium that restricts the passage of ions and metabolic products from the urine into the bladder interstitium (38). The urothelium is stratified and is composed of three cell types: polarized umbrella cells, which form a single cell layer that faces the interior of the bladder; intermediate cells, which lie just underneath the umbrella cell layer; and basal cells, which form a single-cell stratum that is attached to the basement membrane (38). Although multilayered, it is the outermost umbrella cell layer that forms a multifactorial barrier that includes a glycosaminoglycan (GAG) layer with antiadherence properties, an apical membrane with inherently low permeability to urea and water, and relatively impermeable tight junctions (38). Notably, the umbrella cell layer maintains this barrier in the face of the cyclic changes in hydrostatic pressure that take place during the storage and voiding phases.

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a debilitating chronic condition that presents with urinary frequency, urgency, nocturia, and pain in the bladder and/or pelvis in the absence of any identifiable cause (9, 30, 48–50). While the etiology of IC/BPS is unknown, numerous lines of evidence indicate that increased urothelial permeability to urine constituents plays an important role in the onset of the disease (11, 24, 55, 56, 58). Of significance and in good agreement with this notion, it has been reported that the intravesical instillation of KCl triggers urgency and pain in 75–80% of the patients with IC/BPS, but not in asymptomatic controls (57). This observation is consistent with an intrinsic dysfunction of the urothelial barrier in IC/BPS patients, but more importantly reveals that the diffusion of urinary solutes into the interstitium may produce urgency and cause pain. Interestingly, recent studies showed reduced expression of the tight junction-associated protein zona occludens (ZO-1) in IC/BPS patients (32, 43, 71, 83), which is consistent with epithelial barrier dysfunction.

Tight junctions are protein complexes that function as permeability barriers to restrict the movement of solutes through the intercellular space (21). Claudins, a family of integral membrane proteins, constitute the structural and functional core of the tight junctions and define the electrical properties of epithelia (6, 7, 28). To date, 27 mammalian claudins have been identified (47). Based on their effects on transepithelial resistance, claudins are classified as pore-forming, which tend to make tight cell monolayers leakier, or barrier-forming, which tend to make leaky cell monolayers tighter (5, 29). To examine whether urothelial barrier dysfunction is sufficient to promote bladder inflammation (cystitis) and alter bladder function, we overexpressed the pore-forming claudin-2 (CLDN-2) in the rat urothelium using in situ transduction. As anticipated, CLDN-2 overexpression increased the permeability of the urothelium to cations, without affecting the permeability to organic molecules. In vivo studies of bladder function using continuous cystometry revealed higher intravesical basal pressures, decreased bladder compliance, and shorter intervoiding intervals associated with selectively overexpressing the pore-forming tight junction-associated protein claudin-2 (CLDN-2) in the umbrella cells. In rats transduced with CLDN-2 vs. controls transduced with green fluorescent protein. While the integrity of the urothelial barrier was preserved in the rats transduced with CLDN-2, we found that the expression of this protein in the umbrella cells initiated an inflammatory process in the urinary bladder characterized by edema and the presence of a lymphocytic infiltrate. Taken together, these results are consistent with the notion that urothelial barrier dysfunction may be sufficient to trigger bladder inflammation and to alter bladder function.

Materials and Methods

Reagents and Antibodies

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal antibody to
CLDN-2, rabbit polyclonal antibody to CLDN-2, and rabbit polyclonal antibody to ZO-1 were purchased from Life Technologies (Grand Island, NY), whereas mouse monoclonal antibody to cytokeratin-20 (CK-20) was purchased from Dako (Carpinteria, CA). Mouse monoclonal antibody to uroplakin 3a (UPK3a) was described previously (74). Alexa Fluor 488-coupled and horseradish peroxidase-coupled secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). ToPro-3, streptavidin Alexa Fluor 647, phalloidin Alexa Fluor 488, and phalloidin Alexa Fluor 546 were purchased from Life Technologies.

**Experimental Animals**

Female Sprague-Dawley rats (250–300 g; Harlan Laboratories, Indianapolis, IN) were used in this study. Rats were euthanized by CO2 inhalation, followed by a thoracotomy. Care and handling of the animals were in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee.

**Preparation of Recombinant Adenoviruses and In Situ Transduction of Umbrella Cells**

Replication-defective adenoviruses coding for GFP (AdGFP) or rat CLDN-2 (AdCLDN-2) were constructed by subcloning the coding region of the respective proteins into the plasmid pAdlox. Adenoviruses were generated at the University of Pittsburgh Vector Core facility. In situ transduction of umbrella cells was accomplished via direct intravesical instillation of replication-defective adenoviruses under isoflurane anesthesia as described previously (40). Briefly, the urinary bladder was catheterized with a 22-gauge teflon catheter under isoflurane anesthesia as described previously (40). Briefly, the direct intravesical instillation of replication-defective adenoviruses was performed through the urethra and the bladder was emptied. The urinary bladder was infused thereafter with the 1–2 ml of PBS, and then infused with 450 μl of 0.1% (wt/vol) n-dodecyl-β-D-maltoside dissolved in PBS. The catheter was removed immediately afterward, and the urethral orifice was clamped with a metal clip to prevent leakage. After 5 min, the catheter was reintroduced through the urethra and the bladder was emptied. The urinary bladder was infused thereafter with the 1–2 × 108 infectious virus particles (ivp) of AdGFP or AdCLDN-2 diluted in 450 μl PBS, and the external urethral orifice was clamped again. After a 30-min incubation, the catheter was reintroduced through the urethra. The bladder was emptied and washed one time with PBS and the rats were allowed to recover. In most cases, the animals were euthanized after 24 h and the urinary bladder was surgically removed for further studies.

**Western Blot Analysis of CLDN-2 Expression in Rat Urothelium**

Samples were obtained from rats transduced with AdGFP or AdCLDN-2 by gently scraping the epithelium into RIPA buffer [40 mM Tris, 150 mM NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 0.2% (wt/vol) SDS, and a Protease Inhibitor Cocktail Set III (EMD Biosciences, Billerica, MA), pH 7.6]. Extracts were rotated for 20 min at 4°C and then centrifuged at 25,000 g for 20 min at 4°C. The supernatant was collected in a new tube and kept on ice for Western blot analysis. The BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA), used to normalize the protein concentration of the samples, was performed according to the manufacturer’s instructions. Samples were mixed in a 1:1 ratio with loading buffer [Laemmli sample buffer supplemented with 0.277 M SDS and 1.420 M β-mercaptoethanol] and then incubated for 45 min at 37°C. Protein samples, 3 μg of each, were loaded onto Criterion TGX 12% gels (Bio-Rad, Hercules, CA) and resolved by electrophoresis at 180 V for 55 min. The proteins were transferred to nitrocellulose membranes using the Trans-Blot Turbo Transfer system (Bio-Rad) according to the manufacturer’s instructions. The membrane was then blocked with PBS supplemented with 10% (wt/vol) nonfat milk for 1 h. After blocking, the membrane was incubated overnight at 4°C with the mouse anti-CLDN-2 primary antibody (1:1,000). The next day, the membrane was incubated with a goat-anti-mouse secondary antibody labeled with peroxidase (1:5,000, KPL, Gaithersburg, MD), and CLDN-2 reactivity was detected using a Western Lightning Plus-ECL kit (PerkinElmer, Waltham, MA).

**Immunofluorescent Labeling of Urinary Bladders**

The animals were euthanized with CO2, and the urinary bladder was removed, carefully cut open, and pinned mucosal side up onto a rubber sheet submerged in Krebs solution containing (in mM) 110 NaCl, 25 NaHCO3, 5.8 KCl, 1.2 MgSO4, 1.2 KH2PO4, 11 glucose, and 2 CaCl2 buffered at pH 7.4 by gassing with a mixture of 95% O2-5% CO2 (vol/vol). After incubation for 1 h, the tissue was fixed with 4% (vol/vol) paraformaldehyde in 100 mM sodium cacodylate buffer, pH 7.4, for 30 min at 37°C. Alternatively, it was fixed with 10% (wt/vol) TCA (prepared in H2O) for 60 min at room temperature. The TCA-treated tissue was subsequently washed with 100 mM glycine (prepared in PBS), pH 7.5, to remove the fixative. In either case, fixed tissues were rinsed with PBS and then incubated in 30% (wt/vol) sucrose dissolved in PBS for ~2 h at 4°C until the tissue lost its buoyancy and sank to the bottom of the 15-ml tube. The tissues were then embedded in Optimal Cutting Temperature (O.C.T.) compound, before storage at ~70°C. Frozen tissue blocks were sectioned using a CM1950 cryostat (Leica, Buffalo Grove, IL). Sections were labeled with primary antibodies or fluorophore-labeled probes using our previously described techniques (1, 10, 39). Whole-mounted bladders were fixed with paraformaldehyde and processed as described previously (1, 10, 39). Images were captured using a Leica TCS SP5 CW STED confocal microscope (in normal confocal mode) equipped with a ×63 glycerol objective (numerical aperture = 1.3) and low-noise hybrid detectors. The captured images were contrast corrected using Volocity (PerkinElmer), exported as TIFF files, and then assembled in Adobe Illustrator.

**Electrophysiological Measurements**

Urinary bladders were harvested from euthanized animals through an abdominal incision. The tissue was maintained in Krebs solution at 37°C and carefully cut open and pinned mucosal side up onto a rubber sheet. Urinary bladders were mounted on custom-fabricated teflon tissue sliders with an exposed tissue area of 0.65 cm2 and eight sharp pins set at 3 mm from the opening. Silicone grease (Dow Corning, Midland, MI) was carefully applied to the region of tissue impaled by the pins to prevent edge damage, and the tissue sandwiched between an additional tissue slider, which contained an opening but no pins. The assembled tissue sandwich was inserted into the chambers of an EM-CSYS Ussing system (Physiologic Instruments, San Diego, CA) equipped with a heat block for temperature control. The mucosal and serosal hemichambers were filled with 3 and 5 ml of Krebs solution, respectively. The temperature inside the chambers was kept at 37°C. The hemichambers were continuously bubbled with 95% O2-5% CO2 (vol/vol). The tissue was equilibrated for at least 1 h before it was subjected to experimental manipulations. The mucosal and serosal hemichambers were connected to Ag/AgCl electrodes via 5 M NaCl agar bridges for voltage sensing and current passing. These electrodes were connected to a VCC MC6 Multichannel Voltage/Current Clamp (Physiologic Instruments). The asymmetry of voltage-sensing Ag/AgCl electrodes and the liquid junction potentials were compensated using an offset-removal circuit before tissue mounting. The voltage potential difference across the bladder (PD) and the tissue electrical resistance (TER) were measured under current clamp conditions. To calculate TER, a bipolar pulse of ±10 μA of current with a duration of 250 ms was applied every 60 s. Data were acquired at 100 Hz using a Powerlab 8/30 analog-to-digital converter (ADInstruments, Colorado Springs, CO).
Flux Measurements

Rat bladders were mounted in Ussing chambers as described above. Fluorescein (sodium salt) was added to the mucosal hemichamber to give a final concentration of 100 μM. Aliquots were collected at time 0 and after 1 h. The fluorescent signal was measured in a 96-well GloMax microplate reader (Promega, Madison, WI). A standard curve was generated using defined dilutions of fluorescein in Krebs solution, and linear regression was used to determine the amount of fluorescein in the samples. Fluorescein flux was expressed in nmol per tissue area and unit of time (nmol·cm⁻²·h⁻¹).

In Vivo Biotin Permeation Assay

Rats were anesthetized by inhalation of isoflurane [3% (vol/vol)], and subsequently 1.2 g/kg urethane dissolved in H₂O was injected subcutaneously. Isoflurane treatment was ceased, and 2 h later a toe/tail pinch was used to confirm that the animals had reached an appropriate anesthetic depth. A 22-gauge catheter was passed through the urethra to empty the urinary bladder. The bladder was washed three times with 400 μL of Krebs infused at a rate of 200 μL/min using a PHD ULTRA syringe pump (Harvard Apparatus, Holliston, MA). After this, the bladder was infused with 400 μL EZ-link sulfo-NHS-biotin (1 mg/ml, Thermo Fisher Scientific) dissolved in Krebs solution at a rate of 200 μL/min. The catheter was removed immediately after EZ-link sulfo-NHS-biotin infusion, and the urethral orifice was clamped to prevent leakage. After a 15-min incubation, the catheter was reintroduced through the urethra, and the bladder was emptied and then washed three times with 400 μL of Krebs solution. The Tissue was fixed with paraformaldehyde (4%), embedded in O.C.T. compound, frozen, cryosectioned, and labeled with streptavidin Alexa Fluor 647 and phalloidin Alexa Fluor 546 as described above.

Estimation of Permselectivity of the Paracellular Route

Urinary bladders were mounted in Ussing chambers as described above, and changes in PD were recorded in response to the application of bipolar current pulses of increasing magnitude. Current-voltage (IV) relationships were obtained under symmetrical conditions first and then after the mucosal bathing solution was exchanged for one containing a low NaCl concentration ([NaCl]) or a high [KCl]. The apical solution was replaced by simultaneously infusing and removing 30 ml of the prewarmed desired solution using a PHD ULTRA syringe pump. The composition of the low-NaCl solution was (in mM) 5 NaCl, 25 NaHCO₃, 5.8 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 11 glucose, and 2 CaCl₂. The osmolarity of the solution was adjusted with mannitol to 290 mosmol/kgH₂O. The composition of the high-K⁺ solution was (in mM) 115.8 KCl, 7 NaHCO₃, 18 KHC₂O₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 11 glucose, and 2 CaCl₂. Solutions were buffered at pH 7.4 by gassing with a mixture of 95% O₂-5% CO₂ (vol/vol). These experiments were performed in the presence of 1 mM benzamil in the mucosal bathing solution to inhibit transcellular sodium flux. Liquid junction potentials were estimated by adding 0.1% Triton X-100 to the mucosal and serosal compartments. Linear regression was employed to determine the reversal potential (Vrev) in each condition. Relative permselectivities (PCl/PNa, and PK/PNa) were calculated from the Vrev corrected by the junction potential using the Goldman-Hodgkin-Katz (GHK) voltage equation (33):

\[ V_{rev} = \frac{RT}{F} \ln \frac{P_{Na}^{M} + P_{K}^{M} + P_{Cl}^{S}}{P_{Na}^{S} + P_{K}^{S} + P_{Cl}^{M}} \]

where \( P \) represents the permeability coefficient for a given ion, \( a \) represents the ionic activity, \( M \) and \( S \) represent the mucosal and serosal compartments, and \( F \), \( R \), and \( T \) have their usual meaning. Absolute permeabilities were estimated using the Kimizuka-Koketsu equation as described in the literature (27, 80, 82):

\[ P_{Na} = \frac{RTG}{F^{2}a^{2}Na}(1 + \alpha) \]

where \( G \) represents conductance (mS), \( \alpha = PCl/PNa \), and \( \beta = PK/PNa \) obtained as described above.

Histopathology and image analysis. Rat urinary bladders from control rats or from rats transduced with AdGFP or AdCLDN-2 were harvested from euthanized animals through an abdominal incision. The bladders were rinsed with Krebs buffer, cut open, and rested in gassed Krebs buffer for 30 min at 37°C. The tissue was then fixed in neutral buffered formalin (4% wt/vol paraformaldehyde, 45.8 mM Na₂HPO₄, and 30 mM NaH₂PO₄·H₂O) for 30 min at 37°C, and then each animal’s bladder was cut into five to seven ~2-mm-wide strips. To ensure vertical sections through the full thickness of the bladder wall, the strips were oriented along their long axis, cut surface up, and placed side by side in slotted histology cassettes. Tissue was maintained in neutral buffered formalin for at least 24 h before embedding in paraffin. Approximately 5-μm paraffin sections were cut, mounted on slides, and then stained with hematoxylin and eosin. For each bladder, a random slide (containing transects through each of the bladder’s strips) was placed on the stage of a Leica DM6000B upright microscope (fitted with a ×40 HCX PL-APO, 1.25-numerical aperture objective), and a random image was collected from each of the five to seven bladder strips. Images were captured using a QImaging Retiga 4000R color digital camera interfaced with an Apple iMac computer running Volocity Acquisition software (version 6.3). In some cases, the orientation and wall thickness were such that a full thickness view of the bladder wall required stitching two to five images together in Photoshop using the Photo Merge function. The individual composited images were opened in National Institutes of Health Fiji (ImageJ 2.0), and the “Cell Counter” plugin was employed to assist in counting the total number of mononuclear lymphocytes observed in the bladder wall of each composite image. Lymphocytes, characterized by their relatively small size (~6–9 μm in diameter), spindled/ovoid morphology, prominent heterochromatic nuclei, little cytoplasm, and presence in the extracellular matrix (or between epithelial cells), were counted under the supervision of a trained pathologist. For each bladder, the data from each of the five to seven slices were used to calculate an average number of lymphocytes per image. To measure the wall thickness, the images were printed and the average wall thickness was measured using a calibrated ruler. Again, for each bladder, the data from each of the five to seven slices were used to calculate the average wall thickness. For publication, exported images were opened in Adobe Lightroom CC, and adjustments were made to the white point. The images were exported in JPEG format and compiled in Adobe Illustrator CC.

Assessment of Bladder Function by Continuous Infusion Cystometry

Rats were anesthetized with urethane, and their bladders were exposed through an abdominal incision, as previously described (62). Upon being exteriorized, shallow purse string sutures were made around the dome using 6-0 silk suture material. The area within the bladder’s strips was placed on the stage of a Leica DM6000B upright microscope (fitted with a ×40 HCX PL-AP0, 1.25-numerical aperture objective), and a random image was collected from each of the five to seven bladder strips. Images were captured using a QImaging Retiga 4000R color digital camera interfaced with an Apple iMac computer running Volocity Acquisition software (version 6.3). In some cases, the orientation and wall thickness were such that a full thickness view of the bladder wall required stitching two to five images together in Photoshop using the Photo Merge function. The individual composited images were opened in National Institutes of Health Fiji (ImageJ 2.0), and the “Cell Counter” plugin was employed to assist in counting the total number of mononuclear lymphocytes observed in the bladder wall of each composite image. Lymphocytes, characterized by their relatively small size (~6–9 μm in diameter), spindled/ovoid morphology, prominent heterochromatic nuclei, little cytoplasm, and presence in the extracellular matrix (or between epithelial cells), were counted under the supervision of a trained pathologist. For each bladder, the data from each of the five to seven slices were used to calculate an average number of lymphocytes per image. To measure the wall thickness, the images were printed and the average wall thickness was measured using a calibrated ruler. Again, for each bladder, the data from each of the five to seven slices were used to calculate the average wall thickness. For publication, exported images were opened in Adobe Lightroom CC, and adjustments were made to the white point. The images were exported in JPEG format and compiled in Adobe Illustrator CC.

Rats were anesthetized with urethane, and their bladders were exposed through an abdominal incision, as previously described (62). Upon being exteriorized, shallow purse string sutures were made around the dome using a 6-0 silk suture material. The area within the boundary of the sutures was punctured with an 18-gauge needle, and a flame-flanged PE-50 tube was inserted into the hole. The suture was tightened around the tubing, and the tube was gently retracted until the flange was flush with the mucosa. The bladder was returned to the peritoneal cavity, and the surgical incision was closed around the tube. The PE-50 tubing was connected to a three-way port: one branch led to a pressure transducer (ADInstruments), while another was connected to a syringe pump (New Era Syringe Pump Systems, Farmingdale, NY) for continuous infusion with Krebs solution. The pressure transducer was connected to a Quad Bridge Amplifier and Powerlab 4/30 (ADInstru-
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To determine whether CLDN-2 is expressed in the urothelium, we mounted bladders from untreated rats or rats transduced with AdCLDN-2 or AdGFP (control) in Ussing chambers and measured urinary bladder electrical parameters. We found that the TER and PD of bladders transduced with CLDN-2 were significant lower, 0.5 ± 0.1 kΩ·cm² and −1.7 ± 0.2 mV (n = 20), than the TER and PD of the untreated bladders, 5.3 ± 0.4 kΩ·cm² and −7.4 ± 1.6 mV (n = 10), or the bladders transduced with GFP, 4.6 ± 0.3 kΩ·cm² and −12 ± 1 mV (n = 18) (Fig. 2, A and B).

Based on charge and size selectivity, at least two functionally distinct pathways can be defined for the flux of solutes through the paracellular space (69). The first is the high-capacity claudin-based pore pathway, which facilitates the passage of large quantities of small ions based on their charge and size. The second is the so-called leak pathway, a low-capacity paracellular route that does not discriminate on the basis of solute size, and thus large molecules can leak across it. Since the expression of CLDN-2 in the urothelial cells is only expected to increase the permeability of the urothelial membrane to ions, we measured the permeability of monovalent ions (Na⁺), K⁺, and Cl⁻ in urinary bladders, from untreated rats and rats transduced with GFP or CLDN-2, mounted in Ussing chambers. I-V relationships were generated under asymmetric conditions, and the selectivity of the paracellular pathway was estimated from the reversal potential (Vrev) using the GHK voltage equation (33).

As shown in Fig. 3, I-V relationships were linear between the tested current steps. Urinary bladders from untreated rats or transduced with GFP were slightly more selective for anions than cations (Fig. 3A and Table 1). In contrast, the paracellular pathway of urinary bladders transduced with CLDN-2 were more selective for cations than anions (Fig. 3B). In the presence of a basolateral-to-apical NaCl chemical gradient (i.e., low NaCl in the mucosal bathing solution), the Vrev for the bladders transduced with CLDN-2 was 30.3 ± 1.2 mV, reflecting a Cl⁻/Na⁺ selectivity ratio of 0.32 ± 0.02 (Fig. 3C and Table 1). The expression of CLDN-2 in the urothelium also altered the selectivity of the paracellular pathway toward cations. For instance, with a mucosal bathing solution containing high K⁺ (see MATERIALS AND METHODS), the Vrev for the untreated bladders was 0.2 ± 0.8 mV, while the value for bladders transduced with GFP was 0.3 ± 0.2 mV and the value confirm that overexpressed CLDN-2 was still delivered to the tight junction of the umbrella cell layer, we examined whole-mounted and cryosectioned bladder tissue fixed with paraformaldehyde. In whole mounts, CLDN-2 was observed to concentrate at the periphery of the umbrella cell where it colocalized with the tight junction-associated protein ZO-1 (Fig. 1D). In cross sections (see arrows in Fig. 1, E and F), overexpressed CLDN-2 was restricted to the umbrella cells and was observed along the basolateral surface (much like the endogenous protein) and in intracellular accumulations (Fig. 1F, see arrowheads). These latter elements colocalized with TGN-38, a transmembrane protein that marks the trans-Golgi network, a compartment involved in the biosynthetic transport of proteins to the cell surface.

CLDN-2 Expression Increases Permeability of the Urothelium to Ions, But Not to Larger Solutes

To examine whether CLDN-2 overexpression altered the size of the umbrella cell layers, as well as along the lateral surfaces and at the tight junction region of the umbrella cell layer (Fig. 1A, leftmost panels). The latter was marked with CLDN-8. Similar staining was observed using a different CLDN-2-specific antibody (Fig. 1A, rightmost panel). These results indicated that the rat urothelium expresses CLDN-2.

To assess the impact of increased paracellular transport on bladder function, we overexpressed CLDN-2 in the urothelium. Rat bladders were transduced in situ with adenovirus coding for GFP or CLDN-2 as described in MATERIALS AND METHODS. We previously showed that in situ transduction per se does not alter TER or morphology of the tight junctions (40). In the current study, the transduction efficiency was high (80–95%); Fig. 1B).

Western blot analysis confirmed expression of CLDN-2 in AdCLDN-2-transduced urothelium, but no signal was detected in AdGFP-transduced bladders after short incubation times with the ECL reagent (Fig. 1C). However, an identical molecular weight species was detected in the GFP-transduced bladders if the incubation was extended for several hours (data not shown). The latter indicated that endogenous CLDN-2 protein expression is likely to be relatively low. To

Data and Statistical Analysis

Data are expressed as means ± SE (n), where n equals the number of independent experiments or animals. Parametric or nonparametric tests were employed as appropriate. P < 0.05 was considered statistically significant. Fitting and statistical comparisons were performed with Clamfit (Molecular Device, Sunnyvale, CA), Sigmaplot 11.0 (Systat Software, Chicago, IL), or GraphPad 5.03 (GraphPad Software, San Diego, CA).

RESULTS

Expression of CLDN-2 in the Urothelium Using In Situ Transduction

Work to date has established that CLDN-2 forms a cation-selective paracellular pore that decreases transepithelial electrical resistance when expressed in tight epithelia (4, 22, 82). Although normally expressed in the tight junctions of leaky epithelia, such as the proximal tubule in the kidney (19, 41) and the intestinal crypts (63), we previously reported that mRNA message for this claudin isoform was present in the mouse urothelium (1). To determine whether CLDN-2 is expressed in the rat urothelium, we performed immunohistochemistry of paraformaldehyde-fixed tissues. However, no CLDN-2 staining was revealed (data not shown). In contrast, when we fixed the tissue with TCA we observed CLDN-2 expression in the basal and intermediate cell layers, as well as along the lateral surfaces and at the tight junction region of the umbrella cell layer (Fig. 1A, leftmost panels). The latter was marked with CLDN-8. Similar staining was observed using a different CLDN-2-specific antibody (Fig. 1A, rightmost panel). These results indicated that the rat urothelium expresses CLDN-2.

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for the bladders transduced with CLDN-2 was 4.3 ± 0.4 mV. While the paracellular pathway of untreated bladders and those transduced with GFP did not discriminate between Na⁺ and K⁺, the paracellular pathway of bladders transduced with CLDN-2 was slightly more selective for Na⁺ than K⁺ (Fig. 3C). We found that CLDN-2 expression in the urothelium significantly increased the absolute permeabilities to both Na⁺ and K⁺ (P_{Na} and P_{K}, respectively) and concomitantly decreased the absolute permeability to Cl⁻ (P_{Cl}) (Table 1). In summary and consistent with previous studies (4, 82), our results indicate that CLDN-2-based pores are about three times more permeable to monovalent cations than anions and showed limited discrimination between Na⁺ and K⁺.

To determine whether overexpression of CLDN-2 in the urothelium affects the leak pathway, we measured fluorescein (MW: 332.3 Da) flux across urinary bladders mounted in Ussing chambers from rats transduced with GFP or CLDN-2. As shown in Fig. 2C, no significant difference was observed between the fluorescein flux across bladders transduced with GFP (0.28 ± 0.12 nmol·cm⁻²·h⁻¹, n = 10) or transduced with
CLDN-2 (0.80 ± 0.30 nmol·cm⁻²·h⁻¹, n = 10) (P > 0.12). To determine whether CLDN-2 expression changed the permeability of the urothelium to large noncharged organic molecules in vivo, we measured the permeability to sulfo-NHS-biotin (MW: 443.4 Da) (Fig. 4). NHS-biotin reacts with and irreversibly modifies primary amines (e.g., protein lysine residues). The extremely high affinity of biotin/streptavidin interactions allows for the efficient detection of traces of biotinylated proteins. Thus, 24 h after transduction, rats were anesthetized and sulfo-NHS-biotin was infused into their bladders using a transurethral catheter. After a 15-min incubation, bladders were washed and processed as indicated in MATERIALS AND METHODS. We previously showed that EGTA treatment, which opens the tight junction barrier, results in permeation of sulfo-NHS-biotin into the lateral space between adjacent umbrella cells and into the underlying tissues (10). However, no such leakage was observed in rats transduced with GFP or CLDN-2, indicating the presence of an intact barrier to large organic molecules. Taken together, our studies show that the overexpression of CLDN-2 in the urothelium increases the transepithelial transport of ions, without affecting the permeation of large organic molecules through the leak pathway.

Fig. 2. Claudin-2 expression increases the permeability of the urothelium to small ions. Urinary bladders from control (untreated) and rats transduced with adenoviruses coding for GFP or CLDN-2 were mounted in Ussing chambers as indicated in MATERIALS AND METHODS. A: tissue electrical resistance (TER) of urinary bladders from control and rats transduced with GFP or CLDN-2 (n = 10–20, ***P < 0.001, ANOVA following by Dunn’s multiple comparisons test). B: tissue electrical potential difference (PD) of urinary bladders from control and rats transduced with GFP or CLDN-2 (n = 10–20, ***P < 0.001, ANOVA following by Dunn’s multiple comparisons test). C: fluorescein fluxes in urinary bladders transduced with GFP or CLDN-2 (n = 10, P = 0.12, Mann-Whitney nonparametric test).

Fig. 3. Claudin-2 expression alters the ionic selectivity of the paracellular pathway. A and B: current-voltage relationship generated under NaCl symmetrical conditions (○), NaCl asymmetrical conditions (gray circles), and NaCl/KCl asymmetrical conditions (●) for urinary bladders transduced with GFP or CLDN-2 (n = 6–9). Linear regression was used to determine the reversal potential (Vrev) in each condition. C: paracellular permselectivity toward monovalent ions for untransfected control rat bladders (Ctrl) or rat bladders transduced with GFP or CLDN-2. Paracellular permselectivity was calculated from Vrev using the GHK voltage equation as described in MATERIALS AND METHODS. ***P < 0.001, statistically significant difference between experimental conditions (n = 5–8, ANOVA followed by Bonferroni multiple comparisons test).
CLDN-2 Overexpression Increases Bladder Activity

To examine the effect of CLDN-2 expression on bladder function, we performed continuous cystometry on rats transduced with AdGFP or AdCLDN-2. Cystometrograms (CMGs) record the intravesical pressure during continuous bladder filling, providing a functional measurement of the urinary bladder-voiding cycle (44). The bladder maintains a fairly constant pressure during the filling phase, but once the bladder reaches capacity the nervous system triggers detrusor contraction, which is followed by relaxation of the urethral sphincter and voiding. Representative recordings of CMGs obtained 24 h after transduction with GFP (control) or CLDN-2-transduced rats are shown in Fig. 5A. We found that the basal pressure was significantly higher, the compliance of the bladder wall was significantly lower, and the ICI was significantly shorter in rats transduced with CLDN-2 than control animals transduced with GFP (Fig. 5B). In contrast, the threshold pressure and peak pressure were similar in rats transduced with GFP or CLDN-2 (Fig. 5B). In summary, our results indicate that the expression of CLDN-2 in the urothelium promotes voiding at low filling volumes.

DISCUSSION

The function of the bladder is to store urine and to discharge it in a coordinated and controlled fashion. During the storage phase, information about the filling status of the bladder is conveyed from afferent terminals located in the epithelium, subepithelial plexus, and bladder wall to the spinal cord (8, 16, 17, 23). Because the urine contains solutes and waste products that have the potential to influence afferent activity, a critical function performed by the urothelium during the storage phase is to prevent the diffusion of urinary components into the underlying bladder interstitium. Consistent with this function, we and others have reported that the rabbit urothelium achieves a very high junctional resistance in the quiescent state (25,000 Ω·cm²) (42, 77), and even though this resistance decreases during bladder filling, it remains relatively high (2,500 Ω·cm²), and the barrier to other organic solutes is maintained (10). Importantly, reduced urothelial barrier function has been reported in patients with IC/PBS (11, 24, 55, 56, 58), but whether tight junction dysfunction can lead to the onset and perpetuation of symptoms has not been evaluated before.

Table 1. Umbrella cell paracellular pathway absolute permeabilities to Cl⁻, K⁺, and Na⁺

<table>
<thead>
<tr>
<th></th>
<th>Pₖ (10⁻⁷ cm/s)</th>
<th>Pₖ (10⁻⁷ cm/s)</th>
<th>Pₖ (10⁻⁷ cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.32 ± 0.01</td>
<td>1.64 ± 0.01</td>
<td>1.30 ± 0.02</td>
</tr>
<tr>
<td>GFP</td>
<td>1.30 ± 0.03</td>
<td>1.66 ± 0.04</td>
<td>1.20 ± 0.04</td>
</tr>
<tr>
<td>CLDN-2</td>
<td>2.25 ± 0.03*</td>
<td>0.72 ± 0.03*</td>
<td>1.80 ± 0.04*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE of 5–8 independent experiments. GFP, green fluorescent protein; CLDN-2, claudin-2. The permselectivity (P) of the paracellular pathway was calculated from reversal potential (Vₜₙₐ₅) obtained in the current-voltage (I-V) relationships using the Goldman-Hodgkin-Katz (GHK) voltage equation as described in MATERIALS AND METHODS.*P < 0.001 (n = 5–8), statistically significant difference between experimental conditions by ANOVA followed by Bonferroni multiple comparisons test.

CLDN-2 Overexpression Increases Rat Urothelium Promotes Inflammation

The above-noted observed urodynamic changes are similar to those observed in the cyclophosphamide-induced model of cystitis (35, 37, 53, 62). To assess whether rats transduced with CLDN-2 developed cystitis, we examined paraffin-embedded bladder sections stained with hematoxylin and eosin (Fig. 6). For the most part, there was little evidence of edema or inflammation in the mucosa of the bladders transduced with GFP 24 h after viral instillation (Fig. 6A). In contrast, we observed inflammation with diffusive edema in the urothelium and lamina propria of the rats transduced with CLDN-2 (Fig. 6, B and C). Accordingly, we observed a significant increase in the number of lymphocytes and mean wall thickness in bladders transduced with AdCLDN-2 vs. AdGFP (Fig. 6, D and E). Inflammatory cells in the mucosa of the bladders transduced with CLDN-2 were mostly mononuclear (primarily lymphocytes). Despite this inflammation, the integrity of the urothelium was preserved, and we did not observe desquamation. We also observed occasional focal patches of polymorphonuclear leukocytes in the epithelium, lamina propria, and submucosal blood vessels of bladders transduced with AdCLDN-2 (not shown). When in the presence of these cells, the epithelium sometimes appeared vacuolated. In addition to mucosal inflammation, a diffuse inflammation in the serosal regions of the bladder wall including detrusor of bladders transduced with AdCLDN-2 was also observed (Fig. 6B). To determine whether CLDN-2 expression alters umbrella cell polarity and/or differentiation, we examined the expression of the urothelial differentiation markers cytokeratin-20 (CK-20) and uroplakin IIIa (UPK3a) using confocal microscopy (25, 31, 65, 67, 76). Consistent with previous studies, CK-20 and UPK3a were primarily expressed in the umbrella cells in bladders transduced with CLDN-2 or GFP (Fig. 7). Taken together, our findings indicate that selectively increasing the paracellular permeability of the urothelium to small molecules promotes inflammation and edema in the bladder wall with limited alterations in the integrity of the urothelium.

Fig. 4. Claudin-2 expression does not alter the permeability of the urothelium to large organic molecules. An in vivo biotin permeation assay was performed in rats transduced with GFP or CLDN-2. EZ-link sulfo-NHS-biotin (1 mg/ml) was infused into the bladder of anesthetized rats transduced with GFP (left) or CLDN-2 (right). The tissue was fixed with 4% paraformaldehyde, embedded in OCT compound, cryosectioned, and labeled with streptavidin Alexa Fluor 647 (red) and phallolidin Alexa Fluor 546 (blue). GFP fluorescence is shown in green. The position of the tight junctions is indicated by arrows.
Effect of Selectively Increasing Tight Junction Permeability

To examine the effects of increasing tight junction permeability on bladder function, we selectively overexpressed CLDN-2 in the rat urothelium using in situ transduction. CLDN-2 is known to form a cation-selective pore, and, consistent with studies conducted in epithelial cell lines (4, 22, 81, 82), we found that the overexpression of CLDN-2 in the urothelium reduced TER about ninefold, without affecting the permeability to macromolecules. In addition, CLDN-2 expression changed the selectivity of the urothelial paracellular pathway toward ions, increasing the permeability to cations (Na\(^{+}\) and K\(^{+}\)) and reducing the permeability to anions (Cl\(^{-}\)) (Table 1). The consequences of this altered ion flux were considerable. First, we found that overexpression of CLDN-2 in the urothelium increased TER about ninefold, without affecting the permeability to macromolecules. In addition, CLDN-2 expression changed the selectivity of the urothelial paracellular pathway toward ions, increasing the permeability to cations (Na\(^{+}\) and K\(^{+}\)) and reducing the permeability to anions (Cl\(^{-}\)) (Table 1). The consequences of this altered ion flux were considerable.

Fig. 5. Claudin-2 expression increases bladder activity. Continuous cystometry was performed in rats transduced with replication-defective adenoviruses coding for GFP (AdGFP) or CLDN-2 (AdCLDN-2). A: representative tracings of cystometrograms (CMGs) performed in rats transduced with GFP (top) or CLDN-2 (bottom). B: summary bar graphs of basal pressure, threshold pressure, peak pressure, intercontraction intervals (ICI), and bladder wall compliance for rats transduced with GFP or CLDN-2. Values are means ± SE from GFP (n = 11)- or CLDN-2-transduced bladders (n = 8). \(*P < 0.05\) and **\(P < 0.01\), statistically significant differences between sample means (Mann-Whitney non-parametric test).

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What causes the increase in basal pressure and reduction in bladder wall compliance? We assume that the urodynamic changes observed in the rats transduced with CLDN-2 are mainly due to the inflammatory process that occurs in response to the leakage of urinary solutes into the bladder interstitium. The neural pathways that control storage and voiding act as a simple switch that is either “off” during storage or “on” during voiding (8, 15, 16, 20, 66). Thus the increase in basal pressure during filling we observed in cystometrograms is more likely to result from altered detrusor smooth muscle tone and not efferent nerve activity. The urodynamic changes observed in rats transduced with CLDN-2 are similar to those reported for the acute cyclophosphamide-induced model of cystitis (35–37, 53, 62), which also affects the urothelial barrier upon metabolism of the cyclophosphamide to acrolein (12). Cyclophosphamide induces bladder inflammation, which sensitizes afferent sensory pathways (14, 79). Thus we assume that in rats transduced with CLDN-2 the inflammatory process alters the excitability and firing of the bladder afferents neurons, as well as detrusor smooth muscle tone, promoting voiding at low filling volumes.
Why Would Increased Cation Transport Promote Inflammation and Augment Bladder Function?

K⁺ is a potent irritant that can reach a concentration of up to 565 mM in the urine of rats kept on a normal diet (45, 70). Because there is a large chemical gradient for K⁺ between the lumen and the interstitium, we hypothesize that the expression of CLDN-2 in the urothelium could cause the accumulation of K⁺ within the bladder interstitium. It is unlikely a contribution of Na⁺ to the inflammatory process and augmented bladder function, as its concentration in the urine of rats (149 ± 60 mM) is within the range in the interstitial fluid (136–145 mM) (70). We postulate that in the case of extended CLDN-2 overexpression, the accumulation of interstitial K⁺ promotes the release of cytokines and inflammatory mediators from epithelial cells, immune cells, and nerve terminals within the mucosa, which initiate and/or promote the inflammatory process. Moreover, elevated interstitial K⁺ is reported to alter the membrane potential and consequently the excitability of Aδ and C-fiber afferents and smooth muscle cells (3, 13, 34, 46, 52, 64, 72). It has been postulated that urinary K⁺ contributes to the pain and voiding symptoms associated with IC/PBS (57), since even small changes in extracellular [K⁺] can exert profound effects on neuronal excitability and smooth muscle function (3, 13, 34, 46, 52, 64, 72). In this regard, the intravesical instillation of KCl, also known as the potassium sensitivity test, has been used as a diagnostic tool to evaluate the integrity of the urothelium in patients with IC/PBS (54, 57, 59). Even though this test is no longer employed in clinical practice because it causes pain and IC/PBS symptoms, ~75% of IC/PBS patients responded to KCl instillation (54).

If increased K⁺ flux is critical for the responses we report, why did we observe effects when cystometry was performed using Krebs buffer, which only contains 7 mM K⁺? In this regard, it is important to note that the rat bladders were transduced with CLDN-2 24 h before the start of the experiment, and as noted above, rat urine contains relatively high concentrations of K⁺ (45, 70). Thus we believe the changes we observed in our studies were due to the inflammation that resulted from the extended nature of the increased tight junction permeability (we observed increased flux even in quiescent, unstretched CLDN-2-transduced tissue), the magnitude of the increase, and the predominance of cation transport, particularly K⁺, vs. the somewhat anion-selective transport we observed in GFP transduced urothelium.

Summary

Epithelial paracellular barrier dysfunction is thought to initiate intestinal inflammation in Crohn’s disease, inflammatory bowel disease, and ulcerative colitis (2, 18, 26, 51, 60, 61, 68, 73, 75, 78). Furthermore, increased permeability of low molecular-weight substances (e.g., bacterial products) across the paracellular pathway has been proposed as causative of colonic inflammation in these conditions (68, 73). Coupled with our findings, it is becoming increasingly clear that tight junction barrier dysfunction may be a significant cause of inflammatory conditions that affect several organ systems. In particular, our results indicate that augmented paracellular transport of small ions, cations in particular, across the urothelial barrier promotes inflammation and edema, and a concomitant increase in bladder activity. Thus we hypothesize that alterations in the
umbrella cell tight junction barrier may play a central role in the pathophysiology of various forms of cystitis, including IC/BPS.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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