Calcium Inhibits Paracellular Sodium Conductance through Claudin-2 by Competitive Binding

Received for publication, May 19, 2010, and in revised form, August 17, 2010. Published, JBC Papers in Press, August 31, 2010, DOI 10.1074/jbc.M110.146621

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Claudins form paracellular pores at the tight junction in epithelial cells. Profound depletion of extracellular calcium is well known to cause loosening of the tight junction with loss of transepithelial resistance. However, moderate variations in calcium concentrations within the physiological range can also regulate transepithelial permeability. To investigate the underlying molecular mechanisms, we studied the effects of calcium on the permeability of claudin-2, expressed in an inducible MDCK I cell line. We found that in the physiological range, calcium acts as a reversible inhibitor of the total conductance and Na+ permeability of claudin-2, without causing changes in tight junction structure. The effect of calcium is enhanced at low Na+ concentrations, consistent with a competitive effect. Furthermore, mutation of an intrapore negatively charged binding site, Asp-65, to asparagine partially abrogated the inhibitory effect of calcium. This suggests that calcium competes with Na+ for binding to Asp-65. Other polyvalent cations had similar effects, including La3+, which caused severe and irreversible inhibition of conductance. Brownian dynamics simulations demonstrated that such inhibition can be explained if Asp-65 has a relatively high charge density, thus favoring binding of Ca2+ over that of Na+, reducing Ca2+ permeation by inhibiting its dissociation from this site, and decreasing Na+ conductance through repulsive electrostatic interaction with Ca2+. These findings may explain why hypercalcemia inhibits Na+ reabsorption in the proximal tubule of the kidney.

The tight junction is the most apical component of the junctional complex between epithelial cells (1). It acts as a barrier that regulates the permeability of the paracellular transepithelial pathway (2–4). Profound depletion of extracellular calcium to the micromolar range is well known to cause loosening of the tight junction with loss of transepithelial resistance, disorganization of the junctional strands, and internalization of tight junction proteins (5–9). There have been reports that more modest changes in extracellular calcium within the physiological range also affect transepithelial resistance (7, 10, 11). However, the underlying molecular mechanism has not been established.

Claudins are four transmembrane domain proteins located at the tight junction between epithelial cells (12, 13). Their extracellular domains protrude into the paracellular space and form pores that regulate the paracellular permeability to small ions. The first extracellular loop appears to form the lining of the paracellular pore and determines charge selectivity (14, 15). The pore diameter, uniform among claudins, is ~8 Å as estimated from permeability to polyethylene glycols (16).

We have used claudin-2 overexpression in a high resistance strain of MDCK (17) renal epithelial cells (MDCK I) as a model for investigating claudin pore permeability. In these cell lines, claudin-2 increases transepithelial conductance dramatically (17, 18) (10-fold in our hands), behaving as a cation-selective pore with permeability for Na+ relative to Cl– (PNa/PCl) of 7.5 (19). We previously identified a negatively charged residue in the first extracellular loop, aspartate 65, as an intrapore electrostatic cation binding site that is largely responsible for conferring Na+ selectivity (19).

We now show that variations in extracellular calcium concentration within the physiological range regulate claudin-2 conductance and Na+ permeability. This regulation is rapid, reversible, and not associated with morphological derangements of the tight junction, indicating that it is distinct from the changes found with profound calcium depletion. Our experimental data and modeling suggest a mechanism in which calcium (and other polyvalent cations) binds with relatively high affinity to negatively charged site(s) within the pore, thereby reducing Na+ occupancy within the pore. These findings may explain how calcium regulates Na+ reabsorption in the proximal tubule of the kidney.

EXPERIMENTAL PROCEDURES

Tissue Culture and Electrophysiological Studies—The generation and maintenance of MDCK I TetOff cells stably expressing the wild type and the D65N mutant of claudin-2 were as reported previously (19). Cells were plated at confluent density on Snapwell filters (Corning) and cultured for 7–8 days in the presence (Dox+) or absence (Dox−) of 20 ng/ml doxycycline. The filters were then mounted in Ussing chambers, stirred with

* This work was supported, in whole or in part, by National Institutes of Health Grants DK062283 (to A. S. L. Y.) and 1S10RR024754 (shared instrumentation grant to the University of Southern California Multi-Photon Microscopy Core).

† The on-line version of this article (available at http://www.jbc.org) contains supplemental "Methods" and "Results," Figs. S1 and S2, and Table S1.

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2 Supported in part by National Science Foundation Grant CHE-0750332.

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gas lifts in 100% O₂ at 37 °C, current-clamped using Ag/AgCl electrodes bridged by 3 m KCl/3% agar pipettes, and interfaced via head-stage amplifiers to a microcomputer-controlled voltage/current clamp (DM-MC6 and VCC-MC6, respectively; Physiologic Instruments, San Diego). The standard Ringer solution used at base line contained (in mM): NaCl (140), CaCl₂ (2), MgCl₂ (1), glucose (10), mannitol (20), and Tris-HEPES (10), pH 7.4 (4-ml volume in each hemichamber). The conductance was measured at 1-s interval by measuring the voltage evoked by a 20-μA bipolar current pulse.

Solutions with different Na⁺ and Ca²⁺ concentrations were made up with the appropriate concentrations of NaCl and CaCl₂, and the osmolality was balanced by adjusting the concentration of mannitol. Solution exchanges were performed by one of two methods: (a) each hemichamber was continuously perfused with 10 volumes of the new solution for ~1 min or (b) serial exchanges of fixed volumes (up to 2 ml) of high or low Ca²⁺ solution were performed by rapid aspiration and pipetting. For determination of the Ca²⁺ concentration-conductance relationship at fixed Na⁺ concentrations (Fig. 4A), filters were bathed in 2 mM Ca²⁺ plus the indicated Na⁺ concentration (50 or 100 mM), and then solution was exchanged by serial pipetting with solution containing either 130 mM NaCl (with Ringer solution (140 mM NaCl, 2 mM CaCl₂), the standard Ringer solution containing 70 mM NaCl (with 3% DMSO), and buffer containing 1% bovine serum albumin, 5% acetone. They were then blocked by incubation in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, 5% goat serum, and 0.3% Triton X-100 for 1 h. The filters were incubated in primary antibodies (mouse anti-occludin, 1:100, and rabbit anti-claudin-2, 1:100, both from Invitrogen) for 1 h, washed in PBS, incubated in secondary antibodies (Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 555-conjugated anti-mouse IgG, 1:1000, both from Invitrogen), washed again, and mounted in ProLong anti-fade agent (Invitrogen). Slides were visualized with a Leica TCS SP2 multi-photon confocal microscope.

Brownian Dynamics Modeling—Recently we developed a Brownian dynamics (BD) model to investigate the ion permeation characteristics and charge selectivity of different monovalent alkali solutions in claudin-2 (full details can be found in Ref. 19). Briefly, the claudin-2 channel was represented in a simplistic manner as a cylindrical pore with a diameter of 6.5 Å, which was connected to two cone-like vestibules on either end. The critical charged residues at aspartate 65 were modeled as six spheres located hexagonally in the middle of the pore, each sphere characterized by radius Rₖj, charge qₖ, and the distance between the channel centerline and the sphere center, Rᵢ. The critical charged residues at aspartate 65 were modeled as six spheres located hexagonally in the middle of the pore, each sphere characterized by radius Rₖj, charge qₖ, and the distance between the channel centerline and the sphere center, Rᵢ. (see supplemental Fig. S1). The protein channel and water were treated as static continua, but ions were treated explicitly and underwent high friction regime Brownian motion in which the movement of ion k was guided by the following effective potential,

\[ W_k = q_k \phi_k^{stat} + q_k^2 \phi_k^{self} + \sum_{j \neq k} \frac{q_k q_j}{\epsilon_w} \phi^{coul}(r_{kj}) + \sum_{j \neq k} \frac{q_k q_j}{\epsilon_w} \phi^{diele}(r_{kj}) - k_B T \ln(D_k(z)/D_0) \] (Eq. 3)

where \( q_k, q_j, \) and \( D_k \) are the charges of ions k and j and one of the six Asp-65 residues, respectively. \( D_k(z) \) and \( D_0(z) \) are the diffusion constants of ion k in the bulk and at position z in the channel direction, respectively. \( \phi_k^{stat} \) is the electric potential due to effective protein charges (excluding Asp-65) and an externally applied membrane potential. These effective protein charges included all other charged residues (Glu-53, Asp-76, Arg-30, and Lys-48) in the first extracellular domain and were treated as point charges of 0.2e for positively and negatively charged residues, respectively (19). \( \phi_k^{diele} \) is the electric contribution (see below) to the self-energy, or image potential, for an ion with a unit (proton) charge. Both \( \phi_k^{stat} \) and \( \phi_k^{self} \) were obtained by solving Poisson’s equation on a three-dimensional grid using a standard finite difference method (21, 22). \( \phi^{coul}(r_{kj}) \) (or \( \phi^{diele}(r_{kj}) \)) is a truncated Coulomb potential (21), and the term \( \phi^{diele}(r_{kj}) \) (21, 22) arises from the fact that our simulation system is dielectrically inhomogeneous (19), here \( r_{kj} \) is the distance between mobile ions k and j, and \( r_{kj} \) is the distance between mobile ion k and the charge at the center of the sphere representing the i-th Asp-65 residue. The dielectric constant of the aqueous regions (both inside and outside the pore region) is taken to be \( \epsilon_w = 80 \), and the dielectric constant in the protein/membrane regions is taken as \( \epsilon_p = 20 \). The third and fourth terms together account for Coulombic interactions between pairs of ions in a dielectrically inhomogeneous medium. The fifth and sixth terms estimate the Coulombic interactions between a mobile ion and...
the charged residues Asp-65. The effects of the dielectric inhomogeneity of the channel environment on the ion-ion and ion-Asp-65 electrostatic interactions (the fourth and sixth terms in Equation 3) were implemented using an efficient empirical pair potential (22, 23).

\[
\phi_{\text{diele}} = 2 \sqrt{\frac{\epsilon_{\text{diele}} \phi_{\text{self}} \phi_{\text{crom}}}{\kappa L}} \exp(-c r_d L) \tag{Eq. 4}
\]

In the present study, the channel length was taken as \( L = 32 \, \text{Å} \), and the empirically determined value \( c = 2.0 \) was employed (22, 23). The last term in Equation 3 accounts for the variation of the diffusion constant characterizing ion \( k \) along the permeation path way (i.e. the channel z direction) (24).

Radii of 1.8, 0.95, and 0.99 Å were taken for \( \text{Cl}^- \), \( \text{Na}^+ \), and \( \text{Ca}^{2+} \), respectively (25). Bulk diffusion coefficients for \( \text{Cl}^- \), \( \text{Na}^+ \), and \( \text{Ca}^{2+} \) were assumed to be \( 2.0 \times 10^{-5} \), \( 1.33 \times 10^{-5} \), and \( 0.8 \times 10^{-5} \, \text{cm}^2/\text{s} \), respectively (25). We assumed that \( \text{La}^{3+} \) had the same parameters as \( \text{Ca}^{2+} \) except for the charge it carried. Inside the claudin-2 channel, the diffusivities for different ions were assumed to be half of their bulk value (19). All simulation parameters followed our previous BD model (19) except that the effective charge carried on one Asp-65 residue was varied in order to investigate the effects of the strength of the Asp-65 binding site charge on calcium inhibition. Bulk solutions with different \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) concentrations were treated in the BD simulations by distributing fixed numbers of ions within the boundary buffer regions at each Monte Carlo cycle. The desired numbers of \( \text{Na}^+ \), \( \text{Ca}^{2+} \), and \( \text{Cl}^- \) ions in the buffer regions were obtained by integrating the given boundary concentrations over the volumes of the boundary buffer regions. In the present study, the size of the boundary regions was adjusted appropriately to simulate different bath concentrations in an efficient way. Unless otherwise expressed explicitly, a transmembrane potential of −60 mV was applied to calculate the channel conductance and mobile ion density profiles.

**RESULTS**

**Extracellular Ca\(^{2+}\) Inhibits Claudin-2 Conductance**—To determine the effect of extracellular \( \text{Ca}^{2+} \) concentration on claudin-2 conductance, we used MDCK I TetOff claudin-2 cells cultured either in the absence of doxycycline (Dox−) to induce claudin-2 expression or in the presence of doxycycline (Dox+) to suppress claudin-2 (Dox+) expression. Exchanging the extracellular solution from a standard Ringer solution containing 2 mM \( \text{Ca}^{2+} \) to a solution containing 5 mM \( \text{Ca}^{2+} \) caused a prompt increase in transepithelial resistance (TER), reflecting a decrease in transepithelial conductance. Conversely, reducing the extracellular \( \text{Ca}^{2+} \) to the submillimolar range caused a rapid decrease in TER (increase in conductance). Both of these effects were greater in Dox− cells than in Dox+ cells (5 mM \( \text{Ca}^{2+} \) caused a 9.2 ± 3.9% increase in TER in Dox− cells versus 19.0 ± 3.3% in Dox+ cells; 0.5 mM \( \text{Ca}^{2+} \) caused a 8.5 ± 3.6% decrease in TER in Dox+ cells versus 13.4 ± 0.6% in Dox− cells), indicating that these effects were mediated largely by regulation of claudin-2.
conductance. In subsequent studies, we investigated the effect of Ca²⁺ on claudin-2 conductance in isolation by subtracting the conductance in Dox⁺ cells from that in Dox⁻ cells.

Effect of Moderate Changes in Ca²⁺ Is Distinct from That of Profound Ca²⁺ Depletion—Changes in claudin-2 conductance due to moderate changes in extracellular Ca²⁺ concentration (up to 5 mM and down to 0.25 mM) for at least 15 min were rapidly and fully reversible. By contrast, profound extracellular Ca²⁺ depletion (zero Ca²⁺ plus 5 mM EGTA) caused TER to drop to almost zero in both Dox⁺ and Dox⁻ cells; this could not be reversed by restoring the Ca²⁺ concentration to 2 mM Ca²⁺ (Fig. 1) even after up to an hour of observation (not shown). This suggests that the effects of moderate changes in extracellular Ca²⁺ are distinct from those due to profound Ca²⁺ depletion.

Two other lines of evidence are consistent with this hypothesis. First, moderate reductions in extracellular Ca²⁺ (down to 0.25 mM) increased claudin-2 conductance by selectively increasing the permeability to Na⁺ (PNa), which was reflected in an increase in the ratio of permselectivities to Na⁺ relative to Cl⁻ (PNa/PCL). Moderate elevations in extracellular Ca²⁺ had the opposite effect (Fig. 2). However, more extreme reductions in Ca²⁺, down to 0.1 or 0 mM, decreased PNa/PCL progressively to a minimum of 0.71, similar to the ratio of their free solution mobilities (μNa⁺/μCl⁻ = 0.66). This suggests that moderate changes in Ca²⁺ caused a functional interference with Na⁺ permeation through the claudin-2 pore, whereas extreme reductions in Ca²⁺ opened up a free solution shunt pathway.

The second line of evidence for a biphasic effect of Ca²⁺ is shown in Fig. 3. When we performed immunofluorescence staining for claudin-2 or for occludin, another tight junction membrane protein that is constitutively expressed in all epithelial cells, we found a typical chicken wire pattern of staining in cells incubated in normal (2 mM) Ca²⁺ and also in cells exposed to moderate changes in Ca²⁺ (down to 0.25 mM or up to 5 mM). However, after even brief exposure to extreme reductions in Ca²⁺, the tight junction was morphologically grossly disrupted, with separation of the lateral membranes (see Fig. 3, 0 Ca²⁺, 5 mM EGTA). We observed occasional areas with loss of claudin-2 from the tight junction, although using digital image quantitation we were unable to detect a difference in the proportion of claudin-2 at the junction between monolayers exposed to any of the Ca²⁺ concentrations (see supplemental Fig. S2 and Table S1).

Ca²⁺ Competes with Na⁺ for Occupancy within the Claudin Pore—We showed previously that claudin-2 is permeable to Ca²⁺ and that permeating Ca²⁺ ions, like Na⁺, interact electrostatically with the negatively charged side chain of aspartate 65 located within the pore (19). We therefore hypothesized that within the range of moderate variations in Ca²⁺ concentration, Ca²⁺ inhibits Na⁺ conductance by competing with Na⁺ for occupancy at this site within the claudin-2 pore. To test this hypothesis, we looked for intrapore interactions between Na⁺ and Ca²⁺ ions in two ways. First, we determined the Ca²⁺ concentration-conductance relationship at two different Na⁺ concentrations. As shown in Fig. 4A, claudin-2 conductance fell progressively with increasing Ca²⁺ concentrations from 0.26 to 18 mM. The
Calcium Inhibition of Claudin-2

FIGURE 4. Interaction between Na\(^+\) and Ca\(^{2+}\) in the claudin-2 pore. A, effect of Ca\(^{2+}\) on conductance at different Na\(^+\) concentrations. Conductances were normalized to the conductance in 0.26 mM Ca\(^{2+}\). Curves were fitted by nonlinear regression to the Michaelis-Menten equation. Apparent \(K_i\) (best fit value, 95% CI) were 3.5 mM (3.3–3.8 mM) in 50 mM Na\(^+\) and 5.5 mM (5.2–5.7 mM) in 100 mM Na\(^+\). B, claudin-2 exhibits non-ideal mole fraction behavior. Claudin-2 conductance was measured in Ringer solution with the concentration of \([\text{Na}^+ + \text{Ca}^{2+}]\) kept constant at 142 mM. Relative conductance (normalized to conductance at 140 mM Na\(^+\)/2 mM Ca\(^{2+}\)) is plotted against the mole fraction of Ca\(^{2+}\), \([\text{Ca}/\text{Na} + \text{Ca}]\) (white circles, mean ± S.D., \(n = 3\); the solid curve is drawn arbitrarily). The theoretical conductance that would be predicted if \(\text{Ca}^{2+}\) and \(\text{Na}^+\) permeation were independent is indicated by the dashed gray line. Note that the limiting conductances are 1.4 and 0.6, so the midpoint mole fraction at a relative conductance of 1.0 is 0.014, indicating that Ca\(^{2+}\) binds preferentially in the pore.

FIGURE 5. Role of Asp-65 in calcium inhibition. A, effect of changing bath Ca\(^{2+}\) from 2 to 5 mM (left) or 0.5 mM (right) on conductance in wild-type claudin-2 (WT) or the D65N mutant is shown. \(*\), \(p < 0.05\); **, \(p < 0.005\) compared with WT. B, Western blot of cell lysates from MDCK I TetOff cells expressing WT or D65N claudin-2 (predicted molecular mass, 24 kDa) in the presence (D\(^+\)) or absence (D\(^-\)) of doxycycline. L, ladder (molecular mass indicated on the left in kDa).

Concentration of Ca\(^{2+}\) that caused half-maximal inhibition, \(K_i\) (best fit value, 95% CI), was lower in the presence of 50 mM Na\(^+\) (3.5 mM, 3.3–3.8 mM) than in 100 mM Na\(^+\) (5.5 mM, 5.2–5.7 mM) consistent with competitive binding. Second, we measured the conductance while varying the mole fraction of Ca\(^{2+}\), keeping the sum of Na\(^+\) and Ca\(^{2+}\) concentrations constant (Fig. 4B). If Na\(^+\) and Ca\(^{2+}\) ions permeate claudin-2 independently of each other, then conductance should be a linear function of the mole fraction of Ca\(^{2+}\). We found that the claudin-2 conductance was a nonlinear function of the mole fraction of Ca\(^{2+}\) but that it was monotonic (i.e. not anomalous mole fraction behavior). Such non-ideal mole fraction behavior can be explained by preferential binding selectivity of one ion species over another (26, 27). In this model, the mole fraction that produces the average of the limiting conductances (midpoint mole fraction (MMF)) can be used to determine the ion that is preferred by the channel. In claudin-2, we estimated that the MMF was 0.014, suggesting that Ca\(^{2+}\) binds preferentially over Na\(^+\) within the pore.

We showed previously that an acidic residue in the first extracellular domain of claudin-2, aspartate 65, is an intrapore binding site for Ca\(^{2+}\) and a major determinant of its conductance and cation selectivity (19). We therefore tested the hypothesis that aspartate 65 is part of the binding site that mediates Ca\(^{2+}\) inhibition of Na\(^+\) conductance. As shown in Fig. 5, mutating aspartate 65 to a polar, uncharged residue (D65N) reduced the Ca\(^{2+}\) inhibition of conductance by approximately half, suggesting that aspartate 65 is an important Ca\(^{2+}\) binding site.

Inhibition of Claudin-2 by Other Polyvalent Cations—On the basis of our findings, we postulated that other polyvalent cations small enough to enter the pore might also inhibit Na\(^+\) conductance. As shown in Fig. 6, several inorganic cations were able to inhibit claudin-2 conductance (La\(^{3+}\) \(\gg\) Ba\(^{2+}\) = Ca\(^{2+}\) \(>\) Mg\(^{2+}\)). Interestingly, La\(^{3+}\) inhibition was poorly reversible, suggesting that its high charge density leads to particularly tight binding to negatively charged sites within the pore. The organic polycations 2,4,6-triaminopyridine and protamine, which have been used as paracelluar blockers (28, 29), were also found to inhibit claudin-2 conductance moderately.

Insights from Brownian Dynamics Modeling into the Inhibition Mechanism Associated with Multivalent Ions—We recently performed extensive Brownian dynamics simulations of ion permeation through the claudin-2 pore (19). The behavior of claudin-2 was found to be well described by a simple model in which the pore was assumed to be a 6.5 Å diameter cylinder with conical vestibules (supplemental Fig. S1). The negatively charged side chain of Asp-65 was positioned at the center, facing into the lumen. A hexagonally distributed array of such Asp-65 spheres was constructed, each characterized by a partial charge, \(-0.1e\). We then tested whether this model could explain the mechanism of Ca\(^{2+}\) inhibition of claudin-2 conductance.

Using the parameters in our original model, increasing the extracellular Ca\(^{2+}\) concentration from 0 up to 75 mM did not significantly reduce the conductance (not shown). Known binding sites with high affinity for Ca\(^{2+}\) and selectivity over Na\(^+\) generally tend to have high charge density and hence high electrostatic field strength (30, 31). We therefore tested whether increasing the effective charge carried by Asp-65 (from \(-0.1e\) in our original model) would reproduce Ca\(^{2+}\) inhibition. We were indeed able to observe Ca\(^{2+}\) inhibition of Na\(^+\) conductance when we increased the charge carried by Asp-65 to \(-0.3e\) (but not at \(-0.2e\)) (Fig. 7A). The effect on total conductance was saturable with respect to Ca\(^{2+}\) concentration, with maximum inhibition of about 50% of the conductance and a \(K_i\)
for Ca\(^{2+}\) of about 6.5 mM. Decreasing the Na\(^{+}\) concentration caused a leftward shift in the Ca\(^{2+}\) concentration-conductance curve and reduction in \(K_i\) (Fig. 7B). Furthermore, there was a good concordance in the magnitude of the changes in conductance induced by moderate changes in extracellular Ca\(^{2+}\) in the presence of different Na\(^{+}\) concentrations between what was predicted in our simulations and what we observed in our experiments (Table 1).

To investigate the mechanism by which the binding site charge strength affects Ca\(^{2+}\) inhibition, we calculated in silico the predicted single channel conductances in symmetric bath concentrations of 0.15 M CaCl\(_2\) or NaCl for a range of charges on the Asp-65 residues. As the effective charge carried by Asp-65 was progressively increased from \(-0.1\) to \(-0.3\)e, the Ca\(^{2+}\) conductance first increased to a maximum (at \(-0.2\)e) and then dropped significantly (at \(-0.3\)e) (Table 2). By contrast, the Na\(^{+}\) conductance increased monotonically and appeared to saturate as the effective charge was increased up to a value of \(5\)e (cf. Ref. 19, supplemental material therein). Further augmentation of the charge on Asp-65 (i.e. \(\geq-5\)e) produced a decrease in Na\(^{+}\) conductance. The reduction in conductance of Ca\(^{2+}\) as the charge carried by Asp-65 was increased (or in conductance of Na\(^{+}\) at a very high and nonphysiologically relevant electrostatic field (19)) can be explained as arising from excessively strong electrostatic attractions between the cation and claudin-2 so that the rate of its dissociation from the channel is greatly reduced (25). Inspection of the ion density profiles along the claudin-2 pore during our simulations in mixtures of NaCl and CaCl\(_2\) at different ratios revealed that increasing concentrations of Ca\(^{2+}\) reduced occupancy by Na\(^{+}\), predominantly at the site of Asp-65 but also along the entire length of the pore (Fig. 7C and Table 1). Thus Ca\(^{2+}\) inhibits Na\(^{+}\) conductance by competing for binding to Asp-65, presumably through repulsive electrostatic forces. Moreover, because the Ca\(^{2+}\) conductance was almost 12 times less than that of Na\(^{+}\) when the charge on Asp-65 was chosen to be \(-0.3\)e (Table 2), the increase in Ca\(^{2+}\) conductance with increasing Ca\(^{2+}\) concentration was insufficient to offset the loss of Na\(^{+}\) conductance, and consequently the overall channel conductance was inhibited.

We then investigated the inhibition mechanism associated with La\(^{3+}\) ions. BD simulations were performed in symmetric bulk solutions containing 150 mM NaCl and 5 mM LaCl\(_3\). Assuming \(q_D\) (the effective charge carried by Asp-65) to be \(-0.3\), \(-0.35\), and \(-0.4\)e, we found that the total channel conductance was inhibited by 20 ± 10, 52 ± 15, and 85 ± 14%, respectively (Table 3). In the BD simulations with \(q_D\) = \(-0.4\)e, any La\(^{3+}\) ion entering the center portion of the channel pore became bound near Asp-65 throughout the entire simulation (~17 \(\mu\)s), and no La\(^{3+}\) ions were observed to transport through the channel.

**DISCUSSION**

We have found that Ca\(^{2+}\) acts as an inhibitory ion, reducing the overall conductance of claudin-2 when present at high con-

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**FIGURE 6. Inhibition of claudin-2 conductance by polyvalent cations.** A and B, MDCK I TetOff claudin-2 cells under Dox− conditions were bathed in Ringer solution containing Ca\(^{2+}\) and Mg\(^{2+}\) each at 0.25 mM. At the times indicated by the gray bars, the bath was exchanged with solution containing the indicated polyvalent inorganic cation salts (5 mM) (A) or the organic cations, 2,4,6-triaminopyridine (TAP, 5 mM) and protamine (100 \(\mu\)g/ml) (B). C, percentage inhibition of claudin-2 conductance by each polyvalent cation (mean ± S.E.). *, \(p < 0.05\); **, \(p < 0.005\) compared with CaCl\(_2\).
Calcium Inhibition of Claudin-2

A. Effect of extracellular Ca\(^{2+}\) concentration (with total Na\(^+\) concentration maintained at 150 mM) on the partial conductances of Na\(^+\) and Ca\(^{2+}\) and the total conductance. B. Different Na\(^+\) concentrations influence the effect of Ca\(^{2+}\) on total conductance. C. Comparison of the ion density profiles inside the claudin-2 pore when the extracellular Ca\(^{2+}\) concentration is 2 versus 5 mM, with Na\(^+\) concentration fixed at 100 mM.

**TABLE 1**

Comparison between Ca\(^{2+}\) inhibition effects determined experimentally and by BD modeling

<table>
<thead>
<tr>
<th>Na(^+) concentration (mM)</th>
<th>Final Ca(^{2+}) concentration</th>
<th>Change in conductance(^a)</th>
<th>Change in Na(^+) occupancy inside the channel calculated by BD(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
<td>5</td>
<td>-14.2 ± 0.2</td>
<td>-20 ± 5</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>-17.9 ± 0.5</td>
<td>-29 ± 3</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>-19.9 ± 3.1</td>
<td>-33 ± 5</td>
</tr>
<tr>
<td>50</td>
<td>0.25</td>
<td>-0.35 ± 2</td>
<td>-0.26 ± 0.2</td>
</tr>
<tr>
<td>50</td>
<td>0.25</td>
<td>+16.9 ± 2</td>
<td>+0.12 ± 0.5</td>
</tr>
</tbody>
</table>

\(^a\) The base-line condition in each experiment is 2 mM extracellular Ca\(^{2+}\) and the indicated Na\(^+\) concentration. The extracellular solution was then switched to the indicated final Ca\(^{2+}\) concentration, and the change relative to base line is reported.

\(^b\) A Ca\(^{2+}\) concentration of 0 mM was used in the simulation instead of 0.25 mM as in the experimental study, because simulation of these particular experimental conditions would require much more computation time. For the system of 0.25 mM CaCl\(_2\) and 50 mM NaCl in the bath, a minimum of \(n = 403\) ions (\(n_{Ca} = 1, n_{Na} = 200, n_{Ca} = 202\)) must be distributed over a boundary region of \(66 \times 66 \times 1500\) Å\(^3\). But for the system of 50 mM NaCl (and no CaCl\(_2\)), 20 times fewer ions (\(n_{Na} = 10\) and \(n_{Cl} \approx 10\)) can be distributed over a boundary region of \(66 \times 66 \times 75\) Å\(^3\). In the present BD simulations, the computational time scales roughly as \(n^2\), and thus the BD simulation of the experimentally studied system would require 400 times more simulation time.

**TABLE 2**

Comparison of binding site charge strength on the conductance

<table>
<thead>
<tr>
<th>(q_D)</th>
<th>(P_{Ca})</th>
<th>(P_{Na})</th>
<th>(P_{Na}/P_{Ca})</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.1e</td>
<td>1.0 ± 0.2</td>
<td>0.25 ± 0.02</td>
<td>4.0</td>
</tr>
<tr>
<td>-0.2e</td>
<td>2.8 ± 0.5</td>
<td>1.6 ± 0.2</td>
<td>1.7</td>
</tr>
<tr>
<td>-0.3e</td>
<td>3.2 ± 0.3</td>
<td>0.26 ± 0.07</td>
<td>12.5</td>
</tr>
</tbody>
</table>

**TABLE 3**

Comparison of binding site charge strength on La\(^{3+}\) inhibition

Simulations were performed in symmetric bulk solutions containing 150 mM NaCl and 5 mM LaCl\(_3\).

<table>
<thead>
<tr>
<th>(q_D)</th>
<th>Change in conductance(^a)</th>
<th>Na(^+) occupancy</th>
<th>La(^{3+}) occupancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.3e</td>
<td>-20 ± 10</td>
<td>0.81 ± 0.2</td>
<td>0.27 ± 0.1</td>
</tr>
<tr>
<td>-0.25e</td>
<td>-52 ± 15</td>
<td>0.78 ± 0.12</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>-0.4e</td>
<td>-85 ± 14</td>
<td>0.28 ± 0.1</td>
<td>0.88 ± 0.12</td>
</tr>
</tbody>
</table>

\(^a\) The base-line condition in each simulation is 150 mM NaCl with the same \(q_D\).

centation in Ringer solution and increasing overall conductance when its concentration is moderately reduced. The major conducting ion for claudin-2 in Ringer solution is Na\(^+\), and indeed we showed that these effects of Ca\(^{2+}\) were due to changes in Na\(^+\) permeability. Importantly, the effects of mod-
Na⁺ selectivity, did not alter the distribution of the tight junction proteins, and was rapidly and fully reversible (τ₁/₂ ~ 3–6 s).

Diamond and colleagues (10, 11) first described this phenomenon in the gall bladder epithelium. They found that decreasing the extracellular Ca²⁺ concentration from 5 to 0.25 mM increased transepithelial Na⁺ permeability and hence charge selectivity and showed that this was qualitatively different from the effects of exposure to the Ca²⁺ chelator, EDTA, which abolished selectivity altogether. They postulated that there exists a transepithelial permeability pathway in the gall bladder that is lined by fixed negative charges and that Ca²⁺ acts by binding and masking these charges. In a 1980 paper by Martinez-Palomo et al. (7) that investigated the effects of low Ca²⁺ in MDCK cells, it was found that decreasing the Ca²⁺ concentration from 10 mM to “a fraction of a millimole” caused a graded decrease in TER of ~25–30%, whereas a further reduction to nominally zero Ca²⁺ concentration, or the addition of 2.4 mM EGTA, caused a sharp drop in TER (see Fig. 1 in Ref. 7), again consistent with the idea that the effects of Ca²⁺ are biphasic. More recently, Tang and Goodenough (32) observed a similar inhibition of conductance by Ca²⁺ in MDCK II cells but not in MDCK I or T84 cells.

Our findings extend these prior observations by demonstrating that the effects of moderate changes in Ca²⁺ concentration on epithelial permeability are due to effects on claudin-2 and, by implication, on the paracellular pathway at the tight junction. Interestingly, both the mammalian gall bladder (33, 34) and low resistance strains of MDCK cells such as MDCK II (17) express claudin-2, which likely accounts for the majority of the Na⁺ conductance found by previous investigators to be inhibitable by Ca²⁺. However, in our cells, Ca²⁺ also had similar, albeit lesser, effects on Dox⁺ cells that did not express claudin-2 (Fig. 1). This suggests that the effects of moderate changes in Ca²⁺ are probably not specific to claudin-2 but may be shared to some extent by other claudins as well, including those claudins endogenously expressed in MDCK I cells.

The mechanism of Ca²⁺ inhibition is not due to pore block, because we know from radiotracer flux studies that Ca²⁺ itself can pass through the claudin-2 pore (19). However, our finding that the Kᵢ for Ca²⁺ inhibition is dependent on the extracellular Na⁺ concentration suggests that Ca²⁺ and Na⁺ do interact in some way within the pore. Furthermore, we showed previously that Ca²⁺ permeability is strongly dependent on the negative charge at Asp-65, which is also an intrapore Na⁺ binding site (19). Thus, the simplest explanation is that Ca²⁺ competes with Na⁺ for binding to negatively charged site(s) within the pore. Indeed when we measured conductance at different mole fractions of Ca²⁺/Na⁺, we found a monotonic but nonlinear relationship, which is consistent with the preferential binding of Ca²⁺ over Na⁺ at a common site within the pore (26, 27).

Note that we did not find anomalous mole fraction dependence, which is defined as the appearance of a minimum or maximum in the conductance curve and implies the presence of a single file, multi-ion channel (35). Our findings are in contrast to those of Tang and Goodenough (32), who observed apparent anomalous mole fraction behavior of Ca²⁺ versus Na⁺ in MDCK II cells. However, this was based on an inflection point of their curve at ~20 mM Ca²⁺ and a decline in TER as extracellular Ca²⁺ was further increased to 150 mM, concentrations that could have pleiotropic toxic effects on cell function (32). Furthermore, previous estimates of the claudin-2 pore diameter by us (6.5 Å (19)) and by Van Itallie et al. (8 Å (16)) suggest that the pore is too large to be consistent with a single file permeation mechanism (the Pauling ionic diameters of Na⁺ and Ca²⁺ are 1.9 and 2.0 Å, respectively).

We found that mutation of Asp-65 to asparagine partially abrogated Ca²⁺ inhibition, confirming that the side-chain carboxylate group at Asp-65 is part of the binding site shared by Na⁺ and Ca²⁺. That Ca²⁺ inhibition is not totally abolished by the D65N mutation suggests the possibility that there are other negatively charged cation-binding residue(s) within the pore. Alternatively, there could be residual cation binding to asparagine due to strong polarization by the Ca²⁺ ions of its amide group.

Our BD simulations shed light on the probable structural determinants of Ca²⁺ inhibition of claudin-2. Two effects were found necessary for Ca²⁺ inhibition. First, along the permeation pathway there must be a common binding site (presumably at Asp-65) shared by Ca²⁺ and Na⁺, to which Ca²⁺ binds much more strongly than Na⁺. In this way, increases in extracellular Ca²⁺ can decrease Na⁺ ion occupancy within the pore (Fig. 7C and Table 1) and hence Na⁺ conductance. For this condition to be satisfied, our simulations show that the binding site must have a relatively high charge density (at least ~0.3e/ residue), a property that favors Ca²⁺ over Na⁺ binding.

Second, the permeability ratio for Ca²⁺ relative to Na⁺ must be small enough that any increase of Ca²⁺ conductance cannot compensate for the reduction of Na⁺ conductance. In general, the conductance of Ca²⁺ is lower than Na⁺ because its ion diffusion constant is smaller (by almost 50%) and the dielectric energy barrier encountered by Ca²⁺ in partitioning from the bulk fluid to the protein-enclosed pore is higher (4 times higher than for Na⁺ under the assumptions of our model). In addition, however, our model predicts that the binding site charge density is also a major determinant of Ca²⁺ conductance. Specifically, small increases in intrapore negative charge (from ~0.1 to ~0.2e) favor Ca²⁺ conductance, presumably by electrostatically attracting more Ca²⁺ ions into the pore, whereas further increases (to ~0.3e and greater) dramatically inhibit Ca²⁺ conductance (Table 2), presumably by binding tightly to Ca²⁺ ions and preventing their dissociation from the site. Similar principles apply for Na⁺, but because its binding to the site is much weaker, the charge density at which its conductance peaks is much higher (~5e). Thus, a binding site with a moderately high charge density could explain the experimental findings.

Not surprisingly, the effects of Ca²⁺ on claudin-2 could be mimicked by other divalent inorganic cations such as Mg²⁺ and Ba²⁺. Interestingly, even large organic polycations such as protamine and 2,4,6-triaminopyridine were inhibitory, validating their use as tools to inhibit paracellular permeability (28, 29). One prediction ensuing from our model is that cations with extremely high charge density would bind so tightly to the intrapore site that inhibition of conductance would be profound and effectively irreversible. Our findings with La³⁺, a trivalent cation with a small ionic radius, are entirely consistent with this prediction.
Calcium Inhibition of Claudin-2

All of our current simulations (of both Ca\(^{2+}\) and La\(^{3+}\) inhibition) indicate that Asp-65 must bear a higher charge density than suggested by our original model (which only accounted for Na\(^{+}\) and Cl\(^{-}\) permeation) (19). One possible explanation is that the presence of Ca\(^{2+}\) or La\(^{3+}\) near the binding site may significantly change the protein structure and thus the effective charges carried by Asp-65, which would then differ considerably from that generated by the binding of Na\(^{+}\) (36). Asp-65 is an aspartate residue that bears a charge of \(-1.0e\). However, this charge is distributed over more than one atom (e.g. over the side-chain carboxylate group), and may be partly shielded by other protein atoms in the immediate vicinity of the Asp-65 residue, thus reducing the effective charge employed in a simplistic static sphere model of the critical Asp-65 residue. The degree of effective Asp-65 charge reduction may differ slightly for Na\(^{+}\), Ca\(^{2+}\), and La\(^{3+}\), because the deformation/polarization of the Asp-65 residue induced by the approaching ion will undoubtedly be different. Our BD simulations suggest that the effective charge carried by Asp-65 may increase with the charge of the binding ions. This hypothesis is in accordance with MD simulations of Na\(^{+}\) and Ca\(^{2+}\) binding to an acid-sensing ion channel (36), where 40% more oxygen atoms from acidic residues were observed to coordinate the bound Ca\(^{2+}\) than Na\(^{+}\). Such variations at the molecular level may not be appropriately captured by the present BD model, as this model assumes the same static protein structure for all permeant ions. However, the model can, in principle, be refined by calculating the single or even multi-ion potential mean forces for Ca\(^{2+}\) (or La\(^{3+}\)) and Na\(^{+}\) once an atomic level structure of claudin-2 becomes available.

It is worth noting that our findings may well have physiological relevance. Claudin-2 is highly expressed in the proximal tubule of the kidney (37, 38), where it has recently been shown to play a major role in paracellular Na\(^{+}\) reabsorption (39). Acute hypercalciuria, which leads to a high filtered load of Ca\(^{2+}\) in the renal tubule, induces a profound natriuresis, at least in part by inhibiting Na\(^{+}\) reabsorption in the proximal tubule (40). We postulate that this effect may be due to the inhibition of Na\(^{+}\) diffusion through claudin-2 by luminal Ca\(^{2+}\). This is an important protective mechanism for keeping urinary Ca\(^{2+}\) concentrations from exceeding their solubility threshold and thus preventing kidney stone formation in the setting of hypercalciuria.

Finally, the identification of claudin-2 inhibitors may also have pharmacological utility in gastrointestinal diseases. Claudin-2 is expressed in the crypt epithelia (41), where it may mediate paracellular Na\(^{+}\) secretion coupled to transcellular Cl\(^{-}\) secretion. Intestinal claudin-2 is up-regulated in human and mouse models of inflammatory bowel disease, where it is thought to play a role both in the pathogenesis of the disease and in the development of leak flux diarrhea (42–44). Interestingly, bismuth salts have been used for more than 30 years in the treatment of diarrhea (45), and even lanthanum chloride has been found to inhibit intestinal secretions in response to *Escherichia coli* enterotoxin (46). We therefore speculate that orally administered polyvalent cation salts may act by inhibiting intestinal claudin-2 permeability and may have a potential clinical role in the treatment of inflammatory bowel and secretory diarrheal diseases.

REFERENCES