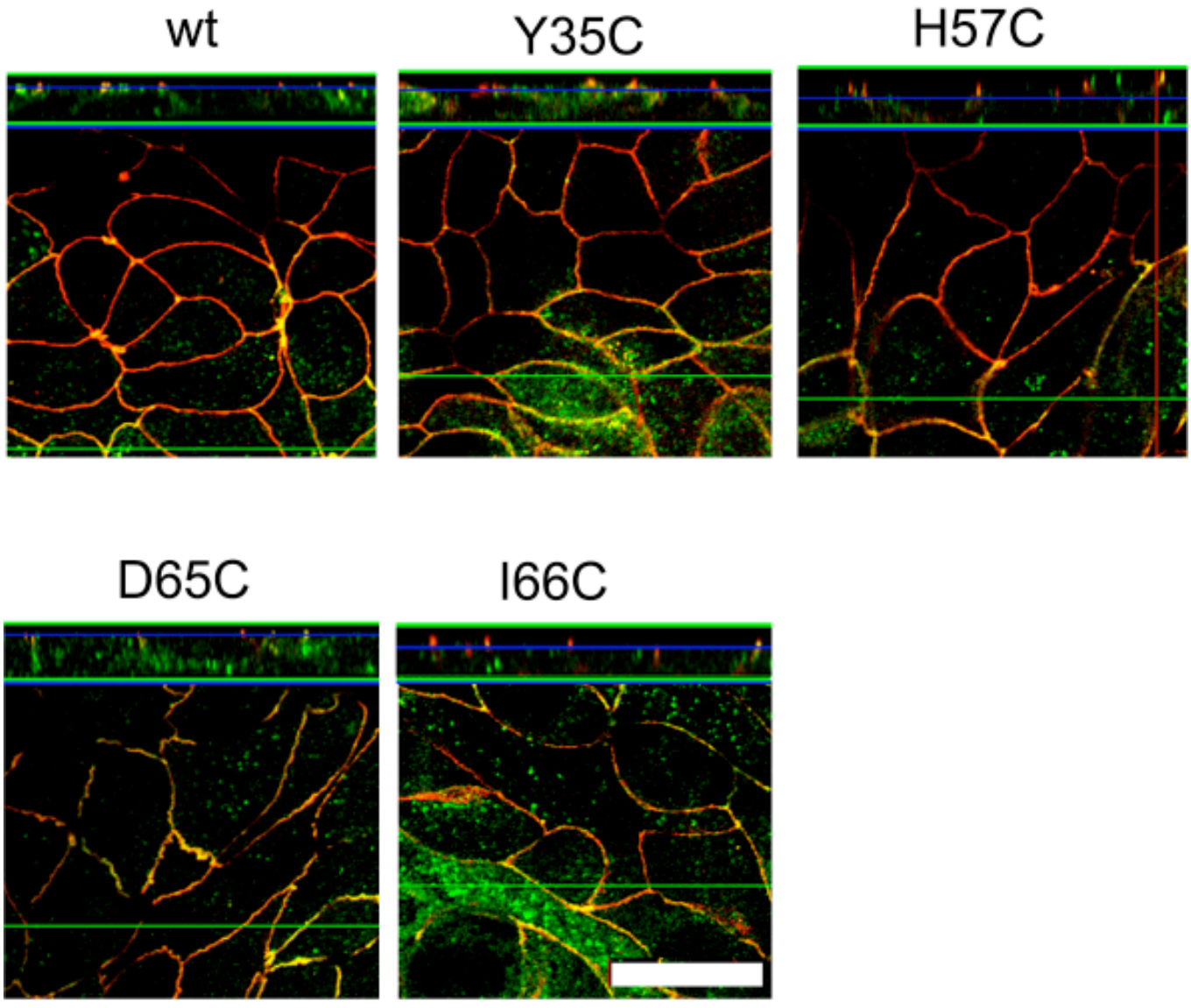
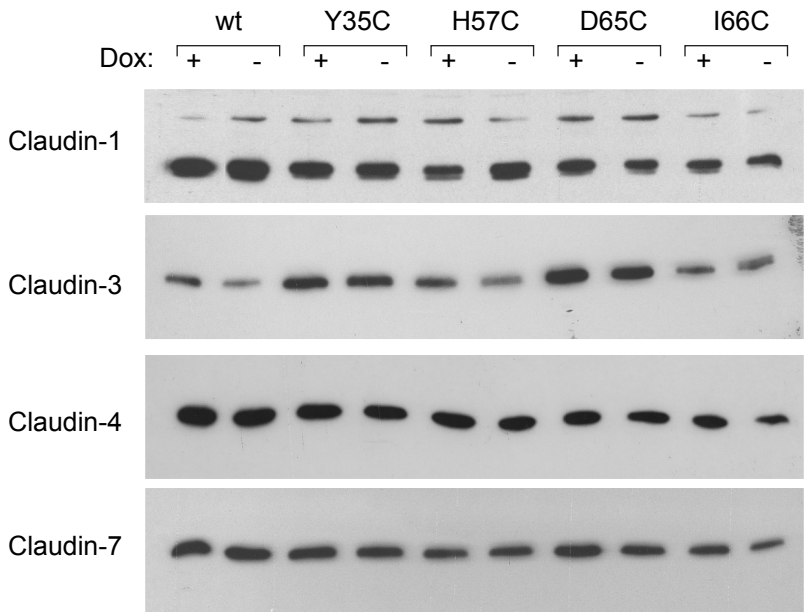
Supplemental Figure S1

Subcellular localization of wild-type and mutant claudin-2. Images show *en face* images (main panel) and vertical sections (strip above each panel) from confocal images of MDCK I cells, transfected with the indicated wild-type (wt) and cysteine mutants of claudin-2 and immunofluorescence double stained for claudin-2 (green) and ZO-1 (red). Scale bar = 10 μm.



Supplemental Figure S2

Western blots of cell lysates from uninduced (Dox+) and induced (Dox-) MDCK I TetOff cell lines expressing wild-type (wt) and the indicated mutants of claudin-2, using antibodies to the indicated claudin isoforms.



Supplemental Material

Appendix: Role of the unstirred layer in the rate of reaction of MTS reagents

Unstirred layers, regions of constant laminar flow parallel to a solid/liquid interface, can limit the rate of diffusion of molecules to the entrance of a channel and, thus, determine the overall kinetics of ion permeation. Accordingly, the rate of the chemical modification of accessible cysteines in paracellular pores by MTS reagents could be limited by the diffusion of MTS molecules to the pore entrance rather than structural determinants within the pore itself. By applying a method suggested by Dainty and House [1] we crudely estimated the thickness of unstirred layers that may hinder the access to paracellular pores of the tight junction. We then calculated the expected rate of diffusion of MTS reagents to the pore entrance and compared the results to data obtained from measurements of changes in conductance caused my MTS.

1. Estimation of the thickness of unstirred layers

We designed a bi-ionic diffusion potential experiment in Ussing chambers and estimated the thickness of unstirred layers from the kinetics of the changes of the transepithelial diffusion potential caused by disturbance of the transepithelial chemical equilibrium. In brief, MDCK I TetOff cells expressing claudin-2 I66C, were exposed from both sides to Ringer solution containing 100 mM NaCl and continuously stirred by a stream of bubbles of 100% O_2. Concentrated CsCl solution was added rapidly to the apical side, to a final concentration of 50 mM. Differences in osmolarity between the apical and basolateral chamber were compensated by simultaneous addition of concentrated mannitol solution to the basolateral side (final concentration = 100 mM). The experiment was designed to mimic the conditions of a typical experiment using MTS reagents, which are also added as concentrated solutions. The concentration gradient of CsCl caused a rapid change in the transepthelial potential, which could be fit to a single exponential decay. The final steady state bi-ionic diffusion potential (V_{TE}) was about -6.6 mV which is close to values calculated from the Goldman-Hodgkin-Katz equation (-6.8 mV) (eq. 1) using previous estimates for the permeability ratios of Na, Cs and Cl (see main text).

$$V_{TE} = \frac{RT}{F} \ln \frac{a_{Na,bl} P_{Na} + a_{X,bl} P_X + a_{Cl,ap} P_{Cl}}{a_{Na,ap} P_{Na} + a_{X,ap} P_X + a_{Cl,bl} P_{Cl}} \qquad (eq.1)$$

The method of Dainty and House is based on an estimate of the time required for half of the total change in solute concentration at the pore entrance to occur (in this case, for CsCl concentration to increase from 0 to 25 mM), $t_{1/2}$. The expected transepithelial voltage when apical CsCl is 25 mM, calculated from Eq. 1, is -3.1 mV. The time taken for the transepithelial potential to reach -3.1 mV ($t_{1/2}$), estimated from the data fitted to an exponential curve, was 1.76 ± 0.7 s.

$$t_{1/2} = \frac{0.38 \bullet d^2}{D} \qquad (eq.2)$$

The thickness of the unstirred layer was then estimated according to eq. 2 (equation 8 in ref. [1]). The diffusion coefficient of CsCl, *D*, is unknown, so we arbitrarily used a value of 1.5 x10^{-5} cm^2/s, which corresponds to the diffusion coefficient of NaCl in free solution [2]. The thickness of the unstirred layer, *d*, in our experimental set-up was found to be approximately 83 μm, which is within the same order of magnitude as was originally reported by Dainty and House [1] in frog skin (30-60 μm) and also quite similar to the unstirred layer thickness (50 μm) observed with cultured epithelial cell monolayers in Ussing chambers [3].

2. Estimation of $t_{1/2}$ for MTS reagents to diffuse through the unstirred layer

Eq. 2 was applied to calculate the $t_{1/2,\ unstirred}$ required for each MTS reagent to diffuse across an unstirred layer with a thickness of 83 μm. The diffusion coefficients used for MTSEA, MTSET and MTS-PTrEA were calculated based on the Einstein-Stokes relationship (eq. 3), with η being the viscosity of water at 37 ˚C and *r* their molecular

radii, estimated as described in *Materials and Methods* from the 3D molecular structure drawn in ChemSketch.

$$D = \frac{RT}{6N\pi\eta r} \qquad (eq.3)$$

The results obtained for *r*, D and $t_{1/2,\ unstirred}$ are listed in Table 1, which also shows data obtained from conductance measurements in Ussing chambers. $t_{1/2,\ inh}$, the time that it took to achieve 50% of the maximum inhibition of conductance by addition of MTS, and k_{inh}, the corresponding second order rate constant, were calculated by fitting our data (e.g. Fig. 7 in the main text) to single exponential curves by non-linear regression. For comparison, the rate constants for covalent reaction of the MTS reagents with a sulfhydryl group, k_{cov}, are also shown.

Supplemental Table 1

Reagent	MTSEA (2.5 mM)	MTSET (1 mM)	MTS-PTrEA (1 mM)
r [Å]	2.4	2.9	4
D [$x10^{-6}$ cm^2/s]	13.5	11.2	8.1
$t_{1/2,\ unstirred}$ [s][a]	1.95	2.36	3.25
$t_{1/2,\ inh}$ [s][b]	2.9 ±0.5	12.7 ±2.4	16.6 ±1.3
k_{inh} [$M^{-1} * s^{-1}$][b]	105 ±21	54 ±9	40 ±4
k_{cov} [$M^{-1} * s^{-1}$][c]	76,300	212,000	unknown

[a]Estimated from Eq. 2.

[b]Determined from measurements of the time course of MTS inhibition of the conductance of claudin-2 I66C.

[c]Determined from measurements of the rate of reactivity of each MTS reagent with 2-mercaptoethanol in free solution (Pascual & Karlin, J. Gen. Physiol 111 (1998), 717–739). There are no published measurements for MTS-PTrEA.

The rate constant for inhibition of conductance, k_{inh}, is a composite of the rate of three sequential processes: (1) Diffusion of the MTS reagent across an unstirred aqueous layer to the cell surface; (2) Diffusion of the MTS reagent from the surface of the cell to the accessible cysteine (presumed to be within the pore); (3) Covalent reaction of the MTS reagent with the sulfhydryl group on that cysteine. The covalent reaction (Step 3)

occurs several orders of magnitude faster than the rate at which the MTS reagents inhibit conductance, so it cannot be rate-limiting. Inspection of Table 1 reveals that the rate of inhibition of conductance by MTSEA is only slightly slower than what would be expected if the rate was limited by diffusion across the unstirred layer.[1] However, the values of $t_{1/2,\ inh}$ for MTSET and MTS-PTrEA are more than 5-fold greater than would be expected if the reactions were diffusion-limited. The results show that in case of the larger MTS reagents the overall rate of reaction is not controlled by diffusion of these molecules across unstirred layers. Instead, the slow reaction rates are most likely determined by the diffusion of these bulky molecules through the narrow pore to the site of reaction.

References

[1] Dainty, J. and House, C. R. (1966) *J. Physiol.* 182 (1), 66-78.

[2] Stephenson, J. L., Jen, J. F., Wang, H. and Tewarson, R. P. (1995) *Am. J. Physiol.* 268 (4), F680-92.

[3] Misfeldt, D. S. and Sanders, M. J. (1982) *J. Membr. Biol.* 70(3), 191-8.

[1] This assumes that there is a roughly linear relationship between MTS concentration and inhibition of conductance (so that at half maximal concentration one would expected half maximal inhibition of conductance). In reality, the MTS reagents were added in concentrations well above that needed to achieve a maximum effect on conductance (a saturating concentration). We know this because subsequent addition of more MTS reagent did not decrease conductance any further. Thus our values of $t_{1/2,inh}$ underestimate the time required for a half-maximal increase in concentration of MTS at the active site, *i.e.* the discrepancy between the rate of diffusion across the unstirred layer and the composite rate of inhibition of conductance is even greater than appears in Table 1.