Online Supplement

Expanded Materials and Methods

Cells: NRK and NRK-CT[1] were grown in DMEM (Sigma D-1152 with 4.5 mg/mL glucose). The NRK-CT medium was supplemented with 500 μg/mL G418 and hygromycin; CT expression was induced in the NRK-CT cells with 1 μg/mL doxycycline for 24-48h. Rin43[2] cells were grown in RPMI 1640 (Sigma R1383) supplemented with 300 μg/mL G418. Re43 (22C-3 or MC:Re43)[3] and Re43-S368A cells (pI8)[4] were grown in DMEM (Sigma D-1152 with 4.5 mg/mL glucose) supplemented with 350 μg/mL zeocin, 6 μg/mL puromycin, 300 μg/mL hygromycin, respectively. hOCT2 transfected CHO cells [5;6] were grown in Ham's F12-Kaighn's modification (F12K, Sigma-Aldrich) supplemented with 1mg/ml Geneticin (Invitrogen). All media were additionally supplemented with 10% FBS (Gemini BioProducts), 300 μg/mL Penicillin G, and 500 μg/mL Streptomycin. Cells were maintained at 37°C in a 5% CO_2, humidified incubator.

Co-cultures: To facilitate formation and identification of heterocellular junctions with hOCT2 expressing CHO cells, NRK or Rin43 cells were co-cultured overnight with DiI (DS - Molecular Probes) labeled hOCT2-CHO cells. **DiI labeling**: Cells in a 100mm plate were incubated for 60-75 minutes at 37°C in medium containing 1μg/mL DiI (DS). Cells were rinsed with fresh medium before lifting (0.05% trypsin, 0.2g/L EDTA in Ca-, Mg-free balanced salts solution) and replating cells in co-culture with either NRK or Rin43 cells.

OCT dye uptake assay: hOCT2-CHO cells co-cultured with Rin43 or NRK cells were exposed at 37°C for 30-60 minutes to 200 μmol/L of NBD-M-TMA in Waymouth's buffer (WB; in mmol/L: 135 NaCl, 13 HEPES, 28 D-glucose, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 0.8 $MgSO_4$, pH adjusted to 7.4 with NaOH) and washed with and viewed in ice-cold WB containing 250 μmol/L

of the OCT blocker tetrapentylammonium (TPeA). Uptake relative to cell type was documented with fluorescence and phase contrast images of the co-cultures (see figure 2 main manuscript).

hOCT2-CHO cells were exposed at room temperature for 10 minutes to 250 μmol/L of NBD-M-TMA in Waymouth's buffer or external solution (see below). Cells were washed with ice cold Waymouth's or external solution supplemented with 250 μmol/L TPeA. Relative uptake under the two uptake conditions was documented with fluorescence images of the cells (see online figure 1).

Junctional Permeability and Conductance, Single Channel Conductance:[7] Cells were plated onto glass coverslips and allowed to adhere for several hours; coverslips were then mounted in a custom-made chamber and superfused with our standard external solution (in mmol/L:142.5 NaCl , 4 KCl, 15 CsCl, 5 Glucose, 1 $MgCl_2$, 2 Na Pyruvate, 10 HEPES, 10 TEACl, 1 $BaCl_2$, 1 $CaCl_2$, osmolarity adjusted to 310 mOsmol/L with H_2O after pH adjustment to 7.2). The cells were visualized on an upright (Olympus BX50WI) microscope equipped for epifluorescence and differential interference contrast (DIC) observation; cell-pairs in which the cells were of similar size and shape were selected for evaluation of junctional function. The donor cell's interior was accessed (in current clamp mode) with a patch-type microelectrode containing our standard internal solution (in mmol/L: 124 KCl, 14 CsCl, 9 HEPES, 9 EGTA, 0.5 $CaCl_2$, 5 Glucose, 9 TEACl, 3 $MgCl_2$, 5 Na_2 ATP, pH 7.2, 310 mOsmol/L),[7;8] and the selected dyes. Images were acquired with a SenSys CCD camera (Photometrics).[7]

Data Analysis: Junctional permeability to a specific dye was quantified as the rate constant for intercellular diffusion of that dye ($k_{2\text{-dye}}$). To determine this rate constant the fluorescence intensity of donor and recipient cells was quantified from the digital images as the mean intensity of equivalent cell areas/volumes using V++ software (Digital Optics Ltd.) according to the

procedures described by Ek-Vitorin & Burt.[7] Briefly, fluorescence at each time point was normalized to the maximum observed donor cell fluorescence, and plotted as a function of time. k_2 was determined by fitting the data with an implicit finite-difference numerical method that relied on intensity of the donor cell as a function of time. The selective permeability of a junction for a specific dye was calculated as $k_{2\text{-dye}}/g_j$.

Junctional conductance (g_j) was calculated from Ohm's law, $g_j=I_j/V_j$, using the applied V_j and the I_j measured (pClamp8, Axon Instruments) from the current record of the non-stepped cell. Channel conductances, measured as transitions between open and closed states (dwell time >100 msec) in current records where only one or two channels were active, were calculated and the results binned in 5pS bins for event amplitude histograms.

Immunohistochemistry:[9] Briefly, tissue sections were deparaffinized, antigen retrieved, blocked and detected using rabbit primary antibodies against Cx43 (1:250, Sigma) or pS368-Cx43 (1:200, Cell Signaling, Inc). Slides were washed and incubated with a biotinylated anti-rabbit secondary antibody (1:250, Vector Labs) and detected with ABC-avidin/biotin conjugate (Vectastain, Vector Labs).

Statistics: Comparisons between groups were performed with a Student's T test and values are given as mean ± SEM; P values of 0.05 or less were considered significant.

Results: Transjunctional diffusion of NBD-M-TMA.

NBD-M-TMA is a substrate for members of the organic cationic transporter (OCTs) family;[10] OCTs mediate facilitated diffusion – as such their activity depends on the magnitude of the electrochemical gradient. When the electrical gradient is absent concentrative transport is not possible.[5;6] All of the studies presented in the main manuscript were done in the presence of our

external solution, which blocks K-channel activity and results in depolarization to near zero levels. This depolarization blocks hOCT2 mediated NBD-M-TMA uptake, as illustrated in online figure 1. In Waymouth's Buffer (WB), which preserves the resting membrane potential, containing 250 μmol/L NBD-M-TMA, uptake of dye by hOCT2-CHO cells is robust (left panel). In contrast uptake of dye by these same cells from external solution containing 250 μmol/L NBD-M-TMA is virtually undetectable (right panel). Clearly, the presence of an inwardly directed concentration gradient is not sufficient for hOCT2 to concentrate dye intracellularly.

Discussion: Transjunctional diffusion (no uptake) of NBD-M-TMA. Although NBD-M-TMA is a susbtrate for OCTs, the appearance of this dye in the recipient cells is the result of its transjunctional diffusion from the donors. This conclusion is supported by several facts. First, NRK and Rin43 cells do not express functional OCTs even when the conditions (absence of other OCT substrates, no OCT blockers, warm temperature) favor dye uptake by these transporters (as evidenced by the hOCT2-CHO dye uptake – see figure 2 main manuscript). Second, in our studies the cells were depolarized by the presence in out internal and external solutions of Cs and TEA (and Ba in the internal), see online figure 1. Third, there was no detectable dye in the extracellular solution (see figure 1 of the manuscript); indeed, the concentration gradient was outwardly directed. Fourth, TEA is an extremely good substrate, indeed the model substrate, for the OCTs: at 10 mmol/L, the TEA concentration was at least 40x greater than the intracellular NBD-M-TMA concentration, much greater than the extracellular NBD-M-TMA concentration (which was virtually zero), and more than 100x greater than the K_m of the OCT for TEA;[11;12] thus, if OCTs were present they would be saturated by TEA at both

faces of the membrane. Thus, under the conditions of our experiments, uptake of dye by the recipient cells from the external solution does not occur.

Discussion: Flux rates per channel. Flux rates per channel (*f*) were calculated as

$$f = (V\ C_1\ N_A\ k_2) / N/t$$

where V is the donor cell volume (L, assumed to be 1 pL), C_1 is the concentration of NBD-M-TMA in the donor cell (mmol/L, assumed to be 1 mmol/L), N_A is Avogadro's number, k_2 is our experimentally determined rate constant (in min^{-1}), N is the number of channels (from the measured junctional conductance and an assumed unitary conductance of 105 pS; i.e. g_j/105 pS) and t is time (to convert minutes to seconds). The range of per channel flux rates observed under control conditions in our studies revealed an ~300 fold difference (f_{max} / f_{min}) in channel permeability for the same permeant (online Table 1). Reduction of pS368 decreased the absolute range and average *f* of the junctions, although a uniform population of fully open channels and a linear $k_{2\text{-NBD}}$ vs. g_j relationship were not achieved.

Discussion: Comparison of NBD-M-TMA vs. K flux. The very high $k_{2\text{-NBD}}$ values reported herein might create the impression that the dye diffuses faster than potassium ions in some of our pairs; this impression would be incorrect. It is generally assumed that the movement of ions through the pore of a gap junction channel can be described by their movement in bulk solution, which is determined by the ion's molecular diffusivity. Judging from their size alone, potassium should be much more mobile in solution than NBD-M-TMA (ionic radius of K = 1.33 Å; NBD size= 11.7x6x3.85 Å). The rate constant $\boldsymbol{k_{2\text{-NBD}}}$ is a measure of concentration-gradient driven flux whereas $\mathbf{g_j}$ is a measure of electrical gradient driven flux. To ascertain the ratio of dye:K flux, one must either determine $k_{2\text{-K}}$ or g_j when only dye is present to carry the current. Unidirectional flux without an electrical driving force for two ions can be related by the

differences in their diffusivity.[13] We measured the conductivity of KBr vs. NBD-M-TMA-Br at equivalent concentrations and found that K^+ conductivity was 5x higher than NBD-M-TMA^+. Thus, the equivalent K^+ flux for an NBD-M-TMA flux of $2x10^6$ molecules/sec would be $1x10^7$ molecules/sec for a ratio of 1 to 5 or 0.2. One can also calculate the equivalent g_j if only NBD-M-TMA were available to carry current, which for a 105 pS channel (determined with K^+ and Cl^- contributing equally to the current) would be 11.3 pS. This conductance can then be used with Ohms law to calculate the voltage equivalent of the 1mmol/L concentration difference (28mV) and the corresponding K^+ flux of $9x10^6$ ions/s (NBD-M-TMA flux:K^+ flux = 0.22). For most of the selective permeabilities measured in our studies the NBD-M-TMA:K flux ratio was far less; the average $k_{2\text{-NBD}}$ corresponds to a ratio of 0.011, a value similar to that reported by Valiunas and colleagues for the ratio of LY to K flux of 0.025.[14]

Reference List

1. Shin JL, Solan JL, Lampe PD. The regulatory role of the C-terminal domain of connexin43. *Cell Commun Adhes.* 2001;8:271-275.

2. Vozzi C, Ullrich S, Charollais A, Philippe J, Orci L, Meda P. Adequate connexin-mediated coupling is required for proper insulin production. *J Cell Biol.* 1995;131:1561-1572.

3. Martyn KD, Kurata WE, Warn-Cramer BJ, Burt JM, Tenbroek E, Lau AF. Immortalized connexin43 knockout cell lines display a subset of biological properties associated with the transformed phenotype. *Cell Growth Differ.* 1997;8:1015-1027.

4. Lampe PD, Tenbroek EM, Burt JM, Kurata WE, Johnson RG, Lau AF. Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. *J Cell Biol.* 2000;149:1503-1512.

5. Kaewmokul S, Chatsudthipong V, Evans KK, Dantzler WH, Wright SH. Functional mapping of rbOCT1 and rbOCT2 activity in the S2 segment of rabbit proximal tubule. *Am J Physiol Renal Physiol.* 2003;285:F1149-F1159.

6. Wright SH, Dantzler WH. Molecular and cellular physiology of renal organic cation and anion transport. *Physiol Rev.* 2004;84:987-1049.

7. Ek-Vitorin JF, Burt JM. Quantification of Gap Junction Selectivity. *Am J Physiol Cell Physiol*. 2005;289:C1535-C1546.

8. Cottrell GT, Wu Y, Burt JM. Functional characteristics of heteromeric Cx40-Cx43 gap junction channel formation. *Cell Commun Adhes.* 2001;8:193-197.

9. King TJ, Lampe PD. The gap junction protein connexin32 is a mouse lung tumor suppressor. *Cancer Res.* 2004;64:7191-7196.

10. Bednarczyk D, Mash EA, Aavula BR, Wright SH. NBD-TMA: a novel fluorescent substrate of the peritubular organic cation transporter of renal proximal tubules. *Pflugers Arch.* 2000;440:184-192.

11. Suhre WM, Ekins S, Chang C, Swaan PW, Wright SH. Molecular determinants of substrate/inhibitor binding to the human and rabbit renal organic cation transporters hOCT2 and rbOCT2. *Mol Pharmacol.* 2005;67:1067-1077.

12. Volk C, Gorboulev V, Budiman T, Nagel G, Koepsell H. Different affinities of inhibitors to the outwardly and inwardly directed substrate binding site of organic cation transporter 2. *Mol Pharmacol.* 2003;64:1037-1047.

13. Hille B. Elementary Properties of Pores. In: Ion Channels of Excitable Membranes. Sinauer Associates, Inc, Sunderland. 2001; 347-377.

14. Valiunas V, Beyer EC, Brink PR. Cardiac gap junction channels show quantitative differences in selectivity. *Circ.Res.* 2002;91:104-111.

Online Table 1. Blocking phosphorylation of Cx43 at S368 reduces NBD-M-TMA flux/channel/s.

Group (n)	Absolute *f* Range (molecules/sec)	*f*max/*f*min	Mean *f* ± SEM (molecules/sec)
Control (58)	6,600-2,000,000	300	205,214 ± 51,854
NRK-CT (13)	3539-286,052	81	56,909 ± 22,712
S368A mutant (8)	1,600-24,000	15	10,791 ± 2,618
BIM treatment (6)	8,600 to 54,000	6	25,746 ± 7,355

Online Figure 1 – Uptake of NBD-M-TMA by hOCT2-CHO cells is blocked in external solution. Uptake of NBD-M-TMA by hOCT2-CHO cells over a ten minute period is robust from WB solution containing 250μmol/L (left panel). When external solution (containing 15 mmol/L Cs, 1mmol/L Ba and 10mmol/L TEA) replaced WB, uptake was essentially blocked (images displayed with the same fixed range of pixel intensity).