Alkali Cation Binding and Permeation in the Rat Organic Cation Transporter rOCT2*

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Organic cation transporters of the OCT family mediate downhill transport of organic cations, compatible with carrier, pore, or gate-lumen-gate mechanisms. We studied rat OCT2 expressed in Xenopus oocytes by the two-electrode voltage-clamp technique, including membrane capacitance (C_m) monitoring. Choline, a transported cationic substrate, elicited the expected inward currents but also elicited decreases of C_m. Similar C_m decreases were caused by the non-transported inhibitors tetrabutylammonium (a cation) and corticosterone (uncharged). Effects on C_m were voltage-dependent, with a maximum at −140 mV. These findings suggest that the empty rOCT2 protein can undergo an electrogenic conformation change, with one conformation highly favored at physiological voltage. Moreover, alkali cations elicited considerable inward currents and inhibited uptake of [14C]tetrabutylammonium with a sequence Cs⁺ > Rb⁺ > K⁺ > Na⁺ > Li⁺. Cs⁺ affected current and capacitance with similar affinity (K_i,5 ≈ 50 mM). Tetraethylammonium inhibited Cs⁺ currents in a concentration-dependent manner. Conversely, Cs⁺ inhibited tetraethylammonium uptake by a competitive mechanism. Activation energy of the currents estimated from measurements between 12 °C and 32 °C was −81 kJ/mol for Cs⁺ and 39 kJ/mol for tetramethylammonium, compatible with permeation of Cs⁺ through rOCT2 along the same path as organic substrates and by a mechanism different from simple electrodiffusion. Rationalization of Cs⁺ selectivity in terms of a pore pointed to a pore diameter of ~4 Å. Intriguingly, that value matches the known selectivity of rOCT2 for organic compounds. Our data show that selective permeability of rOCT2 is not determined by ligand affinity but might rather be understood in terms of the ion channel concept of a distinct “selectivity filter.”

Organic cation transporters of the OCT family contribute to the distribution, uptake, and excretion of numerous endogenous and xenobogenous compounds, including monoamine neurotransmitters, choline, coenzymes, and drugs or xenobiotics (1–4). All known OCTs seem to operate as uniporters, i.e. they mediate strictly equilibrative transport of organic cations (e.g. choline, tetraethylammonium, or guanidine) that is not coupled to any other solute (e.g. Na⁺, K⁺, or H⁺) (1). In contrast, organic anion transporters of the OAT family can operate as antiporters (e.g. dicarboxylate/PAH antiporter by OAT1) (5, 6), and some OCTNs may switch between symporter mode (e.g. Na⁺/carnitine symport by hOCTN2) (7, 8) and organic cation uniporter mode (7, 9). OCTs are “polyspecific” uniporters, i.e. they transport or bind a wide range of structurally diverse molecules (1, 10). The structural basis of polyspecific ligand recognition is an intriguing phenomenon on its own, and some insight into these aspects could be gleaned from crystal structures of polyspecific drug-binding proteins (11–13) and from three-dimensional modeling of organic cation transporters based on mutation studies and homology to LacY permease (14). For transporters, however, ligand binding is only one aspect of polyspecificity. The other side of polyspecificity refers to permeability and its selectivity: What are the factors that make the transporter permeable for some compounds but impermeable to others? Clearly, the structural features of the transporter protein that determine binding affinity need not be the same as the ones that determine permeability; this is even unlikely for rOCT2 in light of the existing data, which show that transport rates and current amplitudes do not increase together with affinity but rather change in the opposite direction (1).

To understand how selectivity is generated in the process of ion permeation through rOCT2, it would clearly be helpful to know the general transport mechanism first. At present, however, we completely lack such knowledge. On thermodynamic grounds, transport of substrate down its electrochemical gradient without coupling to the transport of any other solute is compatible with multiple models, including an alternating access carrier, an ion-selective pore, or a gate-lumen-gate scheme. Classification of OCTs as “uniporters” insinuates a carrier mechanism, but this tentative assignment cannot be bolstered by direct evidence to date. A carrier mechanism is not self-evident, given the existence of alternative, channel-like mechanisms detected in many cotransporters (glutamate, dopamine, γ-aminobutyric acid, serotonin, and norepinephrine cotransporters) (15, 16). In principle, such mechanisms could explain uncoupled downhill transport. However, other interesting transport mechanisms are found in many transport proteins that share with OCTs their polyspecificity and affinity to hydrophobic compounds. For instance, MDR1, an ATP-driven pump, seems to operate as a “flippase” or in a so-called “vacuum cleaner” mode (17, 18).

Importantly, OCTs can be studied by electrophysiological approaches, as opposed to most other uniporters, in which electrical signals are either absent (e.g. GLUTs) or impossible to resolve in sufficient detail with the available techniques. Thus, rOCT2 allows one to study the transport mechanism of a uniporter in detail, including tests for alternative modes of ion conductance. Using electrophysiological approaches, we previously established directly for most of the cloned OCTs that transport is electrogenic, that substrate-induced currents and affinity for various ligands are voltage-dependent, and that OCTs may mediate influx as well as efflux (1, 19–26). Here, we
characterize rOCT2 further and in greater detail. In this refined analysis, we apply for the first time a novel two-electrode voltage-clamp (TEVC)\(^1\)-based method for the continuous monitoring of plasma membrane capacitance (C\(_m\)) (27) in combination with the standard TEVC technique. Whereas others have used capacitance measurements widely as a convenient measure of membrane surface area, here we used C\(_m\) measurements to detect and characterize the conformation changes of a membrane protein. Comparable to measurements of “pre-steady state currents” (28–32), this approach exploits the fact that membrane proteins may move an electrical charge across the membrane electrical field as they switch between conformations. In our assay, these displacement currents manifest as an increased membrane capacitance. This capacitance is easily distinguished from the intrinsic capacitance of the oocyte membrane inasmuch as it is transporter-dependent, modified by specific transporter ligands, and highly nonlinear (with a bell-shaped dependence on membrane potential). As opposed to pre-steady state currents, capacitance monitoring provides a continuous trace of C\(_m\) over time with high time resolution and in parallel with other relevant electrical parameters, such as current and voltage (27).

Our study successfully and fruitfully extends two paradigms to uniporers such as rOCT2 that were established so far only for cotransporters. Firstly, we show that the mentioned displacement currents can be exploited to detect and characterize conformational transitions of the rOCT2 protein. Secondly, we show that rOCT2 has a high and selective conductance for Cs\(^+\), a small, inorganic alkali cation, and that one can rationalize this selectivity in the same terms in which contemporary statistical mechanics can rationalize the selectivity of pores: The pore geometry inferred from alkali ion selectivity correctly predicts the known selectivity of rOCT2 for organic cations as well. Our findings emphasize the frequently blurred distinction between binding and permeation in organic ion transport. Rather, the concept of a “selectivity filter” that is structurally distinct from the “binding sites” and operates similar to the selectivity filters of ion channels may be useful in structure-function studies of OCTs or other uniporers.

MATERIALS AND METHODS

**Expression of rOCT2 in Xenopus laevis Oocytes—**Animal husbandry, partial ovariectomy, defolliculization, complementary RNA synthesis, injection, and incubation were carried out according to standard protocols as described previously (26). The complementary RNA was transcribed with a commercial kit (T7 mMessage mMACHINE; Ambion, Austin, TX) from the pQG vector (22) in which the coding sequence of rOCT2 (24) (GenBank\(^\text{TM}\) accession number X98334) is flanked by the 5’- and 3’-untranslated regions of the Xenopus β-globin gene. Heterologous protein expression was allowed to proceed for 2–4 days at 13 °C or 16 °C.

**Electrophysiology—**TEVC experiments were performed using a feedback amplifier (TEC-05; NPI Electronic, Tamm, Germany) that was controlled by software (PULSE and X-CHART; Heka, Lambrecht, Germany). Amplifier and personal computer communicated via an AD/DA converter (ITC-1R; Instrutech, Port Washington, NY). The custom-built oocyte chamber had a small volume of 6 nanofarads. Superfusion with 10 mM tetrabutylammonium (TBuA), a non-transported inhibitor of rOCT2, was measured in oocytes expressing rOCT2 the fraction of the Cs\(^+\) currents (50 mM CsCl in 50 mM lysine chloride) that was sensitive to 1 mM tetrabutylammonium (TBuA), a non-transported inhibitor of rOCT2. The temperature dependence of the endogenous Cs\(^+\) conductance was determined with un.injected control oocytes using pulses of 100 mM CsCl. The temperature dependence of rOCT2 transport by rOCT2 was measured using 100 mM TMA. To correct for the intrinsically non-electrostatic solution in these latter two experiments, the currents induced by pulses of standard solution were subtracted from the currents induced by Cs\(^+\) or TEA\(^+\) at the respective temperature. For every experiment, activation energy (E\(_a\)) for permeation of the respective ion was computed from the slope of a linear fit to the Arrhenius plot (i.e. natural logarithm of normalized, dimensionless currents versus 1/RT, where R is the universal gas constant and T the absolute temperature in kelvins) as E\(_a\) = −slope × R.

**Uptake of [\(^{14}\)C]TEA into Xenopus Oocytes—**To test for an effect of various alkali ions on substrate transport by rOCT2, we measured in oocytes expressing rOCT2 the uptake of [\(^{14}\)C]TEA in the presence of 100 mM of the chloride salts of lysine (Lys\(^+\), taken as reference) or of the alkali ions Li\(^+\), Na\(^+\), K\(^+\), Rb\(^+\), or Cs\(^+\) (see below). Uptake was initiated by immersion of the oocytes in the respective uptake solution (8–10 oocytes for each condition), allowed to proceed for 7.5, 30, or 60 min, and stopped by transfer to ice-cold modified ND96 solution (see below) supplemented with 10 μM of the non-transported inhibitor TBuA. Oocytes were washed several times with this latter solution and then dissolved in 1 M NaOH containing 1 μM of sevinate (chloroacetate) and counted in a scintillation counter. Using the same method, the effect of Cs\(^+\) was characterized further at various, logarithmically spaced Cs\(^+\) concentrations between 0 and 100 mM. To test for direct competition between Ca\(^2+\) and TEA, these Cs\(^+\) concentration series were repeated at several fixed TEA concentrations (5, 15, 57, and 100 μM).

**Solutions—**As standard solution, we used a modified ND96 solution that contained no monovalent cations except for the reference ion (100 mM lysine chloride, 5 mM HEPES acid, 1.8 mM CaOH\(_2\), and 1 mM MgOH\(_2\), yielding a pH of ~7.4). Test solutions contained other alkali ions (chloride salts) at various concentrations, always replacing lysine chloride stoichiometrically. Where necessary, pH was adjusted to ~7.4 by acetic acid. Test solutions were either pipetted directly into the chamber (for short test pulses of ~20 s) or perfused by switching between perfusion lines (by prolonged application of fluid and counted in a scintillation counter). Using the same method, the effect of Cs\(^+\) was characterized further at various, logarithmically spaced Cs\(^+\) concentrations between 0 and 100 mM. To test for direct competition between Ca\(^2+\) and TEA, these Cs\(^+\) concentration series were repeated at several fixed TEA concentrations (5, 15, 57, and 100 μM).

**Computations and Statistics—**We used the software package GraphPad Prism Version 4.1 (GraphPad Software, San Diego, CA) to compute descriptive statistical parameters (mean, S.D., and S.E.), for linear and nonlinear regression, and for hypothesis tests (t test against fixed value, t test between groups, and Fisher test between models).

**Simulations—**Quantitative modeling of the simple four-state carrier (50) was done using MATLAB in conjunction with Softcell (written by T. F. Weiss, Massachusetts Institute of Technology, Cambridge, MA; freely available at umech.mit.edu/weiss/softcell.zip).

**RESULTS**

**Ligand Binding to rOCT2 Causes a Capacitance Decrease**

When Xenopus oocytes expressing rOCT2 were clamped at −100 mV, superfusion with 10 mM choline induced the previously described inward current, consistent with the influx of this monovalent cation known from radioisotope uptake experiments to be transported by rOCT2 (Fig. 1). In addition, however, choline simultaneously induced a considerable decrease of membrane capacitance of ~6 nanofarads. Superfusion with 10 μM of the monovalent cation TBuA did not induce any currents, as was expected for this non-transported inhibitor of rOCT2. Interestingly, TBuA caused a capacitance decrease of similar magnitude as that induced by choline. A similar capacitance

\(^1\) The abbreviations used are: TEVC, two-electrode voltage-clamp; TEA, tetraethylammonium, TMA, tetramethylammonium; TBuA, tetramethylammonium; NMDG, N-methyl-d-glucamine; MPP, 1-methyl-4-phenyl pyridinium.
monitoring yield a time-resolved signal, with a time resolution bound for the rates of either process (ligand dissociation and association). In any case, the observed value represents a lower estimate that solution exchange occurs faster than ligand dissociation or a solution change that is slower than that rate (although the corresponding time constant may reflect either a true off-rate or a mobile domain of the rOCT2 protein that is either neutralized or immobilized by ligand binding.

The capacitance effects can be explained by a charged, mobile domain of the rOCT2 protein that is either neutralized or immobilized by ligand binding.

Concentration Dependence of Ligand Binding to rOCT2 Studied by Capacitance Monitoring

To study the observed effect of rOCT2 ligands on membrane capacitance more systematically, we first examined its concentration dependence (Fig. 2). For the transported cationic substrate choline, current effects as well as capacitance effects could be studied. Fig. 2A shows that choline affects current and capacitance with comparable affinity (840 ± 252 versus 518 ± 143 μM, respectively; n = 6), in agreement with previous reports (24). Thus, capacitance measurements provide an alternative read-out of substrate binding whose precision compares favorably with the precision obtained using substrate-induced currents as read-out.

The non-transported, uncharged inhibitor corticosterone does not elicit any current effects. Using the effect of corticosterone on capacitance as a read-out of binding, however, we could determine its binding affinity, and we found a $K_d$ of 1.05 ± 0.68 μM (n = 3), in rough agreement with the various reported values (23, 24).

Time Dependence and Voltage Dependence of rOCT2-associated Capacitance

Time Dependence—Binding measurements via capacitance monitoring yield a time-resolved signal, with a time resolution of up to ~10 Hz (27), a considerable advantage as compared with biochemical or other end point assays. We exploited this property to monitor the dissociation of substrates and inhibitors from the rOCT2 protein (Fig. 3A). Near saturating concentrations of choline and TBuA could be washed out within several seconds. The capacitance changes during the wash-out could be fitted by single exponentials, with half-lives of 1.7 s for choline (τ = 0.40) and 4.2 s for TBuA (τ = 0.17). For choline, the corresponding time constant may reflect either a true off-rate or a solution change that is slower than that rate (although the fast onset of the inward current upon substrate addition suggests that solution exchange occurs faster than ligand dissociation). In any case, the observed value represents a lower bound for the rates of either process (ligand dissociation and solution change). For TBuA, the observed rate was too slow to be explained by slow solution change and thus reflects the off-rate of the TBuA-rOCT2 complex.

Dissociation of the non-transported inhibitor corticosterone was even slower. A single exponential provided a reasonable fit for the time course of membrane capacitance during the wash-out (half-life, 68.5 s). A logarithmic plot of the initial phase (data not shown), however, revealed a second, faster component (half-life, 6.94 s). Accordingly, a double-exponential fit improved the goodness of fit significantly (p < 0.0001, F-test). These data show that capacitance measurements are capable of resolving ligand binding or dissociation down to several individual time constants. The molecular underpinnings of the multiphasic wash-out of corticosterone are unclear. Because corticosterone can diffuse directly across the membrane, the slow component may reflect corticosterone de-binding at the cytoplasmic binding site of rOCT2, a process probably governed by the rate of back diffusion across the membrane.

Voltage Dependence—The effect of ligands on membrane capacitance (Fig. 1) can be rationalized by the notion that hyperpolarization and depolarization pull (or push) the rOCT2 protein into two different respective conformations and that ligand binding blocks this conformation change. The question arises at what electrical potential the two conformations are favored equally in terms of thermodynamic potential, and how sensitively protein conformation responds to voltage changes. This information can be gathered from C-V curves, which we obtained, analogously to I-V curves, by measuring the capacitance changes in response to ligand at a fixed concentration at a series of different membrane potentials (Fig. 3B). In non-
Conformational Flexibility and Ion Selectivity in rOCT2

Fig. 3. Time and voltage dependence of rOCT2-associated capacitance. A, time dependence of membrane capacitance after removal of rOCT2 ligands from the bath (●, 10 mM choline; ○, 10 μM tetrabutylammonium; ▽, 10 μM corticosterone). Data are the same as those in Fig. 1; traces were superimposed and fitted by single exponential functions. B, voltage dependence of ligand-induced capacitance decreases in rOCT2. The differences between membrane capacitance with and without 10 mM TEA were determined in uninjected oocytes (○) and in oocytes expressing rOCT2 (●) at various holding potentials. Dashed line, Gaussian distribution fitted to the Cm changes observed with rOCT2. See “Results” for explanation and statistics.

Injected control oocytes, we observed no ligand-induced capacitance changes at all potentials tested. In oocytes expressing rOCT2, the size of the ligand-induced capacitance changes was strongly voltage-dependent. Data for potentials more negative than −120 mV could not be obtained because oocytes did not tolerate sustained hyperpolarization well. However, data over the available voltage range could be described well by a fitted Gaussian distribution, as expected theoretically for a mobile charge with two voltage-dependent states. Mean (μ) and S.D. (σ) of this distribution in five oocytes were μ = −140.6 ± 3.5 mV and σ = 48.5 ± 3.7 mV, respectively. At physiological potentials (e.g., −50 mV), far from equilibrium, rOCT2 is existing predominantly in one particular conformation.

Cs⁺ Interacts with rOCT2

We systematically tested the alkali ions Li⁺, Na⁺, K⁺, Rb⁺, and Cs⁺ for a potential effect on rOCT2 (Fig. 4). Firstly, we studied the currents elicited by superfusion of Xenopus oocytes with alkali ions at 100 mM concentrations (Fig. 4A). In uninjected oocytes, we observed inward currents with relative magnitudes Li⁺ ≈ Na⁺ < Cs⁺ < K⁺ < Rb⁺ (n = 9) and with considerable variability between oocytes regarding absolute current magnitudes (data not shown). In oocytes expressing rOCT2, the inward currents induced by Cs⁺ were larger, and the sequence was changed to Li⁺ ≈ Na⁺ < K⁺ < Rb⁺ < Cs⁺ (n = 26).

Secondly, we tested for a potential interaction of alkali ions with rOCT2 by looking for their effect on the uptake of [14C]TEA into Xenopus oocytes (Fig. 4B). To minimize the confounding effect of these ions on membrane potential, oocytes were preemptively depolarized by adding 5 mM BaCl₂. In medium containing lysine as the sole monovalent cation, uptake of TEA was seen in oocytes expressing rOCT2 but was absent in uninjected oocytes. Moreover, [14C]TEA uptake was abolished completely in the presence of competitor (excess concentration of unlabeled TEA). When lysine was replaced by various alkali ions at 100 mM, uptake of [14C]TEA was partially inhibited by the larger ones, with a potency of Cs⁺ > Rb⁺ > K⁺. Because membrane potential was not monitored in these experiments, it remains unclear whether the effect of K⁺ was directly on rOCT2 or mediated indirectly via membrane depolarization. However, because Cs⁺ has much smaller effects on membrane potential than K⁺, its stronger effect on [14C]TEA uptake must be explained differently. A direct effect of Cs⁺ on rOCT2 would be the simplest explanation for its effects on current (Fig. 4A) and substrate uptake (Fig. 4B).

Cs⁺ Affects Current and Capacitance with Similar, Low Affinity

We thus focused on Cs⁺, and studied its concentration-dependent effects on current and capacitance in oocytes expressing rOCT2 (Fig. 5). We found that inward currents elicited by Cs⁺ depended on concentration in a simple hyperbolic fashion, with relatively low affinity (Kₐ = 53.8 ± 8.8 mM, n = 6, global fit with weighting by 1/y). Interestingly, Cs⁺ also caused decreases of capacitance, again in hyperbolic fashion and with low affinity (Kₐ = 53.5 ± 13.4 mM, n = 6, global fit with weighting by 1/y), as compared with the much higher affinities of most other known ligands of rOCT2 (1) (Fig. 2), which are in the micromolar range. The effects of Cs⁺ were reversible and repeatable (Fig. 5A). In a given oocyte, the currents elicited by 100 mM Cs⁺ were approximately twice as high as the currents elicited by 100 mM TEA and approximately equal to the currents elicited by 100 mM TMA (data not shown). Taken together, these findings suggest that rOCT2 has a considerable
Cs⁺ conductance and that Cs⁺ blocks an electrogenic conformation change or the electronegativity of such a change. A similar mechanism of action of Ca⁺ ions versus organic cations and their interaction with the transporter at similar sites is strongly suggested by the striking similarity of their effects on rOCT2.

**Specificity of the Cs⁺ Currents in Oocytes Expressing rOCT2**

As evident from Fig. 4A, oocytes of X. laevis have a certain endogenous Cs⁺ conductance, possibly due to the presence of nonspecific cation channels. In principle, up-regulation of endogenous channels might account partially or completely for the increased Cs⁺ conductance with rOCT2 (albeit not for the change from K⁺ selectivity to Cs⁺ selectivity; cf. Fig. 4A).

Even in uninjected oocytes, the Cs⁺ currents varied between different batches (data not shown). As shown in Fig. 6A, Cs⁺ currents in uninjected oocytes were not sensitive to TBuA, a specific inhibitor of OCTs. Expression of rOCT2 in oocytes from the same batch resulted in considerable TBuA-sensitive Cs⁺ currents. In the same oocytes, the effect of TBuA was lost when Cs⁺ in the bath was replaced by lysine.

Together, these findings demonstrate that the observed Cs⁺ conductance is at least partially due to rOCT2 (and not to endogenous channels) and that the current is indeed carried by Cs⁺ (as opposed to Cs⁺ merely blocking influx or efflux of some other anion or cation, respectively). Furthermore, these findings illustrate that TBuA can be used to separate rOCT2-specific Cs⁺ currents from an endogenous background conductance.

As a further specificity control, we compared the sensitivity of the Cs⁺-induced current to inhibitors of rOCT2 versus its sensitivity to an inhibitor of a nonspecific cation conductance in X. laevis oocytes (i.e. NMDG). As shown in Fig. 6B, the Cs⁺ currents were inhibited to a large extent by 200 μM TBuA (75 ± 10%), corticosterone (67 ± 18%), and MPP⁺ (40 ± 12%) but were completely insensitive to very high concentrations of NMDG (inhibition of −1 ± 6%). These results further support the conclusion that rOCT2 conducts Cs⁺ and rule out the NMDG-sensitive endogenous cation conductance.

**Cs⁺ and Organic Substrates of rOCT2 Share the Same Permeation Pathway**

We attempted to obtain further evidence that the observed increase of Cs⁺ conductance in X. laevis oocytes expressing...
rOCT2 is indeed due to the rOCT2 protein (rather than to nonspecific effects such as overexpression of endogenous ion channels) and that Cs⁺ herein uses the same pathway to permeate rOCT2 as the known organic substrates (Fig. 7).

**TEA Inhibits Cs⁺ Currents**—If the Cs⁺-induced currents were mediated by proteins other than rOCT2, then Cs⁺ currents and organic cation currents should be simply additive. However, this was not observed (Fig. 7, A and B). When we continuously superfused oocytes expressing rOCT2 with 100 mM Cs⁺ and then added TEA (in the continued presence of Cs⁺), current was reduced (Fig. 7A). The concomitant capacitance trace suggests that occupancy of rOCT2 with ligand simultaneously increased. It is probable that TEA, with its higher affinity (−50 μM versus −50 mM for Cs⁺), readily binds to the transporters but is conducted less efficiently than Cs⁺. Together, the effects on current and capacitance strongly suggest that both ions permeate through the same pathway and that this pathway is rOCT2. The capacitance decrease caused by the non-transported rOCT2-specific inhibitor TBuA confirmed the expression of rOCT2 in this experiment (Fig. 7A) and the approximate magnitude of the rOCT2-dependent portion of membrane capacitance.

Consistent with a competitive mechanism, the inhibitory effect of TEA on the Cs⁺ currents was strictly concentration-dependent (Fig. 7B). Herein, rOCT2 exhibited an affinity for TEA (Kᵢ = 43 ± 3 μM, n = 2) that was indistinguishable from the known affinity of rOCT2 for TEA in assays of [¹⁴C]TEA uptake or TEA-induced currents (24). This similarity suggests that TEA inhibits the Cs⁺ conductance by interacting with the same rOCT2 sites that are involved in TEA transport itself.

**Cs⁺ Inhibits the Uptake of [¹⁴C]TEA in a Competitive Manner**—The notion of a common permeation path through rOCT2 for Cs⁺ and TEA received further strong support from an experiment that demonstrated the competitive nature of the interaction between TEA and Cs⁺ (Fig. 7C). We measured the concentration dependence of the inhibitory effect of Cs⁺ on the uptake of [¹⁴C]TEA repeatedly at various fixed concentrations of [¹⁴C]TEA, and we found that Cs⁺ inhibited TEA uptake in a simple hyperbolic fashion. Most importantly, the IC₅₀ of this inhibitory effect was progressively shifted to higher values with increasing TEA concentrations. This result implies that Cs⁺ and TEA interact with the same sites of the rOCT2 protein and rules out indirect effects of Cs⁺ on TEA uptake (e.g., via depolarization) as an explanation for the observed inhibition.

**TBuA Inhibits Cs⁺ Currents, but Not K⁺ or Rb⁺ Currents**—As shown above, uninjected Xenopus oocytes as well as oocytes expressing rOCT2 exhibited considerable conductances for K⁺, Rb⁺, and Cs⁺ (Fig. 4A). To dissect out the specific current component that can be attributed to rOCT2, we tested the sensitivity of these alkali ion currents to TBuA (Fig. 7D). Micromolar concentrations are sufficient to inhibit rOCT2 nearly completely, whereas block of endogenous transport proteins such as K⁺ channels usually requires millimolar concentrations of TBuA (33, 34) or of other tetraalkylammonium compounds (35, 36). We could not detect any effect of TBuA on the inward currents induced by 100 mM K⁺ or Rb⁺. In contrast, TBuA caused a partial inhibition of the inward currents induced by 100 mM Cs⁺. This result provides further strong
This value is compatible with electrodiffusion mechanisms for findings. The activation energies for transport of Cs with a corresponding high activation energy of 81 kJ. The activation energies for transport of Cs and K are both within the typical range of membrane transport proteins. By revealing ligand-induced capacitance changes, this method was used, firstly, to detect and characterize conformation changes of the rOCT2 protein (discussed in the following paragraph). Secondly, the effects of rOCT2 ligands on capacitance allowed us to employ capacitance monitoring as a read-out of ligand binding. One advantage of such an approach is that the capacitance effects reflect binding rather than transport. Accordingly, the approach yields the dissociation constant ($K_d$) of the transporter-ligand complex. In contrast, $K_m$ values derived from current or influx measurements depend on additional reaction steps and have no simple interpretation in terms of binding. As a second advantage, capacitance monitoring allowed us to study directly the binding of non-transported ligands (e.g. corticosterone, Fig. 2B). In conventional TEVC experiments, inhibitors can only be studied indirectly via their effect on the transport of other compounds. Lastly, capacitance monitoring is suited to study the time dependence of ligand binding and debinding (Fig. 3). Under suitable conditions, capacitance monitoring can thus directly reveal microscopic kinetic constants such as on- or off-rates for substrates or inhibitors, parameters that are again not accessible in conventional current or flux experiments.

Given the underlying physical principle, capacitance effects may be observed with any type of membrane protein and are not restricted to electrogenic ion transporters. Moreover, capacitance monitoring via our paired ramps approach is directly amenable to high-throughput screening using commercially available automated TEVC instrumentation.

Capacitance Effects in rOCT2: Evidence for an Electrogenic Conformation Change—The present study provides the first direct evidence for electrogenic conformation changes in a unipporter (equivalent to the pre-steady state currents seen in cotransporters using voltage step protocols). We interpret the capacitance decrease caused by various ligands as the inhibition of an electrogenic conformation change because the decrease could be triggered by charged and uncharged ligands alike (Fig. 1). In principle, capacitance changes might also be due to an “ion well” effect, i.e. the voltage-dependent binding and debinding of ion ligands at a binding pocket that extends into the membrane electrical field. In our case, however, addition of ionic ligands decreased capacitance (Figs. 1, 5, and 6), rather than increased it (as would be expected with an ion well). Furthermore, ion well effects require charged ligands, but we observed capacitance decreases also with the uncharged corticosterone (Figs. 1 and 2). Lastly, the capacitance decrease might be explained by an ion well effect for unidentified cations that is blocked upon addition of the known rOCT2 ligands. Other than lysine and H$^+$ ions, however, our solutions contained no monovalent cations. A lysine well can be excluded because capacitance decreases are also observed upon switching from Na$^+$ to TEA or Cs$^+$, in the absence of lysine (data not shown). Similarly, a H$^+$ well seems unlikely because H$^+$ ions apparently do not interact with rOCT2 (24, 40). Furthermore, the alkali ions that are chemically close to H$^+$ (such as Li$^+$ and Na$^{+}$) had no effect on capacitance, current, or TEA uptake. Only Cs$^+$ affected capacitance, but the direction of this effect was inconsistent with an ion well mechanism.

Overall, the data provide a firm basis for the conclusion that rOCT2 exhibits conformational flexibility and that at least one mobile protein domain carries a net charge. Our experiments cannot distinguish whether the charge is positive or negative. Speculating that the known cation selectivity of rOCT2 may be brought about by a negative charge within the permeation pathway, one could invoke the same charge to explain the observed electrogenicity of a conformation change, as illustrated in Fig. 10.
The voltage dependence of the observed conformation change suggests that an equilibrium between two (or more) different conformations of rOCT2 is attained only at the highly negative potential of \(-140\) mV. Cells in which rOCT2 is expressed typically have a much less negative potential (e.g. \(-50\) to \(-60\) mV in renal proximal tubule cells), implying that one particular conformation is highly favored over the other.

Several mechanisms might contribute to the capacitance decrease elicited by substrates and inhibitors. The uncharged corticosterone binds tightly to rOCT2 and might decrease capacitance by blocking the transition to another conformation (Fig. 10). Cationic inhibitors (e.g. TBUA) might decrease capacitance by a similar mechanism. However, alternative mechanisms are possible for both cationic inhibitors and transported substrates. Thus, cationic ligands might simply neutralize the negative charge on the mobile portion of the rOCT2 protein and thus make its conformation unsusceptible to membrane potential. Although this mechanism appears intuitive, quantitative analysis in terms of the simple four-state carrier suggests another mechanism. If reorientation of the empty carrier between the two faces of the membrane is slower than reorientation of the substrate-bound carrier (i.e. the basis for trans-stimulation), then extracellular substrate will drive a major fraction of the carrier into its inward-facing, empty conformation. Under these steady state conditions, the distribution of individual carrier states is less sensitive to the voltage effects on the equilibrium between inward- and outward-facing conformations than it would be in the complete absence of substrate (where a genuine equilibrium develops).

Our experiments do not tell whether voltage changes affect the conformational equilibrium by affecting the rate of outward reorientation (\(k_{\text{empty}}^{\text{out}}\) in Fig. 10), the rate of inward reorientation (\(k_{\text{empty}}^{\text{in}}\) in Fig. 10), or both. The greater the relative contribution of the change of \(k_{\text{empty}}^{\text{in}}\), the more efficiently substrate addition will decrease capacitance because this rate has great impact on the orientation of the empty transporter under genuine equilibrium conditions (i.e. in the absence of substrate), but it becomes completely irrelevant under the particular steady state conditions imposed by saturating extracellular substrate.

These considerations are not purely speculative. Firstly, earlier evidence pointed to an alternating access carrier mechanism for rOCT2 as the basis of putative trans-stimulation (41). Secondly, we could recently demonstrate trans-stimulation directly for the isoform rOCT1 under rigorously controlled experimental conditions. Thus, when rOCT1 was purified, reconstituted in artificial liposomes, and studied under voltage-clamped conditions, choline present on the trans-side stimulated the transport of \(^{14}\)C]MPP offered on the cis-side by a factor of 3–5.

Cs\(^+\) Conductance of rOCT2: Evidence for a Pore-like Selectivity Filter—Our second main finding is that rOCT2 conducts Cs\(^+\) ions very efficiently and with selectivity over other alkali ions. This Cs\(^+\) conductance seems to be a property of the regular permeation pathway of the organic substrates of this transporter (Fig. 7).

Importantly, we observed that Cs\(^+\) is a poor ligand (\(K_m \sim 50\) mM) but a good substrate (with currents at saturating concentration that were comparable or greater than the currents induced by organic cations). This finding emphasizes that binding and transport are different things, and one must not confuse these two basic concepts. More specifically, our data strongly suggest to view binding and transport as associated with separate structural components of rOCT2, one governing binding (the “polyspecific binding site”), and the other one governing transport rate (the “selectivity filter”). In the past, researchers have focused primarily on ligand binding to organic cation transporters. However, the interaction between substrate and rOCT1 need not be complex and stERIC SPECIFIC to stimulate the transport cycle, a case proven by the highly hydrophilic, featureless Ca\(^+\) ion (geometrically, a sphere; electrically, a point charge). Studies of ligand binding and the corresponding “binding pocket” (10, 42–48) thus provide no insights as to how OCTs discriminate between transported and non-transported compounds and must not be interpreted to this end. In contrast, the relevant features of rOCT2 that determine whether a ligand is also transported are completely unexplored. In this respect, our study is the first attempt to directly study and rationalize the selective permeability of an organic ion transporter (see below).

The distinction between binding and transport suggested for rOCT2 by our data is in line with general biophysical principles. Namely, the permeation rates (i.e. permeability and thus selectivity) in a pore depend on the energy barrier between binding sites (corresponding in an alternating access carrier to the energy barrier between the two conformations that expose the binding site to the inner and outer face of the membrane, respectively, possibly in series with a second energy barrier represented by a pore-like selectivity filter as shown in Fig. 10). In contrast, ligand affinity under equilibrium conditions is a measure of well depth with respect to the cytoplasmic or extracellular bulk phase. This well depth is not constitutionally related to the barrier separating different binding sites. Selectivity filter and binding sites may thus correspond to separate structures (49). For principal reasons, affinity thus cannot explain selectivity, in channels and carriers alike.

Clearly, however, carriers do exhibit selectivity, but the carrier hypothesis provides explicitly only for a binding site, not for a selectivity filter (50). Different permeation rates (i.e. selectivity) of a carrier can be accommodated in a kinetic scheme via different activation energies for the reorientation step of the loaded carrier. However, no theories exist that would allow extraction from these activation energies of useful information about carrier structure.

In contrast, enticingly rich physical theories of alkali ion selectivity have been developed in the field of ion channel research. Although not customary, we thus tentatively adopted such selectivity theories for rOCT2. Intriguingly, these theories resulted in fruitful and convincing interpretations. It was also relatively straightforward to accommodate a pore-like selectivity filter structurally in a hypothetic alternating access carrier (Fig. 10).

We explored primarily the particularly elegant and useful theoretical framework developed recently by Laio and Torre (49). In a statistical mechanics approach that goes beyond the theories of Eisenman and Hille (but encompasses them as special cases), this theory explains alkali cation selectivity on the basis of a few physical parameters, namely, hydration energy, ion radius, coulombic interactions, pore size, pore size fluctuation, and friction. Whereas the derivation of the theory is complicated, it results in straightforward predictions as to what particular pore geometry produces what type of alkali cation selectivity. Specifically, this theory shows that a pore will be selectively permeable to Cs\(^+\) if it is relatively rigid and has a diameter of \(-4\) Å. Larger cations are excluded due to their size, and much smaller cations are conducted poorly due to their greater dehydration energy (49). The theory explains, for instance, the Cs\(^+\) selectivity of gramicidin A solely on the basis of such a pore geometry (49).

\(^2\) T. Keller, M. Elfeber, V. Gorboulev, H. Reiländer, and H. Koepsell, submitted for publication.
If one assumes that selectivity of rOCT2 is generated by a pore-like selectivity filter, this theory would thus predict that the pore should have a diameter of ~4 Å at its narrowest part. It is easy to test this prediction against numerous organic compounds for which we know both their size and whether they are transported by rOCT2 or not. Thus, Fig. 9 shows the transport rates of various compounds by rOCT2 (isotope uptake or current relative to TEA; data assembled from the literature indicated in Ref. 1) plotted against molecule radii at their smallest cross-section. Interestingly, all transported substrates have radii between 1.5 and 2.0 Å, whereas the ligands with radii of >2.0 Å are not transported and act as inhibitors. Thus, a size-selective pore can explain the selectivity of rOCT2 for its regular organic cation substrates, in addition to explaining the selectivity for the biologically less important Cs⁺ ion over other alkali ions.

A Working Model of Cation Transport by rOCT2—Fig. 10 presents a model of cation transport by rOCT2 that accounts for the available functional data on the basis of a classical four-state carrier model that was modified to accommodate electrogenic conformation changes and a pore-like selectivity filter. Detailed explanations are given in the legend to Fig. 10. Briefly, the model explains the two main findings of this article: 1) electrogenic conformation changes (by a mobile negative charge), and 2) the selective permeability for Cs⁺ over other inorganic (alkali) cations (by a pore-like selectivity filter of 4 Å in diameter).

Moreover, the model explains multiple additional properties that we and others have established elsewhere: A carrier-type transport mechanism is compatible with the observed high activation energies of Cs⁺ and TMA transport (Fig. 8) and with the trans-stimulation we could demonstrate for rOCT1.⁴ The existence of a large vestibulum is strongly supported by three-dimensional modeling of rOCT1 on the basis of the solved structure of the lactose permease LacY, another member of the major facilitator superfamily (14). The surface available for interaction with ligands is very large (extending over a wide vestibulum and into the central cavity) and can easily accommodate regions that are homogeneous with respect to the types of chemical interactions they are favoring (e.g. hydrophobic vestibulum versus hydrophilic central cavity). Such a structure could explain why many large, hydrophobic compounds (e.g. corticosterone and tetraalkylammonium compounds) can bind with high affinity but are not transported, whereas the small and featureless Cs⁺ ion is translocated efficiently by rOCT2. Moreover, the rocker switch-like movement suggested by the model implies that the surface available for ligand binding will be very different in the inward-versus outward-facing conformation, at least inasmuch as the vestibulum is concerned. Indeed, we have previously demonstrated different affinities for corticosterone at the inner and outer face of rOCT2 (23).

General selectivity for cations over anions may be brought about by the negative charge at the binding site, which would favor the passage of cations across the filter and hinder the passage of anions. The selective permeability or impermeability, respectively, for numerous organic cations according to the literature (see also Fig. 9) can be explained by the same selectivity filter we invoked for the explanation of alkali cation selectivity.

In conclusion, the present work suggests that rOCT2 combines a carrier-type alternating access mechanism with a channel-type selectivity filter. This working model generates numerous specific predictions that can be tested independently from a currently unavailable three-dimensional structure by available biophysical and functional methods.

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FIG. 9. A selectivity filter with a 2 Å radius can explain the selectivity of rOCT2 for organic and alkali cations. Plot of transport rate by rOCT2 versus ion radius for various organic and inorganic cations; transport rates are expressed as multiples of transport rate determined for TEA in the same experiment (either as isotope uptake or as substrate-induced currents in voltage-clamped oocytes; data compiled from this work and the literature). Data suggest that ion selectivity in a transporter can be rationalized in terms of a pore with a particular geometry, an approach applied thus far only to ion channels.

FIG. 10. Incorporating electrogenic conformation changes and a selectivity filter into the classical four-state carrier (schematic model). Center of the top row, empty carrier facing the extracellular membrane face. A central cavity is accessible from a large outer vestibule via a narrowing that is acting as selectivity filter (pair of arrows). Ligands may interact both with the vestibule and the cavity (dotted line). A negative charge inside the cavity (△) facilitates or impedes passage through the selectivity filter of cations and anions, respectively. Large ligands such as corticosterone (CORT) can bind with high affinity, but they are not transported because they sterically hinder the transition to another conformation. In contrast, compounds that can cross the selectivity filter and fit inside the central cavity are transported inasmuch as they do not hinder the transition to a conformation that lets them dissociate at the opposite membrane face. For alkali cations, for instance, the selectivity filter allows passage to the central cavity only without hydration shell; herein, the energetic penalty is relatively favorable for larger ions such as Cs⁺ (top right) but is prohibitive for smaller ones such as Na⁺ (top right). The conformation change is associated with a displacement current at least for the empty carrier, increasing membrane capacitance. Inhibitors block this change and thus decrease capacitance. Transported cations can decrease membrane capacitance because they can neutralize the charge on the transporter (top and bottom right) and because they impose a steady state distribution of conformational states that overrides the genuine equilibrium as determined by membrane potential (see “Discussion”).
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J. Biol. Chem. 2005, 280:24481-24490.
doi: 10.1074/jbc.M414550200 originally published online May 6, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M414550200

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