Mechanism of Electrogenic Cation Transport by the Cloned Organic Cation Transporter 2 from Rat*

Thomas Budiman‡, Ernst Bamberg‡, Hermann Koepsell§, and Georg Nagel‡

From the ‡Max-Planck-Institute for Biophysics, Kennedyallee 70, D-60596 Frankfurt/M and the §Institute of Anatomy, University of Würzburg, Koellikerstrasse 6, D-97070 Würzburg, Germany

The organic cation transporter 2 (OCT2) is expressed in plasma membranes of kidney and brain. Its transport mechanism and substrates are debated. We studied substrate-induced changes of electrical current with the patch clamp technique after expression of rat OCT2 in oocytes. Activation of current, corresponding to efflux, was observed for small organic cations, e.g. choline. In contrast, the bigger cations quinine and tetrabutylammonium elicited no change in current. However, transport of choline could be inhibited by applying quinine or tetrabutylammonium to the cytoplasmic side. Inhibition of organic cation efflux by quinine was competitive with substrates. Quinine at the inside also inhibited substrate influx from the outside. Current-voltage analysis showed that both maximal turnover and apparent affinity to substrates are voltage-dependent. Substrate-induced currents with organic cations on both membrane sides reversed as predicted from the Nernst potential. Our results clearly identify the electrochemical potential as driving force for transport at neutral pH and exclude an electroneutral H⁺/organic cation exchange. We suggest the existence of an electroneutral organic cation exchange and propose a model for a carrier-type transport mechanism.

Amphiphilic organic anions and cations are excreted with variable efficiency by the liver and the kidney (1). Excretion involves at least two steps as follows: uptake of the organic substance into the cell over the basolateral membrane and extrusion via the luminal membrane (1, 2). The cloning of the organic cation transporter 1 from rat kidney (rOCT1) (3) was followed by subsequent cloning of many more transporters belonging to the OCT1 family (for reviews see Refs. 2, 4, and 5). This opened the possibility to identify the molecular entities responsible for excretion. For organic cation transporters it proved more difficult than expected to assign to each cloned transporter the cellular function it might fulfill physiologically. Agreement exists that organic cations are taken up by renal cells via a voltage-sensitive pathway and are excreted by a voltage-insensitive organic cation/proton exchange or by the ATP-powered multidrug resistance P-glycoprotein (2, 6, 7).

Although the substrate specificities of different cloned transporters are emerging (8–11), disagreement exists on their exact cellular location and on their transport mechanism. It was proposed by several groups, e.g. that OCT2 serves to take up organic cations, like choline and TEA, at the basolateral cell side (11–14), whereas Gründemann et al. proposed that porcine OCT2 mediated OC⁺/H⁺ exchange (15) and excluded choline as a substrate for OCT2 (16). However, flux and electrical measurements indicated that rOCT1 and rOCT2 can mediate influx and efflux of organic cations, including choline (7–9, 11).

The structures of rOCT1 and the closely related rOCT2 were proposed to consist of 12 transmembrane segments (TM1–TM12) with a large extracellular loop between TM1 and TM2, a large cytoplasmic loop with consensus sites for protein kinase-like enzyme of OCT6 and TM7, and the N- and C-terminal ends on the cytoplasmic side (3). The molecular mechanism of organic cation transport by these recently cloned transporters is far from understood. So far, cloned organic cation transporters were studied, after heterologous expression, in oocytes with the two-electrode voltage clamp technique or by tracer flux measurements in oocytes or cultured cells. To get more detailed information we applied the giant patch technique (17, 18) which allows us to measure electrical current under defined ionic conditions on both sides of the membrane.

EXPERIMENTAL PROCEDURES

Chemicals—Choline chloride, tetramethylammonium chloride, tetraethylammonium chloride, tetrabutylammonium chloride, Hesper, HEPGTA, and reduced glutathione were obtained from Sigma. Quinine hydrochloride dihydrate, Me₂SO, and D-sorbitol were obtained from Fluka (Neu-Ulm, Germany), and NMG, HCl, and NaOH were obtained from Merck. MgCl₂ was purchased from Roth (Karlsruhe, Germany). Tricine was purchased from Sandoz (Basel, Switzerland). Collagenase A was obtained from Roche Molecular Biochemicals.

Expression of rOCT2—Xenopus laevis were anesthetized with 2 g/liter tricine, and parts from ovaries were removed. Oocytes were separated by incubation for 2–3 h in modified Ringer’s solution, containing 1–2 mg/ml collagenase A. Phosphate-arrested oocytes of stages V and VI (19) were selected for injection of rOCT2 cRNA. rOCT2 cDNA cloned in the pOG2 vector was linearized with NotI. The cRNA was prepared using T7 RNA polymerase, and oocytes were injected with 15–25 ng of cRNA as described before (3). The oocytes were incubated for 4–7 days at 16 °C in modified Ringer’s solution containing 9.5 mg/liter penicillin and 10 mg/liter streptomycin.

Solutions—For storing the oocytes a modified oocyte Ringer’s solution was used: 5 mM Hesper, 110 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, pH 7.6. For patch experiments, pipette and bath solutions based on sorbitol or NMG–Cl were used. Sorbitol supported tight seal formation and did not interact with rOCT2. However, addition of high amounts of charged substrates like choline chloride raised the ionic strength of the solution and thus led to increased conductivity in patches from control oocytes. Therefore, in experiments with high or-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 49-69-6303303; Fax: 49-69-6303420; E-mail: nage@mipb-frankfurt.mpg.de.

The abbreviations used are: rOCT, rat organic cation transporter; NMG, N-methylglucamine; OCTU/OCT2, organic cation transporter 1/2; TBA, tetrabutylammonium; TEA, tetracyclammonium; TMA, tetramethylammonium; tricine, 3-amino-2-hydroxy-2-nitro-1-naphthalenesulfonic acid methyl ester; TM, transmembrane.

2 P. Arndt, C. Volk, V. Gorboulev, T. Budiman, C. Popp, I. Ulzheimer, and V. Gorboulev, T. Budiman, C. Popp, I. Ulzheimer, Teuber, A. Akhoundova, E. Bamberg, G. Nagel, and H. Koepsell, submitted for publication.
Mechanism of Organic Cation Transport by rOCT2

organ cation concentrations, e.g. in Fig. 4A, solutions based on NMG were used. In these solutions, iso-osmolar exchange of NMG against other organic cations diminished the effects on conductivity in control oocytes, but inhibition with quinine revealed NMG-induced currents in rOCT2 cDNA-injected oocytes with a magnitude of maximal 15% (for 112 mM NMG) of the current induced by 5 mM choline. The $K_{0.5}$ (apparent binding constant) was estimated to be higher than 300 mM. Although NMG is likely to be transported by rOCT2, the effect was negligible in the performed experiments. Pipette solution based on NMG is as follows: 100 mM NMG, 2 mM MgCl₂, 10 mM Hepes, pH 7.4 (HCl). Bath solution based on NMG is as follows: 62.5–112.5 mM NMG, 0–50 mM substrate as chloride salt, 2 mM MgCl₂, 2.5 mM EGTA, 10 mM Hepes, 2.5 mM glutathione, pH 7.4 (HCl). Pipette solution based on sorbitol is as follows: 190 mM sorbitol, 2 mM MgCl₂, 2 mM BaCl₂, 10 mM Hepes, pH 7.4 (NaOH). Bath solution based on sorbitol is as follows: 170 mM sorbitol, 22.6 mM NMG, 0–20 mM substrate as chloride salt, 2 mM MgCl₂, 2.5 mM EGTA, 10 mM Hepes, pH 7.4 (HCl). Pipette and bath solution based on sorbitol for measurements with choline chloride in the pipette is as follows: 170 mM sorbitol, 0–20 mM choline chloride or TMA chloride, 2 mM MgCl₂, 2.5 mM EGTA, 10 mM Hepes, pH 7.4 (NaOH).

Patch Clamp Experiments—Immediately before an experiment the oocyte vitelline membrane was removed using sharpened watchmaker’s forceps (20). The devitellinized oocyte was placed in a small Petri dish (35 mm), which was mounted on the stage of an inverted microscope (Axiovert 25, Carl Zeiss, Jena, Germany). For a detailed description of the setup see Ref. 21. Borosilicate glass micropipettes, pulled out in two steps to a tip diameter of 20–30 μm with a PP-83 glass micropipette puller (Narishige Scientific Instrument Lab., Tokyo, Japan), were used for excising giant membrane patches. The pipettes were filled with pipette solutions based on sorbitol or NMG, depending on the experiment. The pipette was mounted on a MM-203 micromanipulator (Narishige Scientific Instrument Lab., Tokyo, Japan) and attached to the membrane. By gentle suction (2–6 mm H₂O) Giga-seals with 1–10 GΩ seal resistance formed within 3–10 min. Membrane patches in inside-out configuration could be excised by pulling the pipette away from the oocyte. After transferring the pipette into a polyethylene chamber the inside-out patch was then accessible from the cytoplasmic side for the perfused bath solutions (exchange time 1–3 s). The bath solutions were, like the pipette solutions, based on sorbitol or NMG. The substrates choline (0.2–50 mM) and TEA (0.05–20 mM) were added by equi-osmolar exchange with sorbitol or NMG; the inhibitor quinine was added from a 0.1 mM stock solution in Me₂SO, resulting in a maximal final MeSO₄ concentration of 0.1%. MeSO₄ in this concentration had no effect by itself on patch current. Modified electrical valves (General Valve, Fairfield, NJ) were employed for solution switching. The electrical measurements were performed with an EPC-7 patch clamp amplifier (List Medical, Darmstadt, Germany). All experiments were performed at room temperature (20–23 °C). The broken line in original recordings of Figs. 1, 4, and 8 indicates zero rOCT2-mediated current and is within a few pA close to absolute zero electrical current.

Data Acquisition and Analysis—Patch current was low-pass filtered (20 or 200 Hz) and continuously recorded on an analog chart recorder (Kipp & Zonen, Delft, The Netherlands) and on a personal computer using KAN1 software (MFK, Niedernhausen, Germany) with a sampling rate of 50 Hz. Outward (positive) current is defined according to the usual convention (22), i.e. electrical current flowing in the direction of positive charges from the cytoplasmic to the extracellular side of the membrane. For current-voltage relations alternating voltage jumps of 200 ms duration were applied in 20-mV steps from −60 mV to +60 mV. Current was filtered at 500 Hz and digitally recorded at a sampling rate of 1 kHz on a personal computer using ISOS3 software (MFK, Niedernhausen, Germany). For analysis, the current evoked by each voltage jump was subtracted from a corresponding current before or after perfusion of substrate or inhibitor. Steady-state levels were evaluated by averaging the difference currents between 100 and 180 ms of the voltage jump. MICROCAL ORIGIN Version 5.0 was used for fitting the data on a personal computer.

RESULTS

Effects of Cytoplasmic Organic Cations on Electrical Current—Electric transport activity of rOCT2 was studied in giant plasma membrane patches from oocytes expressing rOCT2 (see “Experimental Procedures”). Fig. 1 shows the effect on patch current upon applying the organic cations choline, TEA, or quinine from the cytoplasmic side. In patches from non-injected or water-injected (control) oocytes the effect of application of organic cations was indistinguishable from the normal current fluctuation (−0.5 pA, n = 15), see Fig. 1B. Patches from OCT2-expressing oocytes, however, showed a distinct outward current upon application of choline, TMA, or TEA that was inhibited by quinine or TBA, see Fig. 1, A and C, whereas quinine and TBA themselves evoked no change in patch current. Choline-induced outward currents that were analyzed in more detail ranged from several pA to maximally 30 pA at 0 mV. This amplitude is dependent on membrane patch size, degree of rOCT2 expression, and site of membrane patch excision on the oocyte. For comparison, oocytes expressing rOCT2 showed inward currents upon application of 5 mM choline at −40 mV of typically 100–400 nA (data not shown). Fig. 2 shows that the choline-induced outward current became smaller during the course of a patch clamp experiment, a phenomenon frequently observed in excised patch experiments and often called rundown. The time constant of this rundown was variable, often in the range from 10 to 50 min. It had to be accounted for when determining constants for half-maximal activation of current by organic cations.

Substrate Dependence of Outward Currents—Previous measurements with no direct access to the cytoplasmic side of the membrane already indicated that rOCT1 and rOCT2 may also mediate electrogenic efflux of substrates (9). The organic cation-induced outward current in excised patches, see Fig. 1, confirmed these suggestions and allowed us to determine precisely the $K_{0.5}$ for organic cations at the cytoplasmic side. Therefore we first measured at membrane potentials of 0 mV the outward current induced by different organic cation concentrations. Fig. 3A summarizes experiments from six different membrane patches where different choline-induced currents were tested. The results could be well described by a simple Michaelis-Menten equation yielding a $K_{0.5}$ of 2 mM choline at 0 mV. Similarly, the apparent affinity of the organic cation TEA
to rOCT2 was tested. Electrogenic transport of TEA saturated at much lower concentrations, resulting in a $K_{0.5}$ of $160 \, \text{mM}$ at 0 mV, see Fig. 3B. Maximal TEA-induced outward current, however, reached only $50\%$ of maximal choline-induced outward current, see Figs. 3 and 4B.

Inhibition by Quinine from the Cytoplasmic Side Is Competitive—Next we tested the inhibition of choline-induced outward current by quinine. The beginning of the upper trace in Fig. 4A shows the concentration dependence of choline-induced outward current (by application of 2, 10, and 50 mM choline), followed by application of 1 $\mu M$ quinine at different choline concentrations. Obviously 1 $\mu M$ quinine inhibits less at 50 mM choline than at 2 or 10 mM choline. A similar recording is shown in Fig. 4B for TEA as transported organic cation. Here 1 $\mu M$ quinine has virtually no effect at 20 mM TEA, whereas it inhibits TEA-induced outward current to about 50% at 0.1 mM TEA. These observations point to a competitive inhibition of choline- or TEA-induced outward current by quinine. Fig. 5 shows Lineweaver-Burk plots for choline- and TEA-induced currents with and without 1 $\mu M$ quinine, confirming competitive inhibition and yielding a $K_I$ (apparent inhibition constant) for quinine of $1 \, \text{mM}$. In two-electrode voltage clamp experiments on rOCT2-expressing oocytes, however, non-competitive inhibition with a $K_I$ of $10 \, \text{mM}$ was found when quinine was applied externally at pH 7.4. This apparent $K_I$ value increased at lower pH and decreased at higher pH indicating membrane permeation of uncharged quinine and inhibition from the cytoplasmic side.

Voltage Dependence of $I_{max}$ and $K_{0.5}$ of Outward Currents—The voltage dependence of outward currents induced by organic cations was studied by applying short (200 ms) pulses to different membrane potentials. Fig. 6 shows as an example current traces in 40 mV steps in the presence (A) as well as in the absence (B) of organic cations in the bath solution. Fig. 6C shows the difference of these currents, i.e. the choline-induced currents at $-60$ to $+60$ mV. A nearly linear current-voltage relationship with positive slope was obtained, see Fig. 7A. To discern the influence of voltage on $I_{max}$ (maximal turnover) and $K_{0.5}$ we measured the voltage dependence of substrate-induced currents at variable concentrations of organic cations. Fig. 7 (B and C) demonstrates that both $I_{max}$ and $K_{0.5}$ are voltage-dependent.

Voltage Dependence of Inward Currents—By loading the pipette with organic cations, we were able to demonstrate electrogeneric inward current, induced by organic cations, see Fig. 8A. This inward current, induced by 20 mM choline in the pipette, was abolished by application of 20 mM choline to the bath solution, i.e. by eliminating the only driving force at 0 mV, the inward-directed concentration gradient. This interpretation is backed by the demonstration that the competitive in-

---

3 T. Budiman and G. Nagel, unpublished data.
hibitor of outward current, quinine, leads to the same current level, see Fig. 8A. This must mean that quinine is able to inhibit at the cytoplasmic side the inward-directed current of organic cations. Fig. 8A also demonstrates the slow release of quinine from its binding site (time constant from 9 to 45 s) and a partial reduction of the (20 mM) choline-induced inward current by application of 2 mM choline to the cytoplasmic side. In the same fashion, inward current, induced by 2 mM choline in the pipette, was abolished by cytoplasmic application of 2 mM choline, see Fig. 8B, to the bath. Establishing an inward-directed concentration gradient by application of 1 mM choline led to a small inward-directed current, whereas outward-directed concentration gradients induced outward-directed currents. The voltage dependence of the choline-induced inward current is shown in Fig. 9A for 20 mM choline and in Fig. 9B for 2 mM choline. Difference curves have a similar shape, caused by the voltage dependence of electrogenic inward transport of choline. The voltage dependence of this inward current mirrors the voltage dependence of outward current as shown in Figs. 6 and 7.

Voltage Dependence of Quinine-inhibitable Current under Conditions of Organic Cation Exchange—The complete inhibition of electrogenic inward current by cytoplasmic application of 100 μM quinine allowed us to test the current-voltage relationships for conditions with different concentrations of choline on both sides of the membrane (see Figs. 8 and 9). The observed relationships are all to a first approximation linear (in the range from −60 to +60 mV) and yield reversal potentials as expected from the Nernst equation. For example, the reversal potential for 20 mM external and 2 mM cytoplasmic choline is near −60 mV, see Fig. 9A, whereas the reversal potential for 2 mM external and 20 mM cytoplasmic choline is near +60 mV, see Fig. 9A, whereas the reversal potential for 2

![Fig. 5. Lineweaver-Burk plot of quinine-induced inhibition.](image)

![Fig. 6. Voltage dependence of substrate-induced currents.](image)
cloned and also partially characterized (7, 8, 10–12, 14). To characterize in more detail the biophysical properties of organic cation transport, we chose to investigate \( \text{rOCT2} \) in excised giant patches of plasma membrane from \( X. \text{laevis} \) oocytes because in our hands it showed the highest substrate-induced electrical current when measured with the two-electrode voltage clamp technique. At present it is not known if this reflects the highest turnover rate or the highest expression in oocytes.

**Signal Magnitude in Excised Patches—** \( \text{rOCT2} \) mediated robust choline-induced currents, typically 100–400 nA inward current were induced by 5 mM choline. Also in the cases where we loaded pipettes with diameters greater than 20 \( \mu \text{m} \), choline-induced outward current in excised patches was small, typically 5–10 pA, and never exceeding 40 pA at 0 mV and 5 mM choline. Even though we used pipettes with diameters smaller than 20 \( \mu \text{m} \), choline-induced outward current in excised patches emerged from the pronounced rundown of electrogenic transport activity with time. The underlying mechanism for this highly variable inactivation was not found. Dephosphorylation by membrane-bound phosphatases in oocytes as observed, *e.g.*, for cystic fibrosis transmembrane conductance regulator (24), seemed a possibility that was tested. In whole oocytes, however, we observed no effect of forskolin (known to increase cytoplasmic [cAMP]) on \( \text{rOCT2} \), but fast inhibition by 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor (data not shown). Therefore we concluded that, at least in oocytes, \( \text{rOCT2} \) activity is not modulated by cAMP-dependent protein kinase and that 3-isobutyl-1-methylxanthine is an inhibitor, mediating its effect by direct interaction with \( \text{rOCT2} \).

**Affinity for Organic Cations at the Cytoplasmic Side**—The observed outward currents, induced by organic cations, were not unexpected as it was already shown previously for \( \text{rOCT1} \) (9) that tracer efflux and electrical outward current, best explained by cation efflux, can be observed in OCT-expressing cells. It is, however, surprising for an “uptake system” that the apparent affinities at the cytoplasmic side for choline, TMA, and TEA are quite similar to the previously measured affinities at the extracellular side (7, 11). We measured a \( K_{\text{0.5}} \) for TEA of 160 \( \mu \text{M} \) at 0 mV at the cytoplasmic side, whereas published \( K_{\text{0.5}} \) values at the extracellular side are in the range of 50–400 \( \mu \text{M} \). This result is in agreement with the assumption that only membrane potential and concentration gradient, *i.e.*, the electrochemical potential, of an organic cation, drive transport via \( \text{rOCT2} \) and that this transport exhibits little rectification.

**Inhibition by Quinine**—Inhibition of organic cation transport

---

**Fig. 7. Voltage dependence of \( I_{\text{max}} \) and \( K_{\text{0.5}} \).** A, the experiment described in Fig. 6 was performed with different choline concentrations (2, 5, 10, and 20 mM choline (CH), and the steady-state levels of the difference currents were plotted **versus** the membrane potential. Maximal current \( I_{\text{max}} \) (B) and apparent binding constant \( K_{\text{0.5}} \) (C) were calculated for each membrane potential by fitting the Michaelis-Menten equation to the averaged data of three experiments (**error bars**, fitting error). Maximal currents were normalized to maximal currents at 0 mV.

**Fig. 8. \( \text{rOCT2} \)-mediated inward current and current under conditions of organic cation exchange.** A, the pipette was filled with solution containing 20 mM choline (see “Materials and Methods”, “Solutions”), and the bath was superfused with 2 or 20 mM choline, 2 mM TEA, 100 mM quinine, or no substrate, and voltage pulses from −60 to 60 mV were applied after complete solution exchange (indicated by **lowercase letters**), see Fig. 8A. Under “symmetric” conditions, *i.e.*, 20 mM choline in pipette and bath (see a and g), zero net current is observed (indicated by **broken line**). Complete removal of substrate from the bath leads to an inward-directed current (downward, see b and e). A pronounced “rundown” of inward current from b to e is observable. Superfusion with 2 mM choline (f) leads to a reduced inward current. Addition of 100 \( \mu \text{M} \) quinine (c) or 2 mM TEA (h) inhibits the inward current almost completely; B, an analogous experiment with 2 mM choline in the pipette. The bath was superfused with 1, 2, 5, or 20 mM choline, 100 \( \mu \text{M} \) quinine, or no substrate. \( \text{rOCT2} \)-mediated currents are seen below (inward-directed) and above (outward-directed) the **broken line**. **Lowercase letters** indicate application of voltage pulses, see Fig. 9B.
8

low-Voltage pulses were applied at times indicated by corresponding lowercase letters in Fig. 8, a between currents taken at

rents are taken against currents in the presence of 100 m

were taken in the presence of 100 m

ing possibility that quinine inhibits from the cytoplasmic side also in experiments with whole oocytes, presumably by cross-

by quinine was already shown in voltage-clamped oocytes for rOCT1 (9) and rOCT22 (7, 11), but it came as a surprise when we found that the affinity for quinine at the cytoplasmic side is even higher and that this inhibition is competitive. Whereas in whole oocytes inhibition of rOCT2 by quinine at the extracellular side has a $K_I$ of about 10 μM and is not competitive with transported substrates2,3 at neutral or elevated pH, we must infer from our experiments that quinine binds to a cytoplasmic substrate-binding site with a $K_I$ of about 1 μM, competitively with the substrates choline or TEA. These numbers make it unlikely that in our experiments quinine inhibits from the extracellular side after crossing the lipid bilayer in its uncharged form. Experiments with TEA-induced currents in whole oocytes at different pH values confirmed this suspicion; at higher pH where more quinine exists in the uncharged form and more quinine is taken up by oocytes,2 the apparent $K_I$ for quinine inhibition appeared smaller, whereas at lower pH it increased.2,3 More details on the nature of inhibition by externally applied quinine can be found in a paper on specific substrates and inhibitors of rOCT1 and rOCT2.

I/V of Outward Current—The current-voltage relationship of organic cation-induced outward current shows a positive slope in its voltage dependence. Such a voltage-dependent efflux of cations was already suggested for rOCT1 (9) and rOCT22 by measuring outward current indirectly by subtracting electrical current of choline-incubated oocytes from quinine-inhibited current. A drawback of these measurements, however, was the lacking knowledge about the internal concentration of organic cations. Now we measure electrogenic organic cation efflux at different concentrations, and we show that even at saturating cation concentration outward current is slightly voltage-dependent. This indicates that a charge transfer step in the transport cycle is rate-limiting or the potential-dependent distribution between two states confers a voltage-dependent limiting rate in the transport cycle (25, 26). The apparent affinity for organic cations at the cytoplasmic binding site also shows a slight voltage dependence, indicating either a voltage drop across the cytoplasmic binding site (25) or a potential-dependent distribution between two states of the empty carrier.

The determined voltage dependence of choline-induced outward currents at different non-saturating concentrations gives clear indication for the feasibility of choline efflux at physiological membrane potentials, provided the external concentration is low enough.

I/V of Inward Current—With organic cations in the solution filling the pipette (external solution), it was possible to measure electrogenic cation influx. This current was visualized by inhibition of rOCT2 (by quinine) or by abolition of the concentration gradient. The magnitude of the inward current was in the same range as the outward current. The voltage dependence shows again a positive slope and seems very similar to electrogenic cation influx. It confirms previously determined current-voltage relationships, determined with whole oocytes for rOCT1 (9) and rOCT2.

I/V of 'Exchange' Current and Transport Mechanism—At least two mechanistic models can explain the obtained data. i) rOCT2 could be a channel for lipophilic cations allowing the passage of these cations without major conformational changes of the protein itself. ii) rOCT2 could be a transporter with a cyclic transport scheme where binding of the substrate is followed by a conformational change of the transporter, then release of the substrate to the other membrane side, and finally a conformational change of the unloaded transporter exposing the binding site back to the original membrane side, see Fig. 10B.

The concentration dependence of the substrate-induced conductance might help to decide between these two models. Conductance from different patches cannot be directly compared, but the $I/V$ values from different experiments can be normalized as in Fig. 10A. Here the inward current induced by 2 mM choline extracellularly and no choline intracellularly was normalized to 0.5 at 0 mV according to an assumed extracellular $K_{0.5}$ of 2 mM (confirmed in preliminary outside-out experiments). The corresponding $I/V$ from the same patch under conditions of 2 mM choline on both sides was also plotted. The inward current induced by 20 mM choline extracellularly and
no choline intracellularly from another experiment was normalized to −0.91 at 0 mV, again according to an assumed extracellular \( K_{\text{ch}} \) of 2 mM. Now also in this case the corresponding 1/V from the same patch under conditions of 20 mM choline on both sides could be plotted and compared with 2 mM choline on both sides. Obviously the conductance at 20 mM choline on both sides is smaller than at 2 mM choline on both sides (ANOVA test, \( p = 0.04 \)). Such a decrease at high substrate concentrations was not observed so far for channels (22).

Rather it was shown for many channels that single channel conductance saturates with increasing substrate concentration in a Michaelis-Menten-type fashion (22). On theoretical grounds, such behavior is, however, possible for multiple substrates, bound in the channel (22). A channel-like mechanism for organic cation transport by rOCT2 therefore cannot be excluded but seems rather improbable.

The most straightforward explanation for the decrease in conductance at high substrate concentrations on both sides is therefore that rOCT2 is a transporter which, under symmetric conditions of nearly saturating concentrations of organic cations, transverses the membrane most of the time with a cation bound instead of as empty carrier, see Fig. 10B. The ensuing organic cation exchange is then necessarily electroneutral. The small residual conductance results from the voltage dependence of the apparent binding constant \( K_{\text{ch}} \). For infinite high substrate concentrations the expected conductance would therefore be zero.

But for trans-zero conditions this demands a transfer step of the empty carrier-binding site from one side of the membrane to the other. It is quite probable that this transfer of the empty binding site involves a (partial) transfer of charge that will be compensated (or overcompensated) by binding of an organic cation. A similar mechanism was proposed, e.g. for the K⁺-translocating ATPase from *Escherichia coli* (27). For rOCT1 it was shown that Asp-475 in TM11 plays a crucial role in substrate binding (28), and the analogous aspartate is present also in rOCT2. Pre-steady-state substrate concentration step or voltage step experiments (29–31) might be able to identify charge translocation of the empty or loaded transporter.

**Conclusions**—Our experiments with defined salt solutions on both sides of the membrane yielded the first insight into intracellular affinity for substrates and inhibitors of rOCT2. We confirmed earlier suggestions of electrogenic efflux of substrates, mediated by rOCT1 (9) and rOCT2 (2) even against an opposing membrane voltage. The exact substrate and voltage dependence of this electrogenic efflux, now at hand, should help to clarify the possible role of OCT2 in different organs and cells. Specifically, we confirm that OCT2 is perfectly suited for substrate uptake, driven by the electrochemical potential, i.e. membrane potential and concentration gradient. However, our results emphasize also that it may serve as an efficient efflux pathway, even at negative membrane potential, as long as the external concentration of the organic cation in question is low enough so that the electrochemical potential still provides a driving force for efflux, see reversal potentials for choline for example in Table I. However, there is no evidence that rOCT2 can transport organic cations against a concentration gradient as this is the case for the still unidentified luminal organic cation/proton exchanger (32). Our observation of decreased substrate-induced conductance at high concentrations of organic cations on both sides suggests that rOCT2 is a transporter that can engage in electroneutral cation exchange. Further experiments are needed to study the exact stimulation of electroneutral efflux, preferably under voltage clamp conditions.

**Acknowledgments**—We thank Doris Ollig and Dagmar Stiegert for expert technical assistance, Dr. Valentin Gorboulev for providing plasmids and advice, and Dr. Klaus Fendler and Dr. Christof Grewe for discussing transport models. We are also grateful to Prof. Karl J. Ullrich for reading the manuscript and helpful comments.

**REFERENCES**

1. Ullrich, K. J. (1997) *J. Membr. Biol.* 158, 95–107
2. Koepsell, H. (1998) *Annu. Rev. Physiol.* 60, 243–266
3. Grundemann, D., Gorboulev, V., Gambaryan, S., Veyhl, M., and Koepsell, H. (1999) *Nature* 372, 549–552
4. Koepsell, H., Gorboulev, V., and Arndt, P. (1999) *J. Membr. Biol.* 167, 103–117
5. Endou, H. (1998) *Toxicol. Lett.* 92, 29–33
6. Ullrich, K. J. (1999) in *Membrane Transporters as Drug Targets* (Amidon, G. L. and Sadee, A., eds) pp. 159–179, Kluwer Academic/Plenum Publishing Corp., New York
7. Sweet, D. H., and Pritchard, J. B. (1999) *Am. J. Physiol.* 277, F890–F898
8. Busch, A. E., Quester, S., Ulzheimer, J. C., Waldegger, S., Gorboulev, V., Arndt, P., Lang, P., and Koepsell, H. (1996) *J. Biol. Chem.* 271, 32599–32604
9. Nagel, G., Volk, C., Friedrich, T., Ulzheimer, J. C., Bamberg, E., and Koepsell, H. (1997) *J. Biol. Chem.* 272, 31963–31966
10. Kekuda, R., Prasad, P. D., Wu, X., Wang, H., Fei, Y. J., Leibach, F. H., and Ganapathy, V. (1998) *J. Biol. Chem.* 273, 15971–15979
11. Urakami, Y., Okuda, M., Masuda, S., Saito, H., and Inui, K. I. (1998) *J. Pharmacol. Exp. Ther.* 287, 380–385
12. Okuda, M., Saito, H., Urakami, Y., Takano, M., and Inui, K. (1996) *Biochem. Biophys. Res. Commun.* 224, 500–507
13. Okuda, M., Urakami, Y., Saito, H., and Inui, K. (1999) *Biochem. Biophys. Acta* 1417, 224–231
14. Gorboulev, V., Ulzheimer, J. C., Akhoundova, A., Ulzheimer-Teuber, I., Karbach, U., Quester, S., Baumann, C., Lang, F., Busch, A. E., and Koepsell, H. (1997) *DNA Cell. Biol.* 16, 871–881
15. Grundemann, D., Babin-Ebell, J., Martel, F., Ording, N., Schmidt, A., and Schomig, E. (1997) *J. Biol. Chem.* 272, 10408–10413
16. Grundemann, D., Liebig, G., Kiefer, N., Koster, S., and Schomig, E. (1999) *Mol. Pharmacol.* 56, 1–10
17. Hilgemann, D. W. (1989) *Pfluegers Arch.* 415, 247–249
18. Hilgemann, D. W., and Lu, C. C. (1998) *Methods Enzymol.* 293, 267–280
19. Dumont, J. N. (1972) *J. Membr. Biol.*) 136, 153–180
Mechanism of Organic Cation Transport by rOCT2

20. Weinreich, F., Wood, P. G., Riordan, J. R., and Nagel, G. (1997) Pfluegers Arch. 434, 484–491
21. Friedrich, T., Bamberg, E., and Nagel, G. (1996) Biophys. J. 71, 2486–2500
22. Hille, B. (1992) Ionic Channels of Excitable Membranes, 2nd Ed., Sinauer Associates, Inc., Sunderland, MA
23. Nagel, G., Mockel, B., Buldt, G., and Bamberg, E. (1995) FEBS Lett. 377, 263–266
24. Weinreich, F., Riordan, J. R., and Nagel, G. (1999) J. Gen. Physiol. 114, 55–70
25. Lauger, P. (1991) Electrogenic Ion Pumps, Sinauer Associates, Inc., Sunderland, MA
26. Hansen, U.-P., Gradmann, D., Sanders, D., and Slayman, C. L. (1981) J. Membr. Biol. 63, 165–190
27. Fendler, K., Drose, S., Epstein, W., Bamberg, E., and Altendorf, K. (1999) Biochemistry 38, 1850–1856
28. Gorboulev, V., Volk, C., Arndt, P., Akhoundova, A., and Koepsell, H. (1999) Mol. Pharmacol. 56, 1254–1261
29. Fendler, K., Grell, E., and Bamberg, E. (1987) FEBS Lett. 224, 83–88
30. Friedrich, T., and Nagel, G. (1997) Biophys. J. 73, 186–194
31. Nakao, M., and Gadsby, D. C. (1986) Nature 323, 628–630
32. David, C., Rumrich, G., and Ullrich, K. J. (1995) Pfluegers Arch. 430, 477–492