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Different Affinities of Inhibitors to the Outwardly and Inwardly Directed Substrate Binding Site of Organic Cation Transporter 2

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ABSTRACT

The rat organic cation transporter 2 (rOCT2) was expressed in *Xenopus laevis* oocytes and cation-induced outward and inward currents were measured in whole cells and giant patches using voltage clamp techniques. Tetrabutylammonium (TBuA) and corticosterone were identified as nontransported inhibitors that bind to the substrate binding site of rOCT2. They inhibited cation-induced currents from both membrane sides. Increased substrate concentrations could partially overcome the inhibition. At 0 mV, the affinity of TBuA from the extracellular side compared with the intracellular side of the membrane was 4-fold higher, whereas the affinity of corticosterone was 20-fold lower. The data suggest that the substrate binding site of rOCT2 is like a pocket containing overlapping binding domains for ligands. These binding domains may undergo separate structural changes. From the extracellular surface, the affinity for uncharged corticosterone was increased by making membrane potential more negative. This implies potential-dependent structural changes in the extracellular binding pocket and existence of a voltage sensor. Interestingly, at 0 mV, an 18-fold higher affinity was determined for *trans*-inhibition of choline efflux by corticosterone compared with *cis*-inhibition of choline uptake. This suggests an additional high affinity-conformation of the empty outwardly oriented substrate binding pocket. A model is proposed that describes how substrates and inhibitors might interact with rOCT2. The data provide a theoretical basis to understand drug-drug interactions at polyspecific transporters for organic cations.

The electrogenic organic cation transporters OCT1–3 are members of the large *SLC22* family containing polyspecific transporters for cations, anions and zwitterions (Koepsell, 1998; Koepsell et al., 1999; Dresser et al., 2001; Koepsell et al., 2003). The three OCT subtypes share several common features, such as broadly overlapping substrate specificities, electrogenicity of transport, Na^+-independent organic cation transport, and ability to translocate cations in either direction (Gründemann et al., 1994, 1998; Busch et al., 1996, 1998; Okuda et al., 1996; Gorboulev et al., 1997; Nagel et al., 1997; Zhang et al., 1997; Kekuda et al., 1998; Wu et al., 1998; Budiman et al., 2000; Arndt et al., 2001). On the other hand, they exhibit different affinities to particular substrates (Arndt et al., 2001; Hayer-Zillgen et al., 2002). In the liver, OCT1 mediates sinusoidal uptake of many organic cations into hepatocytes; this represents the first step in biliary excretion (Koepsell, 1998; Koepsell et al., 2003). However, OCT1 can transport organic cations in either direction and may thus also participate in the hepatocellular release of these compounds. In the kidney, OCT2 is localized in the basolateral membrane of proximal tubules, where it mediates the first step in renal secretion of many small organic cations (Karbach et al., 2000; Sugawara-Yokoo et al., 2000; Pietig et al., 2001; Motohashi et al., 2002). However, it may also participate in organic cation reabsorption by releasing intracellular cations into the interstitial space (Koepsell et al., 2003). In certain locations, the cellular release of cations may be the primary function of the organic cation transporters. For example, the organic cation transporters OCT1 and OCT3 mediate the release of acetylcholine from the syncytiotrophoblast of the human placenta during extraneuronal cholinergic regulation (Wessler et al., 2001). Furthermore, the release of organic cations from sympathetic neurons is mediated by OCT3 (Kristufek et al., 2002).

The structural basis for polyspecificity in OCTs still has not been elucidated, and mutual competition between structurally different ligands has not been sufficiently analyzed. For these reasons, it is not possible to safely predict import and export functions of human OCTs in vivo, where multiple endogenous and exogenous ligands simultaneously act on these transporters. Substrates and inhibitors of OCTs are structurally quite diverse and include organic cations, anionic compounds such as prostaglandins or acidic metabo-

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ABBREVIATIONS: rOCT2, rat organic cation transporter 2; TEA, tetraethylammonium; TBuA, tetrabutylammonium; MOPS, 3-(*N*-morpholino)-propanesulfonic acid.

lites, and uncharged compounds such as corticosterone or estradiol (Dresser et al., 2001; Kimura et al., 2002; Koepsell et al., 2003).

Employing the rat organic cation transporter 2 (rOCT2) as a model, we recently presented a detailed characterization of the transport mechanism of electrogenic organic cation transporters (Budiman et al., 2000; Arndt et al., 2001). The relatively high electrical currents obtained after expression of rOCT2 in *Xenopus laevis* oocytes made it possible to study this transporter subtype in giant patches excised from the oocyte's plasma membrane. An important advantage of this approach is that buffer composition can be controlled on both membrane sides. Comparing the currents induced by either extracellular or intracellular application of the substrates choline or tetraethylammonium (TEA), we observed about the same apparent K_m values at 0 mV. However, the membrane potential exhibited opposite effects on the apparent K_m values that were determined for the inward compared with the outward currents induced by these substrates. In the present study, we investigated the substrate binding site of rOCT2 in its outwardly and inwardly directed conformation by employing two inhibitors that are not transported by rOCT2: the permanently charged, monovalent organic cation tetrabutylammonium (TBuA) and the uncharged inhibitor corticosterone. We observed that both TBuA and corticosterone interact with different affinities to the outwardly and inwardly directed substrate binding sites of rOCT2. Our data show that the substrate binding site of rOCT2 must be rather complex, inasmuch as it exposes different interactive domains for different substrates and inhibitors. The domains may undergo separate structural changes. For example, we found that the outwardly directed substrate binding site exists in a low- and a high-affinity state for corticosterone but not for TBuA. The data provide a theoretical basis to understand drug-drug interactions at polyspecific organic cation transporters.

Experimental Procedures

Expression of rOCT2 in Oocytes of *X. laevis*. Oocytes of *X. laevis* were prepared as described previously (Arndt et al., 2001) and stored in Ori buffer (5 mM MOPS, 100 mM NaCl, 1 mM $MgCl_2$, 3 mM KCl, and 2 mM $CaCl_2$, adjusted to pH 7.4 using NaOH) supplemented with 50 mg/l gentamicin. Oocytes were injected with 10 ng of rOCT2 cRNA in a volume of 50 nl of H_2O. Noninjected oocytes were used for controls. After injection with cRNA, oocytes were stored for 3 to 5 days in Ori buffer at 16°C.

Tracer Uptake Measurements. Uptake of radioactively labeled compounds into *X. laevis* oocytes was measured as described previously (Arndt et al., 2001). rOCT2-expressing and noninjected control oocytes from the same batch were incubated at 19°C in Ori buffer containing various concentrations of radioactively labeled corticosterone or TEA without and with the OCT-specific inhibitor cyanine-863. Uptake was stopped at different time intervals, and oocytes were washed with ice-cold Ori buffer. The amount of substrate taken up into the oocytes was determined by liquid scintillation counting.

Electrophysiological Measurements. For two-electrode voltage-clamp measurements, oocytes were continuously superfused with Ori buffer at room temperature (~2 ml/min). To prepare the substrate solutions, TEA, and choline replaced equiosmolar amounts of NaCl. Substrate-induced inward currents were measured in the absence and presence of inhibitors using the amplifier OC 725 from Warner Instruments (Hamden, CT). Corticosterone was dissolved in ethanol or dimethyl sulfoxide before dilution to the final concentration in Ori buffer. The final concentration of the organic solvent was always less than 0.5%. Preliminary experiments showed that neither solvent affects rOCT2 at these concentrations. For the measurement of choline-induced outward currents, oocytes were preloaded with choline by overnight incubation in Ori buffer supplemented with 10 mM choline. The choline-loaded oocytes were superfused with Ori buffer containing 10 mM choline and voltage-clamped. Under these conditions, outward currents were induced by the decrease in extracellular choline. Giant patch measurements were performed as described previously (Budiman et al., 2000). Oocytes were devitellinated, and giant membrane patches were excised with borosilicate glass micropipettes with a tip diameter of 16 to 25 μm. The electrodes were filled with sorbitol buffer (170 mM sorbitol, 2 mM $MgCl_2$, 2.5 mM EGTA, 10 mM HEPES, titrated to pH 7.4 with NaOH). By gentle suction of 2 to 5 cm of H_2O, gigaseals (i.e., resistance of the membrane patch exceeding 1 GΩ) formed within 3 to 15 min. Inside-out patches could be excised by pulling the pipette gently away from the oocyte. The patch was constantly superfused with bath solution at room temperature. Basic composition of bath and pipette solution was identical, except for substrates and inhibitors that replaced equiosmolar amounts of sorbitol.

Calculation and Statistics. Figs. 1 and 2 show representative recordings. Figs. 3 to 9 present the means of substrate-induced currents from two to eight independent experiments. S.E. is indicated in the graphs if it exceeds the symbol size. Because different batches of oocytes showed large differences in the level of rOCT2 expression, the experiments were normalized to the currents obtained at substrate saturation (I_{max}), or to the currents obtained in the absence of inhibitor (I_o). Apparent $K_{0.5}$ and IC_{50} values were obtained from fits of the Hill equation to uptake at different concentrations of substrate or inhibitor, respectively. Individual experiments were fitted by nonlinear regression using SigmaPlot 4.0 (SPSS Science, Chicago, IL). The results from several such experiments are reported as means ± S.E. of the IC_{50} values, $K_{0.5}$ values, and Hill coefficients (Figs. 3–9). The unpaired Student's t test was used to test differences of the mean values for statistical significance. Whereas in most experiments the calculated Hill coefficients were not significantly different from 1, in a few experiments, Hill coefficients different from 1 were obtained. For these experiments, we also calculated IC_{50} values by fitting the Michaelis-Menten equation to the data (see footnotes 1–6). In most cases, the goodness of these fits was inferior. The calculated IC_{50} values differed by less than 40% from those obtained with the Hill equation. We used the IC_{50} values obtained with the Hill equation to calculate apparent K_i values for competitive inhibition and to perform statistical comparisons between K_i values. Doing this assured that our conclusions concerning significance of differences between K_i values were also valid when the K_i values were calculated from IC_{50} values obtained by fitting the Michaelis-Menten equation to the data. When we calculated K_i values from IC_{50} values and $K_{0.5}$ values for substrate activation, error propagation was taken into account (Papoulis, 1991). The calculated K_i values were compared for statistical significance of differences according to Baumgartner et al. (1998).

Materials. [^{14}C]TEA (1.9 TBq/mmol) was obtained from Biotrend (Köln, Germany) and [^{3}H]corticosterone (3.2 TBq/mmol) from Amersham Buchler (Braunschweig, Germany). Unlabeled corticosterone and TBuA were purchased from Fluka (Neu-Ulm, Germany). All other chemicals were obtained as described previously (Budiman et al., 2000; Arndt et al., 2001).

Results

Inhibitor Assays in Intact Oocytes and Selection of Nontransported Inhibitors. Application of choline or TEA induced inward currents in voltage-clamped oocytes that were expressing rOCT2 but not in control oocytes (Sweet and Pritchard, 1999; Budiman et al., 2000; Arndt et al., 2001)

(Fig. 1). The currents induced by choline or TEA increased as membrane potential became more negative and showed saturation at high substrate concentrations (Arndt et al., 2001).

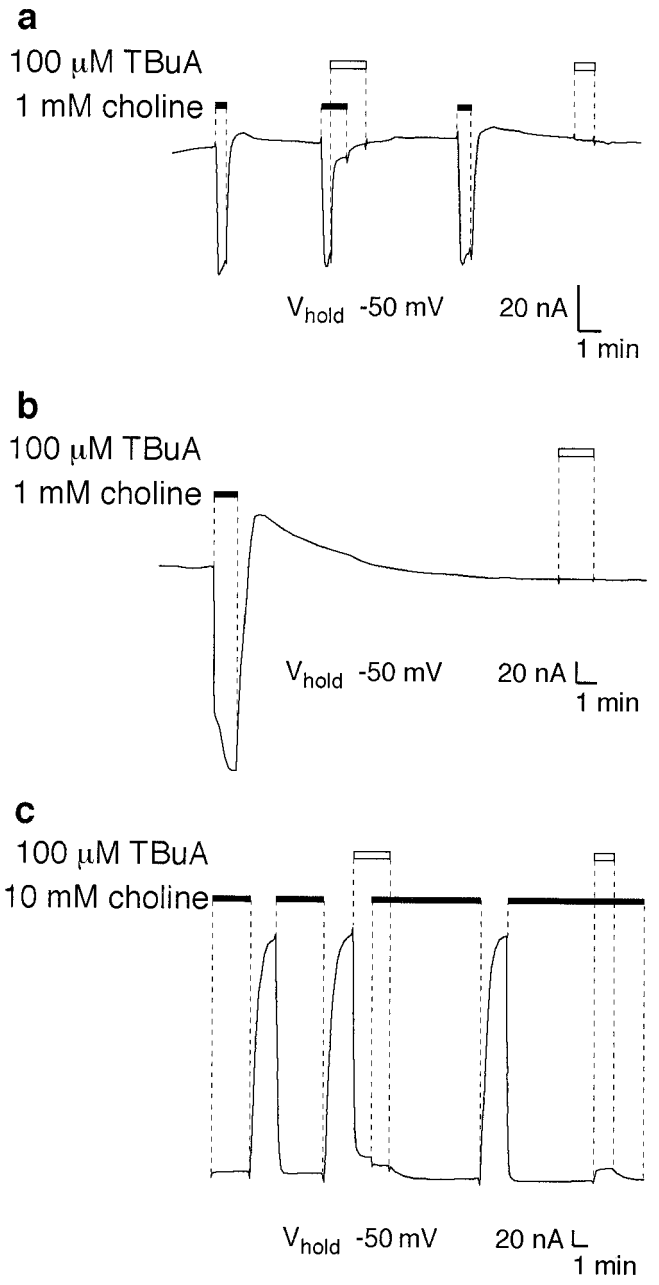

Fig. 1. TBuA inhibits the choline-induced inward and outward currents in oocytes expressing rOCT2. a, effect of TBuA on currents induced by extracellular choline. b, as in a but with ~5-fold higher expression of rOCT2. Oocytes expressing rOCT2 were first superfused with Ori buffer and clamped to −50 mV. Switching the bath solution to 1 mM choline in Ori buffer induced inward currents. Switching the bath solution back to choline-free Ori resulted in a transient outward current; this current probably corresponds to the efflux of choline that entered the oocytes during superfusion with choline. With 100 μM TBuA present in the bath solution, choline-induced currents were reduced by more than 80% (a, *cis*-inhibition). No currents were induced by 100 μM TBuA alone (a and b). c, effect of TBuA on currents induced by intracellular choline. Oocytes were preincubated for 12 h in 10 mM choline, then superfused with 10 mM choline and clamped to −50 mV. Switching the bath solution to choline-free Ori buffer resulted in outward currents corresponding to efflux of choline. Only minor outward currents were observed when the oocytes were superfused with choline-free Ori buffer that contained 100 μM TBuA (*trans*-inhibition).

From various inhibitors, we selected two nontransported inhibitors to study ligand binding to the extracellular and intracellular face of rOCT2, namely TBuA, a permanently charged quaternary ammonium compound, and corticosterone, an uncharged, more hydrophobic compound. Extracellularly applied tetramethylammonium and TEA, but not TBuA, induced inward currents in oocytes expressing rOCT2. In contrast, TBuA inhibited inward currents that were induced by choline or TEA (see Fig. 1, a and b) (Arndt et al., 2001). Inhibition by TBuA was reversible within seconds after removing TBuA from the bath (data not shown). Figure 1c shows that 100 μM TBuA in the bath *trans*-inhibited choline-dependent outward currents in *X.* oocytes. In this experiment, the oocytes were preloaded overnight in 10 mM choline, superfused with 10 mM choline, clamped to −50 mV, and then superfused with choline-free Ori, either in the absence or presence of TBuA. When 100 μM TBuA was added during superfusion of the oocytes with 10 mM choline, a small outward current was observed. This current is probably caused by inhibition of a small choline-dependent inward current.

To test whether corticosterone is transported by rOCT2, we performed tracer flux measurements with radioactive corticosterone, and we tried to *trans*-stimulate choline-induced outward currents by extracellular corticosterone. Figure 2 shows the time course of the uptake of 10 μM [^{3}H]corticosterone into oocytes expressing rOCT2 compared with noninjected control oocytes. We found that uptake of corticosterone was time-dependent and not significantly different between the two groups of oocytes. Uptake of corticosterone by oocytes expressing rOCT2 was not inhibited by 100 μM cyanine-863 (Fig. 2). Within a 1h uptake period, the following amounts of corticosterone were found associated with the oocytes (in picomoles per oocyte): oocytes expressing rOCT2, 36.3 ± 1.1; oocytes expressing rOCT2 in the presence of cyanine-863, 35.5 ± 1.0; noninjected control oocytes, 35.8 ± 1.3. In the same experiment, cyanine-863-inhibitable uptake of 100 μM [^{14}C]-TEA was 161 ± 16. A TEA concentration of 100 μM approximately equals the K_m value for TEA uptake

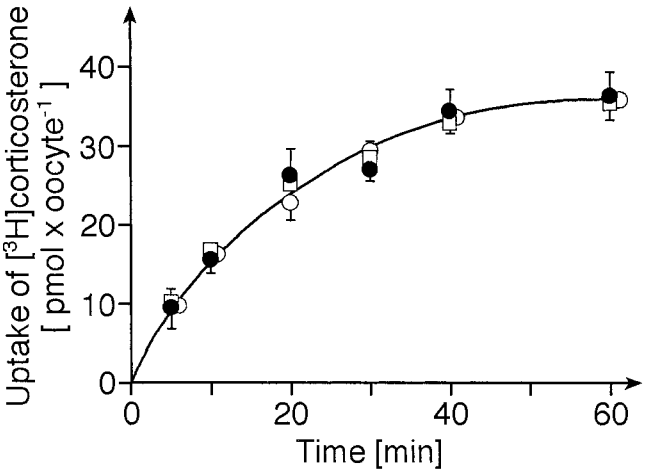

Fig. 2. Uptake of corticosterone in *X. laevis* oocytes without and with expression of rOCT2. Noninjected oocytes (○) and oocytes expressing rOCT2 (●, □) were incubated for different time periods in 10 μM [^{3}H]corticosterone in the absence (○, ●) and presence (□) of 100 μM cyanine-863. Subsequently, the oocytes were washed and corticosterone associated with the oocytes was quantitated using liquid scintillation counting. Data are mean values ± S.E. from 8 to 10 oocytes.

(Arndt et al., 2001), and a corticosterone concentration of 10 μM is within the range of IC_{50} values measured for inhibition of rOCT2 by corticosterone (see Arndt et al., 2001, and below). The sensitivity of the assay was such that it could have discriminated a difference of 4 pmol/oocyte^{-1} h^{-1} between different groups in Fig. 2. This represents 2.5% of the TEA uptake measured at the K_m value of TEA. Because no rOCT2-specific uptake of corticosterone was detected and corticosterone uptake was measured at the apparent K_i value of corticosterone, the experiment suggests that the uptake of corticosterone by rOCT2 is less than 2.5% of TEA uptake by the same transporter. We also found that the uptake of [^{3}H]corticosterone in oocytes expressing rOCT2 did not exhibit substrate saturation up to 0.5 mM (data not shown). Moreover, organic cation-induced outward currents in oocytes expressing rOCT2 were *trans*-inhibited by corticosterone rather than being *trans*-stimulated, as is frequently observed with transported compounds (e.g., Fig. 8). The data strongly suggest that corticosterone is a nontransported inhibitor of rOCT2 (see discussion). As opposed to TBuA, corticosterone is able to permeate the plasma membrane passively and is slowly taken up by the oocytes (see Fig. 2).

Interaction of rOCT2 with Extracellular TBuA. In oocytes expressing rOCT2, we measured the inhibition of choline-induced inward currents by a series of TBuA concentrations (Fig. 3). The measurements were performed with

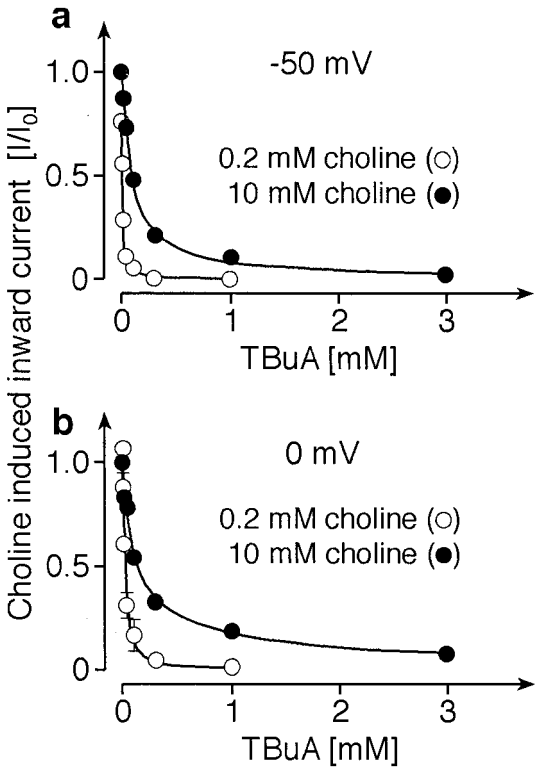

Fig. 3. Effect of extracellular TBuA on currents induced by extracellular choline (*cis*-inhibition) at −50 and 0 mV. a, oocytes expressing rOCT2 were superfused with Ori buffer, clamped to −50 mV, and superfused for 5 to 10 s with Ori buffer containing 0.2 (○) or 10 mM choline (●) plus various concentrations of TBuA. After each superfusion period with choline, the oocytes were superfused for 3 min with choline-free Ori buffer to allow efflux of choline that had entered the oocytes. b, as in a but with oocytes clamped to 0 mV. Mean values ± S.E. of four to six independent experiments are presented. The data show potential dependence of TBuA inhibition and competition by choline.

two concentrations of choline (0.2 and 10 mM) and at two holding potentials (−50 mV and 0 mV). Kinetic parameters were extracted from the Hill equation fitted to the inhibition curves. With 0.2 mM choline at −50 mV, we observed an IC_{50} value of 3.8 ± 0.3 μM and a Hill coefficient of 0.91 ± 0.08, n = 6. With 10 mM choline at −50 mV, we found an IC_{50} of 89 ± 9 μM and a Hill coefficient of 0.97 ± 0.03, n = 4 (Fig. 3a). Assuming Michaelis-Menten kinetics and competitive inhibition and using these values together with the K_m for choline of 381 ± 30 μM at −50 mV (see Fig. 7b), we computed K_i values for TBuA of 2.5 ± 0.2 μM and 3.3 ± 0.3 μM with 0.2 and 10 mM choline, respectively. These K_i values are not statistically different ($P > 0.05$), as expected for a purely competitive type of inhibition.

At a holding potential of 0 mV and a choline concentration of 0.2 mM, we obtained an IC_{50} for inhibition by TBuA of 16 ± 3 μM and a Hill coefficient of 1.14 ± 0.19, n = 6; at 0 mV and 10 mM choline, we found an IC_{50} of 135 ± 5 μM and a Hill coefficient of 0.75 ± 0.07, n = 4 (Fig. 3b).[1] Together with the K_m value of 700 ± 90 μM determined for choline-induced inward currents at 0 mV (Arndt et al., 2001) and assuming competitive Michaelis-Menten inhibition kinetics, we computed K_i values of 12.5 ± 2.8 and 8.8 ± 0.6 μM with 0.2 and 10 mM choline, respectively. Again, these values are not significantly different, demonstrating pure competitive interaction between TBuA and choline also at 0 mV. For both concentrations of choline, however, the K_i values at −50 mV were significantly lower than at 0 mV ($p < 0.05$) (Table 1). This voltage dependence is compatible with two different mechanisms: in the outwardly directed conformation of the rOCT2 substrate binding site, binding and debinding of TBuA may be associated with charge movement into and out of the electric field of the membrane. Alternatively, or in addition, membrane potential may alter the conformation and affinity of the outwardly directed cation-binding site.

To determine the K_i values for TBuA at different membrane potentials without the need to correct for the interaction of rOCT2 with choline, we also measured *trans*-inhibition of choline-induced outward currents by extracellular TBuA. Oocytes expressing rOCT2 were preloaded overnight in 10 mM choline and superfused with 10 mM choline at a membrane potential of −50 or 0 mV, as seen in Fig. 1c. Then, the oocytes were switched to choline-free Ori buffer containing various concentrations of TBuA (Fig. 4). Under these conditions, extracellular choline concentration was close to zero, precluding competition between TBuA and choline. Choline-induced outward currents were inhibited by extracellular TBuA with an IC_{50} of 3.54 ± 0.48 μM at −50 mV (Hill coefficient, 0.72 ± 0.07, n = 6)[2] and an IC_{50} of 6.47 ± 0.37 μM at 0 mV (Hill coefficient, 1.02 ± 0.02, n = 5). Under these conditions, the IC_{50} values and K_i values become identical. The K_i for *trans*-inhibition by extracellular TBuA at −50mV was significantly lower than at 0 mV ($P < 0.01$); this result is in keeping with the K_i values obtained for *cis*-inhibition by extracellular TBuA (Table 1). At a given holding

[1] Setting the Hill coefficient to 1, IC_{50} values of 14 ± 3 μM (0.2 mM choline) and 113 ± 12 μM (10 mM choline) were obtained. Using these values, K_i values of 10.9 ± 1.6 μM and 7.4 ± 0.9 μM were calculated for 0.2 mM and 10 mM choline, respectively. The K_i values are not significantly different.

[2] When the Hill coefficient was fixed to 1, an IC_{50} value of 4.05 ± 0.53 μM was obtained. The K_i values for *trans*-inhibition at −50 and 0 mV are significantly different.

TABLE 1

Calculated K_i values for inhibition of choline-induced currents by TBuA and corticosterone.

Choline-induced currents were measured in the presence of various concentrations of TBuA or corticosterone at the extracellular and intracellular sides of the membrane, and the apparent inhibitor constants (K_i values) were calculated as described under *Materials and Methods* and *Results*. The K_i values for extracellular inhibitors were determined either from the currents induced by extracellular choline (*cis*-inhibition) or from the currents induced by intracellular choline (*trans*-inhibition). The K_i values for intracellular inhibitors were calculated using the currents induced by intracellular choline (*cis*-inhibition). The choline concentrations employed are indicated in parenthesis (in millimolar).

Inhibitor and Application	−50 mV		0 mV	
	cis-Inhibition	*trans*-Inhibition	*cis*-Inhibition	*trans*-Inhibition
	μM (mM)	*μM (mM)*	*μM (mM)*	*μM (mM)*
TBuA				
Extracellular	2.5 ± 0.2 (0.2) 3.3 ± 0.3 (10)	3.5 ± 0.5 (10)	12.5 ± 2.8 (0.2) 8.8 ± 0.6 (10)	6.5 ± 0.4 (10)
Intracellular	N.D.	N.D.	42 ± 13 (1) 48 ± 7.1 (10)	N.D.
Corticosterone				
Extracellular	6–9 (0.2)	0.24 ± 0.06 (10)	19.5–25 (0.2)	1.08 ± 0.18 (10)
Intracellular	0.48 ± 0.06 (1)	N.D.	0.96 ± 0.33 (1)	N.D.

N.D., not determined.

potential, however, the K_i values for *cis*- versus *trans*-inhibition were similar.

Interaction of rOCT2 with Intracellular TBuA. Inside-out–oriented giant patches from oocytes expressing rOCT2 were used to investigate the interaction of TBuA with rOCT2 on the intracellular side. Superfusion with 10 mM choline induced currents between 1 and 10 pA in inside-out patches from oocytes expressing rOCT2 but not in patches from noninjected oocytes, as shown previously (Budiman et al., 2000). No currents were observed in patches from either type of oocyte upon superfusion with 1 mM TBuA. Inside-out patches clamped to 0 mV exhibited IC_{50} values for *cis*-inhibition by TBuA of outwardly directed choline currents of 66 ± 21 μM with 1 mM choline (Hill coefficient, 0.48 ± 0.08, n = 3) and 315 ± 31 μM with 10 mM choline (Hill coefficient, 0.81 ± 0.08, n = 5) (Fig. 5).[3] Assuming competitive Michaelis-Menten-type inhibition and a K_m value of 1.8 ± 0.5 mM for choline efflux (Budiman et al., 2000), we computed K_i values for intracellular TBuA of 42 ± 13 and 48 ± 7.1 μM with 1 and 10 mM choline, respectively (Table 1). The lack of a significant difference between these values is compatible with a purely competitive *cis*-inhibition of rOCT2 by TBuA at the intracellular side. Importantly, at a membrane potential of 0 mV, *cis*-inhibition of choline-induced outward currents by intracellular TBuA exhibits a significantly larger K_i than *cis*-inhibition of choline-induced inward currents by extracellular TBuA ($P < 0.01$) (Table 1). This indicates that the outwardly and inwardly directed conformations of the substrate binding sites have different affinities for TBuA at 0 mV.

Inhibition of TEA-Induced Inward Currents by Corticosterone. For an initial characterization of the interaction of corticosterone with rOCT2, we measured the effect of corticosterone on currents induced by the substrate TEA. In subsequent experiments, we used choline as a substrate, because the maximal currents observable for rOCT2 are higher with choline than with TEA (Arndt et al., 2001). In addition, choline was better suited for preloading experiments. In the experiment shown in Fig. 6a, we superfused oocytes expressing rOCT2 (not preloaded) at −50 mV for 10 min periods with increasing concentrations of corticosterone. In the last 30 s of each 10-min superfusion period, we added 2 mM TEA to the superfusion buffer and recorded the TEA-induced inward currents. An IC_{50} value of 4.7 ± 0.4 μM (Hill coefficient 0.94 ± 0.05, n = 4) was calculated by fitting the Hill equation to the TEA-induced currents

In the experiment shown in Fig. 6b, we assessed the concentration-dependence of TEA-induced inward currents, both in the absence and presence of corticosterone. To do this, we added 4 μM corticosterone 10 min before probing the substrate-induced currents. The currents induced by saturating TEA concentrations (5 mM) were 80 ± 8 nA without corticosterone and were reduced to 23 ± 3 nA in the presence of 4 μM corticosterone ($P < 0.01$ for difference). In contrast to what would be expected for competitive inhibition, the presence of the inhibitor did not increase the apparent $K_{0.5}$ values of the TEA-induced currents (data in the absence versus presence of corticosterone: $K_{0.5}$, 103 ± 12 μM versus 96 ± 11 μM; Hill coefficient, 1.14 ± 0.07 versus 1.25 ± 0.15; n = 4 in either group). To test whether this noncompetitive type of inhibition is caused by a slow off rate of corticosterone that has entered the oocytes, we measured the reactivation of rOCT2 after 10-min incubation in 20 μM corticosterone and performed inhibition experiments in which passive diffusion of corticosterone across the membrane was minimized. Oocytes expressing rOCT2 were clamped to −50 mV and incubated for 10 min in 20 μM corticosterone. Under these conditions, superfusion of 1 mM TEA-induced inward currents that were reduced to 31.2 ± 1.5% (n = 3), compared with TEA-induced currents in the absence of corticosterone. When the oocytes were then superfused with Ori buffer, TEA-induced currents reactivated after 5 min to 56.7 ± 2.4% and after 20 min to 71.1 ± 3.3%.

To minimize the loading of oocytes with corticosterone in Fig. 6c, we superfused oocytes expressing rOCT2 for periods of 5 to 10 s with Ori buffer containing either 50 μM TEA or 2 mM TEA, each with increasing concentrations of corticosterone. Accumulation of corticosterone in the oocytes was minimized by moving from low to high corticosterone concentrations and by washing the oocytes for 5 to 8 min with Ori buffer after each incubation in corticosterone. Under these conditions, the IC_{50} values for inhibition by extracellularly applied corticosterone were 18 ± 6 μM with 50 μM TEA (Hill

[3] Setting the Hill coefficients to 1, IC_{50} values of 90 ± 43 μM (1 mM choline) and 351 ± 41 μM (10 mM choline) were obtained. Using these values and assuming competitive inhibition, K_i values of 58 ± 27 μM (1 mM choline) and 54 ± 10 μM (10 mM choline) were computed.

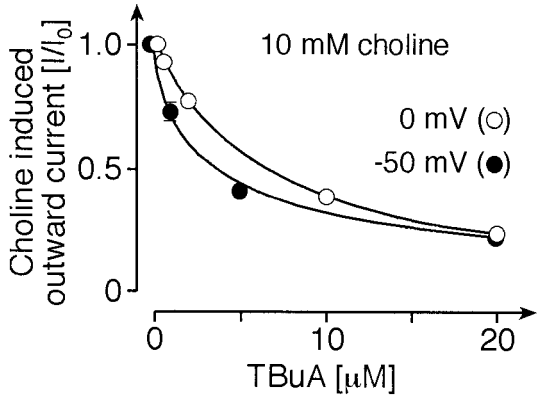

Fig. 4. Effect of extracellular TBuA on currents induced by intracellular choline at 0 and −50 mV (*trans*-inhibition). Oocytes expressing rOCT2 were preloaded by overnight incubation in 10 mM choline, superfused with Ori buffer containing 10 mM choline, and clamped to −50 (●) or 0 mV (○). They were then superfused for 5 to 10 s with choline-free Ori buffer containing the indicated concentrations of TBuA. After each superfusion period with choline-free Ori buffer, the oocytes were superfused for 3 min with 10 mM choline to replenish intracellular choline. Means ± S.E. of five to six experiments are shown. The data show potential-dependence of *trans*-inhibition of choline efflux by TBuA.

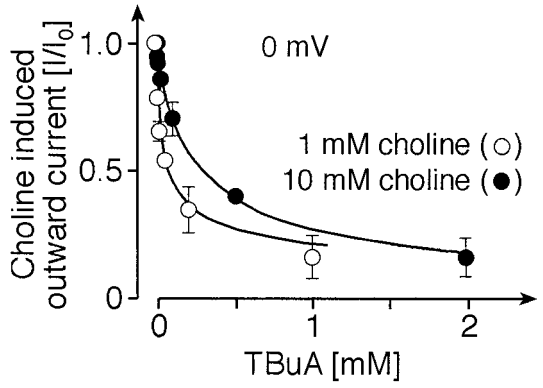

Fig. 5. Effect of intracellular TBuA on currents induced by intracellular choline at 0 mV (*cis*-inhibition). Inside-out patches of oocytes expressing rOCT2 were kept in sorbitol buffer on both sides of the membrane and clamped to 0 mV. We then measured the currents induced by superfusion with sorbitol buffer containing either 1 or 10 mM choline, each plus various concentrations of TBuA. Mean values ± S.E. (n = 3–5) are shown. The data show competition between choline and TBuA from the intracellular compartment.

coefficient 0.68 ± 0.09, n = 7),[4] and 68 ± 7 μM with 2 mM TEA (Hill coefficient 1.05 ± 0.06, n = 7). The finding that the IC_{50} for the inhibitor is ~4 times higher with the higher concentration of substrate ($P < 0.01$ for difference) points to competition at the extracellular substrate binding site between corticosterone and TEA. On the other hand, the substrate effect on the IC_{50} is considerably smaller than expected for a purely competitive mechanism of inhibition: using a K_m value of 100 μM for TEA (Arndt et al., 2001), one would predict a 14-fold increase of the apparent IC_{50} for corticosterone when switching TEA concentration from 50 μM to 2 mM. From the IC_{50} value observed in the presence of 50 μM TEA, the affinity for extracellular corticosterone can be computed assuming either noncompetitive or purely competitive inhibition, resulting in K_i values of 18 ± 6 and 12 ±

[4] An IC_{50} value of 22 ± 6 was calculated when the Hill coefficient was set to 1.

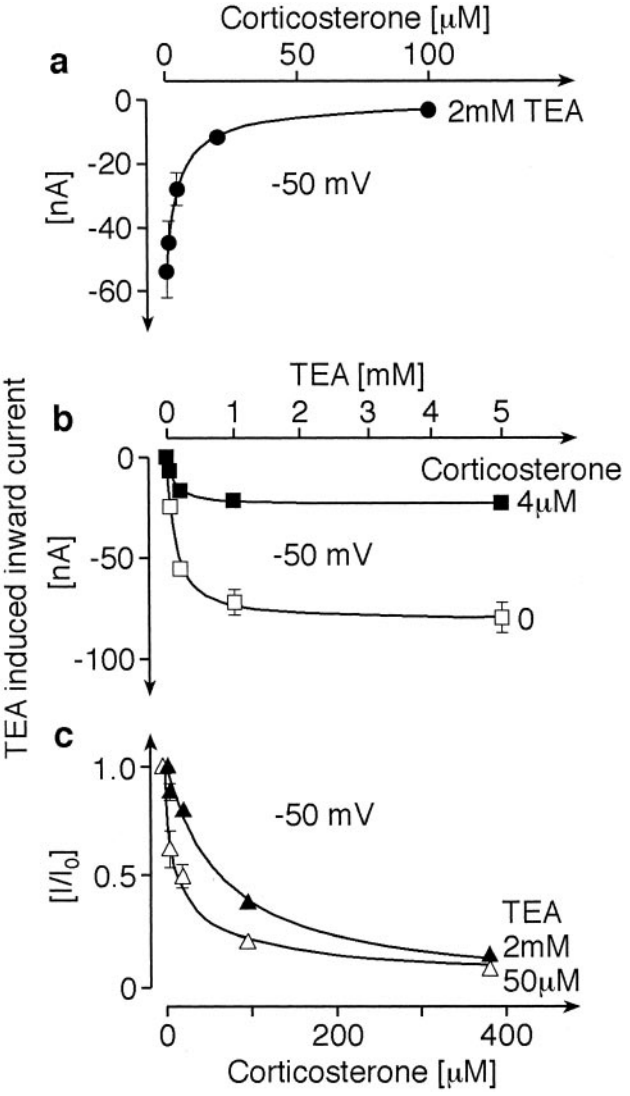

Fig. 6. Effect of incubation time with corticosterone on inhibition of currents induced by extracellular TEA. a, concentration-dependence of corticosterone inhibition after long incubation. Oocytes expressing rOCT2 were voltage-clamped to −50 mV and superfused for 10-min periods with Ori buffer containing increasing concentrations of corticosterone. At the end of each 10-min superfusion period, the oocytes were superfused for 30 s with Ori buffer containing 2 mM TEA plus corticosterone at the indicated concentration, and the respective TEA-induced inward current was measured. Mean values ± S.E. from four experiments (●) are presented. b, substrate dependence of choline uptake after long incubation with corticosterone. Inward currents induced by various concentrations of TEA were measured in absence of corticosterone (□) or after 10-min incubation in 4 μM corticosterone (■). Oocytes expressing rOCT2 were clamped to −50 mV and superfused for 20-s periods with Ori buffer containing increasing concentrations of TEA (□). To allow efflux of intracellular TEA, the oocytes were superfused for 3 min with Ori buffer after each superfusion period with TEA. Thereafter, the same oocytes were preincubated for 10 min with 4 μM corticosterone and then superfused for 20-s periods with Ori buffer containing 4 μM corticosterone plus the indicated concentrations of TEA (■). In this case, the oocytes were superfused for 3 min with Ori buffer containing 4 μM corticosterone after each superfusion with TEA. Mean values ± S.E. (n = 4) are shown. c, concentration dependence of corticosterone inhibition after short incubation. Oocytes expressing rOCT2 were superfused with Ori buffer, clamped to −50 mV, and then superfused for 5 to 10 s with 50 μM (△) or 2 mM (▲) TEA containing the indicated concentrations of corticosterone. After each superfusion period with TEA or TEA plus corticosterone, the oocytes were superfused for 5 to 8 min with Ori buffer to allow for efflux of accumulated TEA. Mean values ± S.E. of seven or eight experiments are presented. The data show higher affinity of corticosterone and absence of competition of corticosterone with TEA after long incubation.

5 μM, respectively. Note that both values are significantly higher ($P < 0.05$) than the IC_{50} value of 4.7 μM that was obtained after prolonged incubation with corticosterone.

Potential-Dependence of the Interaction of rOCT2 with Extracellular Corticosterone. Using short corticosterone pulses as described above and choline as a substrate, we investigated the effect of membrane potential on the interaction of extracellular corticosterone with rOCT2. Oocytes expressing rOCT2 were clamped to either 0 or −50 mV and superfused for 5 to 10 s with Ori buffer containing either 0.2 or 10 mM choline, each with different concentrations of corticosterone. At a holding potential of −50 mV and with 0.2 mM choline, we observed an IC_{50} for corticosterone of 9 ± 1 μM (Hill coefficient, 1.20 ± 0.17, $n = 3$); at −50 mV with 10 mM choline, we observed an IC_{50} of 27 ± 4 μM (Hill coefficient, 0.83 ± 0.06, $n = 6$) (Fig. 7a). The 3-fold higher IC_{50} value obtained with 10 mM choline compared with 0.2 mM choline indicates that choline can partially displace corticosterone from its binding site, as observed with TEA. Assuming purely competitive, Michaelis-Menten-type inhibition and a K_m for choline of 381 ± 30 μM (Fig. 7b), one finds that the apparent IC_{50} for corticosterone observed with 0.2 mM choline corresponds to a K_i of 6.0 ± 0.7 μM. As shown in Fig. 7b, we measured the effect of extracellular corticosterone on the concentration-dependence of the inward currents induced by extracellular choline. As shown in Fig. 7a, oocytes were clamped to −50 mV and superfused for 5 to 10 s with various concentrations of choline and corticosterone. From the results, we calculated the following apparent $K_{0.5}$ values and Hill coefficients for extracellular choline: 1) in the absence of corticosterone, $K_{0.5}$, 381 ± 30 μM; Hill coefficient, 1.07 ± 0.04, $n = 4$; 2) with 10 μM corticosterone, $K_{0.5}$, 1,020 ± 70 μM; Hill coefficient, 1.15 ± 0.12, $n = 5$; and 3) with 40 μM corticosterone, $K_{0.5}$, 3,148 ± 866 μM; Hill coefficient, 0.98 ± 0.12, $n = 4$. Assuming purely competitive, Michaelis-Menten-type inhibition, the apparent $K_{0.5}$ values with 10 and 40 μM corticosterone correspond to K_i values of 6.0 and 5.5 μM, respectively. Similar to the previous result (Fig. 7a), these data suggest that choline can compete with corticosterone for the extracellular substrate binding site of rOCT2. However, the competition was greater in this experimental set up.

Next, we determined the concentration-dependence for corticosterone inhibition at 0 mV (Fig. 7c), using short corticosterone pulses and choline concentrations of 0.2 and 10 mM as shown in Fig. 7a. With 0.2 and 10 mM choline at 0 mV, we observed apparent IC_{50} values for corticosterone of 25 ± 4 μM (Hill coefficient, 0.88 ± 0.18, $n = 3$) and 57 ± 7 μM (Hill coefficient, 1.02 ± 0.07, $n = 7$), respectively. The higher IC_{50} obtained with the higher substrate concentration suggests that corticosterone is partially displaced by choline. Similar to the findings with TEA as a substrate, however, this ~2-fold increase of the IC_{50} is much smaller than expected. Under these conditions, an 11-fold increase would be expected for purely competitive, Michaelis-Menten-type inhibition. The IC_{50} for corticosterone determined with 0.2 mM choline allows the computation of the affinity of rOCT2 for corticosterone at 0 mV. Assuming either competitive or noncompetitive inhibition, this yields K_i values of 19.5 ± 3.2 and 25 ± 4.0 μM, respectively. These K_i values are significantly higher than the values of 5.2 to 6 μM observed at −50 mV ($P < 0.05$ for difference), suggesting that the affinity of rOCT2 for extracellular corticosterone increases with more negative membrane potential.

To study the interaction of rOCT2 with extracellular corticosterone in the absence of extracellular substrate, we measured the *trans*-inhibition of outward currents in rOCT2-expressing oocytes. Oocytes were preloaded in 10 mM choline, superfused with Ori buffer containing 10 mM cho-

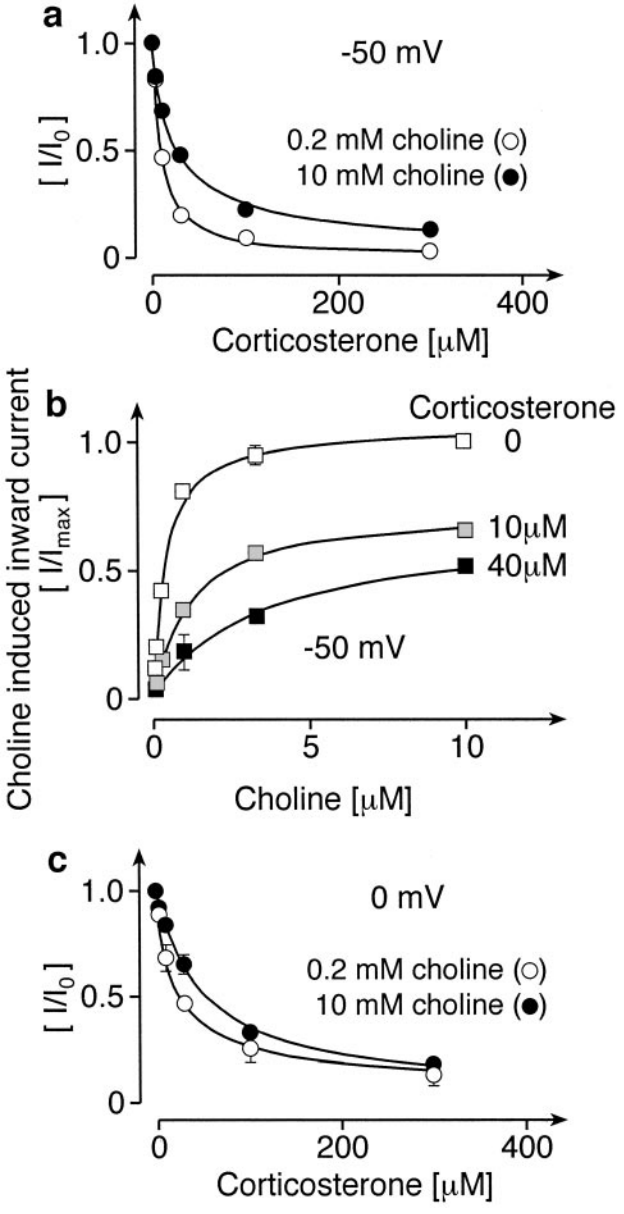

Fig. 7. Inhibition of inward currents induced by extracellular choline by extracellular corticosterone (*cis*-inhibition) at 0 and −50 mV using short incubation. a, inhibitor concentration-dependence at −50 mV using choline concentrations of 0.2 and 10 mM. b, substrate concentration-dependence at −50 mV in the absence and presence of corticosterone (10 and 40 μM). c, inhibitor concentration-dependence at 0 mV using choline concentrations of 0.2 and 10 mM. Oocytes expressing rOCT2 were superfused with Ori buffer and clamped to −50 or 0 mV. Thereafter, the oocytes were superfused for 5- to 10-s periods with Ori buffer containing choline without or with various concentrations of corticosterone. After each superfusion period with choline or choline plus corticosterone, the oocytes were superfused for 5 min with Ori buffer to allow the release of choline and corticosterone. Mean values ± S.E. from four or five experiments are indicated. The data show competition between corticosterone and choline and a higher affinity of corticosterone at −50 mV compared with 0 mV.

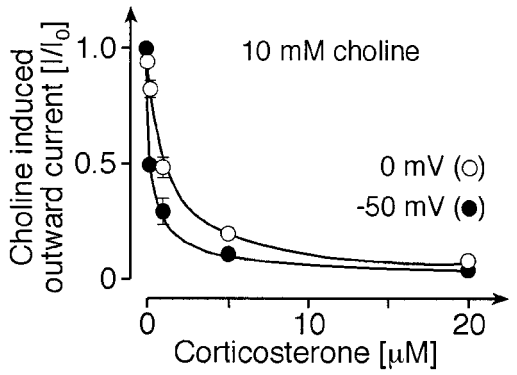

Fig. 8. Effect of extracellular corticosterone on currents induced by intracellular choline (*trans*-inhibition) at 0 and −50 mV using short incubation. rOCT2-expressing oocytes were preincubated overnight in Ori buffer containing 10 mM choline, superfused with 10 mM choline and clamped to either 0 or −50 mV. In either case, the oocytes were superfused for periods of 10 to 20 s with choline-free Ori buffer containing various concentrations of corticosterone. After each superfusion period with corticosterone in the absence of choline, the oocytes were superfused for another 5 to 8 min with 10 mM choline to refill the oocytes with choline and to allow the release of corticosterone that may have entered the oocytes. Mean values ± S.E. of five experiments are shown. The data show a higher affinity for corticosterone at −50 mV compared with 0 mV.

line, and clamped to either −50 or 0 mV. We then recorded the outward currents elicited by superfusion with choline-free Ori buffer supplemented with various concentrations of corticosterone (Fig. 8). Surprisingly, we obtained a much higher affinity for extracellular corticosterone in these *trans*-inhibition experiments than in the experiments using *cis*-inhibition of inward currents induced by extracellular choline. For *trans*-inhibition of outwardly directed choline currents, we obtained, at −50 and 0 mV, IC_{50} values for corticosterone of 0.24 ± 0.06 μM (Hill coefficient, 0.71 ± 0.11, $n = 6$)[5] and 1.08 ± 0.18 μM (Hill coefficient, 0.91 ± 0.08, $n = 5$), respectively. Because the extracellular choline concentration is close to zero, the IC_{50} values obtained in the *trans*-inhibition experiments become identical to the K_i values. Affinity of rOCT2 for corticosterone is significantly higher at −50 than at 0 mV ($P < 0.01$ for difference). At both membrane potentials, the affinity of rOCT2 for extracellular corticosterone was ~20-fold higher in the *trans*-inhibition experiments than in the *cis*-inhibition experiments (Table 1) ($P < 0.001$ for difference).

Interaction of rOCT2 with Intracellular Corticosterone. Inside-out–oriented patches of oocytes expressing rOCT2 were clamped to either 0 or −50 mV and superfused with 1 mM choline supplemented with various concentrations of corticosterone (Fig. 9a). The observed currents yielded IC_{50} values for corticosterone at 0 and −50 mV of 1.5 ± 0.3 μM (Hill coefficient, 0.86 ± 0.06, $n = 3$) and 0.66 ± 0.04 μM (Hill coefficient 0.68 ± 0.16, $n = 4$), respectively.[6] Assuming purely competitive, Michaelis-Menten-type inhibition and using the K_m values for choline efflux at the respective membrane potentials (Budiman et al., 2000), these IC_{50} values correspond to apparent K_i values of 0.96 ± 0.33 and 0.48 ± 0.06 μM at 0 and −50 mV, respectively. These values were not significantly different ($P > 0.05$). In Fig. 9b, the concentration-dependence of outward currents induced at 0 mV by intracellular choline was measured in the presence of either 1 or 4 μM corticosterone. Previously, we found a $K_{0.5}$ value of 1.8 ± 0.5 mM for choline-induced outward currents in the absence of corticosterone (Budiman et al., 2000). In the presence of 1 μM corticosterone, we now observed an increased apparent $K_{0.5}$ for intracellular choline of 2.7 ± 1.3 mM (0 mV; Hill coefficient, 1.01 ± 0.13; $n = 4$). With 4 μM corticosterone, the $K_{0.5}$ for intracellular choline was further increased to 5.7 ± 1.3 mM (0 mV; Hill coefficient, 1.12 ± 0.19; $n = 2$). Assuming purely competitive, Michaelis-Menten-type inhibition, these IC_{50} values for 1 and 4 μM intracellular corticosterone correspond to K_i values of 2.0 and 1.8 μM, respectively. Because these values are not significantly different, the most straightforward interpretation is to assume that corticosterone inhibits rOCT2 from the inside by direct competition with the substrate choline.

Discussion

The interaction of polyspecific cation transporters with their substrates and inhibitors is not well understood. We do not know how these transporters can interact with widely different molecular structures such as choline, 1-methyl-4-phenylpyridinium, *N*- (4,4-azo-*n*-pentyl)-21-deoxyajmalinium, and corticosterone (Arndt et al., 2001; Dresser et al., 2001; Van Montfoort et al., 2001) but discriminate efficiently between compounds

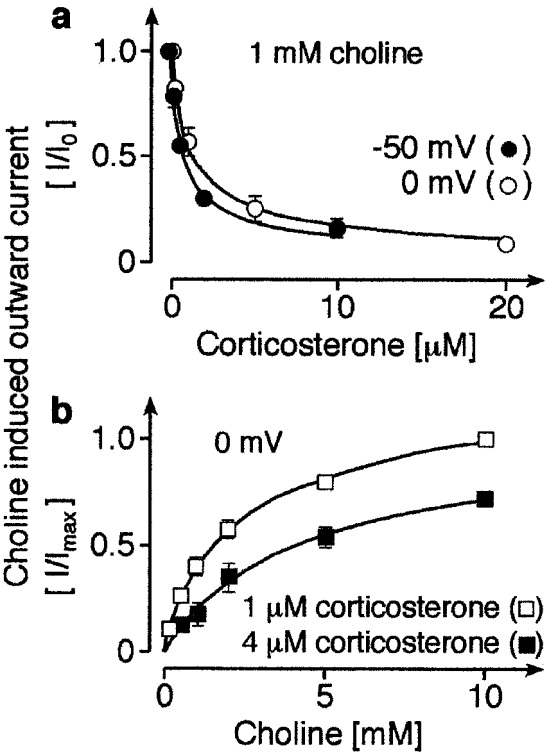

Fig. 9. Effect of cytoplasmic corticosterone on currents induced by cytoplasmic choline at 0 mV using short incubation. Inside-out patches from *X. laevis* oocytes expressing rOCT2 were clamped to 0 mV and exposed to 10- to 20-s pulses of choline and corticosterone at various concentrations. The induced outward currents in the absence and presence of corticosterone are indicated. Mean values ± S.E. of three (●), six (□), or two (■) experiments are shown. The data show a similar affinity for corticosterone at −50 mV compared with 0 mV and competition between corticosterone and choline.

[5] An IC_{50} value of 0.33 ± 0.09 μM was obtained by fitting the Michaelis-Menten equation to the data.

[6] Setting the Hill coefficient to 1, IC_{50} values of 1.9 ± 0.50 μM (0 mV) and 0.90 ± 0.13 μM (−50 mV) were calculated. From these values, apparent K_i values of 1.2 ± 0.3 and 0.70 ± 0.10 μM were calculated that were not significantly different ($P > 0.05$).

exhibiting comparably slight structural differences, such as trimethylamine and betaine or tyrosine and tyramine (C. Volk and H. Koepsell, unpublished data). Moreover, we do not know what structural features determine whether a ligand is a transported substrate like tributylmethylammonium (Van Monfort et al., 2001) or a nontransported inhibitor like tetrabutylammonium (current study). One possibility to explain polyspecificity is to assume complex substrate binding sites for the recognition of structurally different substrates. However, the polyspecificity could also be explained by induced-fit mechanisms.

In the present study, we characterized the interaction of the inhibitors TBuA and corticosterone with rOCT2 from the extracellular and intracellular compartments using intact oocytes of *X. laevis* and inside-out–oriented giant patches from oocyte plasma membranes, respectively. A systematic influence of the employed methods on the measured affinities may be excluded. For example, we measured a higher affinity with TBuA when applied from the extracellular compared with the intracellular compartment and a lower affinity with corticosterone when applied to the extracellular compared with the intracellular side. TBuA and corticosterone were selected as inhibitors because our data suggested that these compounds are not transported by rOCT2 but do interact with the cation binding site of rOCT2. Electrical measurements showed that rOCT2 does not transport TBuA to any detectable extent. However, TBuA can inhibit organic cation transport from either the extracellular or intracellular side of the membrane. Several lines of evidence suggest that TBuA binds directly to the cation binding site of rOCT2. First, TBuA is a permanently charged quaternary alkylammonium cation and therefore structurally similar to tetramethylammonium and tetraethylammonium, which are transported substrates of rOCT2 and rOCT1 (Gründemann et al., 1994; Gorboulev et al., 1999; Arndt et al., 2001). Second, *cis*-inhibition of choline-induced inward and outward currents displayed competitive Michaelis-Menten-type inhibition. Third, the affinity of rOCT2 for extracellular TBuA increased when the membrane potential was decreased from 0 to −50 mV, analogous to increased affinity for the extracellular substrate choline under the same conditions (Arndt et al., 2001). The finding that rOCT2 exhibits a 4-fold higher affinity for extracellular TBuA than for intracellular TBuA at 0 mV shows that the inward and outward conformations of the substrate binding site are structurally different.

Our data strongly suggest that corticosterone is another nontransported inhibitor of rOCT2 that can similarly bind to both the outwardly and inwardly oriented binding sites. Transport of corticosterone by rOCT2 is more difficult to exclude because the relatively hydrophobic, uncharged corticosterone passively permeates the plasma membrane. Our comparison of [^{3}H]corticosterone uptake in noninjected control oocytes and in oocytes expressing rOCT2 showed that rOCT2 does not translocate corticosterone at a rate that would be comparable with the rates of TEA or choline uptake. The absence of appreciable corticosterone transport by rOCT2 was also indicated by the observation that outward currents through rOCT2, elicited by intracellular choline, were *trans*-inhibited rather than *trans*-stimulated by corticosterone. Corticosterone binding to the inward and outward conformation of the substrate binding pocket of rOCT2 rather than to allosteric inhibition sites is strongly supported by the following observations. First, the different affinities for corticosterone determined from the inhibition of rOCT2 by extracellular and intracellular corticosterone indicate that the corticosterone binding sites are accessible from both the inside and the outside of the membrane. Assuming corticosterone binding at the substrate binding site provides a simpler and more likely explanation than postulating two additional sites (one extracellular, one intracellular) for allosteric inhibition by corticosterone. Second, at least partial competition between choline and corticosterone was observed in *cis*-inhibition experiments on both sides of the membrane. Competition by choline would not have been expected with two separate allosteric sites for corticosterone. On the other hand, our finding that choline could only partially displace the inhibitor corticosterone clearly shows that corticosterone binding to the substrate binding site of rOCT2 must be more complex than suggested by the classic model of competitive inhibition. For polyspecific transporters that bind and translocate a variety of structurally different compounds, it is not surprising to find non–Michaelis-Menten–type inhibition between some ligands. For example, TEA uptake by rOCT1 could be only partially inhibited by 1-methyl-4-phenyl-pyridinium, another well transported substrate of this transporter (D. Gorbunov and H. Koepsell, unpublished data).

If it is correct to assume that corticosterone interacts with the substrate binding site of rOCT2, further conclusions may be drawn. The higher affinity of rOCT2 for extracellular corticosterone at −50 mV compared with 0 mV suggests that the membrane potential has an effect on the binding properties of the cation binding site in its outwardly facing conformation. In contrast to the positively charged TBuA, the membrane electrical field should not affect the uncharged corticosterone. Therefore, increased affinity of rOCT2 for corticosterone is supposed to be brought about by structural changes of the substrate binding site. This interpretation implicates that the rOCT2 molecule comprises a voltage sensor that regulates the affinity for certain ligands at the extracellular binding site. The theoretical possibility that the membrane potential influences the apparent affinity of corticosterone by changing the distribution between the inwardly and outwardly directed conformation of the substrate binding site may be excluded for the following reason. Such a mechanism would cause opposite effects of the membrane potential on the affinity at the extracellular and intracellular side of the membrane. However, we observed that the affinity of extracellular corticosterone was significantly increased, with more negative membrane potential, but the affinity of intracellular corticosterone did not decrease. Rather, affinity even increased slightly.

Taken together, the data suggest that the substrate binding sites of rOCT2 and the other polyspecific transporters of the *SLC22* family are pockets containing different interaction domains for different ligands. This view is supported by site-directed mutagenesis experiments. Previously, we reported that an exchange of aspartate 475 to glutamate in rOCT1, located in the putative eleventh transmembrane domain, selectively increased the affinity of rOCT1 for some substrates and inhibitors (Gorboulev et al., 1999). These data suggested that aspartate 475 is part of the substrate binding pocket of rOCT1 but is not required in the binding of all substrates. Recently, X-ray crystallographic structures of ligand transporter-complexes of the AcrB multidrug efflux pump from *Escherichia coli* have been reported (Yu et al.,

2003). The structures show that four ligands bind to partially different subsets of AcrB residues that may be considered interaction domains of a binding pocket.

How can we explain the unexpected observation that the affinity of rOCT2 for extracellular corticosterone was lower for *cis*-inhibition of currents induced by extracellular choline than for *trans*-inhibition of currents induced by intracellular choline? In the model shown in Fig. 10, the substrate binding pocket is pictured with three overlapping interactive domains (for the transported substrate choline and the nontransported inhibitors TBuA and corticosterone). The experimental data can be explained by assuming that the interaction domains for TBuA and corticosterone exist in different conformations during the transport cycle. Moreover, we assume an additional conformation of the unloaded transporter. During an electrogenic transport cycle (reaction steps 1–4), the cationic substrate choline first binds to the substrate binding pocket in its extracellularly oriented conformation. Second, the choline-transporter complex undergoes a conformational change that orients the substrate binding pocket toward the intracellular side. Third, choline is released into the intracellular fluid. Finally, the empty substrate binding pocket reorients toward the extracellular surface. Figure 10 shows an additional conformation of the empty, outwardly directed substrate binding pocket, which is characterized by a very high affinity with the inhibitor corticosterone. We hypothesize that this empty high-affinity conformation does not belong to the normal transport cycle but is in equilibrium with the low-affinity conformation of the outwardly oriented, empty substrate binding pocket. During measurements of inward currents induced by extracellular choline, the fraction of transporters in the empty outward conformation should be small, and the empty outward conformation that has high affinity with corticosterone may not be detectable. When the oocytes are superfused with choline-free solution during the measurements of currents induced by intracellular choline, the concentration of transporters with empty outwardly directed substrate binding sites should be much higher, and the empty outwardly directed conformation with high affinity to corticosterone may become detectable. The observation that both the low- and high-affinity corticosterone binding conformation, observed in *cis*- and *trans*-inhibition experiments, respectively, showed increased affinity when the membrane potential was decreased from 0 to −50 mV supports this model, because a potential-dependent affinity change may involve both outwardly facing conformations of the cation binding pocket.

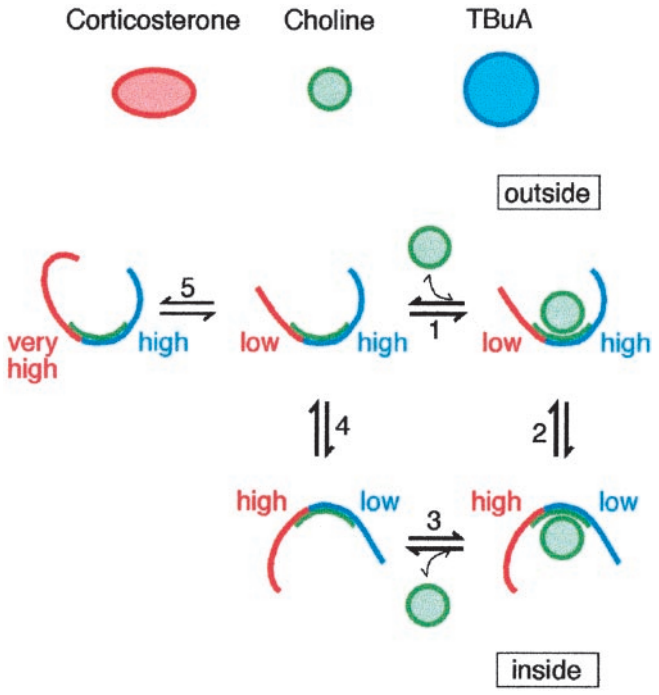

Fig. 10. Model for the binding of choline, TBuA and corticosterone to rOCT2. A modification of a simple transporter model with a monospecific substrate binding site and four reaction steps as described previously (Stein, 1990). In the original model, the substrate binding site of the transporter can exist in different conformations that are accessible either from the extracellular or the intracellular side of the membrane. Binding of a transported compound like choline at the extracellular substrate binding site (reaction step 1) induces a conformational change. During this change, the outwardly oriented cation binding site is exposed to the intracellular side of the plasma membrane (reaction step 2). Next, the substrate is released into the intracellular compartment (reaction step 3). The electrogenic transport cycle is completed by a conformational change of the empty transporter that reorients the organic cation binding site from the intracellular to the extracellular side of the membrane (reaction step 4). To extend the original model, the cation binding site of rOCT2 is depicted as a binding pocket with overlapping interaction domains for different transported cations (green, choline) and nontransported inhibitors (blue, TBuA; red, corticosterone). Different overlapping regions between the binding domains for choline, TBuA, and corticosterone account for the different degrees of competition between choline and the inhibitors. The various degrees of correspondence between ligand shapes and binding domains account for the observed higher affinity of the outwardly directed conformation for TBuA than the inwardly directed conformation of the binding pocket. The affinities of the outwardly and inwardly directed binding pockets are reversed in the case of corticosterone. Finally, the model describes an additional conformational state of the empty, outwardly directed binding pocket of rOCT2 that is characterized by a very high affinity to corticosterone but an unchanged affinity to TBuA. This conformation may form slowly (thin arrows) from the other empty, outwardly facing conformation (reaction step 5). The effect of the membrane potential on the structure of the outwardly directed conformation of the binding pocket is not depicted.

The concept of outwardly and inwardly directed binding pockets with various binding domains that may prevail in different affinity states helps us to understand the effects of membrane permeant inhibitors and complex drug-drug interactions at organic cation transporters. Membrane permeant inhibitors such as quinine and corticosterone may lead to a prolonged inhibition that can persist long after the inhibitor has disappeared from the extracellular fluid. For example, methylprednisolone is applied in very high doses during the treatment of multiple sclerosis [infusions of 1 g/day, (O'Connor, 2002)] and may cause prolonged inhibition of human organic cation transporters according to this mechanism. Comedication of a drug that is transported by an organic cation transporter with a nontransported drug that also interacts with the substrate binding pocket may inhibit or stimulate drug transport. The inhibition may be induced by complete or incomplete competition with the transported drug or by allosteric structural changes of binding domains within the substrate binding pocket or in the transport path. Such structural changes could explain the observation that 1-methyl-4-phenylpyridinium transport by rat OCT3 was increased by low concentrations of some other substrates or inhibitors (Gründemann et al., 2002). Our finding that rOCT2 exhibits different affinities for extracellular corticosterone with respect to the *trans*-inhibition of currents induced by intracellular choline, compared with *cis*-inhibition of currents induced by extracellular choline, prompts the

speculation that it is possible to design inhibitors that may be used as selective blockers of either organic cation efflux or influx. For example, such inhibitors could specifically block excretion or reabsorption of cationic drugs in kidney.

Acknowledgments

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