Molecular Pharmacology Fast Forward. Published on January 20, 2005 as doi:10.1124/mol.104.008821
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Subtype-specific Affinity for Corticosterone of Rat Organic Cation Transporters rOCT1 and rOCT2 Depends on Three Amino Acids within the Substrate Binding Region

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Molecular Pharmacology Fast Forward. Published on January 20, 2005 as DOI: 10.1124/mol.104.008821
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Running title:

Amino Acids in the Corticosterone Binding Site of OCTs

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Number of text pages (without title pages, abstract, references and legends): 14

Number of tables: 2

Number of figures: 7

Number of references: 23

Number of words in the abstract: 247

Number of words in the introduction: 454

Number of words in the discussion: 1511

ABBREVIATIONS: OCT, organic cation transporter; OAT, organic anion transporter; MFS, major facilitator superfamily; TMH, transmembrane α-helix; TEA, tetraethylammonium; MPP, 1-methyl-4-phenylpyridinium.

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The affinity of corticosterone to organic cation transporters (OCTs) is subtype and species-dependent. For example, the IC_{50} values for corticosterone inhibition of cation uptake by transporters rOCT1 and OCT2 are ~150 and ~4 μM, respectively. By introducing domains and amino acids from rOCT2 into rOCT1, we found that exchange of three amino acids in the presumed 10^{th} transmembrane α-helix is sufficient to increase the affinity of rOCT1 for corticosterone to that of rOCT2. Replacement of these amino acids in rOCT2 decreased the affinity for corticosterone. These amino acids (A443, L447, Q448 in rOCT1 and I443, Y447, E448 in rOCT2) are probably located within the substrate binding region because in rOCT1 mutants the Michaelis-Menten constant (K_M) values for uptake of tetraethylammonium (TEA) and 1-methyl-4-phenylpyridinium (MPP) were decreased in parallel with a decrease of the IC_{50} values for inhibition of cation uptake by corticosterone. In mutant rOCT1(L447Y/Q448E) the IC_{50} value for the inhibition of [^{3}H]MPP (0.1 μM) uptake by corticosterone (24 $\pm$ 4 μM) was significantly higher compared to the IC_{50} value for inhibition of [^{14}C]TEA (10 μM) uptake (5.3 $\pm$ 1.7 μM). This finding suggests an allosteric interaction between transported cation and corticosterone. Since this substrate-specific effect cannot be explained by differential replacement of corticosterone by MPP vs. TEA, and was observed after point mutations within the presumed substrate region, the data suggest that MPP or TEA bind to the substrate binding region simultaneously with corticosterone and cause a short range allosteric effect on the corticosterone binding site.

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The polyspecific organic cation transporters OCT1-3 (SLC22A1-3) are involved in the elimination and distribution of drugs, environmental toxins and endogenous organic cations including monoamine neurotransmitters (Koepsell et al., 2003; Koepsell, 2004). Together with the organic anion transporters (OATs) and the sodium carnitine cotransporters OCTN1 and OCTN2, the OCTs belong to the SLC22 transporter family which is a member of the major facilitator superfamily MFS (Pao et al., 1998; Koepsell et al., 2003). The three OCT subtypes have overlapping substrate specificities but differ in tissue distribution, regulation, and selectivity for substrates and inhibitors (Koepsell et al., 2003). For example, steroid hormones inhibit organic cation transport by the three OCT subtypes with different affinities showing distinct species difference (Koepsell et al., 2003). Steroids are also involved in the long term regulation of OCT2 but not of OCT1 (Urakami et al., 2000; Shu et al., 2001). For the inhibition of OCTs by corticosterone, half-maximal inhibitory concentration (IC_{50}) values are ~10 μM (human OCT1), ~30 μM (human OCT2), ~0.2 μM (human OCT3 also called EMT), ~150 μM (rat OCT1), ~4 μM (rat OCT2) and ~5μM (rat OCT3) (Zhang et al., 1998; Gründemann et al., 1998; Wu et al., 1998; Arndt et al., 2001; Hayer-Zillgen et al., 2002). Recently we expressed rat OCT2 (rOCT2) in oocytes of *Xenopus laevis* and investigated the inhibition of cation induced inwardly and outwardly directed currents after short term addition (30 sec) of corticosterone to the extracellular or intracellular side of the plasma membrane, respectively (Volk et al., 2003). From both sides corticosterone inhibited cation-induced currents with different affinities and could be partially or totally competed away by transported cations. Our interpretation of these data was that corticosterone binds to inwardly as well as to the outwardly oriented substrate binding site of rOCT2. Since previous point mutations in the 11th transmembrane α-helix (TMH) of rat OCT1 (rOCT1) led to an affinity increase for some but not all transported substrates (Gorboulev et al.,

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1999), we raised the hypothesis that the substrate binding sites of the OCTs contain overlapping interaction domains for structurally different substrates (Gorboulev et al., 1999; Volk et al., 2003).

The aim of the present study was to identify amino acids of rOCT2 that are responsible for the higher affinity of corticosterone (IC_{50} ~ 4 μM) as compared to OCT1 (IC_{50} ~150 μM). We introduced polypeptide stretches of rOCT2 into rOCT1, exchanged amino acids between both transporters, and measured IC_{50} values for inhibition of uptake of tetraethyl-ammonium (TEA) and 1-methyl-4-phenylpyridinium (MPP) by corticosterone as well as K_M values for TEA and MPP. Our data indicate that amino acids A443, L447, and Q448 in the 10th transmembrane α-helix form part of the substrate binding region of OCTs and are critically involved in corticosterone binding.

Experimental Procedures

Construction of Chimeras and Point Mutants. The overlap extension method of polymerase chain reaction (PCR) (Ho et al., 1989) was used to construct chimeric transporters or to introduce point mutations. To generate chimeras, two PCR-fragments of rOCT1 that flank the region to be inserted, and a PCR-fragment of rOCT2 that contains the region to be inserted, were amplified. The 5´ ends of the primers used for the amplification of the rOCT2 fragment contained nucleotide sequences which corresponded to the respective flanking regions of rOCT1. Next, the three PCR products were combined and fused by PCR which yielded the desired rOCT2 fragment flanked with parts of rOCT1. This PCR-amplificate was digested with two unique restriction endonucleases and ligated to the rOCT1/RSSP plasmid (Gründemann et al., 1994). To introduce point mutations, two overlapping fragments of the respective transporter were amplified into which the desired

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point mutation was introduced at overlapping ends. The amplificates were fused by PCR and the product was inserted into the rOCT1/RSSP plasmid. All chimeric and mutant proteins were sequenced partially to confirm the presence of the desired modifications and the absence of any PCR errors.

Expression of rOCT1, OCT2 and mutants in oocytes of *Xenopus laevis*. For injection into *Xenopus* oocytes, m7G(5′)ppp(5′)G-capped cRNAs were prepared from the cDNAs of rOCT1 (Gründemann et al., 1994), rOCT2 (Okuda *et al.*, 1996) and their mutants using the "mMESSAGE mMACHINE" kit (Ambion, Cambridgeshire, UK). The restriction enzymes used for linearization of the respective cDNA vectors and the RNA polymerases used for transcription were described earlier (Arndt et al., 2001). Stage V-VI oocytes were defolliculated with collagenase A and stored for several hours in Ori buffer [5 mM 3-(*N*-morpholino)propanesulfonic acid-NaOH, pH 7.4, 100 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$] supplemented with 50 mg/l gentamicin. The oocytes were injected with 50 nl H_2O/oocyte containing 10 ng of the respective cRNA and incubated for 3-5 days at 16°C in Ori buffer supplemented with 50 mg/l gentamicin.

Tracer Uptake Measurements. *X.* oocytes injected with cRNAs and noninjected control oocytes were incubated for 30 min at room temperature with Ori buffer containing [^{14}C]TEA or [^{3}H]MPP, washed in ice-cold Ori buffer supplemented with 100 μM quinine, and analyzed by liquid scintillation counting (Arndt et al., 2001). Expressed uptake was calculated by substracting the uptake measured in noninjected control oocytes. TEA and MPP uptake in noninjected oocytes was identical to the uptake in oocytes expressing transporter that was measured in the precence of 100 μM quinine or 10 μM cyanine 863 (inhibitors of OCTs). To determine concentration-inhibition curves, we measured the uptake of 10 μM [^{14}C]TEA or 0.1 μM [^{3}H]MPP in the presence of various concentrations of corticosterone or of unlabelled MPP or TEA. For inhibition by corticosterone, oocytes were preincubated for 10 min with corticosterone at the respective concentrations. Cortico-

sterone was first dissolved in ethanol and then added to Ori buffer keeping the final ethanol concentration below 1%. Uptake with 2 mM TEA or 200 μM MPP was taken as maximal transport velocity (V_{max}).

Calculation and Statistics. For each substrate concentration or combination of substrate and inhibitor, uptake rates were calculated from 7-10 oocytes, and uptake in 7-10 noninjected oocytes measured in parallel. From uptake measurements with 8-10 different concentrations of TEA or MPP, the Michaelis-Menten constant (K_M) values were determined by fitting the Michaelis-Menten equation to the data. Half-maximal inhibitory concentrations (IC_{50} values) were determined from uptake with TEA or MPP and 8-12 different concentrations of the nontransported inhibitor corticosterone or competing substrates. IC_{50} values were calculated by fitting the Hill equation for multisite inhibition to the data. To compare inhibition at a given concentration of corticosterone, IC_{50} values, or K_M values between transporters, 3-9 independent experiments were performed and the respective degrees of inhibition, K_M values, and IC_{50} values were calculated from the individual experiments. The data are presented as means $\pm$ SEM. One-way ANOVA with post-hoc Tukey test was used to evaluate differences where indicated. For comparison of two values, unpaired two-sided Student´s *t*-test was employed. Statistical calculations were done with GraphPad 4.0 (San Diego,USA).

Materials. [^{14}C]TEA (1.9 TBq/mmol) and [^{3}H]MPP (3.1 TBq/mmol) were obtained from Biotrend (Köln, Germany). All other chemicals were obtained as described earlier (Gorboulev et al., 1999; Arndt et al., 2001).

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Results

Functional Characterization of Chimeras Containing rOCT1 Backbone with Inserted Domains of rOCT2. We replaced individually 16 successive amino acid stretches by the the respective regions of rOCT2 (Fig. 1a), and tested for transport of 10 μM [^{14}C]TEA (Fig.1b), K_M value for TEA uptake (Fig.1c), and inhibition of TEA uptake by 4 μM corticosterone (Fig.1d). Eight chimeras (N,3,5,6,iL,8,12,C) had uptake rates of 10 μM [^{14}C]TEA similar to wild-type. Six chimeras (1,eL,2,7,9,10) had uptake rates between 13 and 49% of rOCT1 wild-type. With chimera 11 the expressed uptake rate was 4.5% of wild-type, and chimera 4 showed no significant TEA uptake at all (Fig.1b). The low transport activity expressed by chimera 11 and the absence of transport observed with chimera 4 suggest that the exchanged domains exhibit interactions with other domains of rOCT2 wild-type. These interactions may be disturbed when they are inserted into rOCT1 resulting in inactivation or defective membrane targeting (Koepsell et al., 2003). For the other chimeras, we measured the K_M values for TEA uptake (Fig.1c). They were similar to rOCT1 and rOCT2 wild-types in chimeras N,1,eL,2,3,6,iL,8,9,10,12,C and about two times lower in chimeras 5 and 7. The data suggest functional integrity of the chimeras with the exception of chimeras 4 and 11.

For the functionally active chimeras we measured the uptake of 10 μM [^{14}C]TEA with and without 4 μM corticosterone present (Fig. 1d). 4 μM corticosterone represents the previously determined K_i value for the inhibition of rOCT2 (Arndt et al., 2001; Volk et al., 2003). With the exception of chimera 10, 4 μM corticosterone did not significantly inhibit TEA uptake by wild-type rOCT1 or the chimeras. TEA uptake by chimera 10 was inhibited by the same degree as rOCT2 wild-type (63 $\pm$ 6% vs. 66 $\pm$ 6%). The inhibition observed with chimera 11 (34 $\pm$ 5%, n=3) was not statistical significant according to ANOVA with post-hoc Tukey´s test. Further investigations focussed on chimera 10.

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The concentration-dependence for corticosterone inhibition of [^{14}C]TEA (10 μM) uptake by rOCT1, rOCT2, and by chimera 10, confirmed that high affinity for corticosterone was conveyed to rOCT1 by transferring the presumed 10th TMH of rOCT2 to rOCT1 (Fig. 2a, Table 1). The IC_{50} values for corticosterone inhibition of TEA uptake were 198 ± 10 μM for rOCT1 (n=9), 5.9 ± 1.4 μM for rOCT2 (n=5), and 4.5 ± 0.8 μM for chimera 10 (n=4). The IC_{50} values are similar to the respective K_i values because the employed substrate concentration (S) of 10 μM TEA is 7 times (rOCT1, rOCT2) or 5 times lower (chimera 10) as the respective K_M values (see Fig. 1c and Table 2). Assuming competitive inhibition and Michaelis-Menten type substrate dependence the determined IC_{50} values are 13% (rOCT1, rOCT2) or 17% (chimera 10) higher than the respective K_i values ($K_i = IC_{50} / (1 + S/K_M)$).

Corticosterone Inhibition of rOCT1 Mutants Containing Selected Amino Acids from rOCT2. To identify the amino acids that are responsible for the higher affinity of corticosterone in rOCT2 compared to rOCT1, we exchanged individual amino acids in the presumed 10th TMD of rOCT1 for the amino acids in the respective positions of rOCT2. With mutants L447Y and Q448E, we observed significantly lower IC_{50} values for corticosterone inhibition of TEA (10 μM) uptake than with rOCT1 wild-type (Fig. 2b, Table 1). The IC_{50} values obtained with mutants L447Y (42 ± 9) and Q448E (40 ± 11) were, however, still significantly higher than with rOCT2. When both amino acids were replaced simultaneously (rOCT1(L447Y/Q448E)), the IC_{50} value for corticosterone inhibition of TEA uptake decreased further to 5.3 ± 1.7 μM which is significantly lower ($P<0.01$) than in the mutants with a single amino acid exchange but not different from rOCT2 (Fig. 2b, Table 1).

Recent experiments indicated that rOCT2 contains a substrate binding region with overlapping binding sites for structurally different substrates which may be exposed to the extracellular or intracellular side of the plasma membrane, and that corticosterone binds to

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either conformation of the substrate binding region (Gorboulev et al., 1999; Koepsell et al., 2003; Volk et al., 2003). In such a model, substrates and corticosterone may interact within the binding region, either via direct partial replacement or indirectly via short-range allosteric effects.

To investigate putative differential effects of substrates on the affinity of the inhibitor corticosterone, we measured the inhibition of MPP uptake by corticosterone (Fig. 3, Table 1). MPP was selected because it has little structural similarity to TEA. The measurements were performed with 0.1 μM MPP, a concentration that is at least 10times below the respective K_M values of rOCT1, rOCT2, or the mutants (for K_M values see Table 2). At this concentration, a potential replacement of corticosterone by MPP should not increase the IC_{50} by more than 10%. In the measurements employing 10 μM TEA, a replacement of corticosterone by TEA should not increase the IC_{50} values of rOCT1, rOCT2 or rOCT1(L447Y/Q448E) mutant by more than 15% (for K_M values see Table 2). For rOCT1, rOCT2 and chimera 10, the IC_{50} values for inhibition of TEA uptake by corticosterone vs. inhibition of MPP uptake by corticosterone were not significantly different (Figs. 2, 3a, and Table 1). Remarkably, however, with the double mutant rOCT1(L447Y/Q448E) the IC_{50} value of corticosterone for inhibition of MPP uptake was higher than for inhibition of TEA uptake (5.3 $\pm$ 1.7 (n=3) vs. 24 $\pm$ 3.8 (n=4), respectively, $P< 0.01$ for difference). These data suggest that the affinity for corticosterone is modulated differentially by transport and/or binding of TEA or MPP. Since the substrate effects cannot be explained by a differential replacement of corticosterone by each of the two substrates, they imply a different allosteric interaction of TEA vs. MPP with corticosterone.

Trying to reconstitute in rOCT1, the same high affinity inhibition by corticosterone of both TEA and MPP uptake as exhibited by rOCT2, we studied triple mutants of rOCT1 carrying additional amino acids from the 10th TMH of rOCT2 (Fig. 3b, Table 1). In one of these (A443I/L447Y/Q448E) the IC_{50} value for corticosterone inhibition of MPP uptake

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was similar as in rOCT2. In this triple mutant, the IC_{50} values for corticosterone obtained with TEA and MPP were not significantly different from each other.

Interaction of Cationic Substrates with rOCT1-Mutants Exhibiting High Affinity to Corticosterone. We observed previously for rOCT2 that choline-induced currents were inhibited after brief application of corticosterone from either the extracellular or intracellular side of the plasma membrane (Volk et al., 2003), and that the presence of choline prevented inhibition from either side partially or totally. We thus hypothezised that corticosterone binds to the substrate binding region of rOCT2 which can exist in an extracellularly and an intracellularly oriented conformation. To investigate whether corticosterone binds to the substrate binding region we examined whether the rOCT1 point mutants with increased corticosterone affinity also exhibit altered affinities for transported substrates.

We determined the K_M values for MPP and TEA in rOCT1 wild-type and different mutants as well as the IC_{50} values for inhibition of [^{14}C]TEA (10 μM) uptake by MPP and the IC_{50} values for inhibition of [^{3}H]MPP (0.1 μM) uptake by TEA (Figs. 4 and 5, and Table 2). The K_M values measured for MPP uptake were not significantly different from the IC_{50} values measured for inhibition of TEA uptake by MPP, and the K_M values for TEA uptake were not significantly different from the IC_{50} values for inhibition of MPP uptake by TEA (Table 2). Compared to rOCT1 wild-type, the K_M for MPP uptake and the IC_{50} for inhibition of TEA uptake by MPP were significantly decreased in both, the rOCT1(L447Y/Q448E) double mutant and the rOCT1(A443I/L447Y/Q448E) triple mutant. At variance, the K_M values for TEA uptake and the IC_{50} values for inhibition of MPP uptake by TEA were not significantly different between rOCT1 wild-type and rOCT1(L447Y/Q448E). In the rOCT1(A443I/ L447Y/Q448E) triple mutant, however, the K_M value and the IC_{50} value were significantly decreased. Since combined mutation of L447Y, Q448E, and/or A443I leads to increased affinity for corticosterone and for either

TEA or both TEA and MPP, our data suggest that A443, L447 and Q448 are localized within the substrate binding region of rOCT1 and that corticosterone binds to the same region.

We also measured substrate uptake by rOCT1 wild-type, double mutant L447Y/Q448E, and triple mutant A443I/L447Y/Q448E at saturating concentrations of TEA (2 mM) vs. MPP (0.2 mM). The measurements with TEA and MPP were performed simultaneously (within one hour) using identical batches of oocytes and the same cRNA preparations. The ratios between maximal transport velocities for TEA and MPP were: 11.6 $\pm$ 3.5, n=3 (rOCT1); 9.8 $\pm$ 2.2, n=3 (rOCT1(L447Y,Q448E)); and 11.7 $\pm$ 2.6, n=3 (rOCT1(A443I, L447Y, Q448E)). Taken together, the data indicate that in the double mutant rOCT1(L447Y, Q448E) the apparent selectivity for MPP vs. TEA is increased whereas the maximal velocity for MPP vs. TEA is not changed.

Corticosterone Inhibition of rOCT2 Mutants Containing Individual Amino Acids from rOCT1. To confirm the critical role of amino acids I443, Y447, E448 in rOCT2 for the higher corticosterone affinity of rOCT2 vs. rOCT1 we tested whether the high affinity of rOCT2 can be switched to low affinity by replacing these amino acids with the corresponding amino acids of rOCT1. We generated the double mutant rOCT2(Y447L/ E448Q) and the triple mutant rOCT2(I443A/Y447L/ E448Q) and measured the inhibition of MPP uptake by corticosterone (Table 1, Fig.6). In the double mutant rOCT2(Y447L/ E448Q), the IC_{50} value for corticosterone inhibition (IC_{50} = 36 $\pm$ 6.1 μM, n=3) was significantly increased compared to wild-type rOCT2 (5.2 $\pm$ 1.9, n=4, $P<0.01$ for difference). For corticosterone inhibition of MPP uptake expressed by the triple mutant rOCT2 (I443A/Y447L/E448Q) an IC_{50} of 382 $\pm$ 100 μM was determined. This value was not significantly different from rOCT1.

Discussion

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In this work we identified three amino acids in the presumed 10th TMH (A443I, L447Y, Q448E) of rOCT2 which are responsible for the higher affinity of corticosterone compared to rOCT1. We present evidence that these amino acids are located within the substrate region of OCTs by demonstrating that the affinity of transported cations was increased together with the affinity of corticosterone. In one mutant an allosteric interaction between transported substrate and corticosterone was detected suggesting that more than one compound can bind simultaneously to the substrate binding region.

Our interpretation that the identified amino acids are localized within the substrate binding region of rOCT1 or rOCT2, is based on two arguments. Firstly, we consider it highly improbable that a combined mutation of three amino acids within one α-helix (A443, L447 and Q448) can increase the affinity of transported substrates if they are not localized within and/or close to the substrate binding region. Secondly, using the crystal structure of the lactose permease from *Escherichia coli* (Abramson et al., 2003) that belongs to the MFS superfamily like the OCTs (Pao et al., 1998) we modelled the presumed twelve transmembrane domains of rOCT1 (Popp et al., 2005). In this model seven amino acid residues that are critical for substrate binding and/or substrate specificity are localized in one region surrounding a large cleft that is formed by the 1st, 2nd, 4th, 5th, 7th, 8th, 10th, and 11th TMH and are accessible from the aqueous phase. These seven amino acids are D475 in the 11th TMH (Gorboulev et al., 1999), three amino acids on one side of the presumed 4th TMH (W218, Y222 and T226, Popp et al., 2005), and A443, L447 and Q448 that are described in the present paper. In the 3-dimensional model of rOCT1 the 10th α-helix protrudes into the cleft allowing an interaction of corticosterone with the two succeeding amino acids L447 and Q448.

In Fig. 7 we aligned the presumed 10th TMH and parts of the flanking loops of rOCT1, rOCT2, rOCT3 and human OCT3 (hOCT3) and indicated the IC_{50} values that were determined for inhibition of the OCTs by corticosterone (Gründemann et al., 1998; Wu et

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al., 1998; Arndt et al., 2001). G439, E456, L457, Y458, P459, and T460 (rOCT1 numbering) are conserved in nearly all SLC22 members whereas W430, R440, G442, T444, V453, and N454 are conserved within the OCTs. Note, that rOCT3 has about the same affinity for corticosterone as rOCT2. In the three positions critical for the higher affinity of corticosterone to rOCT2 vs. rOCT1, rOCT3 contains the same amino acids (I443, E448) or a similar amino acid as rOCT2 (F443 in rOCT3 vs. Y443 in rOCT2). This supports the critical role of the amino acids in positions 443, 447 and 448 for the affinity of corticosterone. The comparison suggests that the amino acids in positions 432, 433, 435, 437, 441, 449, 451, 455 that are different between rOCT2 and rOCT3 are less critical for corticosterone binding. Note that the amino acid sequence in the 10^{th} TMH and the flanking loops are identical between rOCT3 and hOCT3 whereas the IC_{50} value for corticosterone inibition of cation uptake by hOCT3 vs. rOCT3 is more than twenty times lower. We conclude that corticosterone interacts with a second domain of the OCT transporters and that differences between hOCT3 vs. rOCT3 within this second domain are responsible for the difference in affinity.

Previous short-term inhibition experiments employing electrical measurements with intact oocytes and inside-out oriented giant patches indicated that the substrate binding region of OCTs can be exposed to the extracellular or intracellular side of the plasma membrane (Volk et al., 2003). These experiments showed that the affinities of corticosterone and tetrabutylammonium were different from both sides. Since the inhibition by corticosterone from either side of the plasma membrane was dependent on the membrane potential similar to the K_M values determined for the uptake and efflux of cations (Budiman et al., 2000; Volk et al., 2003), we hypothesized that corticosterone binds to extracellularly and intracellularly oriented conformations of the substate binding region. The inhibition experiments performed in the present study do not allow to distinguish whether the mutations changed the affinity of corticosterone at the extracellular or

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intracellular orientation of the substrate binding region or whether the affinity to both orientations is changed. Since we used a 30 min-incubation period for the uptake measurements in the presence of corticosterone and pre-incubated the oocytes with the respective corticosterone concentrations, corticosterone was equilibrated across the plasma membrane. Most probably, our measurements characterized the corticosterone binding site at the inwardly directed conformation of the substrate binding region because this site has a higher affinity for corticosterone (Arndt et al., 2001; Volk et al., 2003). Allowing equilibration of corticosterone across the plasma membrane we previously measured an IC_{50} value for the inhibition of cation uptake by corticosterone that was identical to IC_{50} value obtained after short application of corticosterone to the intracellular side of the plasma membrane.

The rOCT1(L447Y/Q448E) double mutant exhibited functional properties which indicated allosteric interaction between transported cation (TEA and/or MPP) and the inhibitor corticosterone which requires simultaneous binding of substrate and inhibitor. IC_{50} for the inhibition of TEA uptake by corticosterone was 4.5 times lower than the IC_{50} for the inhibition of MPP uptake (see Table 1). Since the concentration of 10 µM [^{14}C]TEA used for the uptake measurements was ~ 1/7 of the K_M values of rOCT1 wild-type and rOCT1(L447Y/Q448E) (Table 2), and the concentration of 0.1 µM [^{3}H]MPP used for the uptake measurements was ~ 1/50 of the K_M of rOCT1 wild-type and ~ 1/10 of the K_M of rOCT1(L447Y/ Q448E), differences in competitive replacement of corticosterone by TEA and MPP cannot explain the observed differences in the IC_{50} values. Competitive replacement of corticosterone by TEA or MPP would increase the IC_{50} values for inhibition of TEA uptake by about 14% and the IC_{50} values measured with MPP by about 2% (rOCT1 wild-type) or 10% rOCT1(L447Y/ Q448E). In conclusion, the different IC_{50} values determined for corticosterone inhibition of TEA uptake vs. MPP uptake in the rOCT1(L447Y/Q448E) mutant indicate differential allosteric effects of TEA vs. MPP on

corticosterone binding or an allosteric effect by only one of the two substrates. We cannot distinguish whether the allosteric effect is triggered by substrate binding or by substrate dependent conformational change during the transport cycle.

The above described allosteric effect can be due to interaction between substrate binding regions in monomers of a dimeric transporter, to interaction between coexisting substrate binding regions in a monomeric transporter, or to short-range interaction between TEA (and/or MPP) and corticosterone within one substrate binding region. Notwithstanding that additional experiments are necessary to make an unequivocal distinction between these possibilities, we think that a short-range allosteric interaction within one substrate binding region is the most probable explanation. The 3-dimensional model of rOCT1 supports the existence of only one substrate binding region in the rOCT1 monomer and shows that the amino acids in the binding region that are critical for the binding of TEA and corticosterone are distant enough to allow simultaneous binding of both compounds. An allosteric interaction between cation binding to one rOCT1 monomer and corticosterone binding to another rOCT1 monomer in a dimer or oligomer is less probable because the interaction was only observed after a mutation within the binding region of rOCT1 i.e. in the rOCT1(L447Y/Q448E) mutant. In the present study an allosteric effect between two ligands was observed in the rOCT1(L447Y/Q448E) mutant but not in rOCT1 wildtype. This indicates that some cooperation between 447Y and 448E with the rest of the rOCT1 structure was required for the observed allosteric interaction. However, since we observed allosteric interactions of MPP and other substrates also in wildtypes of human OCT1 and human OCT3 (unpublished data) we interprete the allosteric interaction observed in the rOCT1(L447Y/Q448E) mutant as demonstration of principle i.e. that ligands of OCT binding regions can exhibit allosteric interactions.

Mapping the surface of substrate binding regions in OCTs by crystallisation of ligand transporter complexes and by characterization of point mutations will help to design drugs

that are transported by specific OCT subtypes, inhibit specific OCTs, or do not interact with them. This will allow to influence absorption of drugs in small intestine and their renal and hepatic excretion and thereby modulate physiological functions that are controlled by the OCTs. The interaction of glucocorticoids with OCTs can be of clinical importance. For example, OCT1 and OCT2 are responsible for the luminal release of acetylcholine from bronchial epithelia in the lung and aerosols containing the glucocorticoids budesonide probably inhibit this function (Lips et al., 2004). The interaction of corticosteroids with hOCT3 may be most relevant because this transporter has the highest affinity to corticosterone among the OCTs. Like other OCTs, hOCT3 translocates monoamine neurotransmitters. Among many others, hOCT3 is expressed in smooth muscle cells of blood vessels and neurons throughout the brain (Slitt et al., 2002; Schmitt et al., 2003; Horvath et al., 2003; Vialou et al., 2004). Inhibition of hOCT3 may lead to an increase of blood pressure and to alterations in behaviour (Vialou et al., 2004).

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Molecular Pharmacology Fast Forward. Published on January 20, 2005 as DOI: 10.1124/mol.104.008821
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Footnotes

This study was supported by Deutsche Forschungsgemeinschaft SFB 487/A4.

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Molecular Pharmacology Fast Forward. Published on January 20, 2005 as DOI: 10.1124/mol.104.008821
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LEGENDS TO THE FIGURES

Fig. 1. Identification of a domain in rOCT2 that is responsible for higher affinity for corticosterone of rOCT2 vs. rOCT1. a, Schematic representation of polypeptide regions that were replaced by the respective domains of rOCT2 to rOCT1 (N, 1-20; 1, 21-42; eL, 43-149; 2, 150-173; 3, 174-198; 4, 199-238; 5, 239-264; 6, 265-283; iL, 284-347; 7, 348-377; 8, 378-403; 9, 404-426; 10, 427-463; 11, 464-487; 12, 488-516; C, 517-556). b, Uptake rates of 10 μM [^{14}C]TEA in oocytes expressing rOCT1 or rOCT1 with inserted domains of rOCT2. c, Apparent K_M values measured for TEA uptake by rOCT1, by rOCT1 with inserted domains of rOCT2, and by rOCT2. d, Inhibition of TEA uptake by 4 μM corticosterone. Uptake of 10 μM [^{14}C]TEA was measured in the absence and presence of 4 μM corticosterone and the relative inhibition was calculated. Mean ± SEM are shown. The numbers of the performed experiments are indicated in parentheses. **P<0.01, ***P<0.001, ANOVA with post-hoc Tukey´s test for difference to rOCT1 wild-type. The data indicate that domain 10 of OCTs is important for corticosterone affinity.

Fig. 2. Inhibition of TEA uptake by corticosterone in oocytes expressing OCT wild-types and mutants. a, Concentration-inhibition curves in oocytes expressing rOCT1, rOCT2, or rOCT1 carrying the presumed 10th TMH of rOCT2 (chimera 10, see Fig.1a). b, Concentration-inhibition curves in oocytes expressing rOCT1, rOCT2 or rOCT1 mutants in which one or two amino acids in the 10th TMH were replaced by the corresponding amino acids of rOCT2. Expressed uptake of 10 μM [^{14}C]TEA was measured in the presence of corticosterone at the indicated concentrations. Mean ± SEM of typical experiments with 7-10 oocytes. The curves were obtained by fitting the Hill equation to the data. The data indicate that the replacement of two amino acids (L447, Q448) of rOCT1 by the corresponding amino acids of rOCT2 is sufficent to increase the affinity of corticosterone for inhibition of TEA uptake to the level of rOCT2.

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Fig. 3. Inhibition of MPP uptake by corticosterone in oocytes expressing OCT wild-types and mutants. a, Concentration-inhibition curves in oocytes expressing rOCT1, rOCT2, or chimera 10. b, Concentration-inhibition curves in oocytes expressing rOCT1 or mutants of rOCT1 in which the indicated amino acids in the presumed 10th TMH were replaced by the corresponding amino acids of rOCT2. The experiments were performed and are presented as in Fig. 2 with the exception that the transported substrate was 0.1 μM [^{3}H]MPP. The data indicate that the replacement of three amino acids of rOCT1 (A443, L447, and Q448) by the corresponding amino acids of rOCT2 is required to increase the affinity of corticosterone for inhibition of MPP uptake to the level of rOCT2.

Fig. 4. Substrate dependence of MPP and TEA uptake. rOCT1 wild-type, the double mutant rOCT1(L447Y/Q448E), and the triple mutant rOCT1(A443I/L447Y/Q448E) were expressed in oocytes, and uptake rates of [^{14}C]TEA (a) or [^{3}H]MPP (b) were measured in the presence of substrates at various concentrations. Typical experiments are shown. Data points represent mean $\pm$ SEM of cation uptake measured in 8-10 oocytes expressing the indicated transporter which was corrected for cation uptake measured in 8-10 noninjected control oocytes. The curves were obtained by fitting the Michaelis-Menten equation to the data. The K_M values for TEA uptake in the double mutant rOCT1(L447Y/Q448E) and rOCT1 wild-type were similar, however, they were higher compared to the triple mutant rOCT1(A443I/L447Y/Q448E). At variance, the K_M values for MPP uptake in the double and the triple mutant were similar. The K_M values for MPP uptake in the mutants were lower compared to rOCT1 wild-type.

Fig. 5. Inhibition of MPP uptake by TEA and of TEA uptake by MPP. a, Uptake rates of 0.1 μM [^{3}H]MPP in the presence of various concentrations of TEA. b, Uptake rates of 10

μM [^{14}C]TEA in the presence of various concentrations of MPP. Measurements and data presentation as in Fig. 2. The IC_{50} values for inhibition of MPP uptake by TEA in rOCT1 wild-type and the double mutant were similar. They were higher compared to triple mutant. At variance, the IC_{50} values for inhibition of TEA uptake by MPP were similar in the double and triple mutant. The IC_{50} values of both mutants were lower compared to rOCT1 wild-type.

Fig. 6. Inhibition of MPP uptake by rOCT2 wild-type and mutants containing amino acids of rOCT1. Uptake of 0.1 μM [^{3}H]MPP in the presence of various concentrations of corticosterone. Experiments and data presentation as in Fig. 2. The data show that the transformation between low and high corticosterone affinity by the exchange of three amino acids in positions 443, 447 and 448 between rOCT1 and rOCT2 works in both directions.

Fig. 7. Alignment of the 10th transmembrane α-helix (TMH10) and parts of the flanking loops of organic cation transporters that exhibit different affinities to corticosterone. The sequences of OCT subtypes from rat and OCT3 from human (hOCT3) are aligned. IC_{50} values for inhibition of cation transport by corticosterone are indicated (Gründemann et al., 1998; Wu et al., 1998; Arndt et al., 2001). Amino acids that are conserved between rOCT1 and at least one of the other transporters are indicated in bold face, similar amino acids are shadowed, amino acids that are conserved throughout the SLC22 family are indicated by (×), amino acids that are conserved throughout the organic cation transporters are indicated by (○). The three amino acids in rOCT1 and rOCT2 that determine the difference in affinity to corticosterone between these two subtypes are indicated by arrows.

Molecular Pharmacology Fast Forward. Published on January 20, 2005 as DOI: 10.1124/mol.104.008821
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TABLE 1

Comparison of IC_{50} values for corticosterone inhibition of TEA and MPP uptake expressed by rOCT1, rOCT2 and mutants.

Uptake of 10 µM [^{14}C]TEA or 0.1 µM [^{3}H]MPP by the indicated transporters and mutants was measured in the presence of 8-10 different corticosterone concentrations. IC_{50} values are indicated that were calculated by fitting the Hill equation to the data (mean ± SEM from 3-9 independent experiments). Hill coefficients for inhibition of TEA uptake by corticosterone were not significantly different from 1. Hill coefficients for inhibition of MPP uptake by corticosterone were 1.1 ± 0.3 (rOCT1), 0.6 ± 0.1 (rOCT2), 1.0 ± 0.2 (rOCT1-ch10), 2.2 ± 0.3 (rOCT1-L447Y/Q448E), 1.1 ± 0.1 (rOCT1-A443I/L447Y/Q448E), 1.5 ± 0.1 (rOCT2-Y447L/E448Q), and 1.5 ± 0.1 (rOCT2-I443A,Y447L,E448Q). ***P<0.001, ANOVA, difference compared to rOCT1 using TEA or MPP as substrate; ●●P<0.01, ANOVA difference compared to rOCT2 using TEA or MPP as substrate; □□P<0.01, ANOVA, difference to rOCT1(L447Y/Q448E) using TEA as substrate; ■■P<0.01, ■■■P<0.001, ANOVA difference to rOCT1(L447Y/Q448E) using MPP as substrate; ○○P<0.01, ANOVA, difference between corticosterone inhibition of TEA uptake vs. corticosterone inhibition of MPP uptake by rOCT1(L447Y/Q448E), ΔΔP<0.01, Student´s *t*-, difference to rOCT2(Y447L/E448Q) using MPP as substrate .

	IC_{50} for inhibition of transport by corticosterone [µM]	
transporter	inhibition of TEA uptake	inhibition of MPP uptake
rOCT1	198 ± 10 (9)	174 ± 23 (5)
rOCT2	5.9 ± 1.4 (5) ***	5.2 ± 1.9 (4) ***, ■■■
rOCT1 (ch10)	4.5 ± 0.8 (4) ***	4.8 ± 3.3 (3) ***, ■■■
rOCT1 (L447Y)	42 ± 9 (4) ***, ●●, ¶¶	
rOCT1 (Q448E)	40 ± 11 (4) ***, ●●, ¶¶	
rOCT1 (L447Y/Q448E)	5.3 ± 1.7 (3) ***, ○○	24 ± 3.8 (4) ***, ●●
rOCT1 (A443I/L447Y/Q448E)	7.5 ± 1.4 (3) ***	8.6 ± 1.4 (4) ***, ■■
rOCT2 (Y447L/E448Q)		36 ± 6.1 (3) ●●
rOCT2 (I443A/Y447L/E448Q)		382 ± 100 (3) ●●, ΔΔ

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TABLE 2

Comparison of K_M values for TEA uptake, K_m values for MPP uptake, IC_{50} values for inhibition of MPP uptake by TEA, and IC_{50} values for inhibition of TEA uptake by MPP between rOCT1 wild-type and mutants of rOCT1. The transporters were expressed in *Xenopus* oocytes and uptake was measured at various concentrations of TEA or MPP. In addition, uptake of 0.1 µM [^{3}H]MPP was measured in the presence of various concentrations of TEA, uptake of 10 µM [^{14}C]TEA was measured in the presence of various concentrations of MPP. K_M and IC_{50} values were determined by fitting the Michaelis-Menten equation or the Hill equation to the data, respectively. Hill coefficient varied between 0.75 and 1.25 but was not significantly different between different groups. Mean ± SEM from 3-7 independent experiments are indicated. *P<0.05, **P<0.01, ANOVA, for difference to rOCT1 wild-type; •P<0.05, ANOVA, for difference to rOCT1(L447Y/Q448E).

K_M or IC_{50} [µM]	rOCT1		
	wildtype	L447Y/Q448E	A443I/L447Y/Q448E
K_M for TEA uptake	75 ± 11 (7)	72 ± 6 (3)	28 ± 3 (3) *,•
IC_{50} for inhibition of MPP uptake by TEA	115 ± 18 (4)	97 ± 21 (4)	26 ± 8 (4) **,•
K_M of MPP uptake	5.6 ± 1.0 (6)	1.1 ± 0.1 (3)*	1.1 ± 0.2 (3) *
IC_{50} for inhibition of TEA uptake by MPP	9.2 ± 1.7 (4)	0.9 ± 0.1 (3)**	0.6 ± 0.2 (3) *

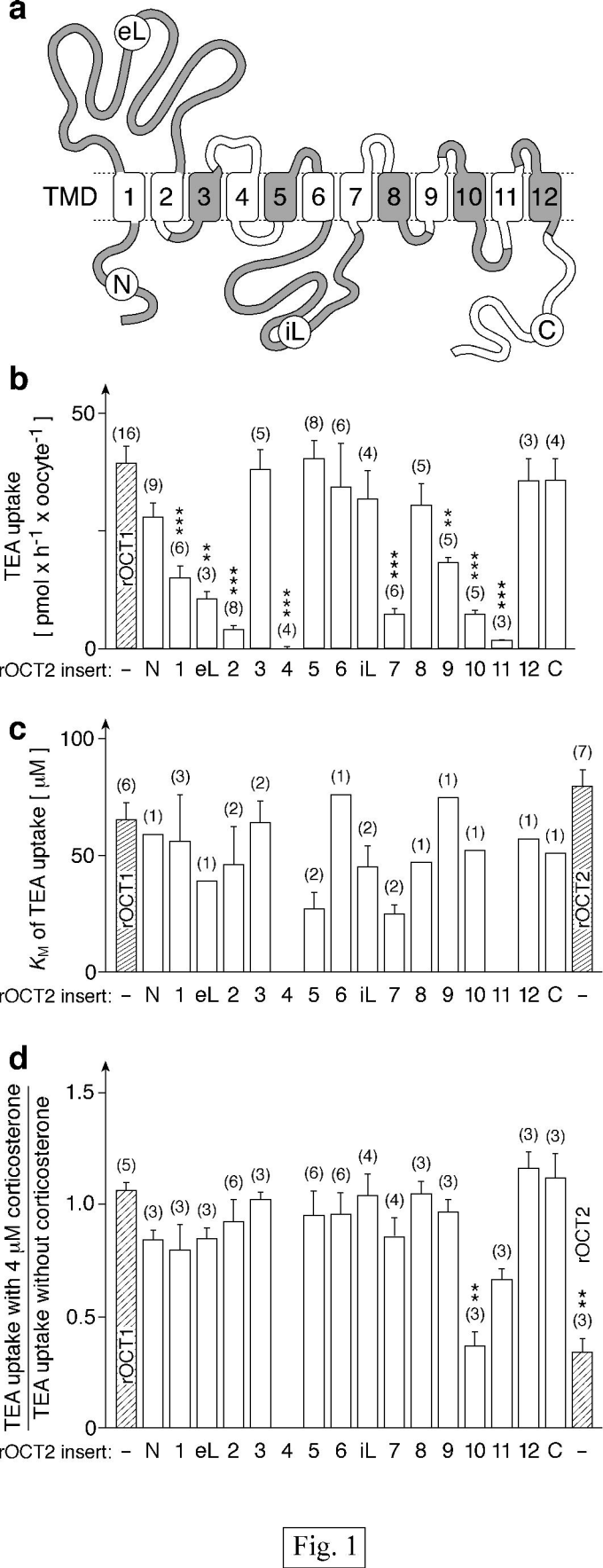
a
eL
TMD
1
2
3
4
5
6
7
8
9
10
11
12
N
iL
C
b
TEA uptake
[pmol x h-1 x oocyte-1]
50
0
rOCT1
rOCT2 insert: – N 1 eL 2 3 4 5 6 iL 7 8 9 10 11 12 C
c
KM of TEA uptake [µM]
100
50
0
rOCT1
rOCT2
rOCT2 insert: – N 1 eL 2 3 4 5 6 iL 7 8 9 10 11 12 C –
d
TEA uptake with 4 µM corticosterone
TEA uptake without corticosterone
1.5
1.0
0.5
0
rOCT1
rOCT2
rOCT2 insert: – N 1 eL 2 3 4 5 6 iL 7 8 9 10 11 12 C –

Fig. 1

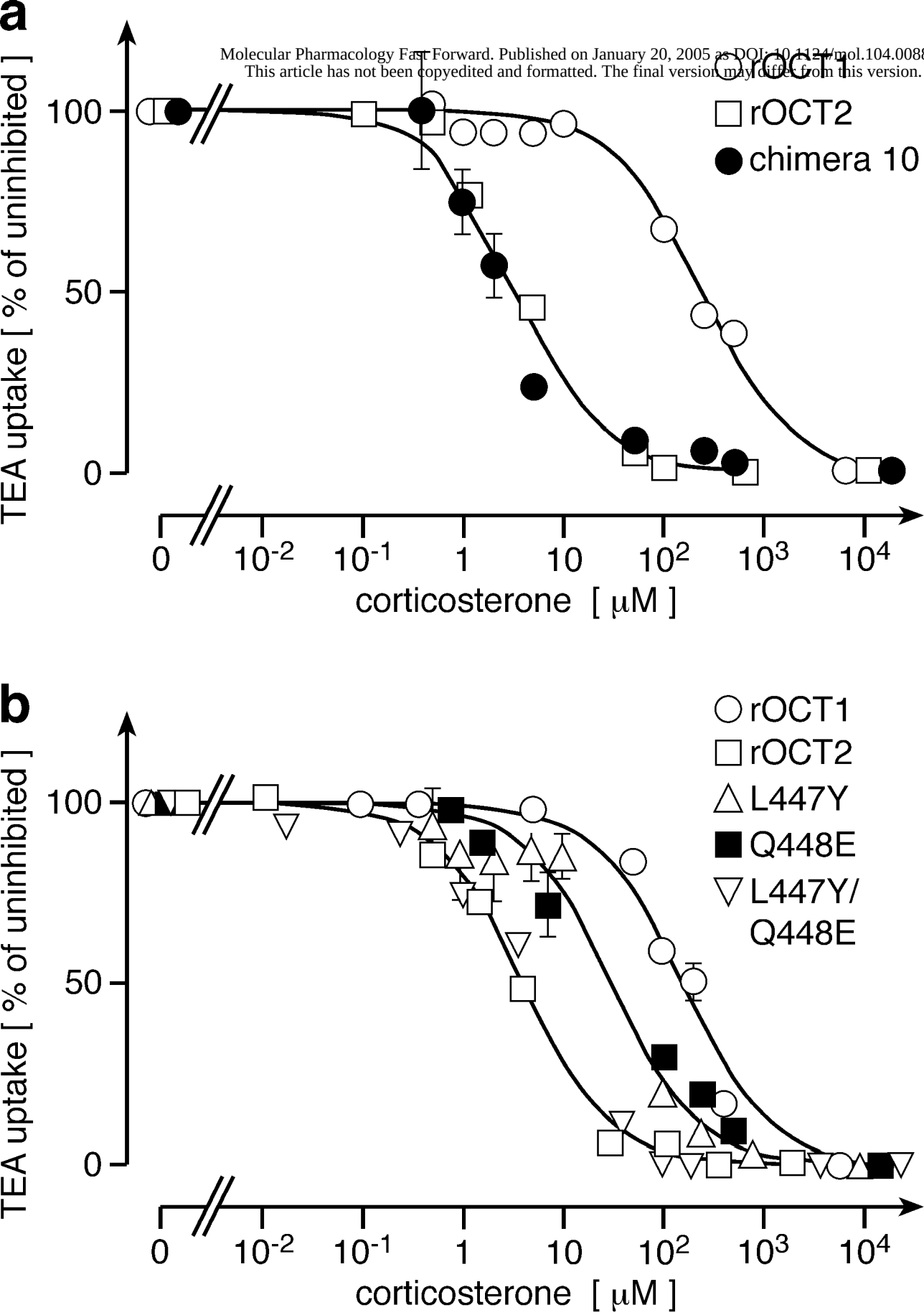

Fig. 2

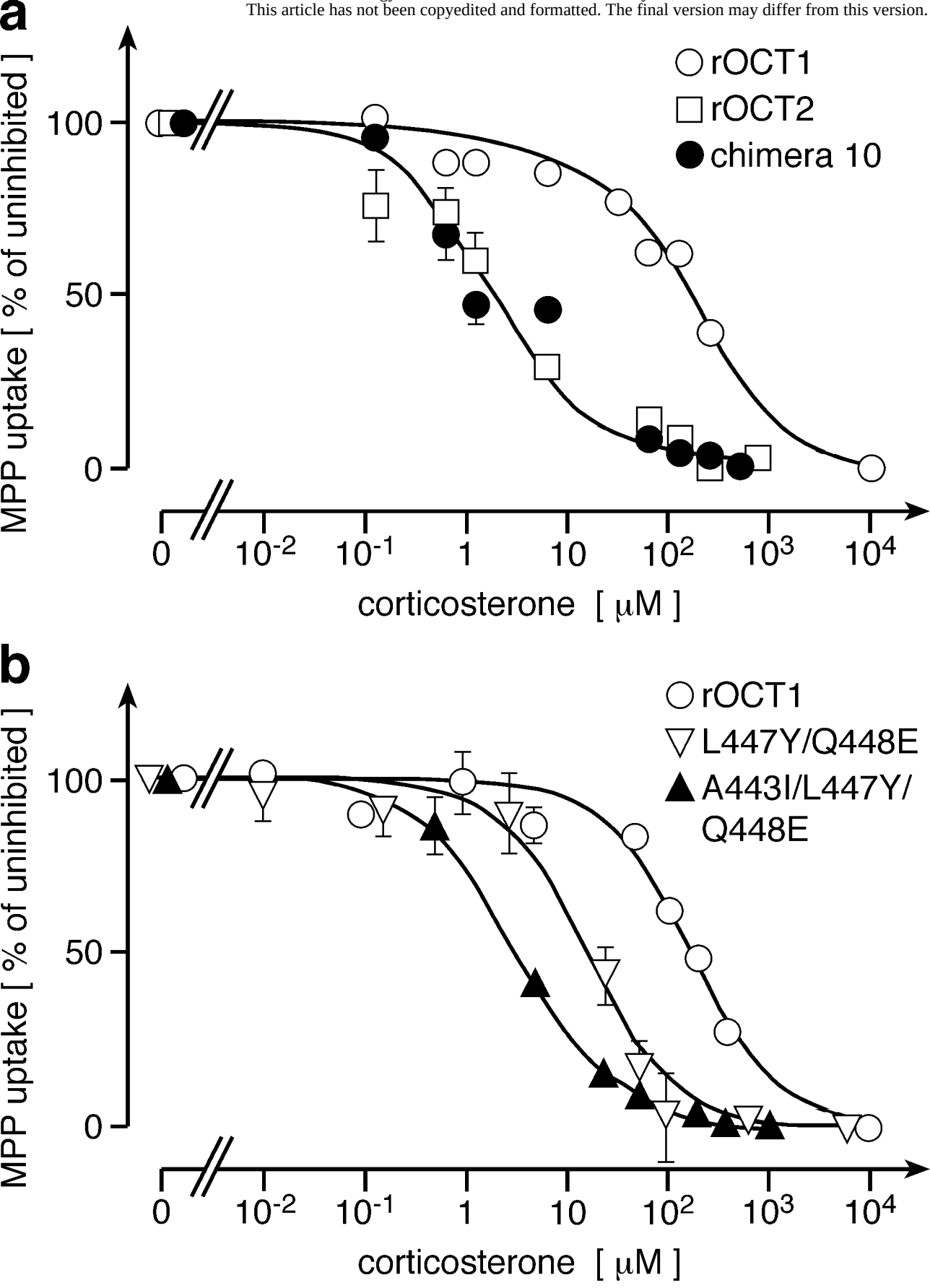

Fig. 3

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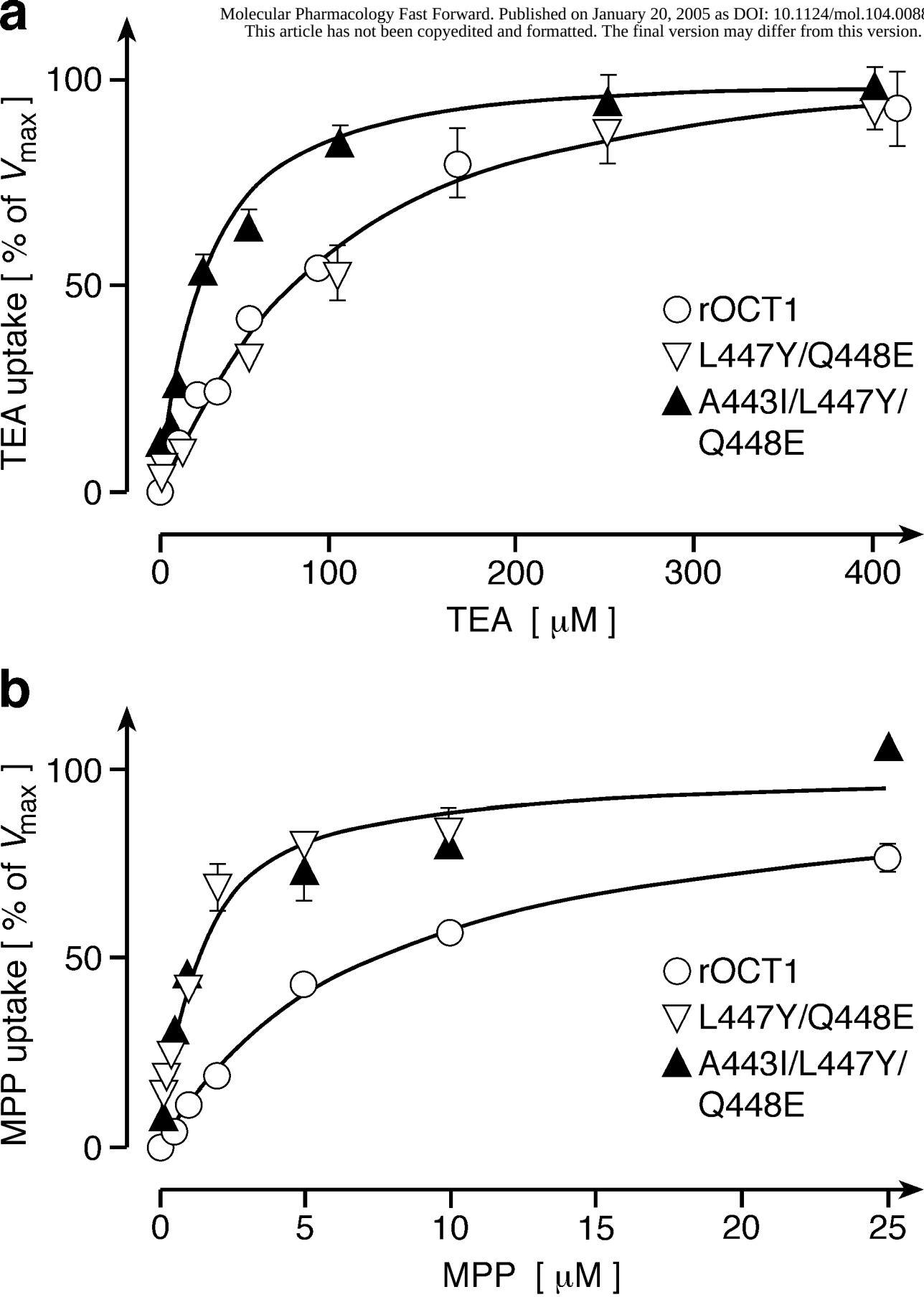

Fig. 4

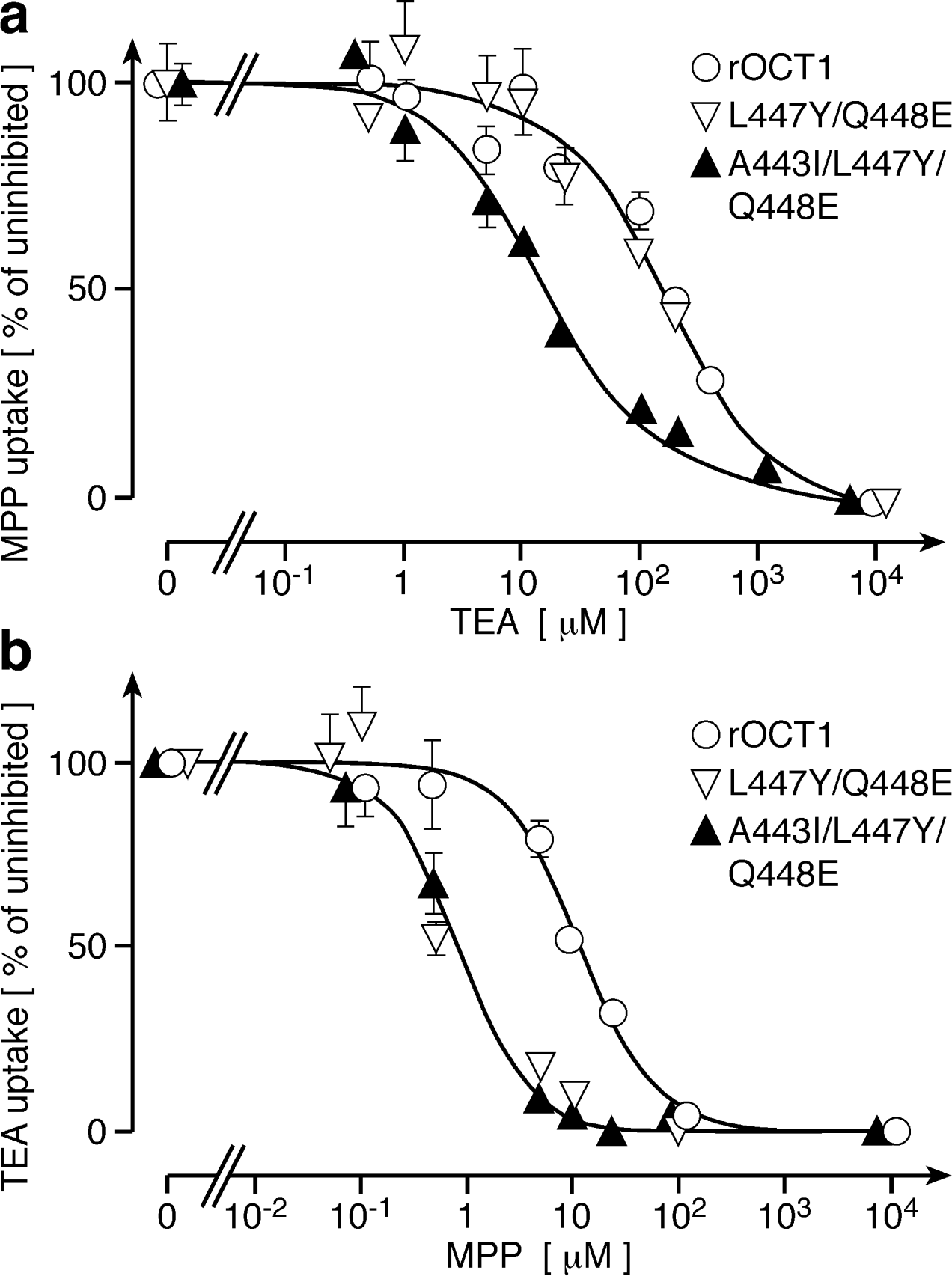
a
MPP uptake [% of uninhibited]
100
50
0
rOCT1
L447Y/Q448E
A443I/L447Y/
Q448E
0
10-1
1
10
102
103
104
TEA [μM]
b
TEA uptake [% of uninhibited]
100
50
0
rOCT1
L447Y/Q448E
A443I/L447Y/
Q448E
0
10-2
10-1
1
10
102
103
104
MPP [μM]

Fig. 5

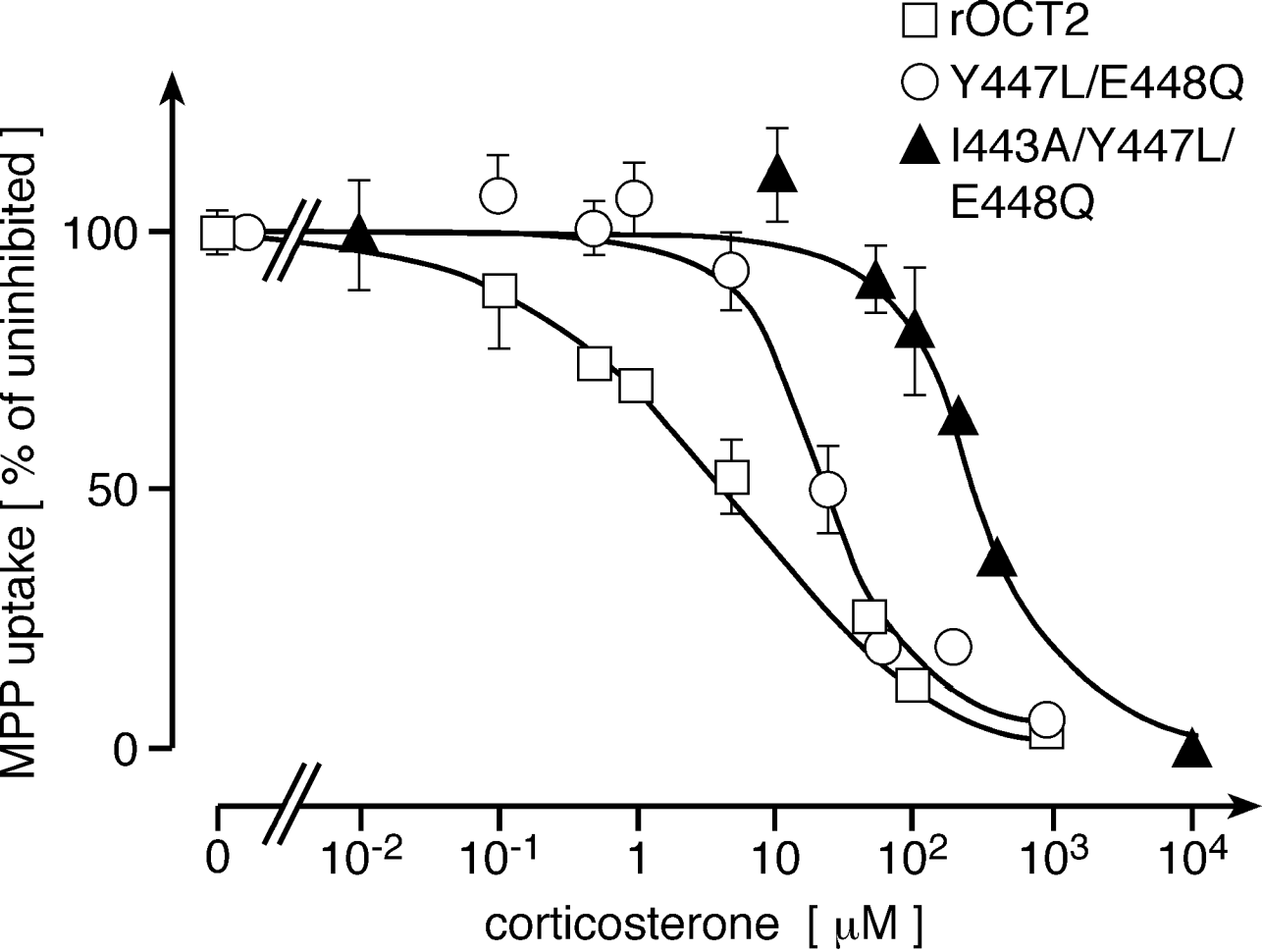
rOCT2
Y447L/E448Q
I443A/Y447L/
E448Q
MPP uptake [% of uninhibited]
100
50
0
0
10-2
10-1
1
10
102
103
104
corticosterone [μM]

Fig. 6

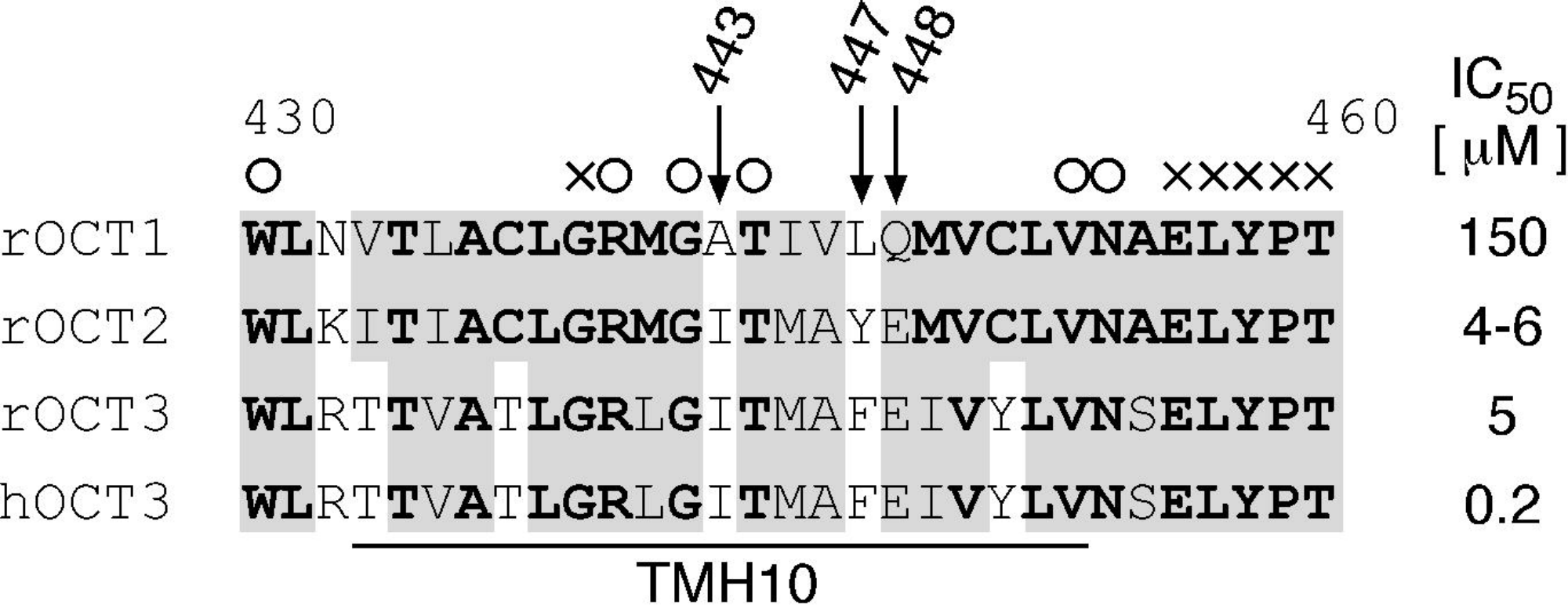
443
447
448
430
460
IC50
[μM]
rOCT1 WLNVTLACLGRMGATIVLQMVCLVNAELYPT 150
rOCT2 WLKITIACLGRMGITMAYEMVCLVNAELYPT 4-6
rOCT3 WLRTTVATLGRLGITMAFEIVYLVNSELYPT 5
hOCT3 WLRTTVATLGRLGITMAFEIVYLVNSELYPT 0.2
TMH10

Fig. 7