Identification of amino acids within the substrate binding region of organic cation transporters (OCTs) that are involved in binding of corticosterone.

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1. Introduction.

Transporters are essential tools that maintain the life and adapt the living beings to changes in the environment. They supply cells with nutrients and ions and thus influence their metabolism. The end products of metabolism are removed from cells by transporters as well. Transporters in liver and in kidney are critical in the detoxification and elimination of xenobiotics from the systemic circulation, and thus are major determinants of drug response and sensitivity. Their malfunction results in diseases, for example, cystinuria, which may lead to death. Because of their critical function, they are often the targets of therapeutic intervention; in some cases, they are responsible for the difficulties encountered in cancer chemotherapy and resistance of microorganisms to antibiotics (Ambudkar et al. 2003, Paulsen 2003). Renal excretion is the principal pathway for elimination of many clinically used drugs and is the exclusive pathway for eliminating many end products of drug-metabolizing enzymes (Leabman et al., 2002; Pritchard et al., 1993; Koepsell et al 2003; Wright et al., 2004). A large fraction of these agents fall into the chemical class commonly referred to as organic cations (OCs), that is, a diverse array of primary, secondary, tertiary, or quaternary amines that have a net positive charge on the amine nitrogen at physiological pH. Although a number of endogenous OCs have been shown to be actively secreted by the proximal tubule (e.g., N1-methylnicotinamide, choline, epinephrine, and dopamine), it is generally accepted that the principal function of this process is clearing the body of xenobiotic agents (Dantzler and Wright, 1997; Pritchard and Miller, 1993; Wright and Dantzler, 2004), including a wide range of alkaloids and other positively charged, heterocyclic compounds of dietary origin; cationic drugs of therapeutic or recreational use; or other cationic toxins of environmental origin (e.g., nicotine).

Transport of organic cations has been studied for more than forty years employing various approaches, including transport measurements in intact animals, isolated organs, tissue slices, perfused renal tubules, and isolated plasma membrane vesicles (Eisenhofer 2001; Elferink et al. 1995; Graefe et al.1988; Roch-Ramel et al. 1992;Turnheim and Lauterbach 1977; Ullrich 1994). The development of molecular biology techniques such as expression cloning allowed the identification and characterization of numerous organic cation transporters over the last twelve years.

1.1 Transporters of SLC22 family.

Transport proteins, an important class of integral membrane proteins, are classified into two large subsets. One subset - transporters of the SLC-group (Koepsell and Endou 2004) - uses the energy of an electrochemical potential of substrate(s). SLC transporters may act as uniporters, symporters or antiporters. Another subset of transport proteins [P-type adenosine triphosphatases (ATPases) and ABC transporters] uses the energy released from ATP hydrolysis to drive solute accumulation or efflux. In contrast, channel proteins — a third important class of proteins associated with transport across the membrane — do not transduce energy but function as selective pores that often open in response to a specific stimulus, allowing movement of solute down an electrochemical ion gradient (Miller 2000).

The MSF superfamily of the SLC transporter group represents the largest group of ion-coupled transporters (Saier 2000). Despite intense interest in these proteins and a large number of laboratories experimenting with several of them, structural information at the atomic level was not available until very recently. The MFS was originally believed to function primarily in the uptake of sugars (Henderson and Maiden 1990).

Pao et al. summarized the properties of transport protein families found within the current MFS. They have classified members of the MFS into 18 distinct families, between them: sugar porters (that include OCTs); drug:H^+ antiporters; organophosphate:P_i antiporters; oligosaccharide:H^+ simporters; metabolite H^+ simporters and so on (Pao et al 1998).

In 1994, our laboratory identified the first polyspecific organic cation transporter rOCT1 from rat kidney by expression cloning (Gründemann et al. 1994). Subsequent expression cloning of the first organic anion transporter OAT1 (SLC22A6) from rat and flounder (Sekine et al. 1997; Sweet et al. 1997; Wolff et al. 1997) and homology cloning of further family members revealed that rOCT1 was the first prototypical member of a large transporter family within the major facilitator superfamily (Pao et al. 1998; Koepsell et al. 2004). The family is named SLC22 (official gene symbol assigned by the Human Genome Nomenclature Committee) and includes three distinct subfamilies: transporters for organic cations (OCT), carnitine transporters (OCTN) and transporters for organic anions (OAT).

The OCT family is now represented by at least 12 distinct homologous transport proteins that, based upon phylogeny, can be organized into several evolutionarily distinct lineages (Fig. 1), including the OCTs (incl. hOCT1-3), the OCTNs (organic cation transporters-novel; incl. hOCTN1-2) and the OATs (organic anion transporters; incl. hOAT1-4).

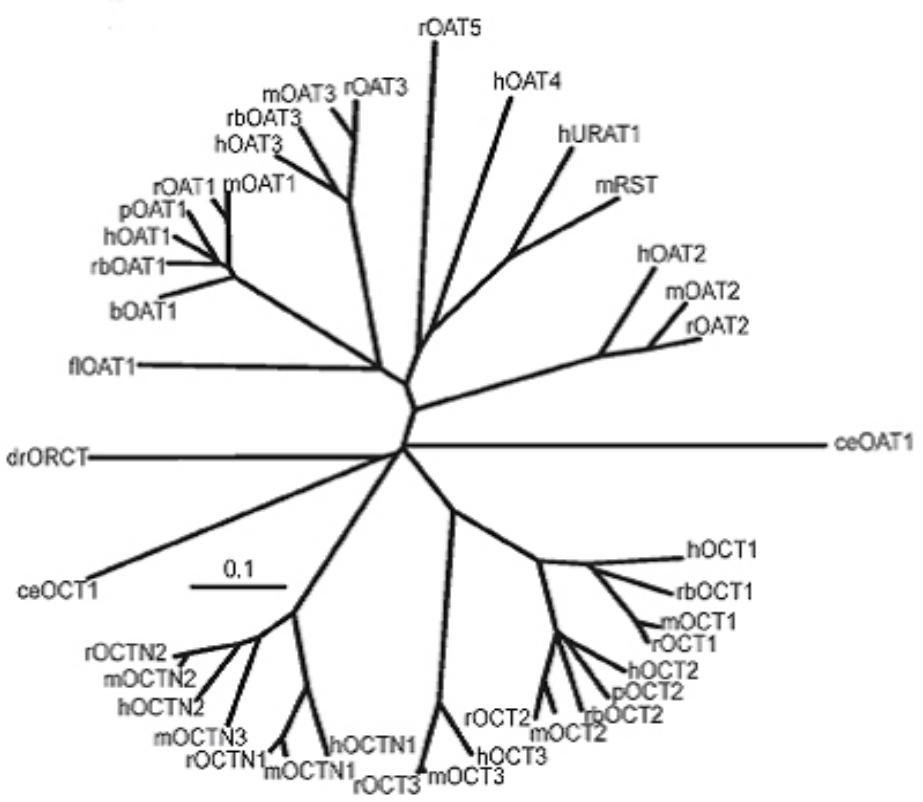

Figure 1. Phylogenetic tree of the human transporters that belong to the SLC22 family. Distance along the branches is inversely related to the degree of sequence identity. Picture is taken from (Wright S. and Dantzler W., 2004).

Organic cations are transported by three electrogenic organic cation transporter subtypes OCT1, OCT2, and OCT3 (Gorboulev et al. 1997; Gründemann et al. 1994, 1997, 1998a; Kekuda et al. 1998; Mooslehner and Allen 1999; Okuda et al. 1996; Schweifer and Barlow 1996; Terashita et al. 1998; Zhang et al. 1997) and by the transporters OCTN1 and OCTN2 (Sekine et al. 1998; Tamai et al. 1997, 1998, 2000; Wu et al. 1998a, 2000a). A large group of transporters within the SLC22 family is engaged mainly in organic anion transport: Six organic anion transporters have been identified in humans, comprising OAT1–OAT5 and URAT1 (Cha et al. 2000, 2001; Enomoto et al. 2002; Youngblood et al. 2004; Hosoyamada et al. 1999; Reid et al. 1998). Flipt1, hUST3,

OCTL1, and OCTL2 are gene products with unknown function (Eraly and Nigam 2002; Nishiwaki et al. 1998; Sun et al. 2001).With the exception of splice variants (Bahn et al. 2000; Urakami et al. 2002; Zhang et al. 1997b), all members of the SLC22 family are approximately 550- 560 amino acids in length and, by hydropathy analysis, have 12 presumed transmembrane-spanning domains (TMDs) and two large hydrophilic loops. The N- and C-termini are cytoplasmic (Meyer-Wentrup et al., 1998), and there is a long (extracellular) loop between TMDs 1 and 2 and a long (cytoplasmic) loop between TMDs 6 and 7 (see Fig. 2).

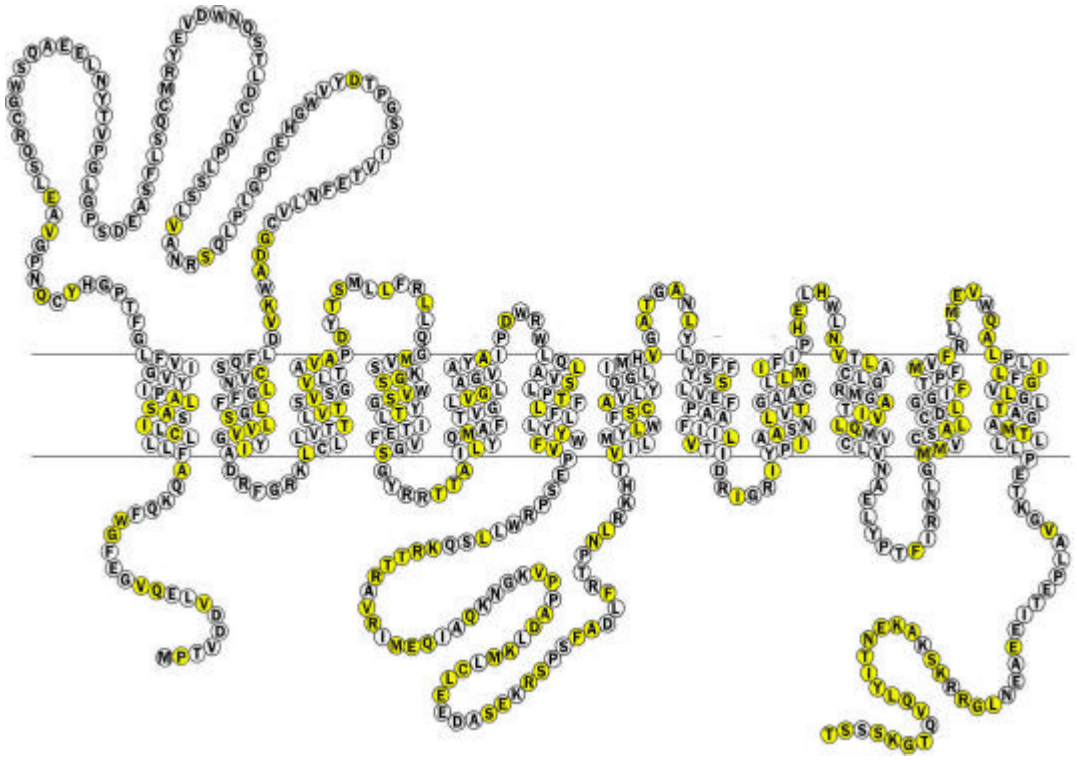

Figure 2. The presumed topology of transporters of SLC22 family on example of rOCT1. Amino acids which are different between rOCT1 and rOCT2 are highlighted in yellow.

The three subtypes of polyspecific electrogenic cation transporters, OCT1, OCT2, and OCT3, have been isolated from rat (Gründemann et al. 1994; Kekuda et al. 1998; Okuda et al. 1996), mouse (Mooslehner and Allen 1999; Schweifer and Barlow 1996; and Gen-Bank accession no. AF078750), and human (Gorboulev et al. 1997; Gründemann et al.1998a; Zhang et al. 1997a). In addition, OCT1 was cloned from rabbit (Terashita et al.1998), and OCT2 from rabbit and pig (Gründemann et al. 1997; Zhang et al. 2002). In human, the genes coding for OCT1, OCT2, and OCT3 are localized within a

cluster on chromosome 6 (q26–27) (Gründemann and Schömig 2000; Gründemann et al. 1998a; Koehler et al. 1997; Eraly et al. 2002). Each of the three genes comprises 11 exons and 10 introns (Gründemann and Schömig 2000; Hayer et al. 1999; Verhaagh et al. 1999). On the protein level, individual transporter subtypes from human, mouse, and rat exhibit cross-species identities of 78–95% (OCT1), 81–91% (OCT2) and 87–93% (OCT3). Within a given species, the amino acid identities between different subtypes are 67–70% between OCT1 and OCT2, 47–57% between OCT1 and OCT3, and 49–51% between OCT2 and OCT3. Splice variants have been identified for OCT1 from rat and human (Hayer et al. 1999; Zhang et al. 1997b) and for human OCT2 (Urakami et al. 2002). One splice variant of rat OCT1, rOCT1A (Zhang et al. 1997b), lacks the first two transmembrane helices and the large extracellular loop that connects them. When expressed in Xenopus oocytes, rOCT1A exhibited TEA uptake with a Michaelis—Menten constant (Km) value of 42 µM that was similar to the Km of wild-type rOCT1 (95 µM, Gründemann et al. 1994), albeit at ~10% of the wild type's maximal transport rate. Four splice variants of human OCT1 showed no activity (Hayer et al. 1999), whereas hOCT2A, a splice variant of human OCT2, has a truncated C-terminus lacking the last three proposed transmembrane domains, transported TEA with ~5% of the wild type's maximal rate, but revealed at higher affinity for a variety of cations (Urakami et al. 2002).

1.2 Functional characteristics of OCT transporters.

Cation transport by OCTs has been investigated in several heterologous expression systems including *Xenopus laevis* oocytes, human embryonic kidney (HEK) 293 cells, HeLa cells, MDCK cells, chinese hamster ovary (CHO-K1) cells, human retinal pigmented epithelium (HRPE) cells, Sf9 insect cells, and others (Koepsell et al. 2003). Expressed transport was determined as uptake of radioactively labelled compounds, as electrical current across the entire oocyte membrane in the two electrode voltage-clamp configuration, or as current across giant patches excised from the oocyte plasma membrane in the patch clamp configuration (Budiman et al. 2000, Volk et al. 2003), and as a cytoplasmic fluorescence change elicited by uptake of a fluorescent substrate (Ciarimboli et al. 2004; Mehrens et al. 2000; Pietig et al. 2001). The apparent Km values for substrates and the apparent Ki values for inhibitors were largely

independent from the employed expression system. In contrast, absolute transport rates differed largely between laboratories. Since the affinities for substrates and inhibitors are dependent on membrane potential and regulatory state of the transporter (Mehrens et al. 2000), different experimental conditions are likely to cause a certain variability of these parameters. In this respect, two-electrode voltage-clamp and patch-clamp approaches have important advantages: because the membrane potential in these experiments is not only known, but also controlled efficiently (usually to -50 mV), this source of variations can be eliminated making the kinetic parameters obtained in such experiments more reproducible.

Several properties are common to all OCTs and independent from subtype or species:

1. OCTs translocate a variety of organic cations with widely differing molecular structures, and are inhibited by other, nontransported compounds

2. OCTs translocate organic cations in an electrogenic manner. Electrogenicity of transport has been shown for the rat transporters rOCT1, rOCT2, and rOCT3 (Arndt et al. 2001; Busch et al. 1996a; Gründemann et al. 1994; Kekuda et al. 1998; Nagel et al. 1997; Okuda et al. 1999), and for the human transporters hOCT1 and hOCT2 (Busch et al. 1998; Dresser et al. 2000; Gorboulev et al. 1997).

3. OCTs operate independently from Na^+ and Cl^- ions (Busch et al. 1996a; Gorboulev et al. 1997; Kekuda et al. 1998).

4. OCTs are able to translocate cations across the plasma membrane in either direction. In addition to cation influx, cation efflux has been demonstrated for rOCT1, rOCT2, hOCT2, and rOCT3 (Busch et al. 1996a, 1998; Kekuda et al. 1998; Nagel et al. 1997).

Most substrates translocated by the OCT transporters are organic cations, but there are also several weak bases and noncharged compounds among the transported substrates. Transported substrates as well as inhibitors of the OCT transporters may be endogenous compounds, drugs or xenobiotics. Transported endogenous substrates of the OCTs include the monoamine neurotransmitters acetylcholine, dopamine, serotonin, histamine, and compounds such as creatinine, choline, guanidine, and thiamine. Many drugs and xenobiotics interact with the OCTs, either as transported substrates or as inhibitors. When the interaction of a compound with OCT was tested indirectly via its effect on tetraethylammonium (TEA) or 1-Methyl-4-phenylpyridiniumiodid (MPP)

uptake (Koepsell et al., 2004), it is important to keep in mind that inhibition does not provide any clues as to whether the compound is transported or not. Competitive binding of a second ligand can provide a sufficient explanation for an observed inhibition. However, whether this second ligand is transported or not has no predictable effect on the uptake of the first ligand.

Examples for drugs that are transported by human OCTs include the histamine receptor antagonist cimetidine (Barendt and Wright 2002; Zhang et al. 1998), the antidiabetics metformin and phenformin (Dresser et al. 2002), the antiviral agents acyclovir and ganciclovir (Takeda et al. 2002), the muscle relaxant memantine (Busch et al. 1998), and the antiarrhythmic quinidine (van Montfoort et al. 2001). Many other drugs are known to inhibit human OCTs but have not been tested for transport, including agonists and antagonists of a-adrenoreceptors, ß-adrenoreceptor antagonists, blockers of Na^+- and Ca^{2+}-channels, and antidepressants. Although organic cations are clearly the preferred ligands of the OCTs, several uncharged compounds are known to be inhibitors or even transported substrates of these transporters. For example, transport of the weak base cimetidine by hOCT2 is only partially dependent on the degree of ionization (Barendt and Wright 2002). Moreover, a number of anionic anti-inflammatory drugs such as indomethacin, diclofenac, ketoprofen, mefenamic acid, piroxicam, and sulindac are inhibitors of human OCT1 and OCT2 (Khamdang et al. 2002), and the organic anions probenecid, PAH, and a-ketoglutarate are inhibitors of the rat organic cation transporters rOCT1 and rOCT2 (Arndt et al. 2001).

1.3 Substrate specificities of OCTs.

The issue of substrate selectivity of OCT1 has been examined in three ways:

1) directly, through measurement of transmembrane flux of labeled compounds (or transport-induced current);

2) indirectly, either through determination of the extent of inhibition of transport of a model substrate (e.g., TEA) produced by coexposure to a test agent, or as noted above, by gauging the stimulatory (or inhibitory) effect on transport of the model substrate following imposition of a *trans*-gradient of the test agent; and

3) by means of introducing mutations into the transporter sequence to gauge the influence of physicochemical alterations in protein structure on interaction with transported substrates.

Some substrates and inhibitors have similar affinities for the different OCT subtypes (see Table 1) in humans, rats (Arndt et al. 2001), and mice (Kakehi et al. 2002; Koepsell 2004). For example, cations like the xenobiotic MPP are transported by all OCTs. Also, similar IC_{50} values were found for the inhibition of hOCT1, hOCT2, and hOCT3 by phenoxybenzamine, of hOCT1 and hOCT2 by procainamide, of hOCT2 and hOCT3 by metformin, and of hOCT1 and hOCT3 by ß-estradiol. Furthermore, the Km values for MPP uptake by OCT1 and OCT2 are similar.

Table1. The substrate and inhibitor affinities for different OCT subtypes.

Compound	**Km or (IC_{50}) [µM]**			**References**
	rOCT1	**rOCT2**	**rOCT3**	
Corticosterone	(151)	(4), (4,2)	(4,9)	Arndt et al. 2001; Wu et al. 1998b
Dopamine	19, 51	2100, (2300)	(384), (620)	Wu et al. 1998; Busch et al. 1996b; ründemann et al. 1998b
Estradiol		(85)	(1.1)	Wu et al. 1998
Guanidine	1660, (4470)	172, (171)		Arndt et al. 2001
Norepinephrine		(4400), (11000)	(432)	Wu et al. 1998; Gründemann et al. 1998b
o-Methylisoprenaline	(37)	(2620)		Arndt et al. 2001
MPP (1-Methyl-4-phenylpyridinium)	17	3-19		Arndt et al. 2001
Procainamide	(20)	(445)		Arndt et al. 2001

Serotonin	38	(3600)	(970)	Wu et al. 1998; Busch et al. 1996b; Gründemann et al. 1998b
TEA (Tetraethylammonium)	91	125		Arndt et al. 2001; Chen et al. 2002
	mOCT1	**mOCT2**	**mOCT3**	
Cimetidine	(0.59)	(8.0)		Kakehi et al. 2002
Procainamide	(3.9)	(312)		Kakehi et al. 2002
Quinine	(0.28)	(2.8)		Kakehi et al. 2002
	hOCT1	**hOCT2**	**hOCT3**	
Corticosterone	(7, 22)	(34)	(0.12, 0.29)	Zhang et al. 1998; Hayer-Zillgen et al. 2002; Gründemann et al. 1998a
Progesterone	(3.1)	(27)	(4.3)	Hayer-Zillgen et al. 2002
Desipramine	(5.4)	(16)	(14)	Zhang et al. 1998; Gorboulev et al. 1997; Wu et al. 2000b
Metformin		(2010)	(1700)	Dresser et al. 2002
o-Methylisoprenaline	(>100)	(570)	(4.4)	Gorboulev et al. 1997; Hayer-Zillgen et al. 2002

Procainamide	(74), (107)	(50)	(738)	Gorboulev et al. 1997; Zhang et al. 1998;Wu et al. 2000b; Zhang et al. 1999
MPP (1-Methyl-4-phenylpyridinium)	15 (12)	19 (2.4)	47 (54)	Gorboulev et al. 1997; Zhang et al. 1998;Wu et al. 2000b; Zhang et al. 1997a
NMN (N-1-Methylnicotinamide)	(7,700)	340 (270)		Gorboulev et al. 1997; Zhang et al. 1998
TEA (Tetraethylammonium)	229 (158–260)	76 (156)	(1372)	Gorboulev et al. 1997; Zhang et al. 1998b; Wu et al. 2000b
Tetramethylammonium	(12,400)	(180), (150)		Gorboulev et al. 1997; Dresser et al. 2002;

In contrast, the affinity of several other substrates and inhibitors differs significantly between the OCT subtypes. When the affinity differences between the OCT subtypes are large enough, they may be used to distinguish these subtypes experimentally in vivo, in complex environment. General statements concerning the affinity of ligands to the human OCT subtypes can be made only with great caution. Thus, the “liver subtype” hOCT1 has a lower affinity for many substrates as compared to the “kidney subtype” hOCT2, including NMN (28:1), quinine (7:1), tetramethylammonium (70:1), and tetrapentylammonium (5:1). Exceptions to this rule are ß-estradiol and verapamil which have a higher affinity to hOCT1 compared to hOCT2 (1:6 and 1:70, respectively).

Table 1 shows inhibitors and substrates that may be used to distinguish OCT1, OCT2, and/or OCT3 in rat and mouse. For a given subtype of the OCT transporters, distinct species differences in affinity for substrates and inhibitors exist. For example,

tetramethylammonium inhibits the MPP uptake by OCT1 with IC_{50} values of 0.9 mM in rat, of 2 mM in mouse, of 5.8 mM in rabbit, and of 12.4 mM in humans (Dresser et al. 2000). Similarly, corticosterone inhibits MPP uptake by OCT3 with IC_{50} values of 4.9 µM in rats vs. 0.12 µM in humans (Gründemann et al. 1998a; Wu et al. 1998). Compounds that interact with two OCT subtypes may be nontransported inhibitors for one subtype and a transported substrate for another. For example, quinine inhibits TEA uptake by rOCT1 and rOCT2 with IC_{50} values of 4.1 µM and 23 µM, respectively, and is transported by rOCT1 but not by rOCT2 (Arndt et al. 2001; van Montfoort et al. 2001).

1.4 The tissue distribution and cellular localization of OCT subtypes.

Expression of all cloned OCT transporters was studied in selected tissues by Northern blotting (Gorboulev et al. 1997; Gründemann et al. 1994; Kekuda et al. 1998; Okuda et al. 1996; Terashita et al. 1998; Wu et al. 2000b). For the rat OCTs, a more comprehensive and detailed analysis was carried out employing quantitative polymerase chain reactions with reversely transcribed mRNAs from multiple tissues (Slitt et al. 2002). The patterns of mRNA tissue distribution were found to be subtype-dependent within a given species (Busch et al. 1998; Gorboulev et al. 1997; Gründemann et al. 1994; Kekuda et al. 1998; Okuda et al. 1996; Zhang et al. 1997a), and species-dependent for a given subtype (Gorboulev et al. 1997; Gründemann et al. 1994). It was demonstrated that rat OCT1 mRNA is predominantly expressed in kidney, with moderate expression in liver, skin, and spleen, and low expression in the other tissues (Gorboulev et al. 1997; Gründemann et al. 1994, Slitt et al. 2002). The highest level of rOCT2 mRNA was found in kidney, with low expression in other tissues (Slitt et al. 2002). Interesting to note, that OCT2 expression is gender biased in rats, in favor of males (Urakami et al., 1999; Slitt et al., 2002). OCT2 expression is down-regulated to female levels in gonadectomized or estradiol-treated male rats. Also, OCT2 expression is up-regulated several fold in female rats treated with testosterone (Urakami et al., 2000; Slitt et al., 2002). Also gender-specific differences were found in mOCT2 mRNA levels in kidney, in the male mice were twice higher than in female mice (Alnouti et al. 2005). rOCT3 mRNA levels are highest in blood vessel, skin, and thymus (Slitt et al. 2002;

Kekuda et al., 1998; Wu et al., 1998), OCT3 mRNA was low in liver, with detectable transcripts in kidney and intestine. About all other subtypes of OCTs see Table 2.

Table 2 .Tissue distribution of different subtypes of OCTs.

Subtype	Localization	References
rOCT1	kidney, liver, gastrointestinal tract, skin, spleen, lung, thymus, muscle, prostate	Gorboulev et al. 1997; Gründemann et al. 1994, Slitt et al. 2002; Lips et al. 2006
hOCT1	liver, kidney, intestine, lung	Zhang et al. 1997a; Müller et al. 2005; Lips et al. 2006
mOCT1	kidney, liver , small and large intestine	Alnouti et al. 2005
rOCT2	kidney, neuronal tissue, lung	Busch et al. 1998 ; Okuda et al. 1996; Sweet et al. 2001; Lips et al. 2006
hOCT2	kidney, brain placenta and CNS neurons, lung	Busch et al. 1998; Gorboulev et al. 1997; Lips et al. 2006
mOCT2	kidney	Alnouti et al. 2005
rOCT3	in placenta, small intestine, heart, brain, kidney, thymus, blood vessels, skin , hippocampal and cerebellar neurons, neurones of the superior cervical ganglion, lung	Kekuda et al. 1998; Slitt et al. 2002. Schmitt et al. 2003; Wu et al. 1998; Kristufek et al. 2002; Lips et al. 2006
hOCT3	skeletal muscle, liver, lung, placenta, kidney, heart, brain, intestine	Gründemann et al. 1998a; Verhaagh et al. 1999; Wu et al. 2000b, Müller et al. 2005; Lips et al. 2006
mOCT3	placenta , hippocampal and cerebellar neurons, ovary, and uterus	Verhaagh et al. 1999 Schmitt et al. 2003; Wu et al. 1998, Alnouti et al. 2005

Cellular localization studies in kidney, using immunohistochemistry, western blotting, have revealed that OCT1 is localized in the basolateral membrane of S1 and S2 segments of renal proximal tubules (Karbach et al. 2000; Sugawara-Yokoo et al. 2000), whereas rOCT2 is localized to the S2 and S3 segments. Recently, similar basolateral localization was shown for human OCT2 in the renal proximal tubules (Motohashi et al. 2002). In contrast to rat, however, human OCT2 is expressed in all three segments of the proximal tubules.

In rat liver, rOCT1 protein was localized in the sinusoidal membrane of hepatocytes, mainly those around the central veins (Meyer-Wentrup et al. 1998). Although no data are available on the immunolocalization of human OCT1 in liver, human OCT1 is likely to be expressed similarly at the sinusoidal membranes of the hepatocytes.

By immunohistochemistry, OCT1 was detected at the basolateral membrane of the enterocytes in the small intestine of rats (Koepsell et al 2003), but also in serotoninergic neurones of the submucosal and myentric plexus in mice (Chen et al. 2001). In the small intestine, OCTs are supposed to participate in the absorption and secretion of organic cations. Recent study showed that in human OCT1 is localized at the lateral membrane of enterocytes whereas OCT3 is localized at the brush border membrane of human jejunum. This study suggested predominant role of OCT3 among OCTs for the absorption of cationic drugs (Müller et al 2005). In airway epithelia of rats and humans, OCT1, OCT2 and OCT3 were detected at the luminal membrane of ciliated cells. In humans, OCT2 showed the strongest expression in the luminal membrane (Lips et al 2006). Using small specimens from human cerebral cortex OCT2 protein was detected in neurons of the human brain (Busch et al. 1998), and rOCT2 was localized by others to the apical membrane of epithelial cells of the choroid plexus (Sweet et al. 2001).

1.5 Polymorphisms and mutations in OCTs.

A number of mutations and polymorphisms have been identified recently in the human OCT1 and OCT2 genes. In a population of 57 Caucasians, numerous single nucleotide polymorphisms (SNPs) within the hOCT1 gene were detected and further analyzed (Kerb et al. 2002).

Another study presents a comprehensive genetic and functional analysis of hOCT2 gene, in ethnically diverse populations. This study had two major goals: first- they identified variants and variant frequencies in OCT2 and second- determined the significance of common variants (polymorphisms) to transporter function. The occurrence of SNPs in the gene coding for hOCT2 was investigated in 247 individuals of various ethnicity (Leabman et al. 2002). They identified 28 variable sites, 16 of which were localized in coding regions. Eight of these caused single amino acid substitutions at seven positions, and one caused a premature termination of the protein. Met165Ile and Arg400Cys were only observed in African-Americans, and Lys432Gln only in African-Americans and Mexican-Americans. Furthermore, the ratio of synonymous over nonsynonymous nucleotide changes was significantly higher than the reported genetic variations in a population of more than 75 other genes (Cargill et al. 1999; Halushka et al. 1999). The higher degree of amino acid conservation in hOCT2 implicates a higher selective pressure, underlining the biological importance of hOCT2, which is probably related to the elimination and detoxification of organic cations. This view is further supported by the observation that a gain of function was found in three out of four tested frequent, nonsynonymous SNPs (Ala270Ser, Met165Ile, Arg400Cys, and Lys432Gln). Mutants Met165Ile and Arg400Cys took up MPP at saturating concentrations at a rate 2–3 times higher than wild-type hOCT2, and Lys432Gln showed apparent Km values for MPP and tetrabutylammonium that were reduced by 44% and increased by 48%, respectively.

Amino acids that are conserved in all OCT- subfamily but are different in transporters of other subfamilies (OAT and/or OCTN) provided a reasonable start point attempts to identify individual amino acids of rOCT1 that are essential for organic cation transport. Of these, a change of Asp145 in the large extracellular loop to histidine had no effect on cation transport (Chen et al. 2002). In contrast, mutations of Asp475 in the middle of the predicted 11th transmembrane domain changed transport selectivity (Gorboulev et al. 1999); OATs 1–5, URAT1, OCTN1, and OCTN2 all carry an arginine rather than aspartate in this position. When Asp475 of rOCT1 was replaced by arginine, asparagine or glutamate specific transport was observed in HEK-293 cells with the rates that were less than 10% as compared to wild type. In Xenopus oocytes, however, only the Asp475Glu mutant yielded detectable transport, which was reduced to 2.3% for TEA, 3.2% for NMN, 3.5% for choline, and 11.4% for MPP. Interestingly, the Asp475Glu

mutant exhibited considerably higher apparent affinities for TEA, NMN, and choline than wild-type OCT1 (respective Km values reduced by factors of 8, 3.5, and 15), whereas affinity for MPP was unchanged. Similarly, inhibition of TEA uptake by the Asp475Glu mutant occurred with affinities that were higher for some inhibitors, but unchanged for others. Two tentative conclusions were drawn from these observations: firstly, Asp475 is probably close to the substrate binding site of rOCT1; secondly, the cation binding site of rOCT1 behaves as a pocket that offers several, only partially overlapping interaction domains for different substrates. Such a crucial role of Asp475 in the 11th potential transmembrane domain rOCT1 contradicts the reported transport activity of a hOCT2 splice variant that lacked the entire C-terminus including the 10th, 11th and 12th transmembrane domain (Urakami et al. 2002). Because the essential role of the 11th TMD is well established for other members of the SLC22 family, it was hypothesized that the residual activity observed with this splice variant was either due to activation of an endogenous transport activity, or to a second, alternate transport path within the same transporter molecule. Thus, point mutations of the arginine residues in the organic anion transporters OAT1 from flounder (fOAT1) and OAT3 from rat (rOAT3) that correspond to Asp475 in rOCT1 produced similar specific functional changes (Feng et al. 2001; Wolff et al. 2001). In fOAT1, the mutation Arg478Asp decreased affinity and maximal transport rate for para-aminohippurate (PAH) (Wolff et al. 2001), and abolished interaction with glutarate. In rOAT3, the mutations Arg454Asp and Arg454Asn both changed substrate selectivity with respect to the anions PAH and ochratoxin A, the weak base cimetidine and the permanently charged cation MPP (Feng et al. 2001).

Other data suggest that the 8th TMD may also be part of the substrate binding pocket in transporters of the SLC22 family. The mutation Lys370 in the 8th transmembrane domain of rOAT3 to alanine changed substrate selectivity; for example, uptake of PAH was reduced to a considerably higher extent than the uptake of cimetidine (Feng et al. 2001). Moreover, substrate selectivity of fOAT1 was changed when Lys394 (corresponding to Lys370 of rOAT3) was mutated to alanine: trans-stimulation of PAH efflux by extracellular glutarate was abolished, whereas trans-stimulation of PAH efflux by extracellular PAH remained functional (Wolff et al. 2001). In rOAT3, an additive effect on substrate recognition was observed in a comparison of MPP uptake in the double mutant Lys370Ala/Arg454Asp vs. the Arg454Asp mutant.

Finally, recent data from our laboratory show an involvement of the fourth transmembrane domain of rOCT1 in substrate recognition (Popp et al., 2005). Eighteen amino acids in the fourth transmembrane helix of rat OCT1 were mutated, and mutants were tested for its ability to translocate the organic cations TEA and MPP. The replacement of two residues, tryptophan 218 by tyrosine, and tyrosine 222 by leucine, increased the affinity for both TEA and MPP, whereas the threonine 226 to alanine (Thr226Ala) mutant had a higher affinity for MPP alone. These "hot spots" were investigated in further detail using additional organic cations for uptake. Other amino acid replacements (e.g., tryptophan 218) had an impact on the selectivity or preference of OCT1 for different cations. It is interesting that tryptophan 218, tyrosine 222, and threonine 226 are located on one side of the fourth transmembrane domain, together with lysine 215 and valine 229. These five amino acids are conserved in all OCTs and therefore are most probably of great functional importance.

The functional role of the large extracellular loop connecting TMD1 and 2 (ECL-1,2) of the SLC22 transporters is not well understood. On the one hand, organic cation transport was preserved in a splice variant of rOCT1 lacking the N-terminus including TMD-1, TMD-2, and ECL-1,2 (Zhang et al. 1997b). Replacing ECL-1,2 of rOCT1 by ECL-1,2 of rOCT2 did not affect the IC_{50} of rOCT1 for several cations that have largely different affinities in rOCT2 (H. Koepsell et al., 2004). On the other hand, several point mutations within the large ECL-1, 2 inactivate rOCT1. Furthermore, mutation of Cys88 in the ECL-1, 2 of hOCT1 drastically decreased transport rates and altered the substrate selectivity (Kerb et al. 2002).

In conclusion, site- directed mutagenesis studies in organic anion and cation transporters of the SCL22 family indicate that transmembrane domains 4, 8, 10 and 11, possibly together with additional domains, determine the structure of the substrate binding pocket, either by providing sites of direct interaction with substrates, or by indirectly stabilizing the conformation of those binding sites. The large extracellular loop may indirectly contribute to the formation or stabilization of the substrate binding pocket. No data are available that allow any insight into the translocation mechanism. The interpretation of the mutations is limited by the fact that the membrane topology of these transporters has not been determined biochemically and that it is not known whether these transporters operate as monomers, dimers, or oligomers.

1.6 Clinical relevance of OCTs.

The expression and function of OCTs are regulated by gender and different diseases. Different pathological conditions also impact the regulation of OCTs. Moreover, OCT1 appears to be involved in the absorption of drugs across the epithelium of the small intestine. All OCTs affect the interstitial concentrations of endogenous compounds (e.g., choline and monoamine neurotransmitters), drugs, and xenobiotics in a variety of tissues including brain and heart. OCT1 and OCT3 may mediate the cellular release of acetylcholine from the placenta during nonneuronal cholinergic regulation (Wessler et al. 1999, 2001). For instance, some drugs might be less promiscuous than others with respect to transporters, and comedication may inhibit alternative transport pathways.

Secretory processes also provide sites of clinically significant interactions between organic cations in human. For example, therapeutic doses of cimetidine retard the renal elimination of procainamide (Somogyi et al., 1982, 1983) and nicotine (Bendayan et al., 1990). Inter-individual variation in the renal secretion of many drugs, including anti-arrhythmic and anti-diabetic drugs, as well as in drug-induced nephrotoxicity, has been documented (Reidenberg et al., 1980), and it is hypothesized that this variation is due partly to genetic variation in transporters in the renal epithelium (Leabman et al., 2002). Thus, knowledge of the relationship between molecular structure of renal OC transporters and their physiological function holds the promise of predicting potential drug interactions and the basis of genetic differences in renal OC secretion.

Mutations in OCT1 or proteins that are involved in its targeting or membrane turnover may result in reduced hepatic excretion of OCT1 substrates (including drugs) that are eliminated mainly via biliary excretion. The resulting higher-than-normal plasma levels may make similar therapeutic effects possible with lower dosage, or may cause untoward side effects. Low expression of hOCT2 in kidney, or defect mutations in hOCT2 potentially related to one of four uncharacterized mutations (Leabman et al. 2002) may reduce renal excretion of more hydrophilic cationic drugs. For drugs transported by both OCT1 and OCT2, reduced function of OCT2 may lead to increased hepatic elimination or toxicity. Comedication with drugs that are substrates or inhibitors of OCTs may have severe consequences. For example, the renal and/or hepatic excretion of a drug that is translocated by OCT1 and/or OCT2 will be impeded by comedication

that blocks both OCT1 and OCT2. At variance, hepatic excretion will be increased by comedication with blockers specific for OCT2, but not OCT1. In this context it should be noted that weak bases of hydrophobic compounds may inhibit OCT transporters from the cytoplasmic side and may not be removed easily (Arndt et al. 2001) and that the interaction of two specific drugs at the outwardly or inwardly directed binding pocket of OCT1 or OCT2 cannot be predicted. Substrates and inhibitors may compete by different degrees and their interaction from intracellular or extracellular may be different. In rat, steroid hormones influence the expression of OCT2. Inui and coworkers showed that the reduced excretion of the organic cation cimetidine in 5/6 nephrectomized rats was associated with a 50% decrease of plasma testosterone and downregulation of OCT2 (Ji et al. 2002); the expression of OCT1 and OAT3 was not changed.

Downregulation of OCT2 after nephrectomy was reverted upon intravenous infusion of testosterone. These findings raise the attractive and testable hypothesis that impaired renal excretion of cationic drugs in patients suffering from chronic renal failure may as well be improved by testosterone. Conversely, orchidectomia (e.g., in patients suffering from prostate cancer) may be associated with an increased risk for accumulation of cationic drugs.

Two examples of how substrates of OCT transporters may produce adverse drug effects if their transport by OCTs is impaired or increased are worth noting. It has been reported that the treatment of peptic ulcer with the H2 histamine receptor blocker cimetidine leads to mental confusion in some patients that is reversible after withdrawal of cimetidine (Kimelblatt et al. 1980; Schentag et al. 1979). Since this drug effect was correlated with increased levels of cimetidine in plasma and cerebrospinal fluid, and cimetidine is transported by OCT1 and OCT2, decreased expression or malfunction of these transporters could be the reason for increased cimetidine concentrations and the associated neurological symptoms. Another example is lactic acidosis, which may occur as a rare but lifethreatening side effect in type-II diabetics who are treated with biguanides such as phenformin and metformin (Davidson and Peters 1997). Because of that risk, phenformin was withdrawn from the market in the 1970s (Kwong and Brubacher 1998); metformin, however, is still used. In recent years, metformin has also been introduced for treatment of polycystic ovary syndrome (Nestler 2001; Velazquez et al. 1994). Metformin is mainly eliminated by glomerular filtration and tubular secretion, but part of it may be also excreted into the bile. Its antidiabetic effect is due to improved

peripheral insulin sensitivity, reduced glucose absorption in small intestine, and reduced glucose generation by the liver (Borst and Snellen 2001; Caspary and Creutzfeldt 1971; Hundal et al. 2000). The effect of metformin on glucose generation in hepatocytes was explained by inhibition of mitochondrial complex I (El Mir et al. 2000; Owen et al. 2000). Extensive inhibition of mitochondrial respiration by biguanides may cause lactic acidosis. Renal failure or impaired renal excretion of metformin may entail increased metformin plasma levels and thus cause lactic acidosis. Metformin is transported by OCT1 in rat and humans, and by OCT2 in humans (Dresser et al. 2002). In humans, lower renal expression of OCT2, mutations reducing the activity of hOCT2, or administration of metformin simultaneously with a drug that inhibits OCT2 in kidney but not hOCT1 in the liver, may cause lactic acidosis.

OCT transporters could play a role in carcinogenesis or may be useful for targeting of anticancer drugs. For example, high expression or activity of OCT1 may increase the concentration of carcinogenic xenobiotics in hepatocytes. On the other hand, high expression of OCT1 was found in hepatocarcinomas induced by diethylnitrosamine (Lecureur et al. 1998), and thus OCT1 could help target anticancer drugs into the carcinoma cells. To prevent renal and hepatic toxicity, the drug should not interact with OCT2 and OCT3, and OCT1 expressed in nontransformed hepatocytes should be downregulated. This could be achieved by employing differences in the regulation OCT1 in normal compared to transformed liver cells. The early study reported that bile acid-conjugates of the anticancer drug cisplatin were transported substrates of human OCT1 and OCT2, in contrast to free cis-platin (Briz et al. 2002); though latter investigations have shown that free cis-platin could be transported by OCTs. Recent investigations concerning interaction of free cis-platin with human OCT1 and OCT2 (Ciarimboli et al. 2005), rat OCT1 and OCT2, and pharmacokinetics of cis-platin in rats (Yonezaw et al. 2005), which showed that transport of cis-platin is mediated by OCT2 rather then OCT1, and thus defined OCT2 as the major determinant of cis-platin-induced tubular toxicity.

2. The aim of study.

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, IC_{50} values are ~10 µM (human OCT1), ~30 µM (human OCT2), ~0.2 µM (human OCT3), ~150 µM (rat OCT1), ~4 µM (rat OCT2), and ~5 µM(rat OCT3) (Gründemann et al., 1998; Wu et al., 1998; Zhang et al., 1998; Arndt et al., 2001; Hayer- Zillgen et al., 2002).

The aim of the present study was to identify amino acids of rOCT2 that are responsible for the higher affinity of corticosterone as compared to OCT1.

For this purpose the rOCT1 protein was used as a backbone molecule which was fragmented into 16 pieces containing the presumed individual transmembrane helices or intra-/extracellular loops and each of these pieces was replaced with the respective part of rOCT2. For characterization of these chimeras the uptake of 10µM [^{14}C]TEA was measured and apparent *K*m values for TEA uptake and inhibition of TEA uptake by procainamide or corticosterone were calculated.

3. Materials and Methods.

3.1 Materials.

3.1.1 Chemicals.

Agarose (SeaKem LE)	Biozym, Oldendorf, Germany
Bromphenolblue	Serva, Heidelberg, Germany
$CaCl_2$	Sigma-Aldrich, Taufkirchen, Germany
Chloroform	Roth, Karlsruhe, Germany
Corticosterone	Fluka, Buchs, Schweiz
Cyanine863	Sigma-Aldrich, Taufkirchen, Germany
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich, Taufkirchen, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Taufkirchen, Germany
DNA- ladder	Fermentas, Vilnius, Lithuania
Ethidium bromide	Sigma-Aldrich, Taufkirchen, Germany
EDTA disodium salt dihydrate	AppliChem, Darmstadt, Germany
Gentamycin sulfate	Fluka, Buchs, Schweiz
3-Morpholino-2-Hydroxypropanesulfonic acid (MOPS)	AppliChem, Darmstadt, Germany
Milk powder	Sigma-Aldrich, Taufkirchen, Germany
N,N-bis[2-Hydroxyethyl]-2-aminoethansulfonic acid (BES)	AppliChem, Darmstadt, Germany
1-Methyl-4-phenylpyridiniumiodid (MPP)	
NaCl	Sigma-Aldrich, Taufkirchen, Germany
Procainamide hydrochloride	Sigma-Aldrich, Taufkirchen, Germany
RNA-ladder	Fermentas, Vilnius, Lithuania
Quinine	Sigma-Aldrich, Taufkirchen, Germany
Roti-Phenol/Chloroform	Roth, Karlsruhe, Germany
Sodiumdodecylsulfat (SDS)	AppliChem, Darmstadt, Germany
Tetraethylammonium chlorid (TEA)	Sigma-Aldrich, Taufkirchen, Germany

3.1.2. Radioactive compounds.

[3H]1-Methyl-4-phenylpyridiniumiodid (MPP)	BIOTREND, Germany
[14C]Tetraethylammonium bromide (TEA)	BIOTREND, Germany

3.1.3. Enzymes and kits.

Collagenase type II (Clostridiopeptidase A)	Sigma-Aldrich, Taufkirchen, Germany
Restriction endonucleases	New England Biolabs, Schwalbach Germany and Fermentas, Vilnius Lithuania
mMESSAGE mMASCHINE^{TM} SP6 Kit	Ambion (Europe) Ltd. Huntingdon, United kingdom
DNA Polymerase	Fermentas, Vilnius, Lithuania

3.1.4 Equipment.

Liquid scintillation counter Tri-Carb 1600CA	Packard Instrument Co., USA
Camera Polaroid MP4	Polaroid Corporation, USA
Photometer, Ultraspec3	Pharmacia, Freiburg, Germany
Microinjection pump	Drummond, USA
Micropipette puller P30	Sutter instruments Co, USA
Thermostat IPP-400	Memmert GmbH, Germany
UV transilluminator TM-20	UVP Inc., USA
Biofuge 28RS	Heraeus, Hanau, Germany

3.2 Methods of Molecular Biology.

3.2.1 Wild type rOCT plasmids, construction of chimeras and point mutations.

All manipulations were performed on the basis of rOCT1 and rOCT2 cDNA cloned into pRSSP vector which contains 5'- and 3'-nontranslating regions of Xenopus laevis β-globin gene, unique Mlu I restriction site for linearization of the plasmid downstream from a cDNA insert, the ampicillin resistance and the promoter for SP6 RNA polymerase.

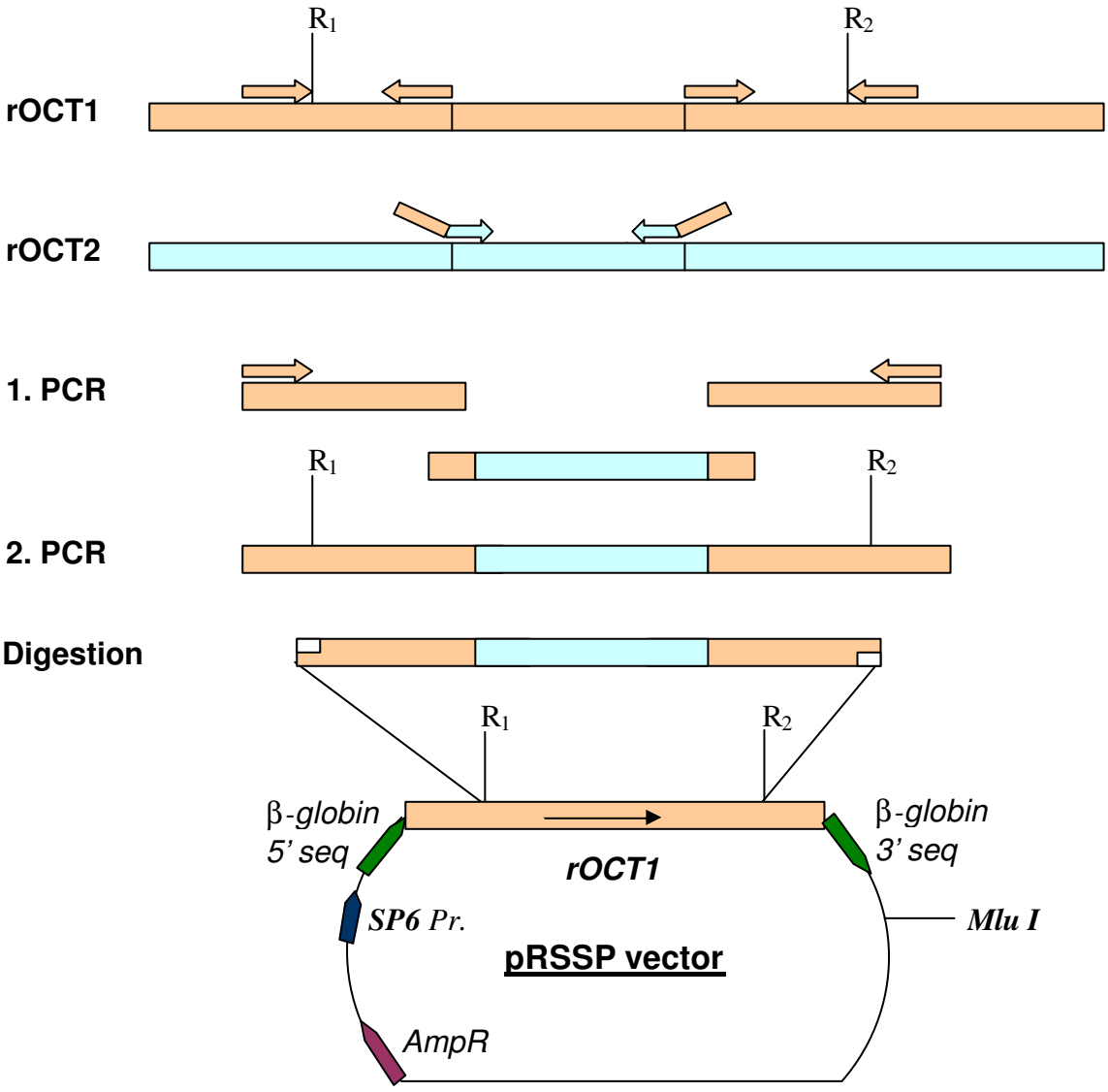

Figure 3. The Sketchy image of construction of chimeras (see explanations in text). R_1 and R_2 –unique sites for restriction endonucleases.

The chimeric rOCT1/rOCT2 transporters and point mutants of rOCT1 and rOCT2 were created by Dr. Gorboulev using the overlap extension method of polymerase chain reaction (PCR) (Ho et al., 1989).

Fig.3 shows schematically the strategy used to generate the rOCT1 chimeras carrying the part of the rOCT2 sequence. To make such a chimera, two PCR fragments of rOCT1 flanking the region to be exchanged and a PCR fragment of rOCT2 containing region to be inserted were amplified. The 5' ends of primers used to amplify the rOCT2 fragment contained nucleotide sequences which corresponded to the respective flanking regions of rOCT1. Next, the three PCR products from the 1st PCR were combined and fused by the 2nd PCR resulting in the rOCT2 fragment flanked with parts of rOCT1.

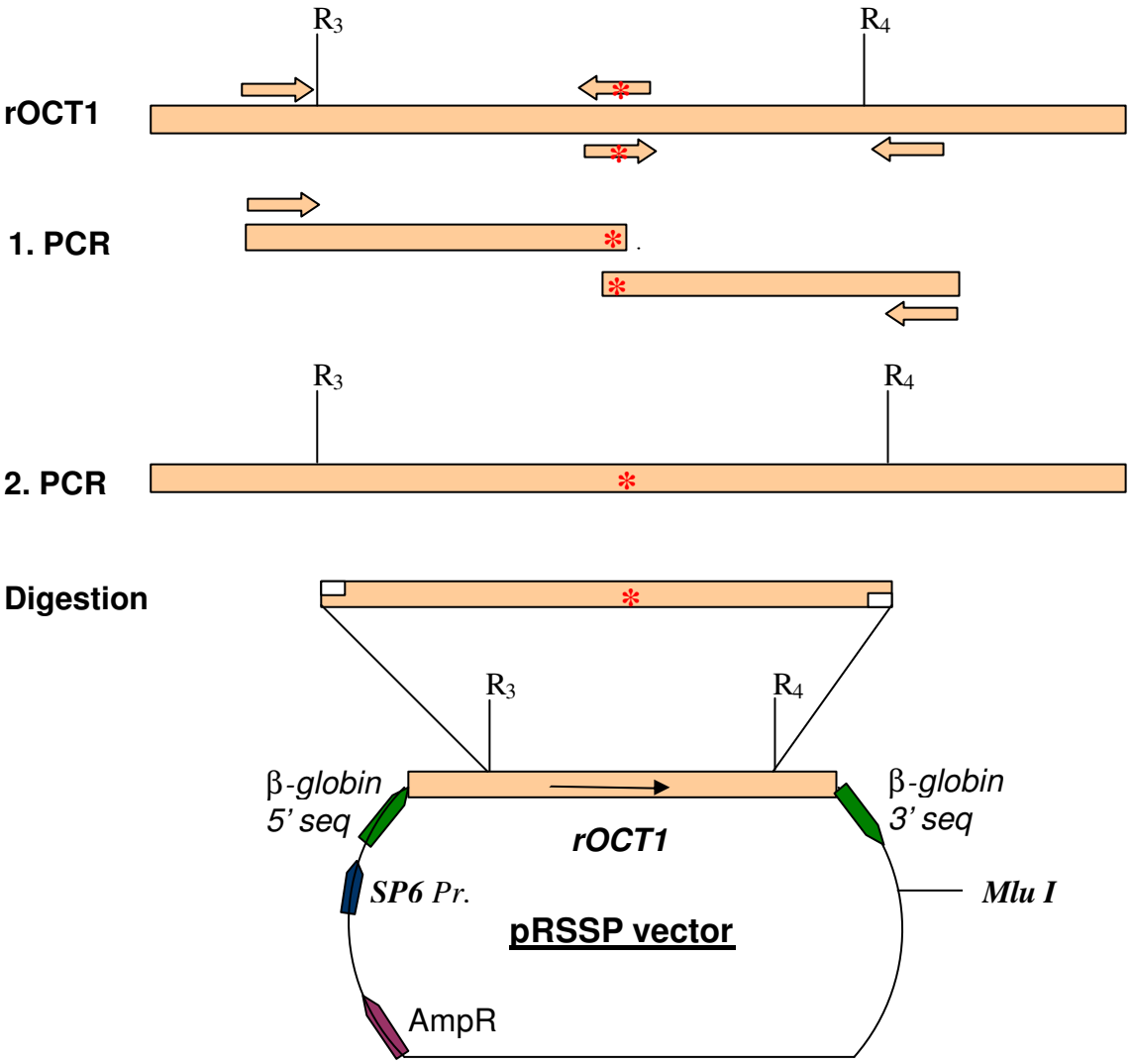

Figure 4. The Sketchy image of construction of point mutations (see explanations in text) R_3 and R_4 –unique sites for restriction endonucleases.

This amplificate was digested with two unique restriction endonucleases (R_1 and R_2 on the Fig. 3 and substituted for the excised rOCT1 fragment by ligation to the rOCT1/RSSP plasmid (Gründemann et al., 1994)

To introduce point mutations, two overlapping fragments of the cDNA for the respective transporter were amplified. The amplificates were used for introduction of a desired point mutation at the overlapping ends (see Fig. 4). The amplificates were fused using the 2. PCR and the resulting PCR product was substituted for the corresponding fragment of the wild type transporter as described for the generation of chimeras. All chimeric and mutant constructs were sequenced to confirm the presence of desired modifications and the absence of any PCR errors.

3.2.2 Linearization of plasmid DNA.

10µg of plasmid DNA (rOCT1, rOCT2, chimeras, mutants, all in pRSSP) were digested with 15 u. Mlu I in 50 µl of R+ buffer (10 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 100 mM KCl, 0,1 mg/ml BSA), overnight at 37^0C. The completion of the digestion was controlled by the agarose gel electrophoresis. DNA was then extracted from reaction mixture by phenol/chloroform extraction. 50 µl phenol/chloroform mixture (1:1 by volume) was added and the tube was vortexed for 20 sec followed by centrifugation for 30 sec at room temperature and at 10000g. The supernatant was taken out and DNA was precipitated by mixing with 3 volumes (150 µl) of ethanol and 1/10 volume (5 µl) of sodium acetate pH 5,0 (Wallace D, 1987). Mixture was vortexed for 20 sec and kept at -20^0 C for 45 minutes. Afterwards, DNA was sedimented by centrifugation for 20 minutes at 4^0 C and 10000g. Supernatant was discarded and the pellet was washed with 500 µl of ice-cold 70% ethanol followed by centrifugation for 10 minutes at 4^0 C, 10000g. DNA pellet was dried on air and dissolved in 10 µl of RNase-free water. Concentration of DNA was determined by spectrophotometry.

3.2.3 Spectrophotometric analysis of DNA.

DNA concentration was measured by absorbance at 260 nm. The purity of DNA was calculated from the ratio between the absorbances at 260 nm and 280 nm. The ratio

should be approximately 1.8 - 2.0 for the pure DNA preparation. It is possible to accept this value as low as 1.5; but if it is lower, sample is thought to be contaminated with proteins.

3.2.4 DNA electrophoresis.

The quality of the Mlu I digestion was analyzed in 1% agarose gel running in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8,0) with 0,1 µg/ml ethidium bromid. Samples contained 0,5 µl restriction mixture (0,1 µg DNA) diluted with 3 µl water and 1 µl bading-buffer (30% Glycerin, 0,25% Bromphenolblue). Electrophoresis was run for 1 hour at 5 V/cm.

3.2.5 Transcription of mRNA from Template DNA.

m7G(5´)ppp(5´)G-capped cRNAs were prepared from the linearized plasmids of rOCT1, rOCT2, their mutants and chimeras by using the "mMESSAGE mMACHINE^{TM}" kit with SP6 RNA polymerase (Ambion, Huntingdon, UK).

20µl of reaction mixture contained 1µg linear template DNA, mixture of triphosphonucleotides, cap analogue, reaction buffer, and RNA polymerase from the kit. Reaction was carried out for 2 hours at 37^{0}C. At the end, the mixture was treated with DNAse I to remove template DNA. Reaction was stopped and mRNA was precipitated with 2,8 M lithium chloride. The mixure was chilled for 1hour at -20^{0}C and centrifuged at 4^{0}C for 15 minutes at 10000g. Supernatant was removed and pellet was washed with 500 µl 75% ethanol followed by centrifugation at 4^o C for 10 minutes at 10000g. Again, supernatant was discarded and pellet was dried on air for about 15 minutes and resuspended in 30 µl nuclease-free water. Concentration of cRNA was determined by agarose gel electrophoresis. All dilutions of cRNA were performed using DEPC-treated water.

3.2.6 RNA electrophoresis.

0,25-1µg RNA in 0,5–1 µl mixed with 3µl loading buffer (71.4 % DMSO, 1.43 M glyoxal, 67 µg/ml ethidium bromid) was heated for 1hour at 50°C to destroy secondary structure of RNA (McMaster and Carmichael,1977, 1980). Samples were subjected to 1% agarose gel containing 10mM iodoacetic acid in BES–buffer (10 mM BES, pH 6,7 and 0,1 mM EDTA). Electrophoresis was run for 1,5 hours at 5 V/cm. Amount of RNA in sample was estimated by comparison to RNA-ladder bands of known concentrations.

3.3 *Xenopus laevis* oocytes expression system.

The oocytes of the South African clawed frog *X.laevis* are widely used for the expression of heterologous proteins. The functional characterization of membrane proteins in particular has significantly profited from the use of this expression system. The oocytes are easily prepared because of their large diameter of 1.1-1.3 mm and easy to handle.

Figure 5. The South African frog Xenopus laevis.

Taxonomy:

Kingdom	*Animalia*
Phylum	*Chordata*
Subphylum	*Vertebrata*
Class	*Amphibia*
Order	*Anura*
Family	*Pipidae*
Species	*Xenopus laevis*

3.3.1. Laparotomy of Xenopus laevis.

Surgical instruments were washed or soaked post usage to remove all debris. They were sterilized by steam autoclaving. If multiple surgeries were done on different animals, then previously sterilized instruments were "quick" disinfected by 70% ethanol.

Female frogs were anaesthesized in solution containing tricaine methanesulfonate (MS-220) and $NaHCO_3$ (1 g/l). MS-220 was used at a dosage range of between 0.5 and 3 g/L. Dosage selection was dependent upon the weight/size of the frog and the duration of anesthesia required. The frog was frequently exposed to water containing dissolved MS-220 to maintain current level of anesthesia. Frogs skin was remained moistened throughout the procedure to prevent desiccation and precipitate complications.

Before operation it was necessary to remove all debris from the frog's skin. The surgical site was rinsed with disinfection spray before the surgical incision had been made. Through a short abdominal incision (1-2 cm in length), small pieces of ovary were removed. Remaining ovarian tissue was placed back into the coelomic cavity and checked for excessive hemorrhage. Both tissue layers were closed separately using a monofilament absorbable suture material (silk). The use of absorbable suture material prevented the need to remove the sutures at a later time.

Post surgery, frog was allowed to recover for approximately 30-60 min in a container with a level of water not to cover the nostrils of the frog. Desiccation of the skin on the dorsum of the frog was prevented by placing moistened paper on any exposed surfaces. When frog became active and mobile the water level was raised. Frogs were monitored daily for at least 5 days post surgery for evidence of excessive inflammation of the incision site, suture dehiscence, or abnormalities indicative of illness (anorexia, listlessness, lethargy, bloating, or "red-leg",).

All procedures with oocytes were performed in ORi–buffer [5 mM MOPS , pH 7.4, 100 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$].

To transfer the oocytes without damaging them, a glass transfer pipette was used.

3.3.2 Preparation of X. laevis oocytes.

After operation ovaries were sliced in small pieces by means of two tweezers. This helps degrade the tissue holding the eggs together. Afterwards oocytes were washed with 50 ml ORi until the solution was clear. Washed oocytes were transferred in the new Petri dish in 10 ml ORi and subjected to the treatment with collagenase (1 mg/ml, 540 units) for overnight at 16^0C to remove follicular layer. Alternatively, digestion was performed for 3 hours at room temperature with solution containing 2 mg/ml collagenase (1080 units). Both procedures gave similar results.

To stop defolliculation and facilitate separation of the cells, oocytes were repeatedly washed with ORi–buffer without Ca. Afterwards oocytes were placed into the standard ORi solution supplemented with 50 mg/l gentamicin and stored at 16^0C.

3.3.3 *The development stages of oocytes.*

For correct selection of oocytes it is necessary to distinguish them according to level of development stage. The oocytes are divided according to development stage into classes I to VI (Dumont 1972). A brief description of the different classes (just for recognition):

Stage I: size is 50 – 300 µM. Oocytes with transparent cytoplasm and big nucleus.

Stage II: size is 300 - 450 µM, the cytoplasm gleams whitely; the vitellogenesis starts, yolk formations begins.

Stage III: size is 450-600 µM, yolk- formed oocytes. The color of cytoplasm is brown-black; the protein-lipid yolk is distributed homogeneously. The oocytes seem homogeneously brown-black

Stage IV: size is 600-1000 µM, yolk-formed oocytes. The polarization in animal (dark) and vegetative (bright) poles is clear to recognize.

Stage V: size is 1000-1200 µM, yolk-formed oocytes. The nucleus is at the animal pole. The volume of the cell is 0.9-1.2 µl, the pigmentation of the animal pole is brown to black and very homogeneous; it is the optimum stage for the microinjection of RNA

Stage VI: size is 1200-1300 µM, yolk- formed oocytes; pigment rings form in the area of the equator.

3.3.4 Microinjection of RNA into the oocytes.

Large oocytes (stage V-VI) showing evenly colored poles and a sharp border between both poles were selected and used for experiments.

The glass capillaries from borosilicate (Hilgenberg, Malsfeld) with internal and external diameters of 0.5x1 mm were pulled with the aim of micropipette puller P30 (Sutter instruments Co, USA). The tips of capillary had melted after this procedure and had to be broken later manually with the aim of fine tweezers. The quality of capillary was controlled under microscope. The injection capillary was filled with mineral oil (Sigma 400-5 Heavy Weight Oil, with a density of 0.88 g/ ml). Injection was performed by mean of the pump for microinjection (Drummond, USA).

For the injection cap with a drop of RNA was positioned under microscope at the injection station. Capillary tip was moved close to the "bubble" of the solution with course controls then inserted into the "bubble" with microcontroller, and the RNA solution was sucked up. The injection volume was 50 nl containing 10 ng of the respective cRNA per oocyte.

Before measurements oocytes were stored in 6-well plates with about 10 eggs per well. The plates were kept in an incubator at 16ºC. Buffer was exchanged for fresh ORi solution daily. A bottle of fresh ORi solution was stored in the incubator with the oocytes.

Oocytes were incubated for 2 - 4 days at 16^{o}C in ORi buffer supplemented with 50 mg/l gentamicin before measurements.

3.3.5 Tracer Uptake Measurements.

The polystyrene reaction vessels had to be rinsed before the transport measurement with a 2 % skim milk powder solution. The proteins of the skim milk blocked the side chains of the polystyrene and prevented the adhesion of oocytes to the walls of the vessels. For the measurements, 7-10 injected with cRNAs or noninjected oocytes were placed into the vessel in 190 µl of ORi buffer. Reaction was started by addition of 10 µl 0,1 µCi [^{14}C]TEA or [^{3}H]MPP. The concentration of compounds in reaction mixture was as indicated in text. Reaction was stopped by rinsing the oocytes three times with ice-cold ORi supplemented with 100 µM quinine. Each oocyte was placed into separate vial and subjected to lysis with 100 µl of 5 % SDS. Afterwards, 1 ml of scintillation cocktail mix Lumasafe^{TM} Plus (Lumac, Netherlands) was added to the vial and radioactivity of samples was analysed by liquid scintillation counting by Tricarb 1600 (Packard-Bell, Dreieich). Amount of transported substrate (in pmol/hour^{-1}oocyte^{-1}) was computed according to formula

$$\frac{\text{Radioactivity per oocyte [DPM]}}{\text{Total radioactivity of uptake mixture [DPM]}} \times \text{Substrate concentration[µM]} \times 200[\mu l] \times 2$$

Specific uptake was calculated as difference between uptake values measured in OCT-expressing and noninjected oocytes. Uptake in noninjected oocytes was identical to the uptake measured in the precence of 100 µM quinine or 10 µM cyanine 863 (specific inhibitors of OCTs) in OCT-expressing oocytes. To determine inhibition curves, the uptake of 10 µM [^{14}C]TEA or 0.1 µM [^{3}H]MPP was measured in the presence of various concentrations of corticosterone or unlabeled MPP or TEA. In case of corticosterone oocytes were preincubated for 10 min with corticosterone at the respective concentrations before radiolabelled compound was added. Stock solution of inhibitors in ethanol was prepared fresh every time. The final ethanol concentration in uptake mixture was less then 1%.

3.3.6 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 10µM TEA or 0.1µM MPP in the presence of 8-12 different concentrations of the nontransported inhibitor corticosterone or procainamide 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 or procainamide, IC_{50} values, or K_M values between transporters, 3-9 independent experiments were performed and the respective degrees of inhibition, IC_{50} values, or K_M values were calculated from the individual experiments. The data are presented as means ± 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. Curve fitting and statistical calculations were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

4. Results.

4.1 Functional characterization of chimeras containing rOCT1 backbone with substituted parts from rOCT2.

Two subtypes of rat organic cation transporters, rOCT1 and rOCT2, have very similar substrate specificity and show nearly the same affinity for the most substrates and inhibitors tested. Nevertheless there are several compounds that have significantly different affinity to these two OCT subtypes (Arndt et al. 2001) (see Table 1 from introduction).

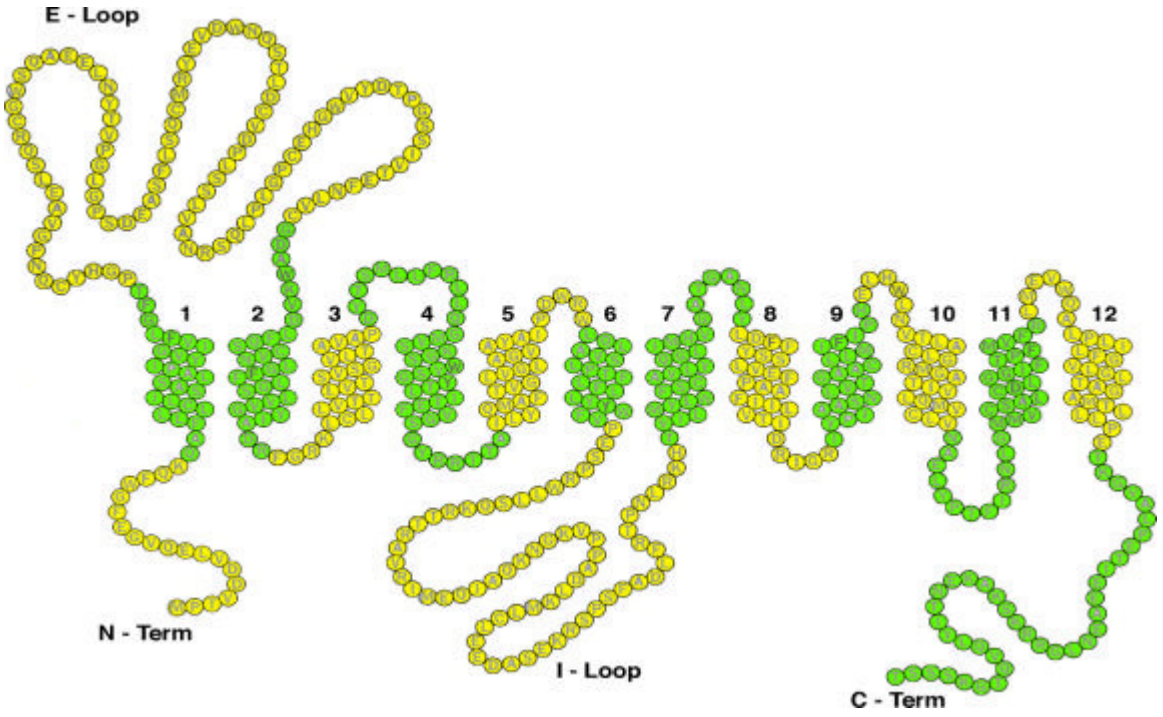

Figure 6. Schematic representation of secondary structure of rOCT1 with individual regions that were replaced by respective domains of rOCT2; insertions are shown in yellow or green. N – and C- term are N- and C-termini, E- and I-Loop are large extra- and intracellular loops, respectively.

The chimeras approach was chosen to elucidate what parts of rOCT1 transporter are responsible for/or contribute to these differences in the affinities. This approach was successfully applied for study of number transporting proteins, for instance, P-

glycoprotein mdr1 (Zhang et al 1995), peptide transporter PEPT1 (Doring F et al 1996), type II Na/phosphate cotransporter (de La Horra et al 2000). Generally, this approach is useful in case of studding homological proteins which nevertheless display different biochemical feature(s).

Table3. The chimeras abbreviations and positions. The names of chimeras derive from the substituted region (name of terminus, of the loop or the number of TMD).

Chimera's name	**Substituted part of rOCT1**
N-terminus (N)	1-20
Chimera 1	21-42
E-loop (eL)	43-149
Chimera 2	143-173
Chimera 3	174-198
Chimera 4	199-238
Chimera 5	239-264
Chimera 6	265-283
I-loop (iL)	284-347
Chimera 7	348-377
Chimera 8	378-403
Chimera 9	404-426
Chimera 10	427-463
Chimera 11	464-487
Chimera 12	488-516
C-terminus (C)	517-556

rOCT1 was used as backbone molecule and its 16 successive parts were individually replaced by the respective regions of rOCT2. The replaced regions were: both terminal sequences (N-terminus and C-terminus), the large extracellular loop

between TMD1 and TMD2 (E-loop), the large intracellular loop between TMD6 and TMD7 (I-loop) and all 12 transmembrane domains (chimeras 1-12).The small parts of the extra- and intracellular loops were substituted together with one of the adjacent transmembrane domains.

The Table 3 and Fig. 6 show all 16 chimeras with the position of the rOCT1 fragments replaced by those from rOCT2.

4.2 Analysis of the activity of chimeras.

First of all the created chimeric constructs were tested for their activity. The activity was tested in uptake experiments using as a substrate 10 µM [^{14}C] TEA. TEA is a good substrate for rOCT1 and rOCT2, and it has a similar affinity to both subtypes (K_m =90 µM and 125 µM respectively). The applied concentration lies significantly below the K_m value, allowing the direct comparison of the activity of chimeras with wild type transporters.

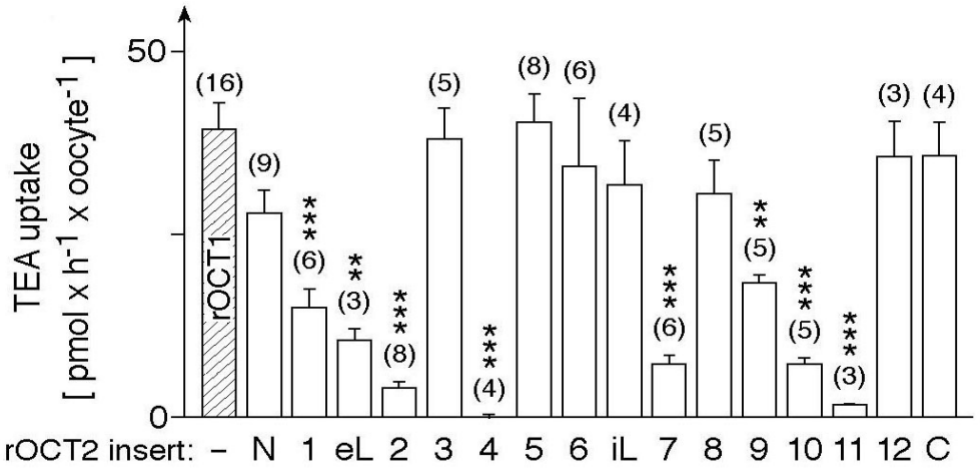

*Figure 7. Uptake rates of 10 µM [^{14}C]TEA in oocytes expressing rOCT1 or rOCT1 with inserted domains of rOCT2. Mean ± S.E.M. values are shown. The numbers of the performed experiments are indicated in parentheses. **, P< 0.01; ***, P< 0.001, according to ANOVA with post-hoc Tukey's test for difference to rOCT1 wild type.*

Eight chimeras (N,3,5,6,iL,8,12,C) showed uptake rates similar to wild type. Six chimeras (1,eL,2,7,9,10) had reduced activities between 13% and 49% of rOCT1 wild type, whereas chimera 11 showed uptake rate of 4.5% of wild-type and chimera 4

showed no significant TEA uptake at all (Fig. 7, Table 4). We suggested that these two domains (TMD 4 and TMD 11) could interact with each other and to prove this hypothesis we constructed the chimera bearing both TMD 4 and TMD 11 of OCT2 in rOCT1 backbone (chimera 4/11). But it was also inactive. The low transport activity of chimera 11 and the absence of transport observed with chimera 4 and double chimera suggest that the exchanged regions are involved in interactions with other domains of rOCT1 wild-type. The interactions between domains may be disturbed when rOCT2 domains are inserted into rOCT1 background which leads to inactivation or defective membrane targeting (Koepsell et al., 2003).

Table 4. Uptake rates of 10 µM [^{14}C]TEA by chimeras and rOCT1 wild type

Transporter	**Uptake of 10 µM [^{14}C]TEA, pmol/h per oocyte**	**Uptake in % from wild type**	**Number of experiments**
wild type rOCT1	37,80 ± 4,01	100 %	16
N-terminus	32,19 ± 3,83	85,2 %	9
Chimera 1	15,20 ± 2,40	40,2 %	6
E-loop	13,88 ± 4,05	36,7 %	3
Chimera 2	4,79 ± 1,18	12,7 %	8
Chimera 3	38,04 ± 3,76	100,6 %	5
Chimera 4	1,1 ± 0,5	2,9 %	4
Chimera 5	37,44 ± 4,67	99 %	8
Chimera 6	34,16 ± 4,98	90,4 %	6
I-loop	31,87 ± 5,28	84,3 %	4
Chimera 7	6,89 ± 1,01	18,2 %	6
Chimera 8	30,49 ± 4,08	80,7 %	5
Chimera 9	18,38 ± 0,91	48,6 %	5
Chimera 10	9,14 ± 1,79	24,2 %	5
Chimera 11	1,71 ± 0,05	4,5 %	3
Chimera 12	35,73 ± 3,80	94,5 %	3
C-terminus	35,34 ± 3,88	93,5 %	4

4.3 Measurements of the apparent K_M values of TEA uptake.

For all active chimeras, the apparent K_M values for TEA uptake (Fig. 8) were measured to check possible changes of properties. The measured constants 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. Thus, replacement of part of the rOCT1 molecule with the respective part from the rOCT2 did not induce significant alteration of apparent affinity for TEA, a substrate for which both transporters have similar affinity. This result is an evidence for similarity of TEA-binding pockets in both rOCT1 and rOCT2. Taken together, the data suggest the functional integrity of the chimeras with the exception of chimeras 4 and 11.

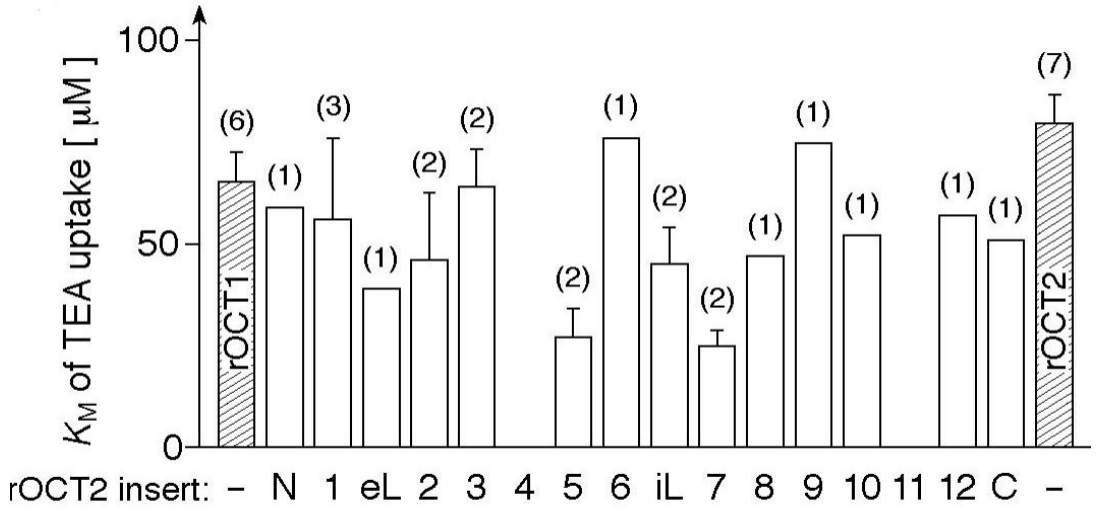

Figure 8. Apparent K_m values measured for TEA uptake by rOCT1, rOCT2 and chimeras. Numbers at the top of bars show the number of repeats.

4.4 Inhibition of 10 µM [^{14}C]TEA uptake of rOCT1, rOCT2 and chimeras by procainamide or corticosterone.

Among the substrates and inhibitors of rOCT1 and rOCT2 there are several compounds which have markedly different affinity to rOCT1 compared to rOCT2 (see Table 1 from Introduction). For further experiments we chose two compounds: procainamide that has about 20 times higher affinity to rOCT1 than to rOCT2 and corticosterone that demonstrates about 40 times higher affinity to rOCT2 than to rOCT1. We wanted to find out what part(s) of OCT molecule is responsible for the respective difference. For the functional active chimeras, we measured the uptake of 10 µM [^{14}C]TEA in presence and absence of two concentrations of procainamide or corticosterone. Concentrations were selected in accordance to IC_{50} values of rOCT1 and rOCT2 for procainamide or corticosterone (see Table 5). Experiments with procainamide were carried out at concentrations 20 µM (=IC_{50} for rOCT1) and 450 µM (=IC_{50} for rOCT2) and with corticosterone – at 4 µM (=IC_{50} for rOCT2) and 200 µM (=IC_{50} for rOCT1).

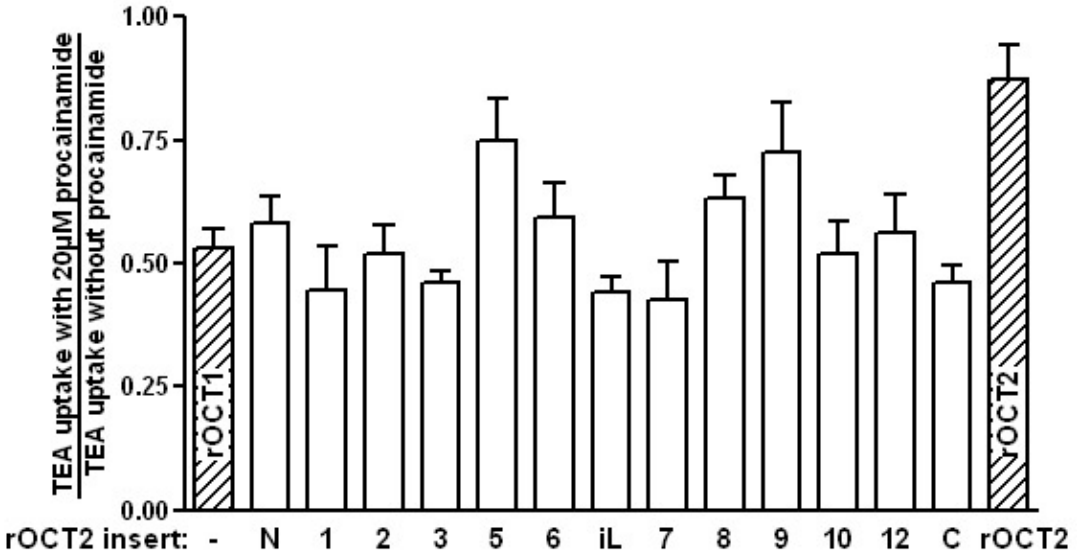

Figure 9. Inhibition of TEA uptake by 20 µM procainamide. Mean ± S.E.M. values are shown. All experiments were done in three repeats.

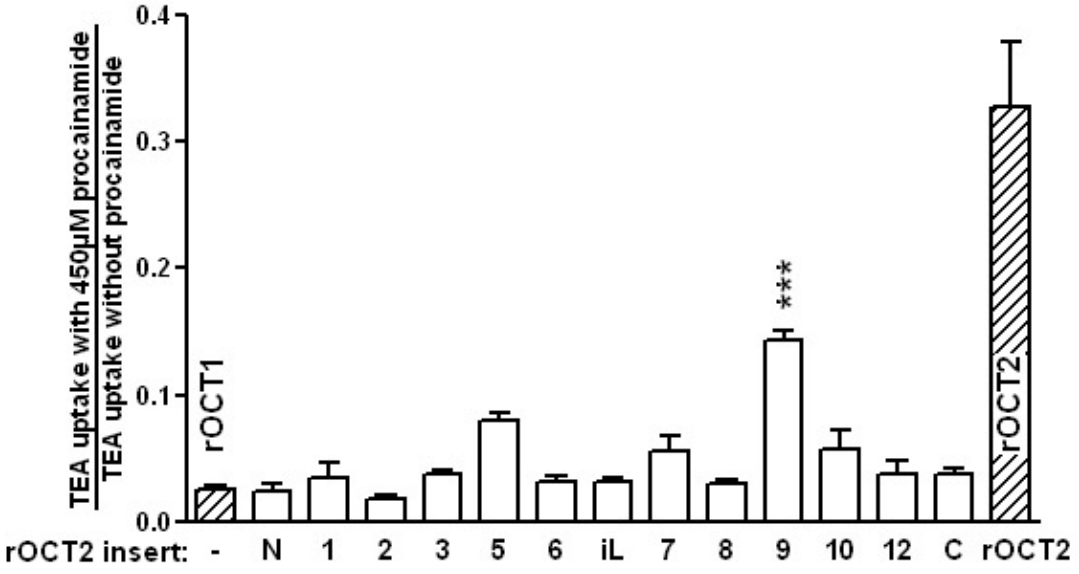

*Figure 10. Inhibition of TEA uptake by 450 µM procainamide. Mean ± S.E.M. values are shown. All experiments were done in three repeats. ***, P<0,001 according to ANOVA with post-hoc Tukey's test for difference to rOCT1 and rOCT2.*

For uptake of 10 µM [^{14}C]TEA in presence of 20 µM procainamide no statistically significant differences of inhibition between chimeras and rOCT1 wild type were found (Fig 9). Procainamide at concentration 450 µM strongly inhibited rOCT2 activity and nearly completely abolished transport of TEA by rOCT1 or chimeras with the exception of chimera 9 which still was inhibited stronger than rOCT2 (14 ± 0.8 *vs.* 33 ± 5%) (Fig 10). The results suggest that TMD9 is involved into the binding of procainamide, but the subtype specific binding of procainamide to rOCT1 compared to rOCT2 involves more than one transmembrane domain.

In case of inhibition by corticosterone, its 4 µM concentration strongly decreased TEA uptake by rOCT2 and did not significantly inhibit TEA uptake by rOCT1 wild type and by most chimeras except for the chimera 10, which activity was inhibited to the same degree as that of rOCT2 wild-type (63 ± 6% *vs.* 66 ± 6%) (Fig 11). Presence of 200µM corticosterone led to strong inhibition of rOCT2 and chimera 10 (2.2 ± 1.3% and 6.7 ± 1.4%, respectively) whereas extent of inhibition of other chimeras was not statistically different from rOCT1 (44.7 ± 5.2%) (Fig 12). It suggests that the difference between

affinities of rOCT1 and rOCT2 for corticosterone is mainly dependent on the 10th transmembrane domain.

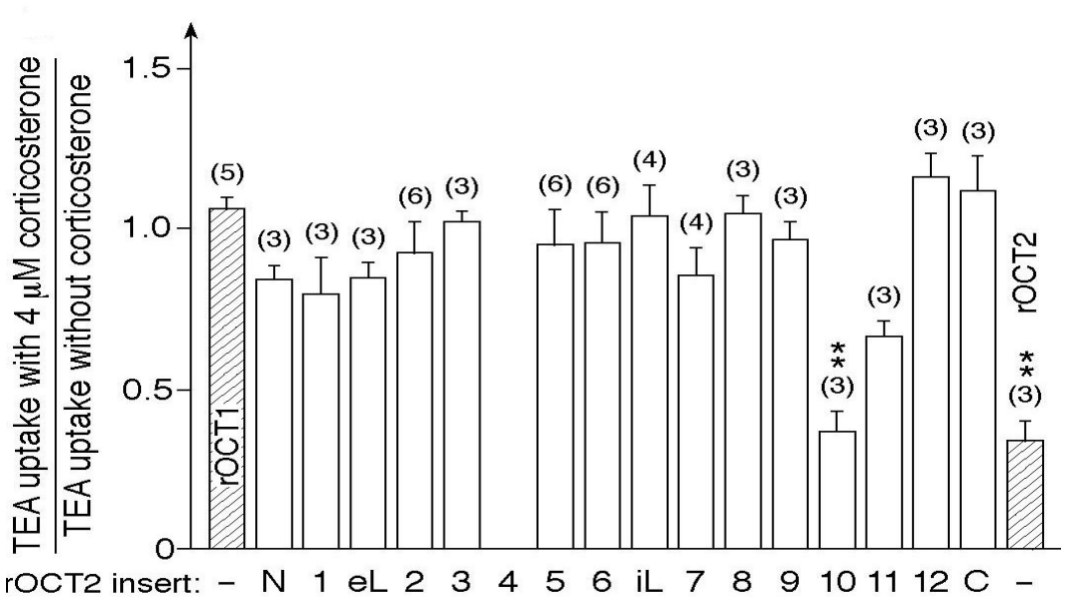

*Figure 11. Inhibition of TEA uptake by 4 µM corticosterone. Mean ± S.E.M. values are shown. The numbers of the performed experiments are indicated in parentheses. **, P< 0.01, according to ANOVA with post-hoc Tukey's test for difference to rOCT1 wild type.*

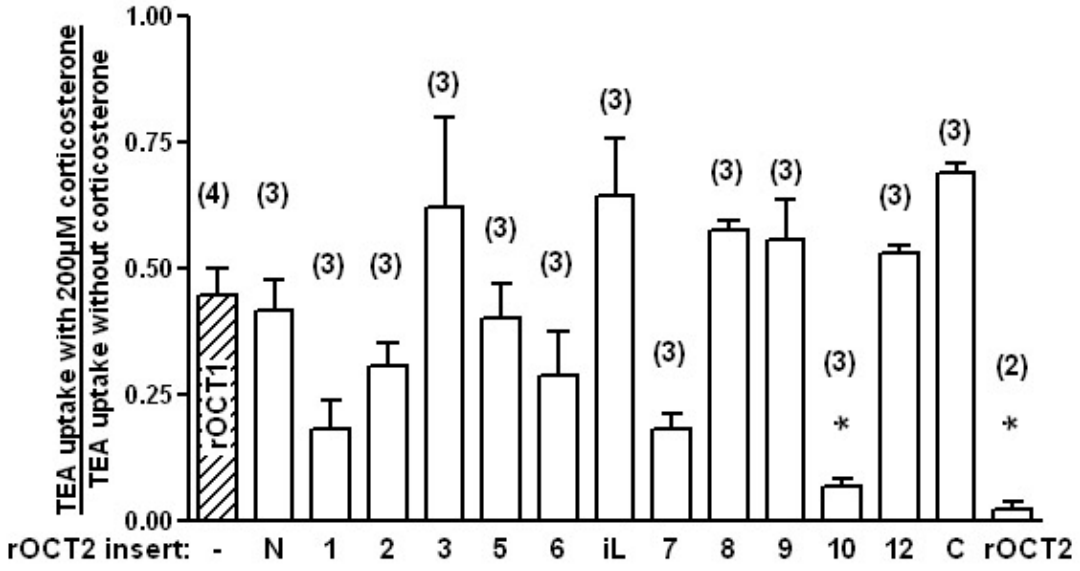

*Figure 12. Inhibition of TEA uptake by 200 µM corticosterone. Mean ± S.E.M. values are shown. The numbers of the performed experiments are indicated in parentheses. *, P< 0.1, according to ANOVA with post-hoc Tukey's test for difference to rOCT1 wild type.*

In next experiments, we analysed uptake of 10 µM [^{14}C]TEA by the chimera 10 at different concentrations of corticosterone and compared the data with the concentration dependence of corticosterone inhibition of [^{14}C]TEA uptake by wild type rOCT1 and rOCT2 (Fig. 13, Table 6). 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. Assuming competitive inhibition and Michaelis-Menten type substrate dependence the determined IC_{50} values are 13% (rOCT1, rOCT2) or 17% (chimera 10) higher as the respective K_i values ($K_i = IC_{50} / (1 + S/K_M)$). These results confirm that high affinity for corticosterone was conveyed to rOCT1 by transferring the presumed 10th TMD of rOCT2 to rOCT1. Our further study was focused on this region of rOCT1.

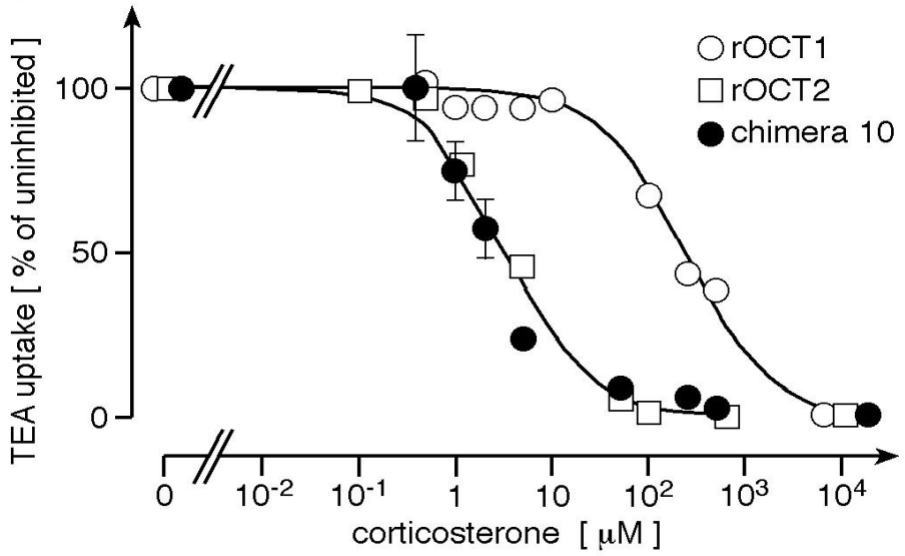

Figure 13. Inhibition of TEA uptake by corticosterone by OCT wild-types and mutants. Mean ± SEM of typical experiments with 7-10 oocytes. The curves were obtained by fitting the Hill equation to the data.

4.5 Interaction of Corticosterone with rOCT1 Mutants Containing Selected Amino Acids from rOCT2.

To identify 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. First we looked on those positions where amino acids showed strong difference of physicochemical properties between them, 447 (leucine in rOCT1 and tyrosine in rOCT2) and 448 (glutamine and glutamate, respectively) (see alignment in Fig 21 in Discussion). We measured concentration dependence for corticosterone inhibition of TEA uptake in oocytes expressing rOCT1, rOCT2 or rOCT1 mutants in which one or two amino acids in the 10th TMD were replaced by the corresponding amino acids of rOCT2.

Table 6. The IC_{50} values for inhibition of TEA uptake by corticosterone (mean ± S.E.M.). The numbers of the performed experiments are indicated in parentheses.
*** P< 0.001, ANOVA, difference compared with rOCT1.
^{oo} P <0.01, ANOVA, difference compared with rOCT2.
$^{++}$ P 0.01, ANOVA, difference compared with rOCT1(L447Y/Q448E).

Transporter	**IC_{50} for inhibition of TEA uptake by corticosterone (µM)**
rOCT1	198 ±17,4 (9)
rOCT2	5,9 ± 1,4 (5) ***
rOCT1 (TMD 10)	4,5 ± 0,8 (4) ***
rOCT1(L447Y)	42 ± 9 (4) ***, $^{oo, ++}$
rOCT1(Q448E)	40 ± 11 (4) ***, $^{oo, ++}$
rOCT1(L447Y/Q448E)	5,3 ± 1,7 (3) ***
rOCT1(A443I/L447Y/Q448E)	7,5 ± 1,4 (3) ***

For mutants L447Y and Q448E there were significantly lower IC_{50} values for corticosterone inhibition of TEA (10 µM) uptake than with rOCT1 wild-type were measured (Fig. 14, Table 6). The IC_{50} values obtained for mutants L447Y (42 ± 9µM) and Q448E (40 ± 11µM), however, were still significantly higher than that for rOCT2.

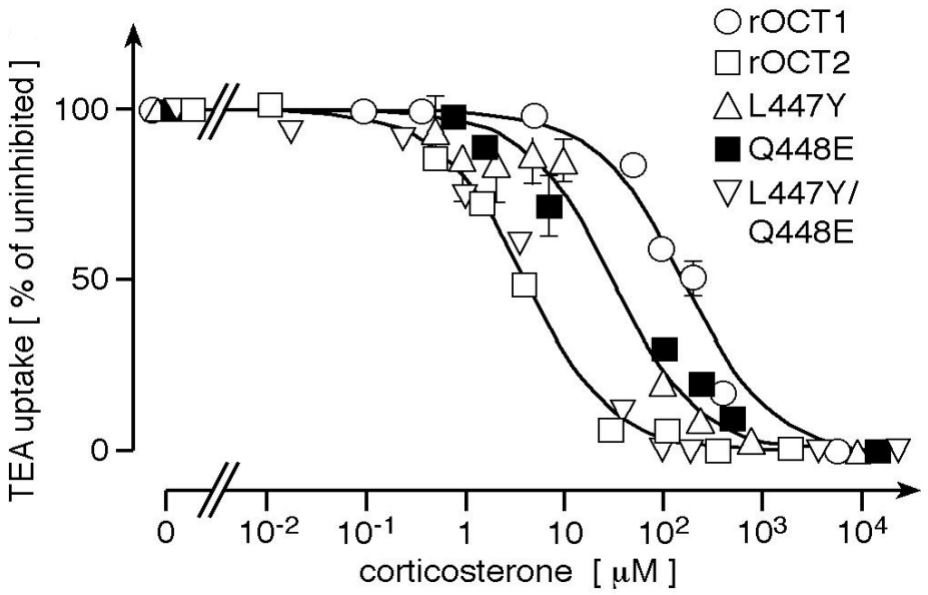

Figure 14. Inhibition of TEA uptake (10µM) by corticosterone by OCT wild-types and mutants. Mean ± SEM of typical experiments with 7-10 oocytes. The curves were obtained by fitting the Hill equation to the data.

When both amino acids were replaced simultaneously (rOCT1(L447Y/Q448E) double mutant), the IC_{50} value for corticosterone inhibition of TEA uptake decreased further to 5.3 ± 1.7 µM, the value that is significantly lower ($P<0.01$) than for the mutants with a single amino acid susbstitution but not different from rOCT2 (Fig 14 , Table 6). These data show that the replacement of two amino acids (L447, Q448) in rOCT1 by the corresponding amino acids from rOCT2 is sufficient to shift a corticosterone inhibition of TEA uptake to the level of rOCT2.

Recent experiments indicated that a substrate binding region of rOCT1 and rOCT2 contain overlapping binding sites for structurally different substrates and that this substrate binding region can be exposed to the extracellular or intracellular side of the plasma membrane (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. This implicates that effect of mutation(s) on a corticosterone inhibition of transport of different substrates may be different. To investigate putative differential effects of substrates on the affinity of corticosterone, we measured the inhibition of MPP uptake by corticosterone (Fig.15, 16). MPP was selected because it has little structural similarity to TEA and it was shown that binding sites for these substrates are overlapped only partially (Gorboulev et al., 1999) (see Figure 22 for structures in Discussion).

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. However, with the double mutant rOCT1(L447Y/Q448E) the IC_{50} value of corticosterone for inhibition of MPP uptake was higher compared to inhibition of TEA uptake (5.3 $\pm$ 1.7µM vs. 24 $\pm$ 3.8µM, respectively; $P<0.01$ for difference) (Figs. 14, 15, 16, and Table 6, 7).

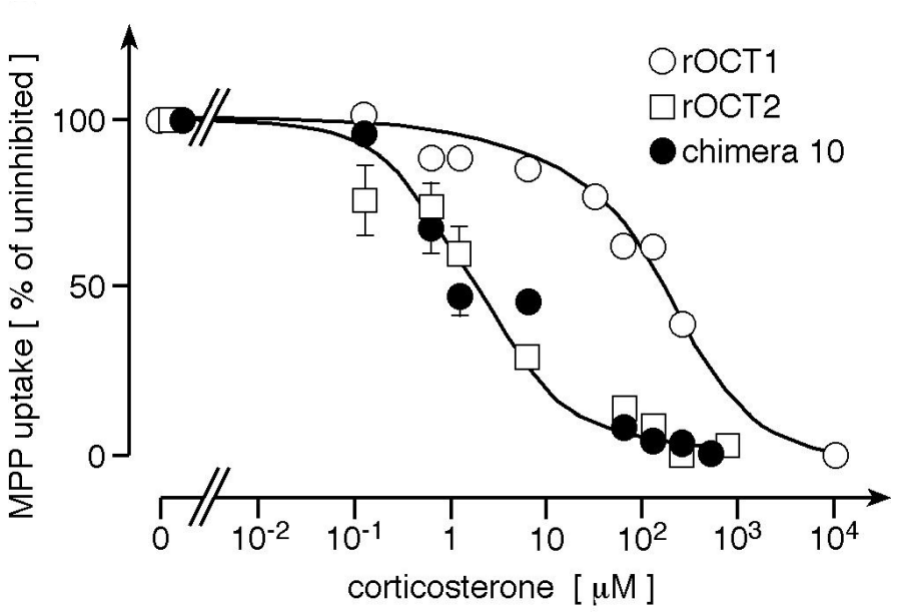

Figure 15. Concentration-inhibition curves in oocytes expressing rOCT1, rOCT2, or chimera 10. Mean $\pm$ SEM of typical experiments with 7-10 oocytes. The curves were obtained by fitting the Hill equation to the data.

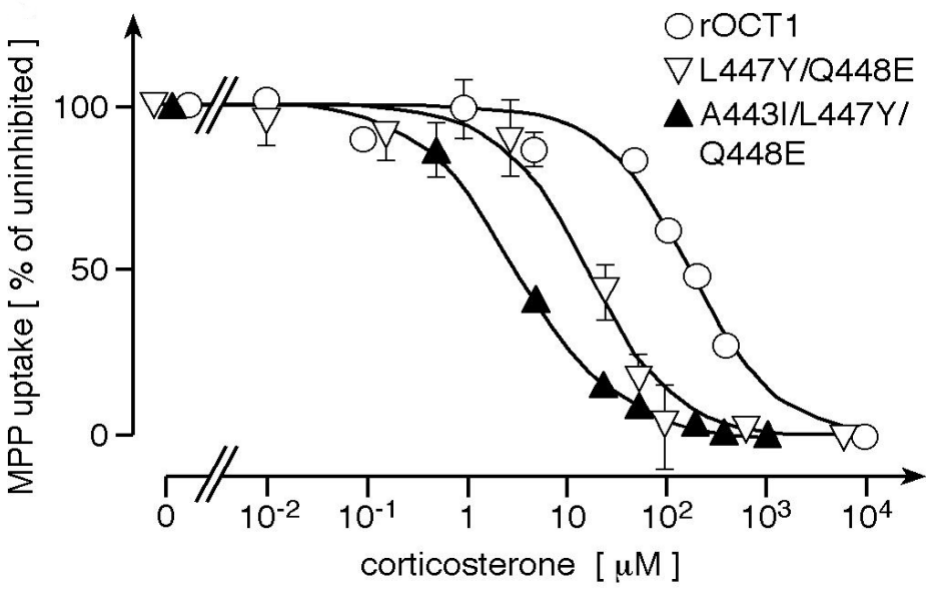

Figure 16. Inhibition curves of MPP uptake by corticosterone in oocytes expressing rOCT1 and mutants of rOCT1 in which indicated amino acids were replaced by the corresponding amino acids of rOCT2. Mean ± SEM of typical experiments with 7-10 oocytes. The curves were obtained by fitting the Hill equation to the data.

Measurements were performed with 0.1 µM MPP, a concentration that is at least 10 times below the respective K_M values of rOCT1, rOCT2, or the mutants (Table 7). Assuming a competitive inhibition by corticosterone, according to formula

$$Ki_{app} = Ki * (1 + S/Km),$$

where Ki and Ki_{app} are true and apparent inhibition constants, respectively, and Km is a Michaelis-Menten constant, 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 6).

Taking these calculations into account one should 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.

Table 7. The IC_{50} values for inhibition of MPP uptake by corticosterone (mean ± S.E.M.). The numbers of the performed experiments are indicated in parentheses.
**** P< 0.001, ANOVA, difference compared with rOCT1.*
^{oo} P <0.01, ANOVA, difference compared with rOCT2.
$^{++}$ P <0.01, $^{+++}$ P< 0.001, ANOVA, difference compared with rOCT1(L447Y/Q448E).
$^{\#\#}$ P< 0.01, ANOVA, difference between corticosterone inhibition of TEA uptake vs. corticosterone inhibition of MPP uptake by rOCT1(L447Y/Q448E).

Transporter	**IC_{50} [µM] for inhibition of MPP uptake by corticosterone**
rOCT1	174 ± 23 (5)
rOCT2	5,2 ± 1,9 (4) ***, +++
rOCT1 (TMD 10)	4,8 ± 3,3 (3) ***, +++
rOCT1(L447Y/Q448E)	24 ± 3,8 (4) ***, oo, ##
rOCT1(A443I/L447Y/Q448E)	8,6 ± 1,4 (4) ***, ++

In order to reconstitute in rOCT1 the same high affinity inhibition by corticosterone of both TEA and MPP uptake as exhibited by rOCT2, we studied inhibition by corticosterone of triple mutants of rOCT1 carrying double mutation L447Y/Q448E and additional amino acids from the 10th TMH of rOCT2. For the mutant rOCT1(A443I/L447Y/Q448E) the IC_{50} value for corticosterone inhibition of MPP uptake (8,6 ± 1,4 µM) was similar to rOCT2 (5,2 ± 1,9 µM). In this triple mutant, the IC_{50} values for corticosterone obtained with TEA and MPP were not significantly different from each other (Tables 6 and 7).

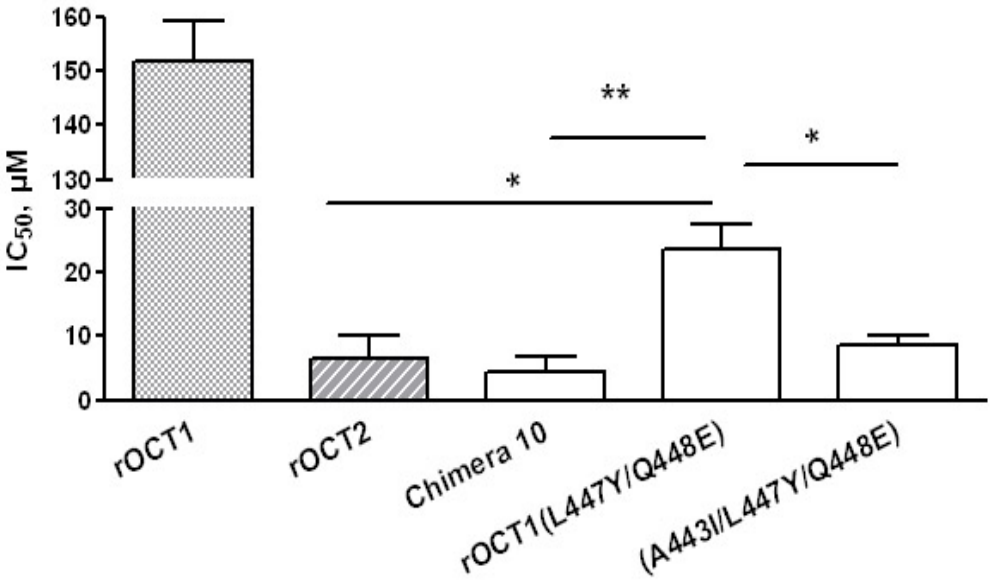

*Figure 17. The IC_{50} values for inhibition of MPP uptake by corticosterone. * P<0.05, ** P<0.01 according to ANOVA with post-hok Tukey's test. Value for rOCT1 was significantly different to all other values (P<0.01).*

4.6 Interaction of Cationic Substrates with rOCT1-Mutants Exhibiting High Affinity to Corticosterone.

It was shown previously for rOCT2 that choline-induced currents were inhibited after short 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 hypothesized that corticosterone binds to the substrate binding region of rOCT2 which can exist in an extracellularly and an intracellularly oriented conformation. To confirm that corticosterone binds to the substrate binding region we examined whether the rOCT1 point mutants with increased affinity to corticosterone also exhibit altered affinities for transported substrates.

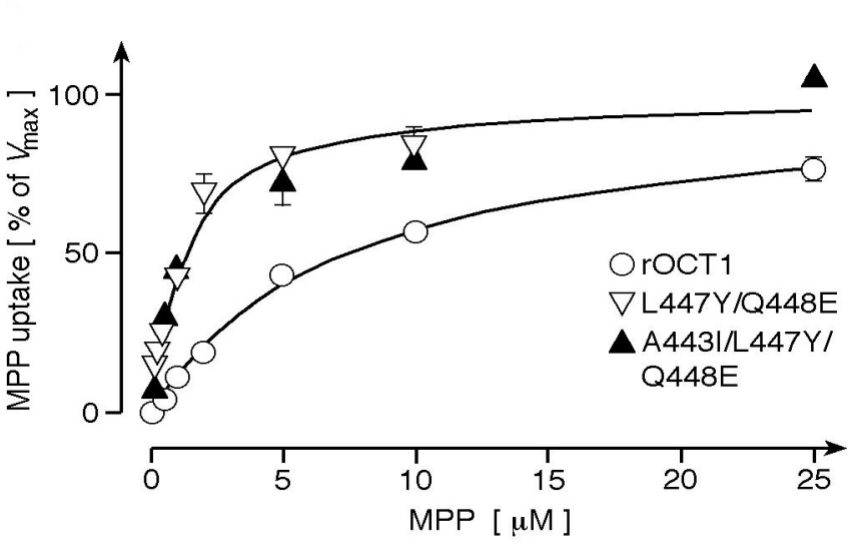

Figure 18. Substrate dependence of MPP uptake. rOCT1 wild-type, the double mutant rOCT1(L447Y/Q448E), and the triple mutant rOCT1(A443I/L447Y/Q448E) were expressed in oocytes, and uptake rates of [^{3}H]MPP were measured in the presence of substrates at various concentrations. Data points represent mean ± SEM. The curves were obtained by fitting the Michaelis-Menten equation to the data.

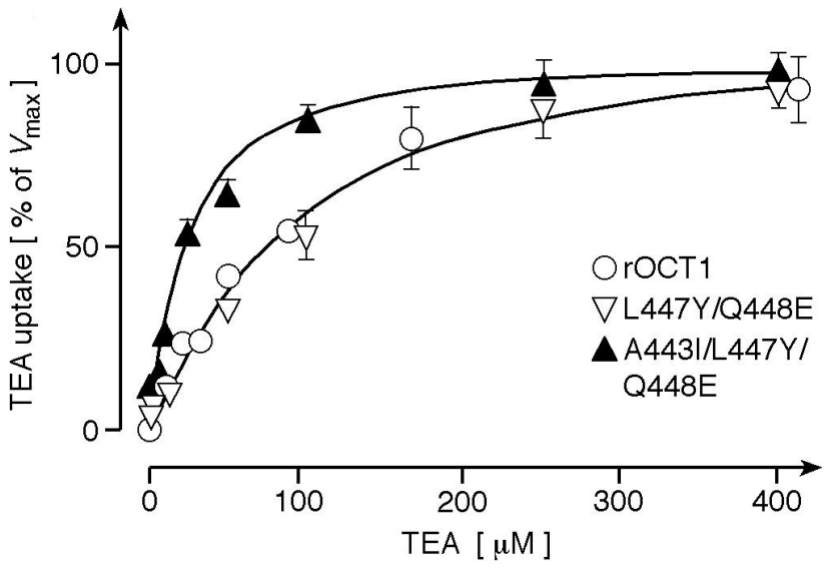

Figure 19. Substrate dependence of TEA uptake. rOCT1 wild-type, the double mutant rOCT1(L447Y/Q448E), and the triple mutant rOCT1(A443I/L447Y/Q448E) were expressed in oocytes, and uptake rates of [^{14}C]TEA *were measured in the presence of substrates at various concentrations. Data points represent mean ± SEM. The curves were obtained by fitting the Michaelis-Menten equation to the data.*

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. 18 and 19, and Table 8). 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 8). 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. In contrary, 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). However, in the rOCT1(A443I/L447Y/Q448E) triple mutant the K_M values and the IC_{50} values 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.

Table 8. The apparent K_m values and the IC_{50} values for rOCT1 and mutants (mean ± S.E.M.). The numbers of the performed experiments are indicated in parentheses.

** P< 0.05; ** P< 0.01, ANOVA, for difference compared with rOCT1 wild type.*

† P<0.05, ANOVA, for difference compared with rOCT1(L447Y/Q448E).

K_m or IC_{50} [µM]	**rOCT1**		
	Wild type	**(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) **, †
$K_m$for 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) *

To test whether the mutants have not only changed affinity to TEA and MPP but also differently changed transport rates for these substrates we measured substrate uptake by rOCT1 wild-type, double mutant L447Y/Q448E, and triple mutant A443I/L447Y/Q448E at saturating concentrations of TEA (1 mM) or MPP (0.1 mM). The measurements with TEA and MPP were performed simultaneously (within one hour) using identical batches of oocytes and the same cRNA preparations.

As can be seen from the Table 9, substitution of selected amino acid residues in rOCT1 decreases maximal transport velocity for both TEA and MPP to the same extent - the ratios between uptake rates are 11,8 $\pm$ 2,2, 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 double substitution L447Y and Q448E leads to increase of the apparent selectivity for MPP vs. TEA whereas the maximal velocity for MPP vs. TEA is not changed.

Table 9. The uptake rates (mean ± S.E.M) of TEA and MPP for rOCT1, rOCT1(L447Y/Q448E) and rOCT1(A443I/L447Y/Q448E). All experiments were done in three repeats.

	Uptake rates [pmol/h*oocyte^{-1}]		
Substrate	**rOCT1**	**rOCT1(L447Y/Q448E)**	**rOCT1(A443I/L447Y/Q448E)**
TEA [1mM]	1018 ± 29	119±20	85 ± 34
MPP [0,1mM]	95±25	13 ± 3	8 ± 2
TEAuptake/ MPPuptake	11,8 ± 2,2	9,8 ± 2,2	11,7 ± 2,6

4.7 Corticosterone Inhibition of rOCT2 Mutants Containing Individual Amino Acids from rOCT1.

To confirm the critical role of amino acids I443, Y447 and 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.

The double mutant rOCT2(Y447L/E448Q) and the triple mutant rOCT2(I443A/Y447L/ E448Q) were generated and the inhibition of MPP uptake by corticosterone was measured (Table 10, Fig.20). 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.

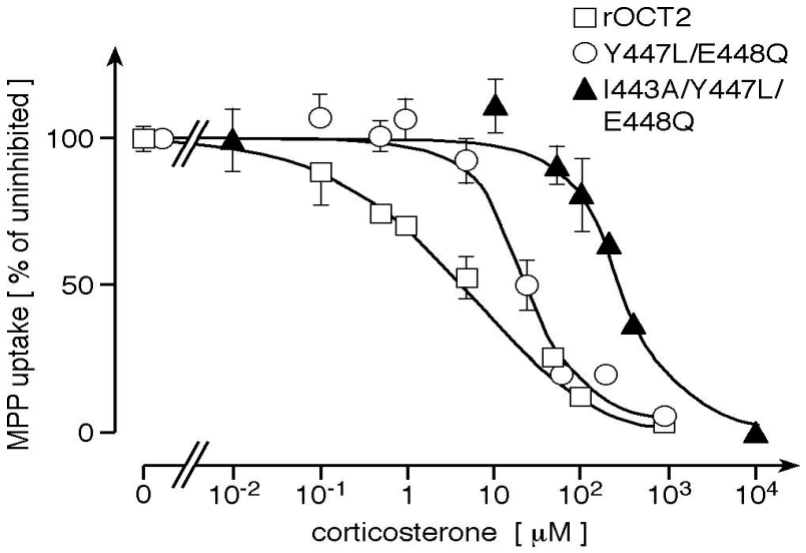

Figure 20. Inhibition of 0.1 µM [^{3}H]MPP uptake by corticosterone by rOCT2 wild-type and mutants containing amino acids of rOCT1. Mean $\pm$ SEM of typical experiments with 7-10 oocytes. The curves were obtained by fitting the Hill equation to the data.

Interesting to note that here we have the same situation with IC_{50} values of double mutant generated in rOCT2 as in rOCT1. The mutant rOCT2(Y447L/E448Q) has the IC_{50} value of corticosterone for inhibition of MPP uptake higher than for inhibition of TEA uptake (1,7 ± 0,6 (n=3) vs. 36,1 ± 6,1 (n=3), respectively).

Table 10. The IC_{50} values of corticosterone inhibition of MPP or TEA uptake for rOCT1, rOCT2 and mutants (mean ± S.E.M.). The numbers of the performed experiments are indicated in parentheses.

*** P <0.01, *** P <0.001, ANOVA, difference compared with rOCT1.*
^{oo} *P <0.01, ANOVA, difference compared with rOCT2.*
$^{++}$ *P < 0.01, Student's t test, difference compared with rOCT2(Y447L/E448Q) using MPP as substrate .*

Transporter	**IC_{50} [µM] of corticosterone inhibition of MPP uptake**	**IC_{50} [µM] of corticosterone inhibition of TEA uptake**
rOCT1	174 ± 23 (5)	198 ±10 (9)
rOCT2	5,2 ± 1,9 (4) ***	5,9 ± 1,1 (5) ***
rOCT2(TMD10)	15,7 ± 8,9 (3) **	
rOCT2(Y447L/E448Q)	36,1 ± 6,1 (3) **, ^{oo}	1,7 ± 0,6 (3) ***, $^{++}$
rOCT2(I443A/Y447L/E448Q)	382,3 ± 99,8 (3) $^{oo, ++}$	

5. Discussion.

Among a variety of substrates and inhibitors including TEA and MPP which interact nearly equally with both rOCT1 and rOCT2, others, for example corticosterone, procainamide and cimetidine show very different affinity for these transporters (Arndt et al., 2001).

We hypothesized that the homologue-specific selectivity of rOCT2 could depend upon a few number of amino acid residues which are different between rOCT1 and rOCT2. For this reason, we systematically and individually replaced different parts of rOCT1 by the respective regions from rOCT2. We have constructed 16 chimeric proteins on the basis of the rOCT1 backbone molecule. In the case of inhibition of TEA uptake by 450 µM procainamide neither substitution changed the inhibition level from the rOCT1-like type to the rOCT2-type. However, the replacement of TMD9 with its flanking region (Chimera 9) decreased the inhibition by 450µM procainamide partially although the inhibition was still significantly different from either rOCT1 or rOCT2. These data suggest that TMD9 participates in procainamide binding but that an additional region with structural difference between rOCT1 and rOCT2 is involved.

At variance, replacement of the only region containing 10^{th} TMD decreased the inhibition level of TEA uptake by 4 µM and 200µM corticosterone significantly from the rOCT1-like type to the rOCT2-like one. The importance of that region was further supported by the fact that detailed inhibition study of both TEA uptake and MPP uptake with corticosterone gave IC_{50} values which were equal to that of rOCT2. Therefore one can conclude that residues which determined higher affinity of rOCT2 to corticosterone were located within this region.

Figure 21 shows the alignment of the presumed 10^{th} TMD and its flanking loops of different members of OCT subfamily: rOCT1, rOCT2, rOCT3 and human OCT3 (hOCT3), and the IC_{50} values that were determined for inhibition of the OCTs by corticosterone (Gründemann et al., 1998; Wu et al., 1998; Arndt et al., 2001). Gly439, Glu456, Leu457, Tyr458, Pro459, and Thr460 (rOCT1 numbering) are conserved in nearly all *SLC22* members whereas Trp430, Arg440, Gly442, Thr444, Val453, and Asn454 are conserved within the OCT subfamily. Note that rOCT3 has approximately the same affinity for corticosterone as rOCT2. In the three positions which we found to be critical for the higher affinity of corticosterone to rOCT2 vs. rOCT1, rOCT3 contains

the same amino acids (Ile443, Glu448) or a similar amino acid as rOCT2 (Phe443 in rOCT3 vs. Tyr443 in rOCT2). This supports the idea that the amino acids in positions 443, 447 and 448 play a critical role in the affinity for corticosterone. At the same time, 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. Interestingly, the amino acid sequences in the 10th TMD and the flanking loops are identical between rOCT3 and hOCT3 whereas the IC_{50} value for corticosterone inhibition of cation uptake by hOCT3 vs. rOCT3 is more than twenty times lower. This means that subtype-specific interactions between corticosterone and OCT transporters involve another domain (or domains) and that differences between hOCT3 vs. rOCT3 within this additional domain(s) are responsible for the difference in affinity.

Replacement of selected residues exerted a significant effect on the apparent affinity of rOCT1 for corticosterone. Both substitutions Leu447Tyr and Gln448Glu decreased IC_{50} for inhibition of TEA uptake significantly, but only a combination of these two mutations gave the IC_{50} value which was not different from rOCT2 (Figure 14, Table 6). At the same time, double substitution decreased the IC_{50} value for inhibition of MPP uptake to the value which was still significantly higher than in the case of rOCT2. Only the combination of three substitutions A443I/L447Y/Q448E could completely create a high affinity for corticosterone in rOCT1. Interestingly, the apparent affinity for TEA and MPP in triple mutant is increased in comparison to wild type (28 ± 3 *vs.* 75 ± 11µM for TEA and 1.1 ± 0.2 *vs.* 5.6 ± 1 µM for MPP) (Figures 18 and 19, Table 8). The data allow one to make two important conclusions: first, these residues are part of binding region of rOCT1 for TEA and MPP since it is highly improbable that a combined mutation of three amino acids within one a-helix can increase the affinity of transported substrates if they are not localized within and/or close to the substrate binding region and hence second, corticosterone interacts with substrate binding site.

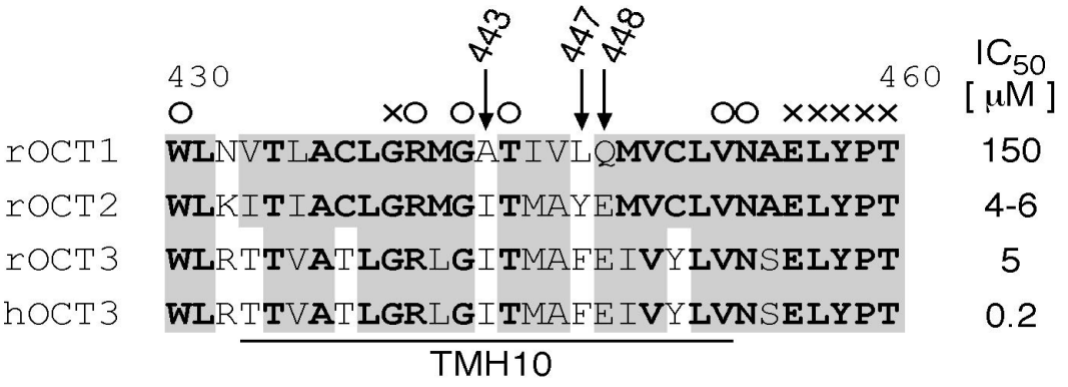

Figure 21. Alignment of the 10th transmembrane a-helix (TMH10) and parts of the flanking loops of OCTs. The sequences of OCT subtypes from rat and OCT3 from human (hOCT3) are aligned. IC_{50} values for inhibition of cation transport by corticosterone are indicated (Gründemann et al., 1998; Wu et al., 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 (x), and amino acids that are conserved throughout the organic cation transporters are indicated by (?).

Difference of the IC_{50} values for corticosterone inhibition of TEA- and MPP-uptake in the case of the double mutant rOCT1(L447Y/Q448E) indicates the presence of allosteric interaction between transported cation (TEA and/or MPP) and the inhibitor corticosterone which requires simultaneous binding of substrate and inhibitor. The 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. 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), 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), the 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). Since this is not the case, one can conclude that 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. It is difficult to distinguish whether the allosteric effect is

triggered by substrate binding or by substrate dependent conformational change during the transport cycle.

Our data about the involvement of residues from 10th TMD in substrate binding are corroborated by recent study of rabbit OCT1 and OCT2 implicating Glu447, which is a homologue of Glu448 in rOCT2, as a key contributor to the higher affinity of rbOCT2 in comparison to rbOCT1 for cimetidine and model substrate NBD-TMA (Zhang X. et al. 2005). Substitution E447Q alone gave the inhibition constants which were still significantly different from that of rbOCT1, and another mutation N353L did not change binding properties of rbOCT2. Combination of E447Q and N353L led to the complete change of affinity from the rbOCT2-like type to the rbOCT1-like type. One should note, however, that in this case substitutions caused a decrease of affinity; thus, interpretations of these data alone as an evidence for involvement of the investigated residues into the binding site should be done with cautions, since this effect could be also a result of indirect influence of mutations on affinity (as a result of conformational disturbances, for example). This is different in our case, where mutations led to increase of affinity and so far allowed making more straightforward conclusions.

The role of charge at the position 447 was supported by the observation that substitution of glutamate with cationic amino acids lysine or arginine completely abolished transport activity and E447L mutant displayed markedly reduced transport and affinity of TEA. At the same time, E447L retained the transport and affinity of MPP at normal level (Zhang X. et al. 2005). This data support the idea of presence in OCTs of multiple partially overlapped binding sites and the role of the residue at position 448 (in rOCT1) as a part of the binding region.

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 for rOCT2 were different from both sides. Corticosterone showed only partially competitive inhibition for the transported substrates choline and TEA. 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), it was

hypothesized that corticosterone could bind to extracellularly and intracellularly oriented conformations of the substate binding region, and the binding sites for corticosterone and other substrates are partially overlapped (Volk et al. 2003).

Recent experiments performed in our group employing electrical measurements of the rOCT1 mutants L447Y and Q448E with increased affinity for corticosterone have revealed that these mutations changed the affinities for corticosterone from both intracellular and extracellular sides. Since it is very unlikely that the same two neighbouring residues could have an impact on two different binding sites (extracellular and intracellular ones), these data suggest the presence of one binding site for corticosterone which can exist in two conformations directed either toward cytoplasmic or extracellular face of the plasma membrane rather than the presence of two different binding sites (Volk C. and Koepsell H., unpublished data).

Due to its hydrophobic nature, in experiments employing cells (or oocytes) corticosterone can penetrate intracellular and so far bind from both sides of the membrane. The inhibition experiments performed in the present study do not allow us to distinguish whether the mutations changed the affinity of corticosterone at the extracellular or 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). This suggestion is supported by the fact that the IC_{50} value for the inhibition of cation uptake by corticosterone under conditions, when corticosterone was equilibrated across the plasma membrane, was identical to IC_{50} value obtained after short application of corticosterone to the intracellular side of the plasma membrane.

The differential influence of selected residues on substrate interactions has been observed in several additional studies from our and others groups. In the assessment of the influence on transport of amino acid residues in TMH4 of rOCT1, it was found that replacement of Trp218 with tyrosine and Tyr222 with leucine resulted in significant decreases in the apparent affinities for both TEA and MPP, whereas the Y222F and

T226A mutants decreased the affinities for only TEA or MPP, respectively (Popp et al., 2005). The earlier study on the influence of acidic residues on the transport activity of rOCT1 showed that D475E mutant displayed a marked increase in apparent affinity for TEA and some inhibitors, with no change in interaction with MPP (Gorboulev et al., 1999). Lending further support to the view that Asp475 plays a key role in defining a binding surface in OCTs is the observation by Wolff and coauthors (Wolf et al., 2001) that the homologous position in the sequence of all members of the OAT subfamily (which are also members of the OCT transport family) is filled with a cationic residue. Although the R478D mutant of the flounder ortholog of OAT1 still supported transport of the monovalent anion PAH (albeit at a reduced rate compared with the wild type protein), this mutation eliminated interaction with another substrate of OAT1, dicarboxylate glutarate. In the rat ortholog of OAT1, similar differential effects on interaction with different substrates have also been reported in the study that used site-directed mutagenesis approach to probe the influence of selected residues on the transport activity of rOAT3. The homologous replacement in rOAT3 (*i.e.* R454D) showed a profound change in interaction with PAH (Feng et al., 2001), and mutations of several hydrophobic residues in TMH7 (W334A, F335A, Y341A, and Y342Q) and one in TMH8 (F362S) resulted in a marked decrease in transport of the hydrophilic substrates PAH and cimetidine, with comparatively little effect on transport of the more hydrophobic substrate estrone sulfate (Feng et al., 2002), leading to the conclusion that the structure of OAT3 includes a general binding domain with no single binding site.

Recently high-resolution crystal structures of transporters belonging to MFS, the lactose permease LacY (Abramson et al., 2003) and the glycerol-3-phosphate transporter GlpT (Huang et al., 2003), became available. Both structures show a highly similar fold and thus led to suggestion that the fold is conserved throughout the superfamily (Vardy et al. 2004).

Based on this idea, a 3D structure of rOCT1 was built in our group by homology modelling (Popp et al., 2005) (Figure 22). As a template for modelling the crystal structure of LacY was used (LacY has 29% of sequence similarity with rOCT1 within regions that are supposed to form transmembrane-spanning domains).

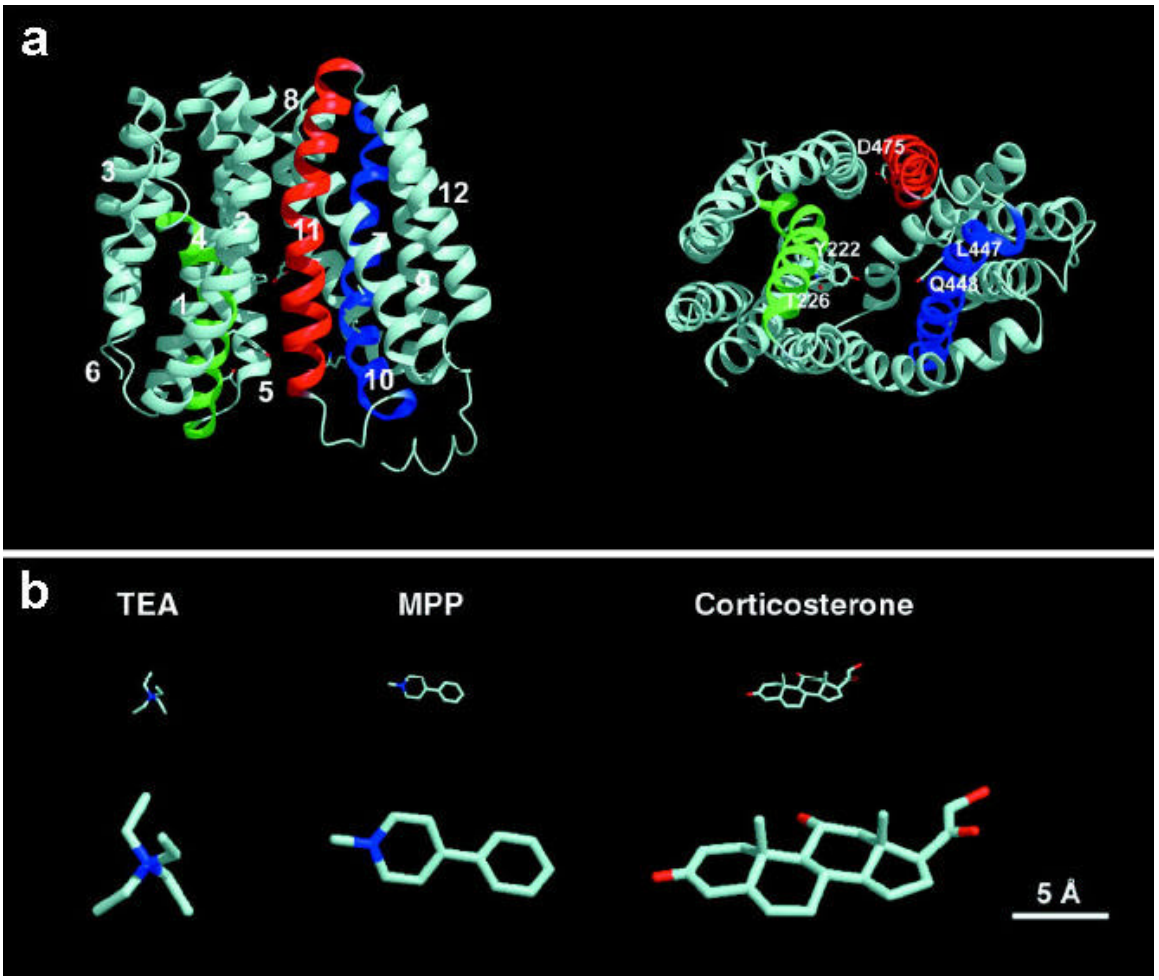

Figure 22. Structure model of rOCT1(from Popp et al. 2005). ***a, left panel-*** *a ribbon presentation of the rOCT1 model. The individual TMDs are numbered. The 4th, 10th and 11th TMD are colored in green, blue and red, respectively. Amino acid side chains on these TMDs that have been localized to the substrate binding region by mutagenesis experiments are depicted (W218, Y222 and T226 on the 4th TMH, A443, L447 and Q448 on the 10th TMH, and D475 on the 11th TMH).* ***a, right panel-*** *a ribbon representation of the rOCT1 model oriented to show the location of all seven amino acids. The side chains of amino acids W218, Y222, T226, A443, L447, Q448 and D475 are indicated. Numbering of amino acids W218 and A443 is omitted for sake of clarity.* ***b,*** *molecular structures of TEA, MPP and corticosterone in two magnifications, the presentation of the upper panel shows the size in respect to the model of rOCT1 in* ***a****, the lower panel shows the enlarged structures, the size bar is for the lower panel.*

As can be seen on the figure, the model of rOCT1 shows a large cleft that is accessible from the intracellular side of the membrane. The cleft in rOCT1 is formed by the 1st, 2nd, 4th, 5th, 7th, 8th, 10th and 11th TMD. Validity of the model is corroborated by the fact that all amino acids that have been identified to be involved in the substrate binding (4th TMD: W218, Y222 and T226 (Popp et al. 2005); 10th TMD: A443, L447

and Q448 – residues described in this work; 11th TMD: D475 (Gorboulev et al. 1999)) are located in the large cleft and accessible from the aqueous phase. Interestingly, according to the model, these amino acids are located at a similar depth within the large cleft. They may be part of a substrate binding region surrounding the cleft. The comparison of the modelled substrate binding region surrounding the cleft with the sizes of TEA, MPP and corticosterone suggests that more than one molecule of these compounds can bind at the same time (Popp et al. 2005).

The above described allosteric effect can be due to interaction between substrate binding regions in monomers of a dimeric transporter, between coexisting substrate binding regions in a monomeric transporter, or 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 three-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. In the 3-dimensional model of rOCT1 the 10^{th} a-helix protrudes into the cleft allowing an interaction of corticosterone with the two succeeding amino acids L447 and Q448.

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 the rOCT1 wild type. This indicates that some cooperation between 447Y and 448E with the rest of the rOCT1 structure was required for the allosteric interaction. However, because we observed allosteric interactions of MPP and other substrates also in wild types of human OCT1 and human OCT3 (U. Roth and H. Koepsell, unpublished data) we interpret the allosteric interaction observed in the rOCT1(L447Y/Q448E) mutant as a demonstration of the principle (i.e., that ligands of OCT binding regions can exhibit allosteric interactions).

Mapping the surface of substrate binding regions in OCTs by crystallization 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 influencing the absorption of drugs in small intestine and their renal and hepatic excretion and thereby modulating physiological functions that are controlled by the OCTs. The interaction of corticosterone and other 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 glucocorticoid budesonide probably inhibit this function (Lips K. et al, 2005). 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; Horvath et al., 2003; Schmitt et al., 2003; Vialou et al., 2004). Inhibition of hOCT3 may lead to an increase of blood pressure and to alterations in behavior (Vialou et al., 2004).

6. Summary.

The polyspecific organic cation transporters (OCT) are involved in the elimination and distribution of drugs, environmental toxins, and endogenous organic cations including monoamine neurotransmitters. Steroid hormones inhibit organic cation transport by the three OCT subtypes with different affinities showing distinct species difference; for example, the IC_{50} values for corticosterone inhibition of cation uptake by transporters rOCT1 and rOCT2 are ~150µM and ~4 µM, respectively.

By introducing domains and amino acids from rOCT2 into rOCT1, we identified three amino acids in the presumed 10^{th} TMD of rOCT2 which are responsible for the higher affinity of corticosterone in comparison to rOCT1. This is the first study which revealed the components of the binding site for corticosterone in OCTs. The evidence is presented that these amino acids (alanine 443, leucine 447, and glutamine 448 in rOCT1 and isoleucine 443, tyrosine 447, and glutamate 448 in rOCT2) are probably located within the substrate binding region of OCTs since the affinity of transported cations was increased together with the affinity of corticosterone. In the double mutant rOCT1(L447Y/Q448E) the IC_{50} value for the inhibition of [^{3}H]MPP (0.1 µM) uptake by corticosterone (24 ± 4 µM) was significantly higher compared to the IC_{50} value for inhibition of [^{14}C]TEA (10 µM) uptake (5.3 ± 1.7 µM), indicating an allosteric interaction between transported substrate and corticosterone. The data suggest that more than one compound can bind simultaneously to the substrate binding region. These results confirm previous suggestion that binding of substrates and inhibitors to OCTs involves interaction with a comparatively large surface that may include multiple binding domains rather than with a structurally restricted single binding site.

Zusammenfassung.

Die polyspezifischen Transporter für organische Kationen (OCT) spielen eine wichtige Rolle bei der Ausscheidung von Medikamenten, Toxinen und endogenen organischen Kationen, zu denen auch monoamine Neurotransmitter gehören.

Der durch OCT vermittelte Transport von organischen Kationen kann von Steroidhormonen gehemmt werden, die für drei OCT Subtypen deutlich unterschiedliche Affinität aufweisen: Zum Beispiel, IC_{50} Werte der Hemmung des Transportes von organischen Kationen durch Corticosteron betragen ~ 150 µM für rOCT1 und ~ 4 µM für rOCT2.

Mithilfe des Austausches von rOCT1 Domänen und einzelnen Aminosäuren gegen die entsprechenden Elemente des rOCT2 Moleküls haben wir in der 10^{th} TMD von rOCT2 drei Aminosäuren identifiziert, die für die höhere Affinität des rOCT2 Transporters zu Corticosteron verantwortlich sind.

In dieser Arbeit wurden zum ersten Mal die Aminosäuren identifiziert, die zu der Bindungsstelle eines OCT Transporters für Corticosteron gehören. Die Ergebnisse der Untersuchungen deuten darauf hin, dass diese drei Aminosäuren (Alanin 443, Leucin 447 und Glutamin 448 in rOCT1 und Isoleucin 443, Tyrosin 447 und Glutamat 448 in rOCT2) hochwahrscheinlich in der Substratbindungstasche von OCT liegen, da zusammen mit der Erhöhung der Affinität zu Corticosteron stieg auch die Affinität zu transportierenden Substraten.

Für die doppelte Mutante rOCT1(L447Y/Q448E) unterscheiden sich die IC_{50} Werte für die Hemmung des [^{3}H]MPP (0,1 µM) und des [^{14}C]TEA (10 µM) Transportes durch Corticosteron um Faktor 4 (24 ± 4 µM bzw. 5,3 ± 1,7 µM), was eine allosterische Interaktion zwischen transportierenden Substraten und Corticosteron vermuten lässt. Die Daten deuten darauf hin, dass mehr als eine Substanz sich gleichzeitig an die Substratbindungsregion binden kann.

Die gewonnenen Erkenntnisse bestätigen unsere vorigen Vorstellungen, dass die Bindung von Substraten und Hemmstoffen zu OCT die Interaktion mit der relativ großen Oberfläche des Proteins vorsieht, die vermutlich nicht auf eine einzige Bindungsstelle beschränkt ist, sondern eher mehrere Bindungsdomäne.

7. List of Abbreviations.

BES	N,N-bis[2-Hydroxyethyl]-2-aminoethansulfonic acid
Bq	Becquerel
Cpm	counts per minute
Ci	Curie ($3.7 \times 10_{10}$ Bequerel)
C-Term	C-terminus
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
E-Loop	extracellular loop
fOCT	Flounder organic cation transporter
hOCT	Human organic cation transporter
I-Loop	Internal loop
kDa	KiloDalton
MBq	MegaBecqerel
Mg	milligramm
MFS	Major facilitator superfamily
MPP	1-Methyl-4-phenylpyridinium
mRNA	Messenger ribonucleic acid
mOCT	Mouse organic cation transporter
MOPS	3-Morpholino-2-Hydroxypropanesulfonic acid
NBD-MTMA	N,N,N-trimethyl-2-(methyl(7-nitro-2,1,3-benzoxadiazol-4-yl)amino)ethanaminium
NMN	N-1-Methylnicotinamide
N-Term	N-terminus
OAT	Organic anion transporter
OC	Organic cation
OCT	Organic cation transporter
OCTN	Organic cation-carnitine transporter
PAH	Para-aminohypuric acid
PCR	Polymerase chain reaction
rOCT	Rat organic cation transporter

rpm	rounds per minute
SDS	Sodiumdodecylsulfat
SLC	Solute carrier superfamily
SNP	Single nucleotide polymorphisms
TEA	Tetraethylammonium
TMD	Transmembrane domain
Wt	Wild type

Amino acids.

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamat	Glu	E
Glutamin	Gln	Q
Glycin	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

8. List of publications:

Kerb R, Brinkmann U, **Chatskaia N**, Gorbunov D, Gorboulev V, Mornhinweg E, Keil A, Eichelbaum M, Koepsell H (2002) Identification of genetic variations of the human organic cation transporter hOCT1 and their functional consequences. Pharmacogenetics 12:591–595

Popp C, Gorboulev V, Muller TD, Gorbunov D, **Shatskaya N**, Koepsell H. (2005) Amino acids critical for substrate affinity of rat organic cation transporter 1 line the substrate binding region in a model derived from the tertiary structure of lactose permease. Mol. Pharmacol. 67(5):1600-1611.

Gorboulev V, **Shatskaya N***, Volk C, Koepsell H (2005) Subtype-specific affinity for corticosterone of rat organic cation transporters rOCT1 and rOCT2 depends on three amino acids within the substrate binding region. Mol. Pharmacol. 67(5):1612-1619.

* - *equally contributed first coauthor.*

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Erklärungen zur Zulassung zum Promotionsverfahren.

Hiermit erkläre ich ehrenwörtlich, dass ich diese Dissertation selbständig angefertigt habe und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Weiterhin erkläre ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegt habe.