L-Carnitine is an essential component of mitochondrial fatty acid β-oxidation and plays a pivotal role in the maturation of spermatozoa within the male reproductive tract. Epididymal plasma contains the highest levels of L-carnitine found in the human body, and initiation of sperm motility occurs in parallel to L-carnitine increase in the epididymal lumen. Using a specific carrier, epididymal epithelium secretes L-carnitine into the lumen by an active transport mechanism; however, the structure-activity relationship comprising the carnitine-permeation pathway is poorly understood. We discovered a novel carnitine transporter (CT2) specifically located in human testis. Analyzing the primary structure of CT2 revealed that it is phylogenetically located between the organic cation transporter (OCT/OCTN) and anion transporter (OAT) families. Hence, CT2 represents a novel transporter family. When expressed in Xenopus oocytes, CT2 mediates the high affinity transport of L-carnitine but does not accept mainstream OCT/OCTN cationic or OAT anionic substrates. We synthesized and tested various carnitine-related compounds and investigated the physicochemical properties of substrate recognition by semi-empirical computational chemistry. The data suggest that the quaternary ammonium cation bulkiness and relative hydrophobicity be the most important factors that trigger CT2-substrate interactions. Immunohistochemistry showed that the CT2 protein is located in the luminal membrane of the epididymal epithelium and within the Sertoli cells of the testis. The identification of CT2 represents an interesting evolutionary link between OCT/OCTNs and OATs, as well as provides us with an important insight into the maturation of human spermatozoa.

* This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports, Culture and Technology, by the Japan Society for the Promotion of Science, and by the High-Tech Research Center, Science Research Promotion Fund of Japan Private School Promotion Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed. Tel.: 81-422-47-5511 (ext. 3451); Fax: 81-422-79-1321; E-mail: endouh@kyorin-u.ac.jp.

** The abbreviations used are: OCT, organic cation transporter; CT, carnitine transporter; OAT, organic anion transporter; OCTN, novel organic cation transporter; GABA, γ-aminobutyric acid; NMDG; N-methyl-D-glucamine; TEA, tetraethylammonium cation; MCPBA, m-chloroperoxybenzoic acid.

Received for publication, April 22, 2002, and in revised form, June 26, 2002
Published, JBC Papers in Press, June 27, 2002, DOI 10.1074/jbc.M203883200

From the ‡Department of Pharmacology and Toxicology and §Department of Pathology, Kyorin University, School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan, the ‡‡Department of Clinical Preventive Medicine, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan, ‡‡‡Novartis Pharmaceuticals Corporation, Drug Metabolism Pharmacokinetics, East Hanover, New Jersey 07936-1080, and the ***Department of Pharmacology and Physiology, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642
A Novel Carnitine Transporter in Human Testis

A human expressed sequence tag (EST) from human testis cDNA libraries (GenBankTM/EBML/DDJB accession no. AA778598) showing nucleotide sequence similarity to rat OAT1 (9, 10) was obtained from the Integrated and Molecular Analysis of Genomes and their Expression (IMAGE) data base (cDNA clone number 1048962). The [32P]dCTP-labeled probe was synthesized from the clone (T7 Quick Prime, Amersham Biosciences) and was used to screen a human testis cDNA library. As described previously, a nondirectional cDNA library was prepared from the human testis poly(A)+ RNA (CLONTECH) with the SuperscriptTM Choice system (Invitrogen) (9, 13). The cDNAs were ligated into ZipLox EcoRI arms (Invitrogen). Replicated filters of the phage library were hybridized overnight at 37 °C in a hybridization solution (50% formamide, 5× standard saline citrate (SSC) (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 3% Denhardt’s solution, 0.2% SDS, 10% dextran sulfate, 0.2 mg/ml denatured salmon sperm DNA, 2.5 mM sodium pyrophosphate, 25 mM MES, and 0.01% Antifoam B, pH 6.5), and washed at 37 °C in 0.1× SSC and 0.1% SDS. The cDNA inserts (CT2) in positive ZipLox phages were recovered in the expression vector, pZL1 (Invitrogen), by in vivo excision.

Sequence Analyses

Sequence analyses and determination of genomic organization were performed with web-based data base searches through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the DNASTAR programs (Hitachi Software Engineering). Multiple sequence alignment and topology prediction were done with the DNASTAR program. The phylogenetic analyses were performed with the CLUSTALW program (clustalw.genome.ad.jp/) and displayed with the TreeView drawtree program (taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Functional Expression of CT2 in Xenopus Oocytes

As previously described, complementary RNA (cRNA) synthesis and uptake measurements were performed (9). Briefly, the cDNA was linearized with SpeI, and the cDNA insert transcribed in vitro with T7 RNA polymerase (Stratagene) in the presence of RNA cap analog (Amersham Biosciences). The resultant cRNA was purified by multiple phenol/chloroform extractions and precipitated with ethanol.

Mature cDNAs from Xenopus laevis (stages IV and V) were isolated by treatment with collagenase A (2.0 mg/ml) (Roche Molecular Biochemicals). Defolliculated oocytes were injected with 30–50 ng of capped CT2 cRNA and incubated at 18 °C in a modified Barth’s solution (88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO3)2, 0.4 mM CaCl2, 0.8 mM MgSO4, 2.4 mM NaHCO3, and 10 mM HEPES) containing gentamicin (50 µg/ml). After incubation for 2–3 days, uptake experiments were performed at room temperature in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES, pH 7.4). The uptake experiment was initiated by replacing the ND96 solution with a solution containing radiolabeled L-carnitine (specific radioactivity, 2.22 TBq/mmol, American Radiolabeled Chemicals) and was terminated by adding ice-cold ND96 buffer after 1 h of incubation. Oocytes were washed five times with ice-cold ND96 solution, solubilized with 10% SDS, and radioactivity content determined.

The kinetic parameters for the uptake of L-carnitine via CT2 were estimated with the following equation: 

\[
\frac{v}{v_{\text{max}}} = \frac{[S]}{K_m} + \frac{1}{K_m} \frac{[S]}{v_{\text{max}}}
\]

where v is the uptake rate of L-carnitine (picomol/oocyte/h), [S] is the substrate concentration (µM) in the medium, and \( K_m \) is the Michaelis-Menten constant (µM). Inhibition constants (\( K_i \)) were calculated as previously described (19).

In Na+ substitution experiments, Na+ in ND96 bath was replaced with an equivalent concentration of Li+ or with an impermeant anion (e.g. N-acetyl-d-glucamine (NDG)). For the inhibition study, uptake rate of 50 mM [3H]-carnitine by oocytes injected with water or CT2 cRNA were measured for 1 h in the absence or presence of 5 or 50 µM test compound in ND96 solution.

To examine trans-stimulatory effects on the efflux of radiolabeled substrates, CT2 expressed oocytes were incubated with [3H]-carnitine (50 nM) for 90 min and transferred into the medium with or without unlabeled L-carnitine. The radioactivity in the medium and within the oocytes was determined after 20-22 min incubation period.

Synthesis of Various Carnitine-related Compounds

To test substrate recognition by CT2, we synthesized using known or slightly modified literature procedures (21–24), a variety of carnitine-related compounds (1–24, and 1–13). The reagents—L-Carnitine (1), ethyl 4-bromobutyrate, ethyl 5-bromovalerate, ethyl 6-bromohexanoate, 4-pentanoic acid, tert-butyl alcohol, sodium, benzophenone, trans-crotyl chloride, NaHCO3, bromoethane, CH3CN, MCPBA, MgSO4, OEt2, acetone, Me2SO, KOH, 2-butanol, triethylamine, tripropylamine, dipropylethylamine, diethylmethylamine, trimethylamine (40% aequous), and ethyl acetate were obtained from Aldrich-Sigma.

(4-Carboxybutyl)trimethylammonium hydrochloride (2A, 48%), (4-carboxybutyl)diethylmethylammonium hydrochloride (2A, 36%), (4-carboxybutyl)triethylammonium hydrochloride (4A, 42%), (4-carboxybutyl)ethylisopropylpropylammonium hydrochloride (5A, 37%), (4-carboxybutyl)trimethylammonium hydrochloride (6A, 40%), (5-carboxypentyl)trimethylammonium hydrochloride (7A, 43%), (5-carboxypentyl)diethylmethylammonium hydrochloride (8A, 46%), (5-carboxypentyl)trimethylammonium hydrochloride (9A, 42%), (6-carboxychexyl)trimethylammonium hydrochloride (10A, 26%), (6-carboxychexyl)diethylmethylammonium hydrochloride (11A, 34%), and (6-carboxychexyl)triethylammonium hydrochloride (12A, 32%).—Ethyl 4-bromobutyrate (25.6 mM, 5.0 g) in 2-butanol (2.0 ml), ethyl 5-bromovalerate (16.7 mmol, 3.5 g) and ethyl 6-bromohexanoate (13.4 mmol, 3.0 g) were in petroleo ether (5 ml), which was mixed with 1 mol eq of amine added. The vials were closed and heated (12 h; 90 °C). The solvent was removed to give oils that were crystallized from EtOAc: 2%; 7%; 9%; 3%; 7%; 4%; 53%; 5, 34%; 6, 67%; 7, 64%; 8, 88%; 9, 51%; 10, 82%; 11, 58%; and 12, 60%. The ethyl esters (2–12) were converted to their carboxyl HCl analogs (2A–12A, and 13–20). Esters (1.0 g) in H2O (20 ml) and concentrated HCl (1 ml) were
heated under reflux (6 h) and solvent removed under reduced pressure and recrystallized from EtOH.

3-Hydroxy-4-(trimethylammonio)butanoic acid (13), 3-hydroxy-4-(triethylammonio)butanoic acid (14), 3-hydroxy-4-(tripropylammonio)butanoic acid (15), and 3-hydroxy-4-(tetraethylammonio)butanoic acid (16)–4-Butyl alcohol (0.59 mol, 56 ml) and TEA (0.40 mol, 56 ml) were added to EtOH (150 ml); trans-Crotyl chloride (90%, 0.38 mol, 40 ml) in EtOH (20 ml) was added dropwise (1 h) and kept below 30 °C. Reaction was stirred (1.5 h), filtered, and solid extracted with OEt2. The organic phase was extracted with ice-cold 5% NaHCO3 (100 ml), H2O (100 ml), 5% HCL (100 ml), 5% NaHCO3 (100 ml), and saturated NaCl (200 ml). The organic phase was dried and red concentrate vacuum distilled (20 mm Hg) to give tert-butyl 3-butenoate (43.5 g, 0.318 mol, 84% yield). Gas chromatography/mass spectrometry (electron ionization) m/z values were 143, 127, 69, and 57. The ester (0.18 mol, 25.2 g) was dissolved in CH2Cl2 (120 ml) and cooled in an ice bath. MCPBA (0.43 mol, 57 g) in CH2Cl2 (600 ml) was added dropwise (3.5 h) with stirring and placed into a refrigerator (4 °C, 4 days). The mixture was filtered and 10% sodium sulfite (500 ml) added and stirred (1 h). The organic phase was extracted with 5% NaHCO3 (200 ml), H2O (200 ml), and saturated NaCl (200 ml). The organic phase was dried, concentrated, and yellow liquid vacuum distilled (0.4–0.5 mm Hg) to afford tert-butyl 3,4-epoxybutanoate (26 g, 0.16 mol, 52% yield). The epoxy-ester was used to prepare 3-hydroxyammoniobutylates. Epoxy-ester (0.1013 mol, 2.1 g) in 30 ml of 70% EtOH was mixed with 1 eq of amine. The vessels were sealed and heated (60 °C) with stirring. After 24 h, the vials were cooled, concentrated, diluted with H2O (20 ml), and extracted with OEt2 (2 × 20 ml). The aqueous layer was placed under vacuum (0.01 mm Hg; 3 day). The products were crystallized from hot, dry acetone, sealed and isolated by vacuum filtration, and dried under vacuum (0.01 mm Hg) to afford: 13, 44%; 14, 54%; 15, 59%; and 16, 48%.

4-Hydroxy-5-(diethylammonio)pentanoic acid (17), 4-hydroxy-5-(triethylammonio)pentanoic acid (18), 4-hydroxy-5-(tripropylammonio)pentanoic acid (19), and 4-hydroxy-5-(tetraethylammonio)pentanoic acid (20)–4-Pentenoic acid (0.20 mol, 20 g) in MeOH (50 ml) was added to powdered ROH (0.299 mol, 16.8 g) in MeOH (200 ml). Bromoethane (0.06 mol, 46 ml) was added by syringe transfer; a mildly exothermic reaction ensued. The mixture was stirred at room temperature for 33 h, poured into H2O (800 ml), and extracted with CH2Cl2 (3 X 250 ml). The organic phase was concentrated and distilled under vacuum (7 mm Hg). The fraction boiling at 52–56 °C was collected to give 25.3 g of ethyl 4-pentenoate (93% yield). Gas chromatography/mass spectrometry (electron ionization) m/z values were 128, 83, 55, and 45. The ester (0.198 mol) was dissolved in CH2Cl2 (500 ml) and MCPBA (0.39 mol, 52 g of 75% solid in CH2Cl2 (150 ml) was added and mixture stirred and heated (50 °C) for 24 h. The mixture was cooled and extracted with 10% NaHSO3 (200 ml), saturated NaHCO3 (2 × 200 ml), and H2O (200 ml). The organic phase was dried, filtered, and concentrated. The product was vacuum distilled (1.0 mm Hg), and the fraction boiling at 58–62 °C was collected to give ethyl 4,5-epoxypentanoate (12.5 g, 86.6 mmol, 44% yield). The epoxy-ester was used to prepare hydroxylated ammoniopentanates. The epoxy-ester (14.6 mmol, 2.10 g) was dissolved in 30 ml of 70% EtOH and 1 eq of amine added. The vessels were sealed and heated (60 °C) with stirring (24 h). The mixtures were cooled, transferred, concentrated, diluted with H2O (20 ml), and extracted with OEt2 (2 × 20 ml). The aqueous layer was frozen and placed on vacuum (0.01 mm Hg; 3 days) and crystallized from dry acetone to afford: 17, 47%; 18, 52%; 19, 63%; and 20, 45%.

Computational Analyses

A Compaq Deskpro EN computer with a Pentium III processor was used. Chemical structures were drawn with CS Chem-Draw Ultra® (version 6.0, Cambridge Soft Corp.). For each molecule, a tight convergence setting, optimized structures (25). Subsequently, optimization using Hartree-Fock/S311G was performed set at “tight convergence” and theoretical atomic charges, electron density, and dipoles (Deybe) were computed. Afterward, the property server (44) was used to compute ClogP and the Connolly accessible area (Å2).

Northern Blot Analysis

A human 16-lane, Multiple-Tissue Northern (MTN™) blot (CLONTECH) was used for the Northern blot analysis of CT2. We used a [14]P]-labeled CT2 cDNA fragment (877–1317) as the probe. According to the instructions from the manufacturer, the master blot filter was hybridized with the probe for 1 h at 65 °C. The filter was finally washed under a highly stringent condition (0.1× SSC and 0.1% SDS at 65 °C).

Immunohistochemistry

Corresponding to the 14 amino acids of the COOH terminus of CT2, we generated a rabbit anti-CT2 polyclonal antibody raised against a keyhole limpet hemocyanin-conjugated synthesized peptide, KTEATT-PRDSSLGIE. The human testis and epididymal tissues were obtained from a patient who had died from bronchial asthma. As described previously, paraffin sections (3 μm) were processed for light microscopic immunohistochemical analysis (14). Regarding the absorption experiments, the tissue sections were treated with the primary antibody in the presence of antigen peptides (50 μg/ml).

Statistical Analysis

The experiments were performed using three different batches of oocytes, and the results from the experiments are expressed as mean ± S.E. Statistical significance was judged from Student’s t tests. Differences were considered significant at a level of p < 0.05.

RESULTS

Structural Features of Human CT2—An EST data base search identified an EST (GenBank™/EMBL/DDBJ accession number AA778598) exhibiting sequence similarity to rat OAT1 (9, 10). Corresponding to the EST as our probe, a human testis cDNA library was screened with a cDNA fragment. As a result, a cDNA encoding a novel carnitine transporter (designated as CT2) was isolated. CT2 cDNA consisted of 2048 base pairs, had an open reading frame of 1632 base pairs, and included the termination codon. The open reading frame was flanked by a 284-bp-long 5′-noncoding sequence and a 132-bp-long 3′-non-coding sequence. The CT2 cDNA encoded a 543-amino acid protein. Fig. 1a shows the deduced amino acid sequence of CT2 aligned with those of human OAT1 (26, 27), OCT1 (28), and OCTN2 (CT1) (16). The amino acid sequence of CT2 shows 36, 38, and 37% identities to human OAT1, OCT1, and OCTN2 (CT1), respectively. Kyte-Doolittle hydrophathy plot analysis (29) predicted 12 membrane-spanning domains in CT2 (Fig. 1, a and b), which are similar to other organic ion transporter family members. Analogously modeled to known mammalian transporters with 12 transmembrane domains, the CT2 amino and carboxyl termini were oriented toward the cytoplasmic side of the membrane. N-Glycosylation sites (residue 74) and protein kinase C-dependent phosphorylation sites (residues 14, 142, 285, 322, 516, and 535) were also predicted in the CT2 sequence (Fig. 1a). Phylogenetic analysis with the CLUSTAL program revealed CT2 to exhibit remote similarity to each member of OCT/OCTNs and OATs (Fig. 2). Hence, CT2 could not be assigned to any of the known families and represents a novel organic solute transporter class.

Genomic Organization of Human CT2 Gene—Using the CT2 cDNA nucleotide, a human genome data base (30) search revealed that the human gene encoding this transporter has been entirely sequenced. The gene is ~50 kbp long and located on chromosome 6q21–22 (2.1). The location was confirmed by fluorescence in situ hybridization using CT2 cDNA as a probe (data not shown). Using the cloned CT2 cDNA with the reported genomic sequence, alignment of the nucleotide sequence enabled us to deduce the exonic-intron gene organization. The CT2 gene consists of 10 exons and 9 introns (Fig. 3). The size of each exon, intron, and nucleotide sequence of the splice junctions is given in Table I. The 5′ and 3′ termini for each intron possess the consensus sequence for RNA splicing (gt/ag). The translation start site ATG is present in exon 2, and the translation termination site TAA is present in exon 10 (Fig. 3). Exon 1 does not code for the protein.
Functional Expression of Human CT2—To test the functional properties of CT2, we injected CT2 cRNA into *X. laevis* oocytes and found that CT2-expressing oocytes showed significant uptake of [3H]L-carnitine (Fig. 4a). Neither typical cationic substrates for OCT/OCTNs such as TEA, 1-methylphenyl-1,2,3,6-tetrahydropyridine, and choline, nor anionic substrates...
transport of 50 nM [3H]L-carnitine. The inhibition constant (Ki)
in absence or presence of extracellular L-carnitine (0.5 and 5
0.1 M) to compete with [3H]L-carnitine uptake (Fig. 5). Distinctly different from OCT/OCTNs and OATs, CT2 showed unique substrate selectivity. Including structural analogs of L-carnitine, the uptake of 50 nM [3H]L-carnitine by CT2 was measured in the presence or absence of non-radiolabeled compounds (5 or 50 μM). As shown in Fig. 5, acetyl-L-carnitine, acetyl-dL-carnitine, octanoyl-L-carnitine, and betaine significantly inhibit CT2-mediated [3H]L-carnitine uptake. In contrast, lysine, leucine, glycine, choline, taurine, GABA, and trimethyllysine did not produce any noticeable effects. Among the precursors of L-carnitine synthesis, only betaine showed an inhibitory effect on CT2-mediated L-carnitine uptake. Fig. 5 illustrates that CT2 failed to accept TEA or choline, which are prototypical substrates for OCT/OCTNs, and para-aminomhippurate and estrone sulfate, which are typical substrates for OATs. Our results demonstrate that the CT2-mediated transport process does not encompass broad substrate specificity.

We tested whether or not CT2 could accept various OCTN2 (CT1) substrates (16, 31). As shown in Fig. 6, L-carnitine uptake by CT2 was not inhibited by cationic xenobiotics such as cephaloridine, procainamide, desipramine, and quinidine. Although both transporters are able to mediate L-carnitine transport, the results show that the property of substrate recognition by CT2 is distinctly different from OCTN2 (CT1).

Examining the mechanism(s) of substrate recognition by CT2, we synthesized various L-carnitine analogous compounds (see “Experimental Procedures”). We tested the ability of carnitine-related compounds (20 μM) to compete with [3H]L-carnitine (20 nM) for the transport process by CT2 (Fig. 7). In addition, to compare the mechanisms of substrate recognition between CT2 and OCTN2 (CT1), we expressed rat OCTN2 (CT1) (19) in oocytes and performed the same inhibition study. As shown in Fig. 7, among 20 synthetic compounds, 1-5A, 7A–10A, and 13 possessed inhibitory effects on both OCTN2 (CT1)- and CT2-mediated carnitine transport. The inhibition of carnitine transport caused by these compounds was in the range of 15–40%. Furthermore, compounds 6A, 11A, 12A, 17, and 18 showed inhibitory effect on carnitine uptake by CT2 but not on OCTN2 (CT1). The inhibitory potency of 6A on CT2-mediated transport was less than 11A, 12A, 17, and 18, although it was statistically significant. These results help to reveal the subtle physicochemical differences between substrate recognition of CT2 and OCTN2 (CT1).

Tissue Distribution of Human CT2—The expression of CT2 messenger RNA (mRNA) in human tissues was investigated by high stringent Northern blot analysis (Fig. 8). The CT2-specific mRNA was exclusively detected in human testis and not in heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, ovary, small intestine, colon, or peripheral blood leukocytes. Two transcript sizes (2.1 and 1.8 kb) were identified in the human testis.

Immunohistochemical Localization of CT2 in Human Testis and Epididymis—For the determination of localization in adult human testis, we generated a rabbit polyclonal antibody raised against COOH terminus of CT2. Using immunohistochemistry techniques, the CT2 protein was detected in Sertoli cells (Fig. 9, a and b); however, the CT2 protein was not detected in germinal cells or in the interstitial tissues such as Leydig cells, blood vessels, and lymph vessels. In the epididymis, CT2 immunoreactivity was prominent in the epithelial
cells of the epididymal tubules (Fig. 9d). In a high magnification view (Fig. 9c), CT2 protein was detected in the luminal membrane of principal cells but not in epithelial cell stereocilium or basal cells of epididymal epithelium. Anatomically, the epididymis is divided into three regions: the caput, the corpus, and the cauda epididymis (32). CT2 immunoreactivity was observed mainly in the caput and corpus regions, whereas CT2 was barely detected in the cauda epididymis regions. CT2 immunoreactivity was faintly present in the luminal membrane of the ductus efference, the rete testis, and spermatozoa (data not shown). In absorption experiments where the tissue sections were treated with primary antibodies in the presence of antigen peptides (50 μg/ml), immunostaining was not observed and confirms the specificity of the immunoreactions (Fig. 9, c and f).

**DISCUSSION**

We describe a testis-specific carnitine transporter (CT2) representing an evolutionary change that shares only 34–37% homology with the other OCT/OCTN and OAT families (Fig. 2). Compared with the multispecific organic ion transporter family members, CT2 has restricted substrate selectivity. CT2 is capable of accepting L-carnitine and analogous compounds but not mainstream OCT/OCTN and OAT substrates (Figs. 5 and 6). Hence, CT2 is unique to other carnitine transporters, OCTN2 (CT1) (16, 18, 19) and OCTN3 (17), which function as carnitine transporters, respectively, had no apparent inhibitory effect on CT2-mediated carnitine uptake. Trimethyllysine and lysine have a carboxylic anion and cationic nitrogen separated by four methylene (–CH2–) units; at physiological pH, trimethyllysine and lysine have molecular formal charges of +1. GABA, like carnitine, is a butanoic acid; however, GABA does not contain a permanent quaternary ammonium cation. On the other hand, choline is a mainstream OCT/OCTN substrate with a trimethyl quaternary ammonium cation but does not possess a carbonyl moiety. Choline has a molecular formal charge of +1 and did not inhibit carnitine uptake with CT2. Hence, the data support the notion that CT2 substrate recognition requires the presence of a carbonyl and quaternary ammonium nitrogen separated by three or fewer carbons.

We synthesized carnitine-related compounds 2A–12A and 13-20 and tested their inhibitory effect on OCTN2 (CT1) and CT2 carnitine uptake (Fig. 7). The experimental and computational analyses, semi-empirical, and *ab initio* data located in the supplemental information (Tables S1 and S2; available in the on-line version of this article) offer insight into OCTN2 (CT1) and CT2 structure-activity differences. Our carnitine-related compounds include two fundamental groups of molecules, those with (13–20) and without a β-hydroxy moiety (2A–12A). As illustrated (Fig. 7) from the gas-phase computational calculations (see supplemental information available in the on-line version of this article), carnitine analogs not possessing a β-hydroxyl group (2A–12A) have considerably more Coulombic attraction between the quaternary ammonium cation and the carboxylic anion. The other carnitine analogs (13–20) contain inherent hydrogen-bonding interaction between the β-hydroxy group and the carboxylic anion (Fig. 10). Electron density values for the carbonyl oxygen atoms (ab *initio*, Hartree-Fock/321G) (Table S2 in on-line supplemental information) help to illustrate these differences, e.g. the butyl series 2A–6A versus 13–16 reveal significant electron density differences between the carbonyl oxygens.

The results summarized in Fig. 7 afford the following observations and conclusions. 1) Carnitine-related molecules not containing a β-hydroxy moiety (2A-12A) can inhibit OCTN2 (CT1) and CT2 carnitine uptake. Exceptions to conclusion one...
are observed with 6A, 10A, 11A, and 12A. Once the substituents on the quaternary ammonium cation has become bulky, i.e., 6A has a tripropyl, the molecule will not readily inhibit OCTN2 (CT1) and CT2 carnitine uptake. Furthermore, once the length of the carbon chain has reached hexyl, 10A–12A, Tris, lack of trans-stimulatory effect of L-carnitine on CT2-mediated efflux of L-carnitine. Oocytes expressed with CT2 were incubated with 50 nM [3H]L-carnitine for 90 min and transferred to the ND96 solution containing 0.5 or 5 μM unlabeled L-carnitine. The amount of L-carnitine efflux for 90 min is shown as the percentage of the preloaded amount. (mean ± S.E.; n = 8–10). N.S., not significant.

Fig. 4. Functional expression of human CT2 in Xenopus oocytes. a, L-carnitine transport via CT2. Uptake of 50 nM [3H]L-carnitine for 1 h was determined in water-injected oocytes (Control) or in oocytes injected with CT2 cRNA (CT2). b, time-dependent uptake of L-carnitine by CT2-expressing oocytes. The uptake of 50 nM [3H]L-carnitine in control oocytes (open circles) and CT2-expressing oocytes (closed circles) was measured during 2 h of incubation (mean ± S.E.; n = 8–10). c, transport kinetics of CT2. L-Carnitine transport (0.1–50 μM) was measured in control oocytes and in CT2-expressing oocytes. CT2-specific transport was calculated by subtracting the transport in control oocytes. These values for CT2-specific transport were used in kinetic analysis. Inset, Eadie-Hofstee plot. V, velocity; V/S, velocity per concentration of carnitine. d, dose-response relationship for the inhibition of CT2-mediated transport of [3H]L-carnitine by unlabeled carnitine. [3H]L-Carnitine (50 nM) uptake via CT2 was measured at different D-carnitine concentrations (1–1000 μM) in the ND96 solution for 1 h (mean ± S.E.; n = 8–10). e, pH dependence of CT2-mediated L-carnitine uptake. Transport rate of 50 nM L-carnitine was measured in control oocytes (open circles) and in CT2-expressing oocytes (closed circles) with the NaCl-containing medium. The pH of the medium was varied between 5.5 and 8.5 by adjusting the concentrations of MES, HEPES, and Tris. g, lack of trans-stimulatory effect of L-carnitine on CT2-mediated efflux of L-carnitine. Oocytes expressed with CT2 were incubated with 50 nM [3H]L-carnitine for 90 min and transferred to the ND96 solution containing 0.5 or 5 μM unlabeled L-carnitine. The amount of L-carnitine efflux for 90 min is shown as the percentage of the preloaded amount. (mean ± S.E.; n = 8–10). N.S., not significant.

Fig. 5. CT2-mediated [3H]L-carnitine transport was inhibited by L-carnitine-related compounds but not other cationic or anionic substrates. The uptake rate of [3H]L-carnitine (50 nM) by CT2-expressing oocytes was determined in the absence or presence of inhibitors at 5 μM and 50 μM. The values were expressed as percentages of [3H]L-carnitine uptake in CT2-expressing oocytes in the absence of the inhibitors (control) (mean ± S.E.; n = 8–10).

Fig. 6. Cationic xenobiotics do not possess inhibitory effects on CT2-mediated [3H]L-carnitine transport. The rate of [3H]L-carnitine uptake (20 nM) by CT2-expressing oocytes was determined in the presence or absence of inhibitors (20 μM), which were previously shown to be substrates of OCTN2 (CT1). The values are expressed as percentage of [3H]L-carnitine uptake in CT2-expressing oocytes in the absence of the inhibitors (control) (mean ± S.E.; n = 8–10).
OCTN2 (CT1) will not be as tolerant to the bulky quaternary ammonium cation as will CT2. 2) Carnitine-related molecules with a β-hydroxy moiety and a quaternary ammonium cation larger than trimethyl- (i.e. triethyl-, tripropyl-), the butane and pentane series (13-20), will not readily inhibit OCTN2 (CT1) and CT2 carnitine uptake. However, compounds 17 and 18 do in fact demonstrate minor differences between OCTN2 (CT1) and CT2 substrate selectivity. It is noteworthy that comparison of the butane and pentane results (14-16 versus 17-20) suggests CT2 can tolerate a pentyl chain with more quaternary ammonium cation hydrophobicity and bulkiness than can OCTN2 (CT1). The butyl series is not as accommodating as the pentyl series, presumably because of differences in relative degrees of freedom and the fact that the pentyl series forms a hydrogen bond in a seven-membered ring whereas the butyl series forms a six-member ring. Hence, the quaternary ammonium cation bulkiness (size) and relative hydrophobicity are important factors that inherently influence the charge on the nitrogen atom and triggers substrate interactions (34).

Immunohistochemistry revealed CT2 to be present in the luminal surface of epithelial cells of human epididymis (Fig. 9). Consistent with previous investigations where L-carnitine uptake was particularly active in isolated epididymal tubules (2, 5, 6, 32, 36), the immunohistochemical result allows one to propose that a CT2-mediated pathway constitutes a mechanism for L-carnitine transport in the luminal membrane of epididymis. In the epididymal lumen, concentrated L-carnitine goes through the sperm plasma membrane by passive diffusion (3) and serves as accessible energy storage when needed (37). When spermatozoa enter the epididymis, they are immotile and L-carnitine content is low. During their transit through the epididymis, spermatozoa initiate flagellar motion in parallel with accumulation of high concentrations (mM) of free L-carnitine from the luminal fluid (2). Therefore, the roles of carnitine are associated with improving sperm quality and fertility. There is a significant positive correlation between carnitine concentration in the genital tract, number of spermatozoa, and percentage of motile normal spermatozoa (38, 39). Furthermore, evidence indicates that clinical administration of L-carnitine or acetyl-L-carnitine to infertile male patients (e.g. idio-
pathic oligoasthenospermia) was followed by increase in sperm number and motility (40–42). Thus, it is plausible that CT2-mediated L-carnitine transport is required for the maturation of spermatozoa and that the human CT2 gene be a potential target for male infertility screening and treatment. Recently, a sperm-specific calcium channel (CatSper) was identified as a requirement for sperm motility and male fertility and represents an excellent molecular target for developing non-hormonal contraceptives (43). CT2 appears to be an additional example of a novel target for developing new antifertility agents. CT2 is only expressed in testis, consistent with its being involved in regulating spermatozoa motility and with the fact that a specific blocker may not influence other tissues.

Essential in the regulation of spermatogenesis, CT2 was also present in Sertoli cells (Fig. 9, a and b), which suggests a potential role of CT2 in these cells. Palmero et al. (35) provides support that l-carnitine facilitates lipid metabolism in Sertoli cells and denote the involvement of l-carnitine in the regulation of Sertoli cell function related to germ cell nutrition.

In summary, we cloned a novel carnitine transporter (CT2) specifically expressed in human testis. CT2 substrate selectivity and substrate recognition mechanism is distinct from other multispecific OCT/OCTN and OAT family members. CT2 represents an interesting evolutionary link between OCT/OCTNs and OATs, and also provides significant insight into the role of carnitine in the maturation of human spermatozoa.

Acknowledgments—We thank Prof. Masakuni Furusato and Dr. Yuichi Terado (Department of Pathology, Kyorin University, School of Medicine, Tokyo, Japan) for discussion in immunohistochemistry, Akie Toki for technical assistance, and Dr. H. Heng for help in the fluorescence in situ hybridization experiment. The anti-CT2 polyclonal antibody was supplied by Transgenic Inc., Kumamoto, Japan.

REFERENCES
1. Bremer, J. (1983) Physiol. Rev. 63, 1420–1480
2. Jeulin, C., and Lewin, L. M. (1996) Hum. Reprod. Update 2, 87–102
3. Jeulin, C., Dacheux, J. L., and Soufir, J. C. (1994) J. Reprod. Fertil. 100, 263–271
4. Hinton, B. T., Snowell, A. M., and Setchell, B. P. (1979) J. Reprod. Fertil. 56, 105–111
5. Bohmer, T., and Johansen, L. (1978) Int. J. Androl. 2, 565–573
6. Radigue, C., Es-Slami, S., and Soufir, J. C. (1996) Arch. Androl. 37, 27–31
7. Grundenmann, D., Gorboulev, V., Gambaryan, S., Veyhl, M., and Koepsell, H. (1994) Nature 372, 549–552
8. Koepsell, H. (1998) Annu. Rev. Physiol. 60, 243–266
9. Sekine, T., Watanabe, N., Hosoyamada, M., Kanai, Y., and Endou, H. (1997) J. Biol. Chem. 272, 18526–18529
10. Sweet, D. H., Wolff, N. A., and Pritchard, J. B. (1997) J. Biol. Chem. 272, 30088–30095
11. Sekine, T., Cha, S. H., Tsuda, M., Ajiwattanakul, N., Nakajima, N., Kanai, Y., and Endou, H. (1998) PFRS Lett. 429, 179–182
A Novel Carnitine Transporter in Human Testis

36271

12. Kusuhara, H., Sekine, T., Utsunomiya-Tate, N., Tsuda, M., Kojima, R., Cha, S. H., Sugiyama, Y., Kanai, Y., and Endou, H. (1999) J. Biol. Chem. 274, 13675–13680
13. Cha, S. H., Sekine, T., Kusuhara, H., Yu, E., Kim, J. Y., Kim, D. K., Sugiyama, Y., Kanai, Y., and Endou, H. (2000) J. Biol. Chem. 275, 4507–4512
14. Enomoto, A., Kimura, H., Cha, S. H., Hosoyamada, M., Sekine, T., Igarashi, T., Matsuz, H., Kikuchi, Y., Oda, T., Ichida, K., Hosoya, T., Shimokata, K., Niwa, T., Kanai, Y., and Endou, H. (2002) Nature 417, 447–452
15. Tamai, I., Yabushiki, H., Nezu, J., Yabuuchi, H., Oku, A., Shimane, M., and Tsuji, A. (1999) FEBS Lett. 419, 107–111
16. Wu, X., Prasad, D. P., Leibach, F. H., and Ganapathy, V. (1998) Biochem. Biophys. Res. Commun. 246, 589–595
17. Tamai, I., Ohashi, R., Nezu, J., Sai, Y., Kobayashi, D., Oku, A., Shimane, M., and Tsuji, A. (2000) J. Biol. Chem. 275, 40064–40072
18. Tamai, I., Ohashi, R., Nezu, J., Yabuuchi, H., Oku, A., Shimane, M., Sai, Y., and Tsuji, A. (1998) J. Biol. Chem. 273, 20378–20382
19. Sekine, T., Kusuhara, H., Utsunomiya-Tate, N., Tsuda, M., Sugiyama, Y., Kanai, Y., and Endou, H. (1998) Biochem. Biophys. Res. Commun. 251, 586–591
20. Wu, X., Huang, W., Prasad, D. P., Seth, P., Rajan, D. P., Leibach, F. H., Chen, J., Conway, S. J., and Ganapathy, V. (1999) J. Pharmacol. Exp. Ther. 290, 1482–1492
21. Charles, R. (1980) J. Org. Chem. 45, 2763–2766
22. Boots, S. G., and Boots, M. R. (1975) J. Pharm. Sci. 64, 1949–1952
23. Boots, S. G., and Boots, M. R. (1975) J. Pharm. Sci. 64, 1262–1264
24. Lloyd, A. W., Olliff, C. J., and Butt, K. J. (1994) J. Pharm. Pharmacol. 46, 704–707
25. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Zakrzewski, V. G., Montgomery, J. A., Stratmann, R. E., Burant, J. C., et al. (1998) Gaussian 98, Revision A.9, Gaussian, Inc., Pittsburgh, PA
26. Reid, G., Wolf, N. A., Dautzenberg, F. M., and Bruckhardt, G. (1998) Kidney Blood Press. Res. 21, 233–237
27. Hosoyamada, M., Sekine, T., Kanai, Y., and Endou, H. (1999) Am. J. Physiol. 276, F122–F128
28. Zhang, L., Dresser, M. J., Gray, A. T., Yost, S. C., Terashita, S., and Giacomin, K. M. (1997) Mol. Pharmacol. 51, 913–921
29. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
30. International Human Genome Sequencing Consortium (2001) Nature 409, 860–921
31. Ganapathy, M. E., Huang, W., Rajan, P., Carter, A. E., Sugawara, M., Iseki, K., Leibach, F. H., and Ganapathy, V. (1999) J. Biol. Chem. 275, 1609–1707
32. Johansen, L., and Bohnet, J. (1979) Arch. Androl. 2, 117–121
33. Wagner, C. A., Luikewille, U., Kaltenbach, S., Moschen, I., Broer, A., Risler, T., Broer, S., and Lang, F. (2000) Am. J. Physiol. 279, F584–F591
34. Flemke, M. F. (2001) J. Mol. Struct. 562, 63–78
35. Palermo, S., Bottazzi, C., Costa, M., Leone, M., and Fugassa, E. (2000) Horm. Metab. Res. 32, 87–90
36. Cooper, T. G., Guerdemann, T. W., and Yeung, C. H. (1986) Int. J. Androl. 9, 348–358
37. Jeulin, C., Soufr, J. C., Marson, J., Paquignon, M., and Dacheux, J. L. (1987) J. Reprod. Fertil. 79, 525–529
38. Sperr, L., Glass, R. H., and Kase, N. G. (1978) in Clinical Gynecologic Endocrinology and Infertility (Sperr, L., Glass, R. H., and Kase, N. G., eds) p. 565, Williams and Wilkins Co., Baltimore
39. Matalliotakis, I., Koumantaki, Y., Evageliou, A., Matalliotakis, G., Goumenou, A., and Koumantakis, E. (2000) Int. J. Fertil. Womens Med. 45, 236–240
40. Vitali, G., Parente, R., and Melotti, C. (1995) Drugs Exp. Clin. Res. 21, 157–159
41. Campaniello, E., Petrarolo, N., Meriggiola, M. C., Valdiserri, A., Pareschi, A., Veci, N., Flamigni, C., and Filicori, M. (1989) Carnitine Administration in Asthenospermia: 4th International Congress on Andrology, Florence, Italy, preliminary program
42. Menzaghi, M. L., Visioli, E., Cimino, C., Calogero, A. E., Mongioi, A., and D’Agata, R. (1992) Acta Eur. Fertil. 23, 221–224
43. Ren, D., Navarro, B., Perez, G., Jackson, A. C., Hsu, S., Shi, Q., Tilly, J. L., and Chlapman, D. E. (2001) Nature 413, 603–609
44. CS CHEM3D 6.0 for Windows and Macintosh (5.0) User’s Guide (2000) CambridgeSoft Corp., pp. 199–203, CambridgeSoft Corp., Cambridge, MA
Molecular Identification of a Novel Carnitine Transporter Specific to Human Testis: INSIGHTS INTO THE MECHANISM OF CARNITINE RECOGNITION
Atsushi Enomoto, Michael F. Wempe, Hiroki Tsuchida, Ho Jung Shin, Seok Ho Cha, Naohiko Anzai, Akiteru Goto, Atsuhiko Sakamoto, Toshimitsu Niwa, Yoshikatsu Kanai, M. W. Anders and Hitoshi Endou

J. Biol. Chem. 2002, 277:36262-36271.
doi: 10.1074/jbc.M203883200 originally published online June 27, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203883200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2002/09/20/277.39.36262.DC1

This article cites 40 references, 11 of which can be accessed free at http://www.jbc.org/content/277/39/36262.full.html#ref-list-1