

## Abnormal Sodium Stimulation of Carnitine Transport in Primary Carnitine Deficiency\*

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**Primary carnitine deficiency is an autosomal recessive disorder of fatty acid oxidation characterized by hypoketotic hypoglycemia and skeletal and cardiac myopathy. It is caused by mutations in the sodium-dependent carnitine cotransporter OCTN2. The majority of natural mutations identified in this and other Na<sup>+</sup>/solute symporters introduce premature termination codons or impair insertion of the mutant transporter on the plasma membrane. Here we report that a missense mutation (E452K) identified in one patient with primary carnitine deficiency did not affect membrane targeting, as assessed with confocal microscopy of transporters tagged with the green fluorescent protein, but reduced carnitine transport by impairing sodium stimulation of carnitine transport. The natural mutation increased the concentration of sodium required to half-maximally stimulate carnitine transport ( $K_{Na}$ ) from the physiological value of 11.6 to 187 mM. Substitution of Glu<sup>452</sup> with glutamine (E452Q), aspartate (E452D), or alanine (E452A) caused intermediate increases in the  $K_{Na}$ . Carnitine transport decreased exponentially with increased  $K_{Na}$ . The E452K mutation is the first natural mutation in a mammalian cotransporter affecting sodium-coupled solute transfer and identifies a novel domain of the OCTN2 cotransporter involved in transmembrane sodium/solute transfer.**

Primary carnitine deficiency (On-line Mendelian Inheritance in Man (OMIM) no. 212140) is a recessively inherited disorder of fatty acid oxidation due to defective carnitine transport (1, 2). Carnitine is essential for the transfer of long-chain fatty acids from the cytosol to mitochondria for subsequent  $\beta$ -oxidation, and the lack of carnitine impairs the ability to use fat as fuel during periods of fasting or stress. This can result in hypoketotic hypoglycemia, Reye's syndrome, and sudden infant death in younger children or in skeletal or heart myopathy with insidious onset later in life.

The gene for primary carnitine deficiency encodes a functional carnitine transporter named OCTN2 (3, 4) that maps to chromosome 5q31.1–32 (3, 5). OCTN2 is a novel organic cation transporter and operates a sodium-dependent transport of carnitine. It belongs to a family of transporters believed important in drug absorption and detoxification, although the physiolog-

ical substrate has been identified only for OCTN2.

Human fibroblasts express the carnitine transporter defective in primary carnitine deficiency (6). This transporter is not inhibited by amino acids, but is competitively inhibited by acetylcarnitine, palmitoylcarnitine, butyrobetaine, and betaine. Noncompetitive inhibition is observed with verapamil and quinidine (6). The carnitine transporter is energized by the sodium electrochemical potential gradient; and in the absence of sodium, only minimal amounts of carnitine enter the cell through a nonsaturable process (6).

Mutations in the OCTN2 gene have been reported in patients with primary carnitine deficiency (7–11), most of whom presented early in life with a severe metabolic decompensation. Identified mutations result in premature termination codons or in nonfunctional transporters when missense mutations have been expressed in mammalian cells (9). We have recently identified a missense mutation (E452K) associated with residual carnitine transport activity in a patient who presented at 7 years of age with severe cardiomyopathy and carnitine deficiency (10). Preliminary experiments indicated that this mutation reduced carnitine transport without affecting the  $K_m$  for carnitine recognition (12). Since the majority of mutations reported in the sodium/glucose cotransporter SGLT1 impair membrane trafficking (13), it was proposed that the E452K substitution could impair insertion of OCTN2 carnitine transporters to the plasma membrane, with the transporters reaching the plasma membrane having normal affinity for carnitine (12).

Here we report that the E452K mutation does not affect membrane insertion of carnitine transporters, but impairs activation of carnitine transport by the cotransported sodium. This is the first natural mutation in a sodium cotransporter affecting energization of substrate transport and identifies a novel domain involved in sodium recognition or transfer.

### EXPERIMENTAL PROCEDURES

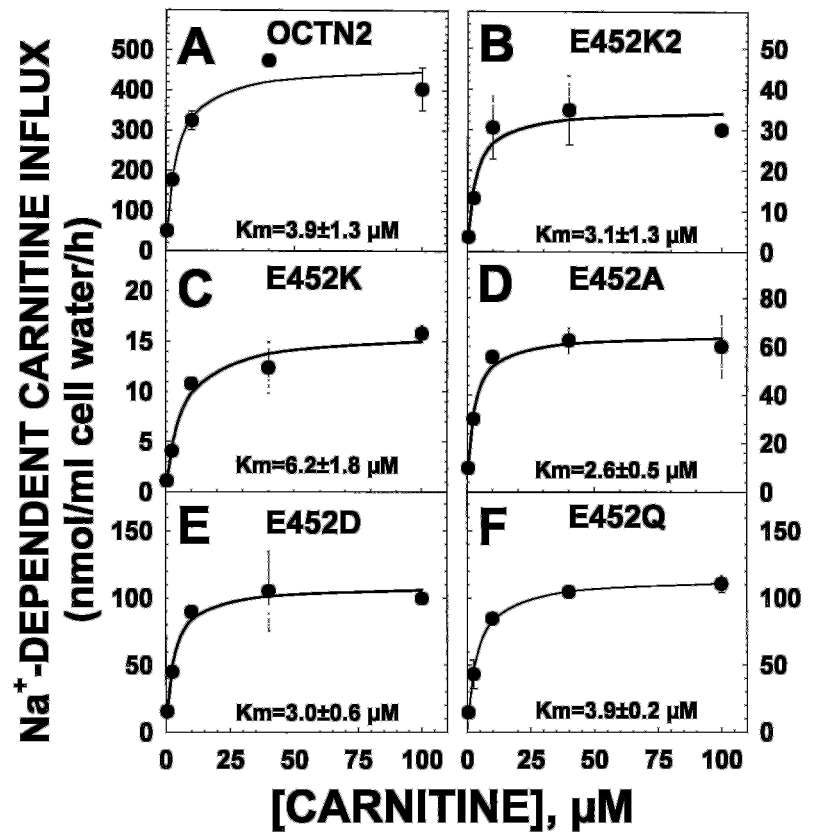
**Cell Culture and Carnitine Transport**—Chinese hamster ovary (CHO)<sup>1</sup> cells were grown in Ham's F-12 medium supplemented with 6% fetal bovine serum. Carnitine transport was measured at 37 °C with the cluster-tray method as described previously (6, 8, 12). Cells were grown to confluence in 24-well plates (Costar Corp.) and depleted of intracellular amino acids by incubation for 90 min in Earle's balanced salt solution containing 5.5 mM D-glucose and supplemented with 0.1% bovine serum albumin. Carnitine (0.5  $\mu$ M, 0.5  $\mu$ Ci/ml) was then added to the cells for 10 min. Nonsaturable carnitine transport was measured in the presence of 2 mM unlabeled carnitine. The transport reaction was stopped by rapidly washing the cells four times with ice-cold 0.1 M MgCl<sub>2</sub>. Intracellular carnitine was then corrected for intracellular water content and expressed as nmol/ml of cell water (6). Saturable carnitine transport was calculated by subtracting sodium-independent carnitine transport from total transport, and values are reported as means  $\pm$  S.E. of three to six independent determinations. Carnitine

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<sup>1</sup> The abbreviations used are: CHO, Chinese hamster ovary; GFP, green fluorescent protein.

FIG. 1. Kinetics of  $\text{Na}^+$ -dependent carnitine transport in CHO cells transfected with normal and mutant OCTN2 cDNAs. Carnitine (0.5–100  $\mu\text{M}$ ) transport was measured for 10 min at 37 °C in the presence of 150 mM sodium.  $\text{Na}^+$ -independent carnitine transport, measured substituting methylglucamine for sodium, was subtracted from each point. Data are means  $\pm$  S.E. of triplicates. Lines represent the best fit of the data to a Michaelis-Menten equation. Parameters for carnitine transport are expressed as means  $\pm$  95% confidence intervals. Note the different scale among panels. E452K and E452K2 are two independent clones of CHO cells expressing different amounts of the E452K mutant carnitine transporter.



transport in the absence of sodium was measured substituting methylglucamine for sodium so that the sum of methylglucamine and sodium remained constant at 150 mM (6). Initial experiments on transfected cells indicated that carnitine accumulation at 0.5  $\mu\text{M}$  was linear for up to 30 min in cells expressing the normal OCTN2 transporter and for up to 4 h in cells expressing mutant transporters, with a roughly inverse correlation between transport activity and time during which transport remained linear (data not shown).

Kinetic constants for carnitine transport were determined by nonlinear regression analysis according to a Michaelis-Menten equation (6).  $\text{Na}^+$ -independent carnitine transport was determined in parallel trays and subtracted from total transport to obtain  $\text{Na}^+$ -dependent carnitine transport. Nonlinear parameters are expressed as means  $\pm$  95% confidence intervals. The  $K_m$  for sodium ( $K_{\text{Na}}$ ) was calculated from the intersection ( $-1/K_{\text{Na}}$ ) of linear regressions of  $1/v$  versus  $1/[\text{sodium}]$  at three different carnitine concentrations (14).

**Construction of Green Fluorescent Protein (GFP)-tagged OCTN2 Expression Vectors**—The OCTN2 cDNA (3) cloned in pcDNA3 (8) was amplified by polymerase chain reaction using high-fidelity *Pfu* polymerase and the primers 5'-GCGAATTCCCAGACCCAGGCCGCGCT-3' and 5'-GCGGATCCAGAAGGCTGTGCTTTTAAGG-3', corresponding to the 5'- and 3'-primers of the OCTN2 cDNA with attached *EcoRI* and *BamHI* linkers, respectively. The 3'-primer removed the physiological stop codon of OCTN2 (TAA). The polymerase chain reaction product was digested with *EcoRI* and *BamHI* and ligated in the corresponding restriction sites of pEGFP-N2 (CLONTECH). The resulting plasmid had the OCTN2 cDNA fused in frame with the green fluorescent protein under control of the cytomegalovirus promoter. The final vector was sequenced, confirming the absence of polymerase chain reaction artifacts and the correct fusion with the green fluorescent protein. This plasmid was transfected into CHO cells by LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. Cells were selected with 0.8 mg/ml G418 (Life Technologies, Inc.) for 2 weeks, and several independent clones of transfected cells were isolated.

**Site-directed Mutagenesis**—The E452K, E452A, E452Q, and E452D mutations were introduced by site-directed mutagenesis using the QuickChange system (Stratagene) following the manufacturer's instructions. The final plasmids were sequenced to confirm the presence of the mutations and the absence of polymerase chain reaction artifacts. The plasmids obtained were then transfected into CHO cells as de-

scribed above.

**Northern Blot Analysis**—Cellular RNA was extracted with guanidinium thiocyanate, separated by formaldehyde-agarose gel electrophoresis, blotted onto nylon, and hybridized under high-stringency conditions with the OCTN2 and actin cDNAs. cpm in each band were determined using a micro-array radioactivity detector (Instant Imager, Packard Instrument Co.). Lane-specific background was subtracted from each lane. OCTN2 cpm were normalized for actin cpm to correct for variations in RNA loading. Linearity of the assay was determined using increasing amounts of control RNA (12). The gel was then exposed to film.

**Confocal Microscopy**—Subcellular distribution of normal and mutant OCTN2 carnitine transporters conjugated with the green fluorescent protein was analyzed by confocal microscopy (Zeiss LSM 410 laser scanning confocal microscope). Cells were seeded on glass slides and covered with medium and a coverslip whose borders were chemically sealed. Images of the cells were obtained at 1- $\mu\text{m}$  sections. Multiple cells were scanned, and the ones shown are representative of the fields observed.

## RESULTS AND DISCUSSION

The initial kinetic experiments indicated that the E452K mutation decreased the  $V_{\text{max}}$  for carnitine transport, without affecting the  $K_m$  (12). This was consistent with the fact that the mutation could impair maturation of the transporter to the plasma membrane, as described for mutations in the sodium/glucose cotransporter SGLT1 (13). To test this possibility, the OCTN2 transporter was tagged with the green fluorescent protein to assess its subcellular distribution. Conjugation with the green fluorescent protein did not affect carnitine transport activity, which remained similar in CHO cells expressing the native OCTN2 cDNA and the normal cDNA with a C-terminal addition of the green fluorescent protein (Fig. 1A). The  $K_m$  for sodium-dependent carnitine transport was  $2.9 \pm 1.3 \mu\text{M}$  in CHO cells transfected with GFP-tagged OCTN2, a value similar to that seen in CHO cells expressing the native OCTN2 cDNA (12) and in human fibroblasts (6). Similarly, the  $V_{\text{max}}$  for sodium-dependent carnitine transport was in the range meas-

TABLE I

Kinetic constants for  $\text{Na}^+$ -dependent carnitine transport in CHO cells expressing normal and mutant carnitine transporters

Kinetic constants for carnitine (0.5–100  $\mu\text{M}$ ) transport were determined in the absence or presence of sodium (150 mM).  $\text{Na}^+$ -independent carnitine transport was subtracted from total transport to obtain  $\text{Na}^+$ -dependent carnitine transport. Parameters are shown as means  $\pm$  95% confidence intervals.

	$V_{\max}$	$K_m$
	nmol/ml/h	$\mu\text{M}$
OCTN2	462.6 $\pm$ 34.1	3.9 $\pm$ 1.3
E452K	15.9 $\pm$ 1.1	6.2 $\pm$ 1.8
E452K2	35.0 $\pm$ 3.1	3.1 $\pm$ 1.3
E452A	65.0 $\pm$ 2.6	2.6 $\pm$ 0.5
E452D	108.9 $\pm$ 4.8	3.0 $\pm$ 0.6
E452Q	114.9 $\pm$ 1.5	3.9 $\pm$ 0.2

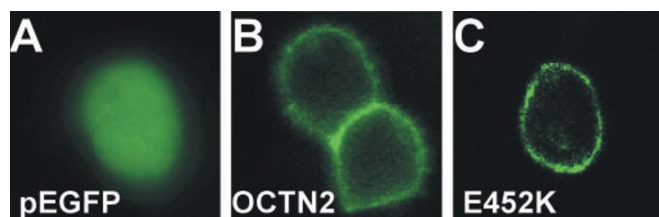


FIG. 2. Subcellular distribution of normal and mutant carnitine transporters tagged with the green fluorescent protein. CHO cells stably transfected with pEGFP-N2 (A), OCTN2-GFP (B), or E452K-GFP (C) were seeded on glass slides and covered with a drop of saline and a coverslip. Confocal images were obtained at 1- $\mu\text{m}$  sections using an emission wavelength of 488 nm. The images shown are representative of the other cells observed. Light microscopy indicated that the images seen in the case of OCTN2 and E452K OCTN2 corresponded to the borders of the cell.

ured in cells expressing the native OCTN2 cDNA (12) (Table I). Two different clones of CHO cells expressing the E452K mutant carnitine transporter (E452K2 and E452K) also retained normal  $K_m$  values for carnitine (3.3  $\pm$  1.6 and 6.2  $\pm$  1.8  $\mu\text{M}$ ) (Fig. 1, B and C), although their  $V_{\max}$  values for sodium-dependent carnitine transport were still markedly reduced compared with that of normal OCTN2 (Table I). These results indicate that modification of the C terminus of the carnitine transporter does not significantly affect carnitine recognition and transfer.

Analysis of the cells with a confocal microscope indicated that CHO cells transfected with the pEGFP vector alone had a diffuse cytoplasmic distribution of green fluorescence (Fig. 2A). By contrast, the fluorescence was concentrated at the periphery of the cell on the plasma membrane in cells in which GFP was conjugated with normal OCTN2 (Fig. 2B). To our surprise, cells expressing the E452K mutant OCTN2 transporter had a distribution of fluorescence identical to that of cells expressing normal OCTN2 (Fig. 2C), indicating that this mutation does not impair insertion of the transporter to the plasma membrane.

The carnitine transporter defective in primary carnitine deficiency is energized by the sodium electrochemical potential (6). We determined the kinetic constants of normal and mutant OCTN2 for sodium to test whether the E452K mutation affected energization of the carnitine transporter by the sodium electrochemical gradient. Sodium was substituted by methylglucamine in the uptake medium (Fig. 3). The dependence of carnitine transport on the extracellular sodium concentration was measured at different carnitine concentrations to estimate the  $K_{\text{Na}}$  from the intersection of the reciprocal plots (14).  $\text{Na}^+$ -independent transport was subtracted from all transport data prior to analysis. CHO cells expressing the normal human OCTN2 cDNA had a  $K_{\text{Na}}$  of 11.6 mM (Fig. 3A). This value

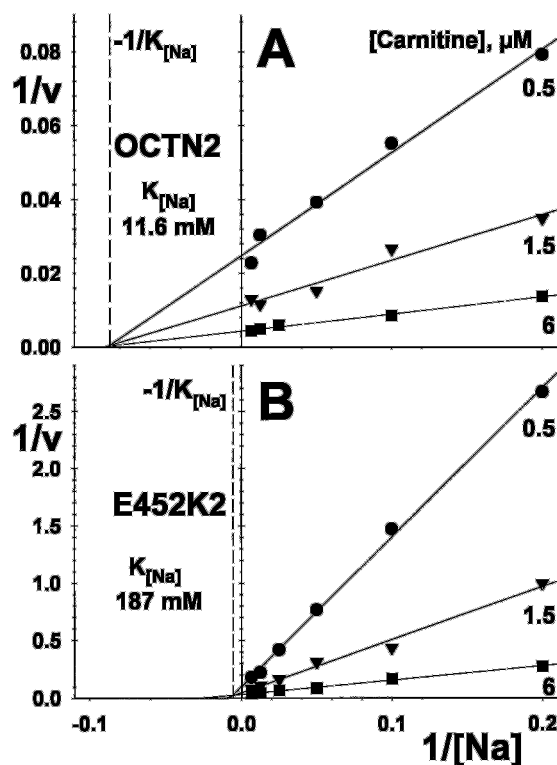


FIG. 3. Sodium dependence of carnitine transport by CHO cells expressing normal (A) and E452K mutant (B) OCTN2 transporters. Carnitine (0.5, 1.5, and 6  $\mu\text{M}$ ) transport was measured for 10 min at 37  $^{\circ}\text{C}$  in the presence of increasing concentrations of sodium (0–150 mM). Cells were washed twice with a sodium-free solution prior to transport. Points are means  $\pm$  S.E. of triplicates. Lines are the best fit to linear regression. The intercept of the regressions is identified by dashed lines representing  $-1/K_{\text{Na}}$ .

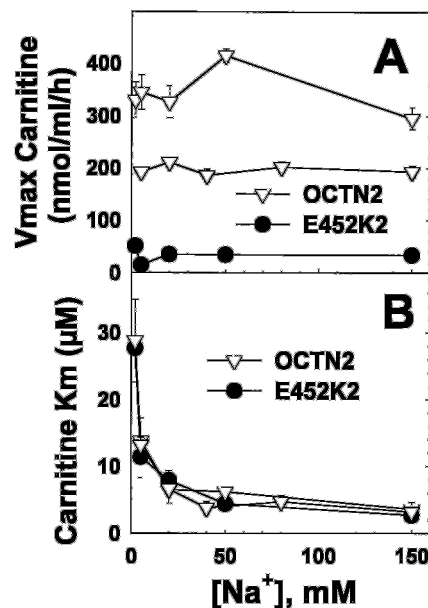


FIG. 4. Kinetic constants for sodium-dependent carnitine transport at increasing concentrations of sodium. Carnitine (0.5–60  $\mu\text{M}$ ) transport was measured for 10 min at the indicated concentrations of sodium (2–150 mM). Sodium-independent carnitine (0.5–60  $\mu\text{M}$ ) transport, measured in parallel trays, was subtracted from each point prior to calculating kinetic constants. Cells were washed twice with a sodium-free solution prior to the transport experiment. The data obtained were subjected to nonlinear regression analysis according to a Michaelis-Menten equation. Data represent the parameters obtained  $\pm$  95% confidence intervals. Two independent clones of CHO cells expressing the normal OCTN2 transporter are reported.



approaches that measured in cultured fibroblasts (14.9 mM) using the same mathematical procedure (from Fig. 2 of Ref. 6). Cells expressing the E452K mutant transporter had a  $K_{Na}$  of 187 mM, which was 16 times higher than that of cells expressing the normal OCTN2 transporter (Fig. 3B). Similar  $K_{Na}$  values were obtained in CHO cells transfected with the E452K mutant OCTN2 cDNA not conjugated to the green fluorescent protein (data not shown), indicating that the abnormal  $K_{Na}$  is not an artifact due to the interaction of the mutant protein with the green fluorescent protein. These results suggest that the E452K mutation impairs sodium stimulation of carnitine

transport by the OCTN2 transporter.

In a random bireactant system, binding of one substrate may affect the affinity of the transporter for the other substrate (14). Since the E452K mutation significantly increased the  $K_{Na}$ , we evaluated the apparent  $K_m$  for carnitine at different extracellular sodium concentrations (2–150 mM). If the E452K mutation were located close to the carnitine-binding site, at low concentrations of sodium, the recognition of carnitine could have been affected, and the  $K_m$  could have been significantly increased. As shown in Fig. 4A, the  $V_{max}$  for carnitine transport remained relatively constant at all extracellular sodium concentrations in two independent clones of CHO cells expressing the normal OCTN2 transporter (*inverted triangles*) and in E452K2 cells (*circles*). By contrast, the apparent  $K_m$  for carnitine decreased as the sodium concentration increased in cells expressing both the normal and E452K mutant carnitine transporters. However, most of the decrease occurred at relatively low concentrations of sodium and was complete at 50 mM sodium (Fig. 4B). This indicates that only minimal amounts of sodium are required to improve recognition of carnitine by this transporter. Importantly, the decrease in the apparent  $K_m$  for carnitine was identical in cells expressing the normal and E452K mutant transporters, indicating that the E452K mutation does not affect initial binding of sodium to the transporter and carnitine recognition.

The structure of the carnitine transporter is unknown. Hydrophathy analysis of the amino acid sequence of OCTN2 by the method of Kyte and Doolittle (26) as described by Wu *et al.* (3) or with TopPred2 (4) predicts 12 transmembrane domains. When OCTN2 is modeled according to other similar membrane transporters, both the amino and carboxyl termini face the cytoplasm (3, 15). This model places on the correct side of the membrane potential glycosylation and protein kinase C phosphorylation sites as well as the sugar transporter protein signature sequence motif and the ATP/GTP-binding motif (3, 4, 15). According to this model, the E452K mutation affects an intracellular loop located between predicted transmembrane domains 10 and 11. In view of the marked increased  $K_{Na}$  of the E452K mutant transporter (Fig. 3) and the normal decrease in the  $K_m$  for carnitine with increased sodium concentration (Fig. 4), we formulated the hypothesis that Glu<sup>452</sup> was needed for the correct transfer of the carnitine-sodium complex inside the cell. To test whether the negative charge of Glu<sup>452</sup> was required for this function, alanine (E452A), glutamine (E452Q), or aspartate

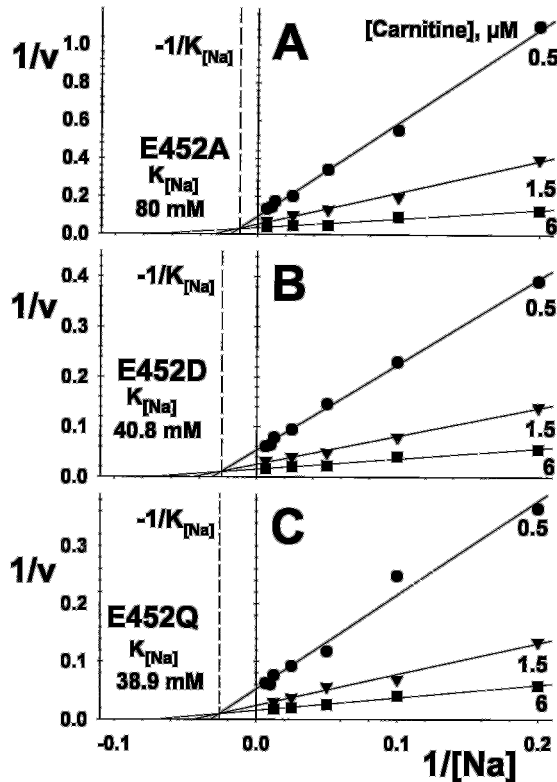
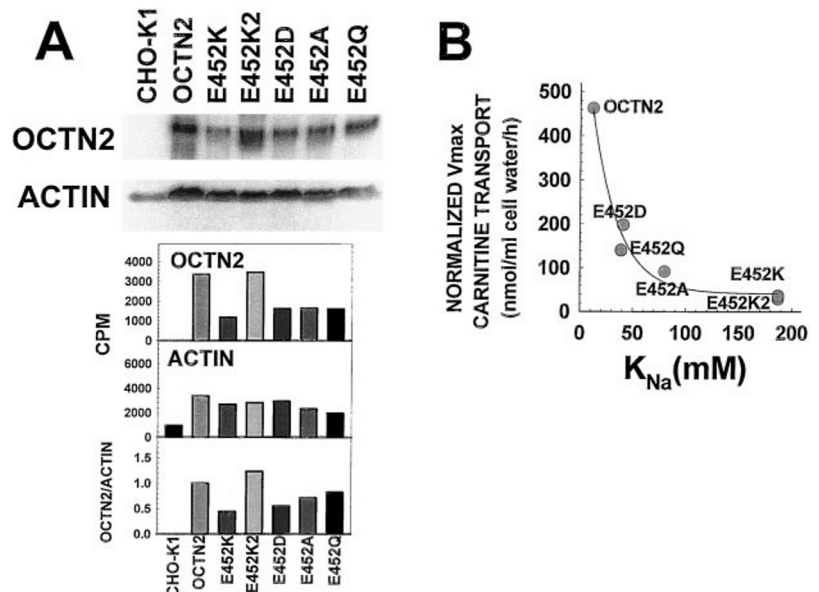


FIG. 5. Sodium dependence of carnitine transport by CHO cells expressing E452A (A), E452D (B), and E452Q (C) mutant OCTN2 transporters. Carnitine transport and  $K_{Na}$  were obtained as described in the legend to Fig. 3.

FIG. 6. Correlation between carnitine transport and  $K_{Na}$  in CHO cells expressing normal and mutant carnitine transporters. A, RNA was extracted from CHO cells and analyzed by Northern blot analysis with hybridization to the full-length OCTN2 cDNA and to a human actin cDNA fragment. cpm in each band were counted on an Instant Imager and normalized for actin cpm. B, the  $V_{max}$  for carnitine transport (Table I) was normalized for OCTN2 mRNA levels (using expression of normal OCTN2 as a standard) and plotted against  $K_{Na}$ . The line represents the best fit of the data to an exponential decay equation ( $p < 0.01$  with analysis of variance;  $r^2 = 0.976$ ).



(E452D) was inserted in position 452 by site-directed mutagenesis, and the resulting plasmids were expressed in CHO cells.

Fig. 1 shows that all mutant transporters conserved a normal  $K_m$  for carnitine, but the  $V_{max}$  for sodium-dependent carnitine transport decreased progressively as follows: wild-type OCTN2 > E452D and E452Q > E452A > E452K (Table I). The lack of changes in the  $K_m$  for carnitine confirmed that Glu<sup>452</sup> is probably not involved in carnitine recognition. By contrast, the  $K_{Na}$  decreased progressively as follows: E452K (187 mM; Fig. 3B) > E452A (80 mM; Fig. 5A) > E452D (40.8 mM; Fig. 5B) > E452Q (38.9 mM; Fig. 5C) > normal Glu<sup>452</sup> (11.6 mM; Fig. 3A). The  $K_{Na}$  values of normal and mutant carnitine transporters were then plotted against the  $V_{max}$  for carnitine transport normalized for OCTN2 mRNA levels (Fig. 6). There was a significant ( $p < 0.01$ ) exponential decrease in the  $V_{max}$  for carnitine transport with the increase in  $K_{Na}$  ( $r^2 = 0.98$ ). The inverse correlation between carnitine transport and the  $K_{Na}$  supports the hypothesis that decreased carnitine transport by the E452K mutation is due to altered sodium stimulation of carnitine transfer by OCTN2. It also indicates that glutamate in this position is more effective than aspartate for sodium handling and that the length of the side chain is more important than the net charge of this residue since glutamine was similar to aspartate in approaching normal function.

Most of our knowledge of sodium/solute cotransporters derives from the study of bacterial proteins and the Na<sup>+</sup>/glucose cotransporter SGLT1 (13). In this latter protein, the N terminus seems devoted to sodium recognition and transfer, whereas the C terminus forms a pore for glucose (13). Most natural mutations of SGLT1 identified in patients with glucose/galactose malabsorption impair membrane insertion of the carrier protein (16–18). In SGLT1 and other sodium cotransporters, tyrosyl residues on the extracellular side of the transporter have been involved in sodium binding based on studies with tyrosyl group-modifying agents (19, 20). However, site-directed substitution of tyrosyl residues in the OCTN2 transporter failed to affect sodium affinity (15).

Conserved acidic and polar residues in transmembrane domains have been proposed to participate in sodium binding and progression across the plasma membrane (21, 22). Only selected transmembrane acidic residues are essential for this process (21). There is no information on the role of acidic residues putatively facing the cytoplasmic side of mammalian sodium cotransporters.

Bacterial sodium/solute cotransporters usually have a higher affinity for cotransported cations than their mammalian counterparts, with  $K_{Na}$  values in the  $\mu$ M rather than the mM range (23). Acidic residues in the N terminus of these transporters are responsible for sodium binding, whereas polar residues in the transmembrane domain of the bacterial Na<sup>+</sup>/proline cotransporters are important for sodium transfer as in their mammalian counterparts (23, 24). An acidic residue (Asp<sup>187</sup>) located in the cytoplasmic side of the Na<sup>+</sup>/proline cotransporter of *Escherichia coli* is essential for sodium and solute transfer and may play a role in the release of Na<sup>+</sup> to the cytoplasmic side of the membrane (24). As with substitution of Glu<sup>452</sup> in the OCTN2 carnitine transporter, substitution of Asp<sup>187</sup> in the bacterial Na<sup>+</sup>/proline cotransporter with Glu, Asn, or Cys resulted in a progressive decrease in the  $V_{max}$  for proline uptake, without major changes in the  $K_m$  for proline (24). However, this decrease in  $V_{max}$  was not correlated with altered affinity for sodium, which was only minimally affected by the substitutions (24), mitigating the analogy with the E452K mutation. However, a role of Glu<sup>452</sup> in the release of sodium to the cytoplasm

remains a possibility.

An additional possibility is that Glu<sup>452</sup> participates in the conformational change of the transporter during the transport process. In facilitative glucose transporters such as GLUT1 and GLUT4, the interaction of the negative and positive side chains of glutamic acid and arginine in the cytoplasmic side of transmembrane domains seems to play a key role in alternating the transporter between an outward- and inward-facing conformation (25). Substitution of some of these amino acids can lock the facilitative transporter in an outward- or inward-facing configuration, as assessed by the binding of ligands specific for the two conformations of the transporter (25). The OCTN2 carnitine transporter has a GLUT signature motif between transmembrane domains 2 and 3 (3, 4), and the domain affected by the E452K mutation could interact with a neighboring positive residue (Arg<sup>459</sup>) to modify the conformation of the carnitine transporter. In summary, the E452K mutation represents the first natural mutation affecting sodium-stimulated substrate uptake in a mammalian cotransporter and identifies a novel functional domain of mammalian cotransporters, possibly related to the release of sodium in the cytoplasm or to the dynamic alteration of transporter conformation necessary for transmembrane solute/co-substrate transfer.

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