MINIREVIEW

Choline Transport for Phospholipid Synthesis

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Choline is an essential nutrient for all cells because it plays a role in the synthesis of the membrane phospholipid components of the cell membranes, as a methyl-group donor in methionine metabolism as well as in the synthesis of the neurotransmitter acetylcholine. Choline deficiency affects the expression of genes involved in cell proliferation, differentiation, and apoptosis, and it has been associated with liver dysfunction and cancer. Abnormal choline transport and metabolism have been implicated in a number of neurodegenerative disorders such as Alzheimer's and Parkinson's disease. Therefore, the study of choline transport and the characteristics of choline transporters are of central importance to understanding the mechanisms that underlie membrane integrity and cell signaling in such disorders. Kinetic studies with radiolabeled choline and inhibitors distinguish three systems for choline transport: (i) low-affinity facilitated diffusion, (ii) high-affinity, Na^+-dependent transport, and (iii) intermediate-affinity, Na^+-independent transport. It is only recently, however, that the proteins having transport characteristics of at least one of these systems have been identified. They include (i) polyspecific organic cation transporters (OCTs) with low affinity for choline, (ii) high-affinity choline transporters (CHT1s), and (iii) intermediate-affinity choline transporter-like (CTL1) proteins. CHT1 and CTL1 but not OCT transporters are selectively inhibited with hemicholinium-3 and essentially display characteristics of specialized transporters for targeted choline metabolism. CHT1 is abundant in neurons and almost exclusively supplies choline for acetylcholine synthesis. The focus here is more on newly-discovered CTL1 choline transporters. They are expressed in different organisms and cell types, apparently not for the biosynthesis of acetylcholine but for the production of the most abundant metabolite of choline, the membrane lipid phosphatidylcholine.
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Importance of Choline

In 1998, choline was identified as an essential nutrient for humans by the National Academy of Sciences, United States (1). Choline is a quaternary amine (trimethyl-β-hydroxy-ethylammonium) (2) and its concentration in plasma has a physiological range of 10–50 μ*M* (3). Choline is predominantly utilized for the synthesis of essential lipid components of the cell membranes, phosphatidylcholine (PC) and sphingomyelin, and for the production of potent lipid mediators such as platelet-activating factor and lysophosphatidylcholine (4). Quantitatively, PC is the most important metabolite of choline and accounts for approximately one-half of the total membrane lipid content (5). Similarly to folate, choline is a source of labile methyl groups (6). In the liver and kidney mitochondria, choline is oxidized to betaine (trimethylglycine), which then enters the one-carbon cycle and serves as a methyl donor in the remethylation of homocysteine to methionine, to ultimately generate the methylation agent S-adenosylmethionine (SAM; Ref. 7). Finally, choline is best known for its key role in neurons as the precursor of the neurotransmitter acetylcholine (8, 9). Acetylcholine could also be synthesized and released from nonneuronal cells; however, its role outside of neurons is not clearly defined (10).

The adequate intake level for choline is 550 mg/day for

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men and 425 mg/day for women (11). Choline content varies considerably among common foods (12) and dietary choline supplementation could improve multiple body functions, including cognition (13), learning, and memory (14, 15). Therefore, choline is an essential dietary component for the normal functioning of organisms. Here, we focus on specific aspects of choline research in recent years, including choline deficiency and choline metabolic pathways and transport, with emphasis on the emerging new families of transporters necessary for the production of PC, a lipid essential for the structural integrity and functioning of cell membranes.

Choline Deficiency

Healthy individuals meet or exceed the adequate intake levels for choline (16); however, certain population groups are at risk for choline deficiency. These include pregnant and lactating women, infants, cirrhosis patients, and patients depending on parenteral nutrition (17). Dietary choline deficiency in animal models causes spontaneous carcinoma of the liver and generally increases sensitivity to carcinogens (18). It is the only type of nutritional deficiency known to have such deleterious effect (19). A gradual reduction in choline supplementation initially causes apoptosis in rat hepatocytes (20, 21); however, when cells adapt to a low choline intake, they become resistant, and a tumorigenic transformation is frequently observed (21). Apoptosis induced by choline deficiency is p53-independent and is linked to TGFβ1 signaling and generation of reactive oxygen species, which disrupt the membrane potential and eventually lead to mitochondrial dysfunction and cell death (21, 22). Adaptive responses to deleterious effects of choline deficiency include increased transcription of the proto-oncogenes *c-myc*, c-Ha-*ras* and *c-fos*, and increased levels of proinflammatory cytokines such as TNF-α (23). Furthermore, it has recently been shown that choline deficiency affects the expression of 1000 genes in neural precursor cells, with one-third of the genes involved in the cell proliferation, differentiation, methyl-group metabolism, and apoptosis (24), demonstrating the essentiality of choline for proper cell function.

Because it is predominantly utilized for membrane PC, choline has a significant impact on liver function and systemic lipid metabolism. In experimental animals as well as in humans (25–28), choline deficiency results in the formation of "nonalcoholic fatty liver," associated with accumulation of hepatic lipids and an increased sensitivity to inflammation (29). In knockout mouse models lacking liver CTP:phosphocholine cytidylyltransferase (Pcyt1), which is the major regulatory enzyme in PC synthesis, hepatic PC decreases and an accumulation of triglycerides in the liver is observed. At the same time, plasma lipids as well as plasma lipoprotein content become reduced (30). It is well established that homocysteine levels are elevated after a methionine load in choline-deficient individuals (26). The interaction of choline with other nutrients in one-carbon metabolism and homocysteine production is becoming an increasingly important issue to be examined more actively in the future (31). The close relationship between choline and folate/methionine metabolism offers an opportunity for improved assessment of human susceptibility to choline status, development of nonalcoholic fatty liver, and development of cancer. The risk assessment based on genetic variations may offer novel preventive strategies for similar diseases. The relationship between folate deficiency and polymorphisms of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene is well-known; it resulted in widespread supplementation of food products with folate to prevent development of birth defects (32). Choline deficiency is not related to the variations in the MTHFR gene; however, the polymorphism of 5,10-methylenetetrahydrofolate dehydrogenase (MTHFDH) is a significant risk factor. When placed on a choline-deficient diet, carriers of the most common allele, MTHFDH1958A, become depleted of choline faster than noncarriers (27).

During pregnancy, fetal plasma choline levels are several-fold higher compared with choline levels in maternal plasma, which may result in a depletion of maternal choline stores (29), implicating the importance of choline for the developing fetus. In a normal pregnancy, plasma choline levels rise constantly throughout the gestation period (33), coinciding with elevated homocysteine levels (34), which indicates that the high fetal demand for choline may stimulate an increased choline synthesis in the maternal liver (34). Choline supplements during pregnancy have been shown to improve lifelong memory in rats (35). Adult offspring of mothers that had received choline supplements were more adept at tasks involving spatial and temporal memory and attention (35–37), and these behavioral effects persisted beyond the age of 2 years. These data suggest that choline has a permanent developmental effect on brain organization and function (1). Furthermore, consuming foods high in choline during pregnancy may reduce the risk of neural tube defects; this is attributed mainly to the role of choline as a methyl-group donor (38). More on choline deficiency and the relationship between choline, the one-carbon cycle, and lipid metabolism is discussed in the following sections on choline metabolic pathways and transport.

Choline Metabolic Pathways

The majority of cellular choline is phosphorylated by choline kinase (CK) to phosphocholine, to which CTP can then be added by Pcyt1 to yield CDP-choline. The formation of PC results from the reaction of CDP-choline with diacylglycerol (DAG), catalyzed by CDP-choline: DAG cholinephosphotransferase (CPT; Fig. 1). This pathway is known as the Kennedy (CDP-choline) pathway for *de novo* synthesis of PC and is essential for the formation of membrane PC in all nucleated cells. Since its discovery in

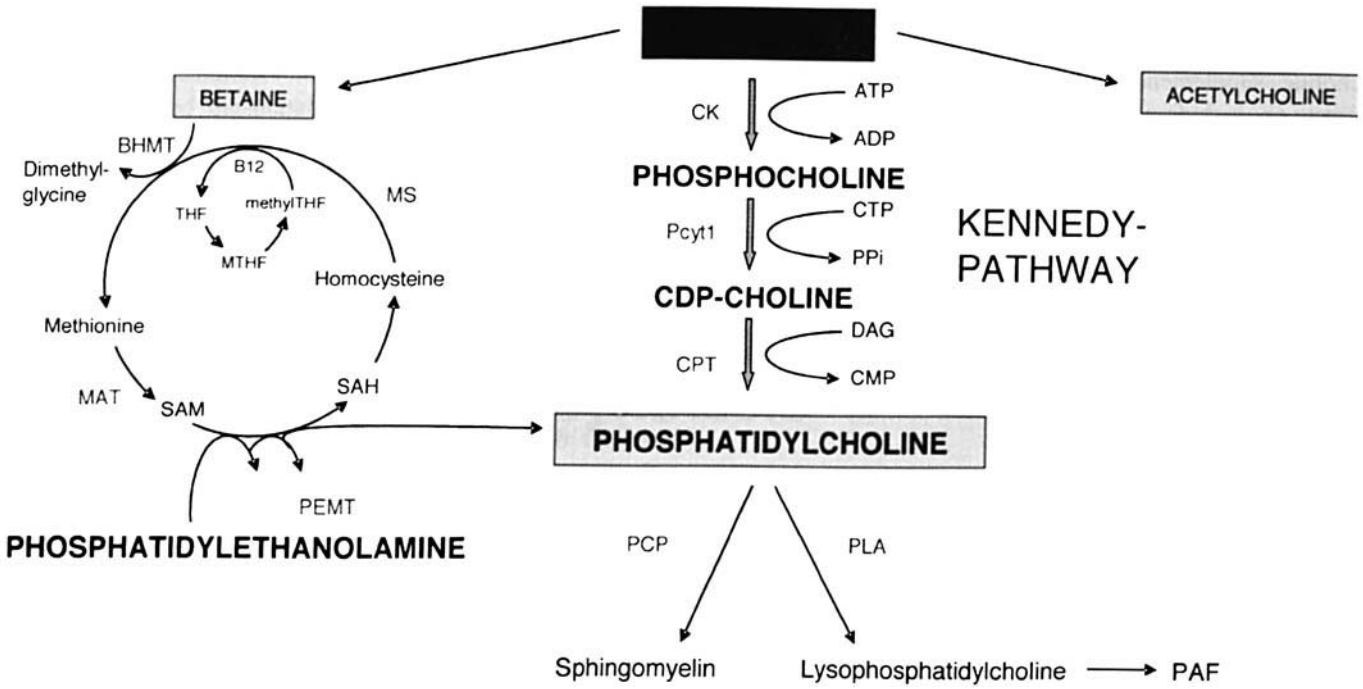

Figure 1. Choline metabolic pathways. ADP, adenosine diphosphate; ATP, adenosine triphosphate; BHMT, betaine:homocysteine methyltransferase; B12, vitamin B12; CDP, cytidine diphosphate; CK, choline kinase; CPT, CDP-choline:DAG cholinephosphotransferase; CMP, cytidine monophosphate; CTP, cytidine triphosphate; DAG, diacylglycerol; MAT, methionine adenosyltransferase; methylTHF, 5-methyltetrahydrofolate; MS, methionine synthase; MTHF, 5,10-methylene-tetrahydrofolate; PAF, platelet-activating factor; PCP, phosphatidylcholine:ceramidecholine phosphotransferase; Pcyt1, CTP:phosphocholine cytidyltransferase; PEMT, phosphatidylethanolamine *N*-methyltransferase; PLA, phospholipase A2; PPi, pyrophosphate; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate.

the 1950s by Eugene Kennedy, this pathway has been extensively studied, and most aspects of its regulation are well established. In recent years, focus is more on cloning and characterization of genes participating in the pathway (39–43), with emphasis on the rate-regulatory gene Pcyt1 (44–46); the Kennedy pathway is not considered to be regulated by other steps in the pathway, nor by the rate of choline transport.

During evolution, the liver has retained a backup pathway for choline production from the second most abundant membrane phospholipids, phosphatidylethanolamine (PE; Fig. 1), to provide this essential metabolite when dietary choline is limited, as, for example, during starvation, embryonic development, pregnancy, or lactation. PE is transformed to PC in a three-step methylation by SAM, which is catalyzed by phosphatidylethanolamine-N-methyltransferase (PEMT). The PE methylation pathway has been extensively studied by Dr. Dennis Vance and his collaborators (for reviews see Refs. 47, 48). More recently, his laboratory developed a *PEMT* knockout mouse model, providing valuable data on the role of this pathway in the liver, PC synthesis, lipoprotein secretion, and bile and homocysteine production (42, 49).

Liver choline/phospholipid and methionine/homocysteine pathways are interrelated by their need for methyl groups provided by SAM (Fig. 1). When PE methylation is blocked, as it is in *PEMT* knockout mice ($PEMT^{-/-}$), plasma homocysteine is 50% lower than in wild-type $PEMT^{+/+}$ mice (50). On the other hand, mice having $Pcyt1\alpha$ conditionally deleted in macrophages have elevated plasma homocysteine because of an increased methylation of PE, to compensate for the dysfunctional CDP-choline pathway (49). When fed a choline-deficient diet, the $PEMT^{-/-}$ mouse dies because of hepatic depletion of PC. When fed a regular diet, the mouse is normal, because the $PEMT^{-/-}$ liver can utilize dietary choline *via* the CDP-choline pathway. The $PEMT^{-/-}$ animals develop an anomalous lipoprotein profile and a "fat-liver" phenotype when fed a high-fat/high-cholesterol, "Western-style" diet (51). Surprisingly, double-knockout $PEMT^{-/-}/MDR2^{-/-}$ mice (MDR2 is a hepatic P-glycoprotein that exports PC into bile) survive on a choline-deficient diet (52). In these animals, choline is recycled *via* increased PC metabolism and increased flux through the CDP-choline pathway, as well as by suppression of choline oxidation to betaine (Fig. 1). Therefore, it becomes apparent that $PEMT^{-/-}$ mice die on a choline-deficient diet because these pathways do not compensate the PC drain into bile. Together, these invaluable studies strongly demonstrate the importance of proper metabolic balances between choline, SAM, PE, and PC in liver function, and make the pathways (shown in Fig. 1) for their regulation significant risk factors for development of "fatty-liver" steatosis, hyperlipoproteinemia, hyperhomocyteinemia, and cardiovascular disease.

Importance of Choline Transport

Separately from choline metabolism, disturbed choline transport has been proposed to play an important role in multiple clinical manifestations, particularly in neurological, muscular, and immunological disorders (53, 54) and in various cancers, including breast and ovarian cancer (55, 56). However, choline transport has been studied predominantly in neurodegenerative disorders, particularly in Alzheimer's disease. Alzheimer's disease is manifested with the specific loss of cholinergic neurons, and choline transport is an attractive target for drug development to treat the disease (57). It is now clear that choline is provided to cholinergic neurons by a high-affinity transport system, which controls the formation of acetylcholine (57) and as such is of the greatest interest in this disorder (58). In addition, a low-affinity choline transport system unrelated to cholinergic transport has often been irregular in the erythrocytes of Alzheimer's disease patients (59). A reduced choline level in the cerebrospinal fluid of Parkinson's disease and Huntington's disease patients is a result not only of impaired choline transport but also of a modified phospholipid output (60, 61). A lower phospholipid output could result in membrane damage, in addition to the disturbances in neurotransmitter production that are very common in neurological disorders. In Friedreich's ataxia, the PC molecules incorporated into the membrane of certain cells are abnormal in that they contain a low percentage of linoleic acid (62). Emerging new data (discussed below) suggest that choline carriers that participate in PC metabolism, not only in neuronal cells but in most cells, are distinct from cholinergic carriers. These cells typically have a medium to low affinity for choline and could be choline-specific, polyspecific for various organic cations, or both. Therefore, besides the independent regulation of intracellular choline utilization for PC production in the Kennedy pathway and in liver PE methylation, complex transport systems for choline to enter and/or exit specialized cells are required in order to adjust free choline with specific cellular demands for choline, which, when disturbed, could have serious implications for membrane fluidity and function in all tissues.

Systems for Choline Transport

Choline is a positively charged quaternary amine and requires a protein-mediated mechanism to effectively pass the membrane lipid barrier. The mechanisms underlying choline transport have not been completely elucidated; however, three protein-mediated and thus saturable uptake systems, following Michaelis-Menten kinetics, are well documented (63–68). The first mechanism is the facilitated diffusion that has an apparent K_m of 10 μM of choline, driven by a choline concentration gradient as described in red blood cells (64). The second is a high-affinity, Na^+- and energy-dependent transport system with a K_m of 0.5–3 μM that is sensitive to inhibition by low concentration of hemicholinium-3 (HC-3, K_i of 1–3 μM; Ref. 65). This "active transport" system is coupled to the biosynthesis of acetylcholine and is primary to neuronal tissues (65). The third, also "active transport," system, which is somewhat lower in affinity for choline ($K_m \approx$ 20–200 μM), is ubiquitously distributed, and less effectively inhibited by HC-3 ($K_i \approx$ 20–200 μM) than the neuronal system (66–68). This system operates in most cells as a means of choline uptake for the purpose of phospholipid synthesis.

Many organs, such as lung, brain, placenta, kidney, and liver have acquired one or a combination of several choline transport systems, which allow them to fulfill specific functions. The alveoli of the lungs are lined with pulmonary surfactant, which is a PC phospholipid-protein complex that promotes lung stability (69). The physiological activity of the surfactant is largely attributable to the unique PC component of the surfactant, disaturated phosphatidylcholine (69). In all other cells in the lung, the major source of PC is the Kennedy (CDP-choline) pathway, which requires the circulating choline to be transported into the lung by the alveolar epithelium. It has been suggested that this transcellular transport process may be rate-limiting for the biosynthesis of pulmonary PC (70, 71). In addition, alveolar type II epithelial cells have developed two types of "active" choline transport mechanisms to regulate the pulmonary choline content (68): The first is a high-affinity, Na^+-dependent system (K_m of 1.5 μM; K_i of 1.7 μM for HC-3) resembling the neuronal transport, and the second is a Na^+-independent system that has a lower affinity for choline (K_m of 19 μM; K_i of 12 μM for HC-3), resembling the choline-specific but nonneuronal transport. There is also a third, very slow residual transport, which is presumed to be by passive diffusion.

The central nervous system requires choline for the synthesis of membrane phospholipids and for acetylcholine. In the brain, choline that is transported *via* the "high-affinity" system is destined for acetylcholine synthesis, whereas the "lower-affinity" system supplies choline for phospholipid synthesis (72). The ranges of affinity for these systems could vary and depend on the region of the brain in which they are located, suggesting that a combination of choline carriers could be involved. Because the brain cannot synthesize *de novo* choline, choline uptake from extracellular fluids is essential (73). Choline enters the brain from the systemic circulation by a saturable transport at the blood-brain barrier (BBB) at a rate proportional to changes in the blood choline concentration, thus representing a facilitated diffusion process (72). Human keratinocytes express a Na^+-independent, polyspecific choline transport system similar to the choline carrier in the BBB and intestinal epithelial cells. This choline carrier has relevance both for the biosynthesis of skin lipids and for the uptake of cationic drugs into the keratinocytes, as it does at the BBB (28).

The importance of placental choline has been demonstrated in studies in which mouse embryos showed growth

retardation and developmental defects of the neural tube and face during neurulation when either choline uptake or metabolism was inhibited (7). The placenta possesses a choline transport mechanism, which is essential because a growing fetus has to derive all its metabolic choline requirements from its mother *via* the placenta (74). It has both a passive diffusion system and an active carrier-mediated process of low affinity with a K_m of 350 μM choline (75). Passive diffusion is the least physiologically relevant type of choline transport, considering positively-charged choline molecules and the blood concentration of choline, which is in the 10–50 μM range. More relevant is the active choline transport, as present at the apical side of BeWo and JEG-3 epithelial cells of the human placenta (76). The uptake is Na^+-independent and saturable, with a K_m of 108 μM and 206 μM, respectively. Several cationic drugs, including diphenhydramine, etilefrine, clonidine, ranitidine, and butylscopolamine interact with this transport system and could be instrumental in the drug's transferring from the maternal blood to the fetal circulation (76).

All ingested choline and free choline generated by phospholipid metabolism enters the hepatic circulation, making the liver, where, as discussed earlier, there are very active biochemical pathways for choline metabolism, a significant "sink" for choline (Fig. 1). The liver contains a transport system resembling the "low-affinity" facilitated diffusion, but multiple systems are also detectable under different conditions (77). Studies of rat liver plasma membrane vesicles have demonstrated the presence of a basolateral transport for both choline and organic cations (77). It seems that the murine transporter organic cation transporter (OCT) 1 is responsible for the most of the hepatic choline and cationic drug circulation (78). At present, it is unknown whether a choline-specific transport is operating in the liver or whether different systems could be present at different membrane sites.

Renal tubular transport of choline is of importance because it maintains the plasma choline concentration within relatively narrow limits by employing both net secretion and reabsorption (79). When choline is presented to the kidney in excess of a species-specific threshold concentration, it will be excreted from the kidney into the urine (80). If the plasma concentration is below this level, choline will be reabsorbed into the kidney, not excreted (80). Membrane transport of choline is elevated in renal failure in erythrocytes and cerebral tissue, but the origins and clinical importance of that are unknown. (53). OCT1, for example, is localized in the basolateral membrane of renal proximal tubules, in intestinal enterocytes, and in the sinusoidal membrane of hepatocytes, where it probably mediates the efflux of many organic cations, including choline. The substrate specificity of rat OCT1 is for more hydrophilic (type 1) cations like tetra-ethyl ammonium and choline. Quinine, quinidine, and cyanine 863, which have been classified as type 2 cations, are not eliminated with rat OCT1 (81). The secretion (efflux) of organic cations by renal proximal tubule is electrogenic, facilitated diffusion, and it is associated with basolateral uptake of type 1 organic cations. These processes are also believed to support electroneutral organic cation/organic cation exchange. An extensive presentation of renal polyspecific transport of organic cations is described by Wright and Dantzler (82).

Choline Transporters

Different families of proteins that have either been demonstrated or thought to play a role in choline transport have been isolated in a variety of species. The most prominent are (i) the high-affinity choline transporter family (CHT), (ii) the family of OCTs, and (iii) the choline transporter–like family (CTL). They exhibit varying affinities not only for choline transport but also for transport of a number of other organic cations (Table 1). Studies of the effect of various organic cation inhibitors revealed that hemicholinium-3 is the most effective inhibitor of choline uptake. Although CHT transporters are mostly important for choline transport for acetylcholine synthesis rather than for lipid synthesis, we want to include a brief summary of these high-affinity transport systems to complete the picture of choline transport. Comprehensive reviews of CHT are presented by Ferguson and Blakely (83) and Sarter and Parikh (84).

High Affinity Choline-Specific Transporters

Cholinergic neurotransmission plays an important role in regulation of many physiological functions (85–88). It is well known that basal forebrain cholinergic neurons are involved in learning and memory processes, and that hypoactivity of the cholinergic system is responsible for the cognitive deficit in Alzheimer's disease (4). Na^+-dependent and HC-3-sensitive choline transport is very active in presynaptic terminals, and it is mediated by a high-affinity choline transporter. After many years of searching, this transporter, named CHT1, has recently been successfully cloned, first from *Caenorhabditis elegans* (*cho-1*) and rat (*rCHT1*; Ref. 89), and subsequently from humans (*hCHT1*; Refs. 90, 91), Limulus (LchCoT; Ref. 92), and mouse (*mCHT1*; Ref. 93). Based on the cDNA sequence, there is a high degree of conservation among CHT1 proteins, and their closest structural similarity is with the Na^+-dependent glucose transporter SGLT (89). *hCHT1* is located on chromosome 2q12. The entire gene is $\approx$25 kb long and contains 9 exons. hCHT1 protein is made of 580 amino acids with a predicted molecular mass of 63 kDa and could form 13 transmembrane domains (90, 91, 94). CHT1 mRNA expression is restricted to the brain regions rich in cholinergic neurons, like the forebrain, striatum, brain stem, and spinal cord (89–93). Recently, however, CHT1 transcripts have been also detected in nonneuronal cells, but the function of CHT1 outside of neurons is not known (95–97).

Now it is clear that CHT1 provides choline for the synthesis of acetylcholine in all cholinergic neurons (92–

Table 1. Choline Transporters: Affinity, Localization, and Expression

	Name	Chromosome	Organism	Tissue distribution[a]	References
Choline-specific					
I. CHT1s	hCHT1[b]	2q12	Human	Brain, spinal cord	90, 91
	mCHT1	17D	Mouse	Brain, spinal cord	93
	rCHT1[b]	9q11	Rat	Brain, spinal cord	89
	CHO-1	IV	*C. elegans*	Brain synaptic vesicles	89
II. CTL1s	hCTL1[b]	9q31.2	Human	Multiple tissues[ac]	149, 150
	mCTL1	4B2	Mouse	Muscle	151
	rCTL1	5q24	Rat	Brain, spinal cord, colon[d]	148, 149
	tCTL1		Torpedo	Central nervous system, myelin	149,152
Choline-nonspecific					
OCTs	hOCT1	6q26	Human	Liver,	120, 140
	hOCT2	6q26	Human	Kidney, brain	120, 140
	mOCT1	17A1	Mouse	Intestine, liver, kidney	121
	mOCT2	17A1	Mouse	Kidney	124
	rOCT1[b]	1q11–q12	Rat	Liver, kidney, intestine[d]	119, 129, 130, 141
	rOCT2[b]	1q11	Rat	Kidney[d]	122, 125, 130, 139

[a] Detection by mRNA.
[b] Antibody being developed.
[c] Detection by Western blotting.
[d] Detection by either RT-PCR, immunohistochemistry, or *in situ* hybridization.

94). Functional expression of rat CHT1 in Xenopus oocytes or COS-7 cells produces typical high-affinity choline uptake that is identical with the high-affinity choline uptake in synaptosomes: (i) K_m for choline of 2 μM, (ii) inhibition by CH-3 with K_i of 2–5 nM, and (iii) Na^+ and Cl^- ion dependence (94). Apart from these cholinergic transport characteristics, the regulation of CHT expression is becoming increasingly important. It has been shown that CHT1 could be regulated by neuronal depolarization (98–102), second messengers (103–105), and acute drug treatments (106–108). CHT1 contains serine and threonine residues for regulation of function and surface expression by protein kinase C (PKC; Refs. 92, 94) and protein phosphatase 1/2A (PP1/PP2A; Ref. 92). It seems a very common phenomenon that PKC and PP1/PP2A regulate neurotransmitter transporter proteins *via* rapid changes in transporter surface expression and phosphorylation (109). Recently it was reported that prostate apoptosis response-4 (Par-4), a leucine zipper protein that plays an important role in neuronal dysfunction and cell death in neurodegenerative disorders such as Alzheimer's disease (88, 110–116), inhibits CHT1-mediated choline uptake activity and reduces the cell surface expression of CHT1 by directly interacting with CHT1 (88). The importance of the biological function of CHT1 has been further confirmed by the fact that disruption of CHT1 in mice is lethal to the neonate (117).

Low-Affinity Polyspecific Organic Cation Transporters

The transport of endogenous organic cations, clinical drugs, and exogenous toxic cations is generally mediated by the family of OCTs also known as the solute carrier 22 family of proteins (118). This family is comprised of five members, including OCT1, OCT2, OCT3 (119–126), OCTN1 (127), and OCTN2 (128), and has been subject to ongoing intensive research since the initial cloning of OCT1 from a rat kidney library in 1994 (119). OCT1 is expressed in the liver, kidney, and small intestine in rodents (119, 121, 129, 130); however, in humans, it is mainly expressed in the liver (120). OCT2 has substrate specificity similar to that of OCT1, but its expression is limited to the kidney and specific regions in the brain (122, 131). OCT3 is expressed most abundantly in the placenta and moderately in the intestine, heart, and brain (Table 1; Ref. 132).

The functional characterizations from different species strongly suggest that OCT1, OCT2, and OCT3 facilitate polyspecific cationic transport (119–121, 128, 131–133). Regarding their physiological role in choline transport, however, the data are less extensive and the information varies in different experiments. In transiently transfected human embryonic kidney (HEK) 293 cells, choline was transported exclusively by OCT1, but not by OCT2 (134). When expressed in Xenopus oocytes, both OCT1 and OCT2 could facilitate choline uptake with low affinity, that is, K_m of 346 μM for rOCT1, 441 μM for rOCT2, and 102 μM for hOCT2 (135). OCT-1 and OCT2-mediated choline uptake could be inhibited by cimetidine (136) and larger, more hydrophobic cations like the antiarrhythmics quinine and quinidine (81, 135, 137). Disprocynium 24 and decynium 22, the most potent inhibitors recognized so far, inhibit OCT2 with K_i values around 10 nM (134, 138), and corticosterone, another potent inhibitor, inhibits OCT2 with K_i values around 200 nM (137). Disprocynium 24, decynium 22, and corticosterone are less effective with OCT1 (134, 137). The major driving force for choline transport *via* OCT1 and OCT2 is the membrane potential

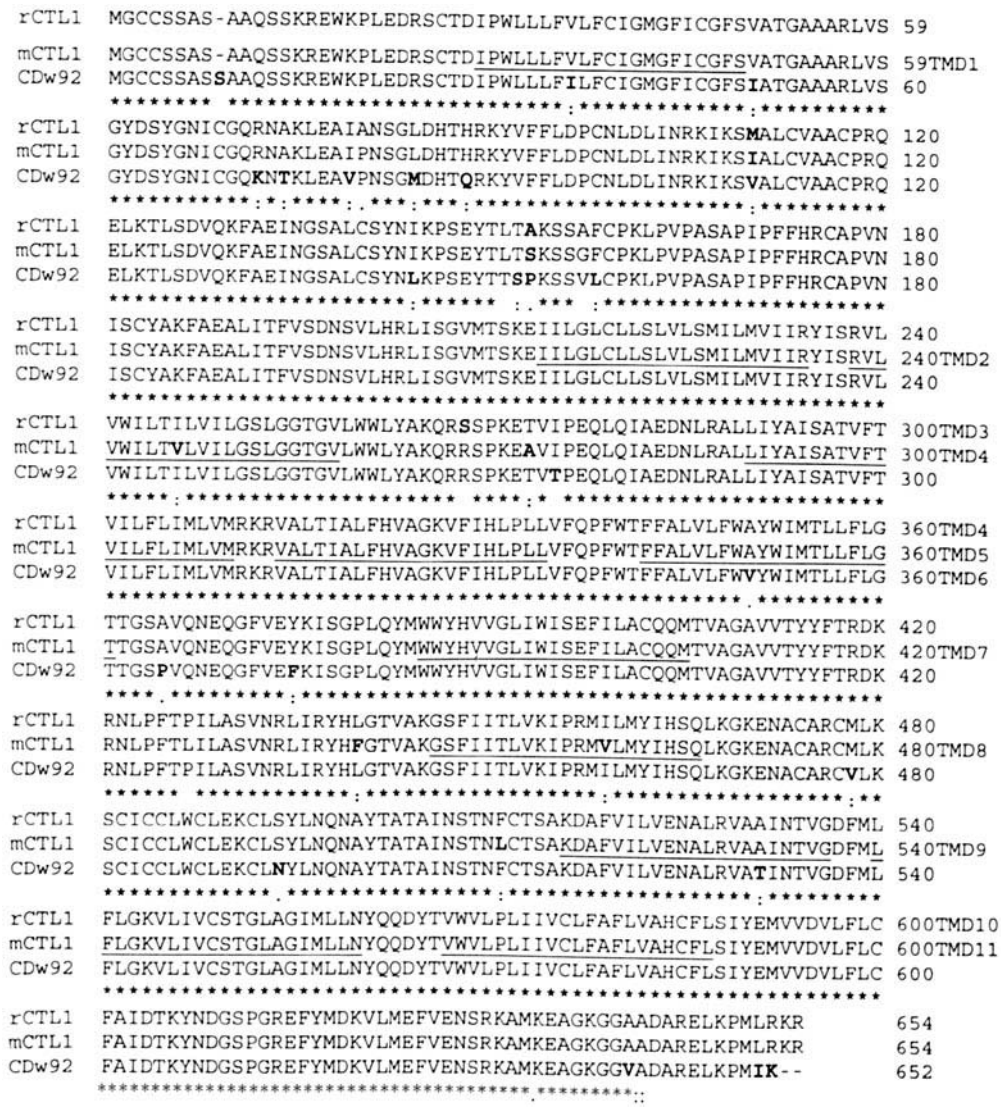

Figure 2. CTL1 amino acid sequence in rat (rCTL1), mouse (mCTL1), and human (hCTL1/CDw92). Underlined stretches represent transmembrane domains; bold letters indicate nonconserved amino acids.

(135, 139). An *in vivo* study indicates that OCT2 plays an active role in brain choline homeostasis and that choline is not a substrate for OCT3 (135).

A most recent summary of the OCT1 and OCT2 family is shown in Table 1. The genes of *OCT1* and *OCT2* have been mapped on chromosome 6q26 in human (138, 140). Rat and mouse forms, *rOCT1* and *mOCT1(Lx1)*, have been localized on chromosome 1q11-q12 and chromosome 17A1, respectively (121, 141). The primary structure of rOCT1 and a tentative model of its membrane topology predict 12 transmembrane domains (TMDs) and three potential glycosylation sites located in the large hydrophilic loop between the first and second transmembrane domains (reviewed in reference 133). Some data indicate the existence of substrate-binding pockets in rOCT1 (142) and rOCT2 (143). There are four potential protein kinase C phosphorylation sites on the large intracellular loop between TMD6 and TMD7 (133).

OCT1 knockout mice ($OCT^{-/-}$ mice) are healthy and fertile (118). $OCT^{-/-}$ mice have reduced hepatic uptake and intestinal excretion of various organic cations, and although OCT1, OCT2, and OCT3 have overlapping substrates, the loss of *OCT1* is not compensated for by upregulation of either *OCT2* or *OCT3*. Interestingly, the levels of radiolabeled [^{14}C] choline in the livers of $OCT^{-/-}$ mice were unaffected compared to those in the livers of wild-type mice, suggesting that an efficient choline transport system other than the OCT type exists in the liver (118). A deficiency in OCT2 and a combined deficiency in OCT1 and OCT2 had no obvious effect on the physiology of mice (144, 145), but in $OCT1/2^{-/-}$ mice, renal secretion of TEA was completely abolished (144), which implies that OCT1 and OCT2 together are essential for renal secretion of small organic cations; however, their role in choline transport is not known (144). OCTN1 and OCTN2 have been identified

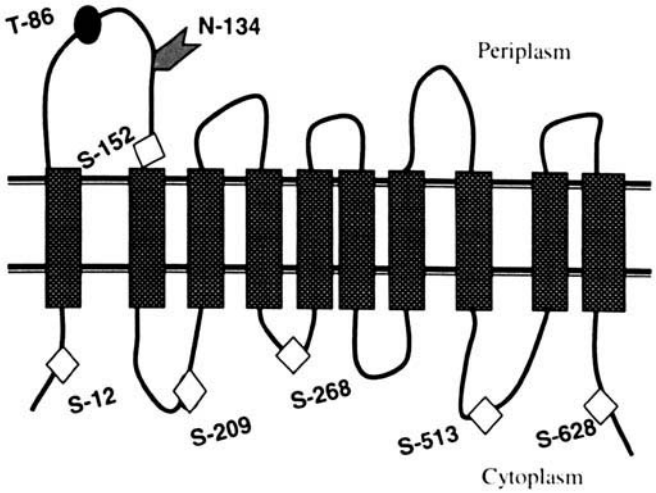

Figure 3. mCTL1 presents 10 transmembrane domains (TMDs) with a large and variable loop between TMDs 1 and 2, which is potentially glycosylated ($N^{134}GSA^{137}$). Potential protein kinase C phosphorylation sites were identified in the cytoplasmic domain ($S^{12}SK$, $T^{86}HR$, $T^{151}SK$, $S^{209}KE$, S^{268} PK, $S^{513}AK$, $S^{628}RK$).

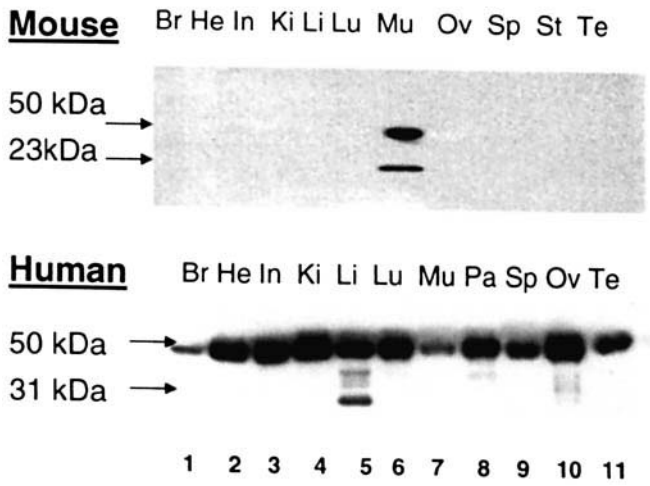

Figure 4. (Top) Western blot of a mouse tissue protein panel showing that mCTL1 is expressed only in the skeletal muscle (Mu), but not in brain (Br), heart (He), small intestine (In), kidney (Ki), liver (Li), lung (Lu), ovary (Ov), stomach (St), spleen (Sp), and testis (Te). (Bottom) Western blot of a human tissue protein panel showing hCTL1 expression as a major 50 kDa and a minor 23 KDa band in multiple tissues, including brain (BR), heart (He), small intestine (In), kidney (Ki), liver (Li), lung (Lu), skeletal muscle (Mu), pancreas (Pa), spleen (Sp), ovary (OV), and testis (Te).

in human and rat (127, 128, 146, 147). However, choline is not a substrate of OCTN1 and OCTN2 (128, 147).

Choline-Specific Transporter-Like Proteins

The choline transporter–like proteins of the CTL family are comprised of the five genes, CTL1-CTL5, with CTL1 being the main member of the family (148). In 2000, O'Regan *et al.* (149) first cloned and characterized *Torpedo marmorata* CTL1 (*tCTL1*) and rat CTL1 (*rCTL1*). Subsequently the human form was cloned by Dr. Stockinger's laboratory (*hCTL1*/CDw92; Ref. 150), and we have cloned the mouse form, *mCTL1* (151). It is interesting that the tCTL1 was isolated by complementation of a yeast strain defective in choline transport and that hCTL1 was cloned from a cDNA expression library using a monoclonal antibody for the human antigen CDw92. CDw92 has a role in immune cell function as part of an auto-regulatory signaling loop that controls expression and maintenance of IL-10 production in dendritic cells (150). The function assigned to CDw92 as a choline transporter has been based on the cDNA sequence similarity to rCTL1 and other family orthologs.

Mouse CTL2 and CTL4 cDNA have been initially identified in the Genbank libraries as members of this family (149); however, the mouse form of CTL1 was not detected, suggesting that this form could be rare or not present in the mouse. We searched for this rare form and were able to successfully clone the mCTL1 cDNA from the mouse embryonic fibroblasts, to fully characterize the mouse gene that is localized at chromosome 4B2. Thus, we added a new member to the CTL1 family of proteins that is a choline transporter abundantly expressed in the mouse skeletal muscle, the function of which would be in transporting choline for phospholipid synthesis and/or choline recycling at the neuromuscular junctions. The protein sequence alignment of mouse, rat, and human CTL1 is shown in Figure 2. They are all transmembrane proteins containing 10 highly conserved TMDs, possibly with intracellular N- and C-termini. Figure 3 shows that mCTL1 protein contains three potential N-glycosylation sites ($N^{134}GSA^{137}$, $N^{179}ISC^{182}$, $N^{506}STN^{509}$) and seven protein kinase C phosphorylation sites ($S^{12}SK$, $T^{86}HR$, $T^{151}SK$, $S^{209}KE$, S^{268} PK, $S^{513}AK$, $S^{628}RK$), five of which are distributed within the N-terminal domain. Additionally (not shown), there is one cysteine-rich region ($C^{473}ARC^{476}MLKS$-$C^{481}IC^{483}C^{484}LWC^{487}LEKC^{491}$) and one cadmium-resistance transporter domain between L^{220} and F^{341}, covering the membrane domains 3, 4, and 5.

The *hCTL1* gene is located at chromosome 9q31.2, the *rCTL1* gene at chromosome 5q24, and the *mCTL1* gene at chromosome 4B2. (Table 1). tCTL1 mRNA is present throughout the central nervous system and along the electric nerves (152); mCTL1 RNA is expressed in testis, brain, heart, and skeletal muscle, but the mCTL1 protein is abundantly present only in the skeletal muscle (Fig. 4; Ref. 151). hCTL1 protein is expressed as two major polypeptides of 50 and 23 kDa in a variety of tissues, including brain, heart, small intestine, kidney, liver, lung, skeletal muscle, pancreas, spleen, ovary, and testis (Fig. 4; Ref. 153). Rat rCTL1a mRNA is expressed in the ileum and colon, whereas rCTL1b mRNA is expressed exclusively in the brain (148, 149). Both isoforms of rCTL1 were recently functionally expressed and identified as an intermediate-affinity, Na^+-independent choline transport system in N18 neuroblastoma cells (148) and in astrocytes (154).

Two different splice variants have been identified in rat

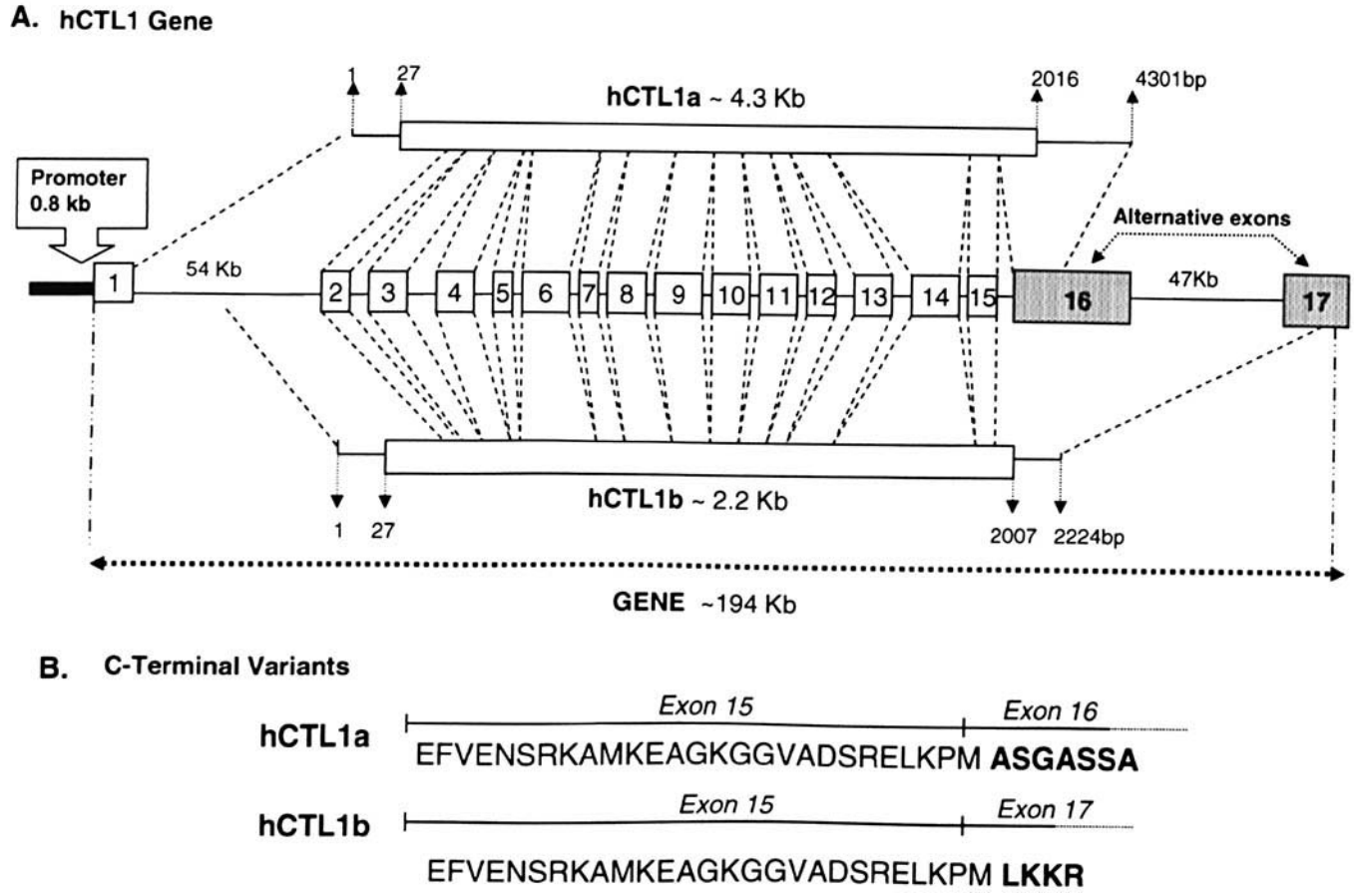

Figure 5. Organization of the hCTL1 gene and its 3′ end splicing. (A) The ≈194-kb hCtl1 gene is made of 17 exons and 16 introns. The hCTL1a transcript is encoded by exons 1–16 and the hCTL1b transcript by exons 1–15 and exon 17. The 5′-flanking (regulatory promoter) region is 0.8 kb. The first two exons are separated by the large (54 kb) intron I. The alternatively expressed exons 16 and 17 at the 3′ end are also divided by the large (47 kb) intron XVI. (B) Structure of the C-terminal peptides of the hCTL1a and hCTL1b proteins, showing that they are encoded by different exons.

(rCTL1a and rCTL1b) and humans (hCTL1a and hCTL1b; Refs. 148, 149, 153). The hCTL1 gene spans over 194 kb at chromosome 9q31.2 and consists of 17 exons and 16 introns (Fig. 5). The last two exons, 16 and 17, are alternatively spliced in the two isoforms hCTL1a and hCTL1b; hCTL1a is made of exons 1–16, whereas hCTL1b uses exons 1–15 and exon 17, resulting in distinct 3′ UTRs in both isoforms (153). The alternative splicing produces proteins with differing C-terminal peptides, A^{651}SGASSA^{657} peptide in hCTL1a and L^{651}KKR^{654} peptide in CTL1b. Noteworthy, KKXX is a short carboxy-terminal signal that plays a crucial role for the localization in the endoplasmic reticulum. The existence of KKXX motif for hCTL1b would support the identification of the additional site for choline transport at the endoplastic reticulum (148, 153, 155). Our human tissue expression profiles indicate that the hCTL1a mRNA is highly expressed in placenta and brain and the hCTL1b mRNA is most abundant in the liver (153). The significance of both hCTL1 isoforms has yet to be discovered.

The function of CTL1 as a choline transporter has recently been disputed. CTL1 was initially identified to restore choline transport in a triple yeast mutant strain (*ctr ise URA3Δ*), which is deficient in both choline transport and neosynthesis under selective conditions (149, 156). Furthermore, expression of tCTL1 in Xenopus oocytes increased sodium-independent high-affinity choline uptake (156). However, Zufferey *et al.* reported that the loss of PNS1, a CTL1 homolog in yeast, does not impair choline transport, and that overexpression of both PNS1 and tCTL1 in transport-deficient mutants did not restore choline uptake in the yeast strain (157).

In our laboratory, we demonstrated a 2.5-fold increase in choline transport by overexpression of mCTL1 in Cos-7 cells (151). We also identified hCTL1 to be the predominant choline transporter in THP-1 cells and demonstrated that choline uptake is high in monocytic cells and severely reduced after PMA treatments as a result of diminished hCTL1 protein trafficking to the plasma membrane (Fig. 6). Our choline transport studies using [^{3}H]-choline revealed that choline uptake in THP-1 cells was almost completely inhibited by HC-3, whereas OCT-specific inhibitors had no effect on choline transport. The transport was of intermediate affinity for choline (K_m = 68 ± 12 μ*M*), PMA-dependent and CTL1-related, because we strongly excluded

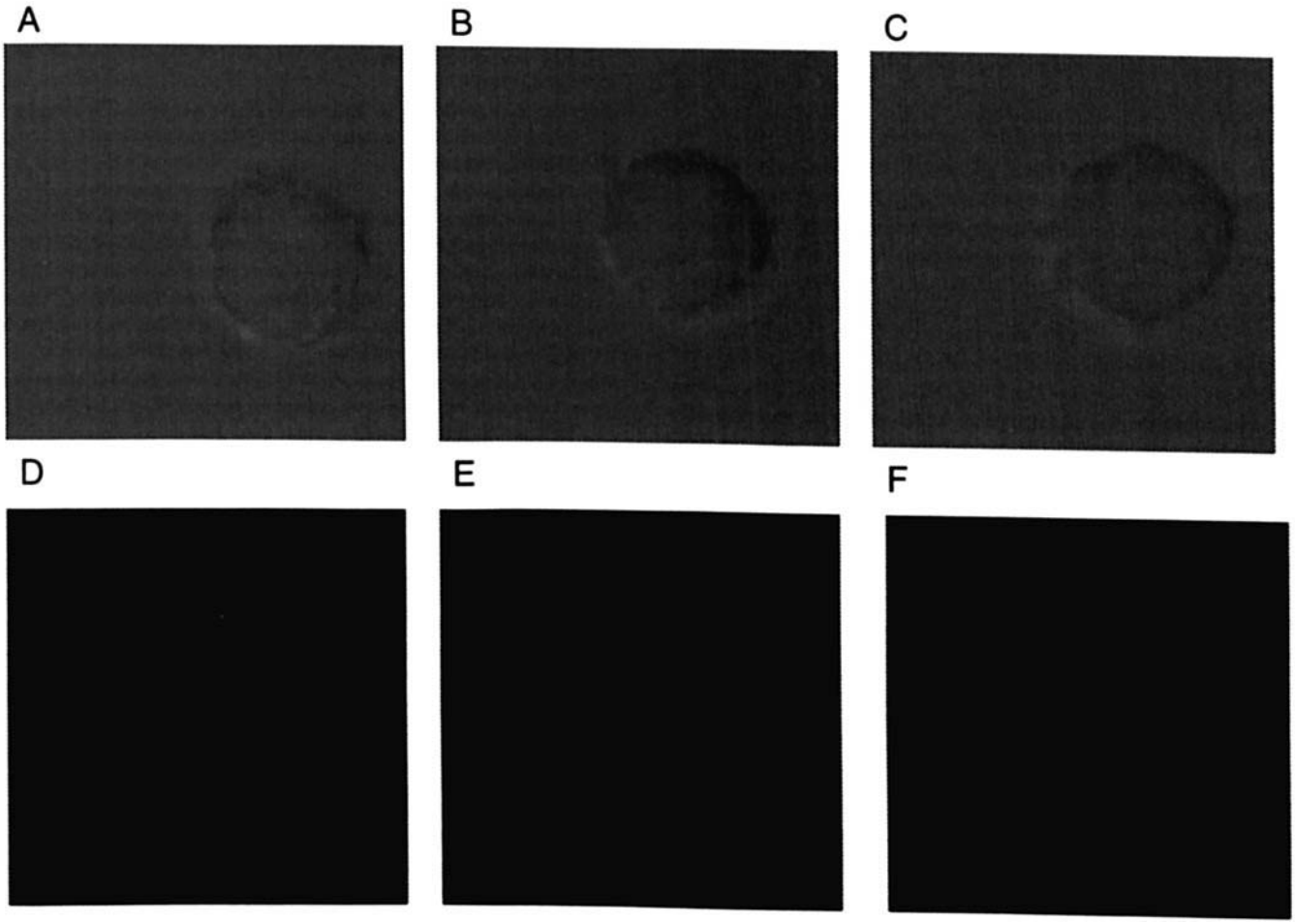

Figure 6. CTL1 disappearance from the membrane of THP-1 cells after treatment with 160 n*M* phorbol myristate 13-acetate (PMA). CTL1 was detected with a monoclonal anti-CDw92 antibody and a secondary AlexaFluor 568 antibody (Molecular Probes, Burlington, Canada). (A–C) Differential interference contrast (DIC) microscopy in (A) untreated cells, (B) 24-hr PMA treated cells, and (C) 48-hr PMA treated cells. (D–F) Confocal laser scanning microscopy of the same cells in (D) untreated cells, (E) 24-hr PMA treated cells, and (F) 48-hr PMA treated cells.

OCT and CHT1 as possible transporters (for recently published data, see ref. 158).

Three recent studies have given further strong support to the idea that CTL1 is in fact a choline transporter, as suggested by O'Regan's studies on rCTL1 and our studies on mCTL1. Inazu *et al.* (154) identified a Na^+-independent, saturable choline transport system in rat astrocytes with an intermediate affinity for choline ($K_m = 37.7$ μM), which was likely CTL1. Interestingly, they reported an impaired choline transport with a decrease in pH, which may give an indication of a possible proton dependency of CTL1-mediated choline transport. Fujita *et al.* (159) report a Na^+-dependent, saturable choline transport in mouse neurons of the cerebral cortex with an affinity for choline of $K_m = 26.7$ μM. The only member of the OCT group expressed in these cells is OCT3, which does not have choline as a substrate. Although CHT1, which is expressed by these mouse neurons, likely accounted for the Na^+-dependent choline transport, CTL1 is most likely responsible for the Na^+-independent choline transport observed in this study. Both of these recent studies imply that CTL1 could also play an important role in the choline transport of neuronal cells. Choline transport by CTL1 has also recently been associated with amyloid-β-peptide (160), a fragment of amyloid precursor protein that shows an altered metabolism in Alzheimer's disease. A long-term treatment of NG108–15 cells with amyloid-β-peptide resulted in a downregulation of choline uptake by CTL1. Although CTL1 mRNA levels did not change and the amyloid-β-peptide therefore did not affect gene expression, the reduced choline uptake may be caused by changes in mechanisms downstream of gene expression. Importantly, similarly to our studies with THP-1 monocyte-macrophages cells (158), protein kinase C could be involved in the regulation of CTL1, because inhibition of PKC increases choline uptake, whereas after PKC activation choline uptake is impaired. Recently, both CTL1 and CHT mRNA expression has been shown to be upregulated by leukemia inhibitor factor, which further supports a regulation of CTL1 expression by PKC (161). Therefore, these data suggest that CTL1 may be involved in the pathogenesis of Alzheimer's disease, which further underlines its importance in the human metabolism.

Interestingly, we also demonstrated that hCTL1 protein is overexpressed in the skeletal muscle of muscular dystrophy patients (153). The CTL1 promoter contains a binding site for the MyoD transcription factor, which typically regulates muscle myogenesis by maintaining fast muscle fiber phenotypes as well as other regulatory functions (162). The remarkable regenerative capacity of skeletal muscle depends largely on the number of available satellite cells and their proliferative capacity (163). An increased macrophage accumulation can also be observed in the diseased or injured muscle, with the extent of macrophage accumulation correlating with protein clearance following injury and the efficiency of regeneration (164). The increase of hCTL1 protein in muscular dystrophy is a sign that the hCTL1 gene is responsive to processes common to degenerated muscle, and it could represent a target for improving muscle function.

Conclusions and Prospects

With the identification of three kinds of choline transport systems—facilitated diffusion (64), low-affinity (68), and high-affinity (65)—and the identification of three families of choline transport proteins—CHT, OCT, and CTL—it is now possible to further elucidate the role of choline transport in health and disease, and to identify possible practical applications in the treatment of disorders associated with abnormal choline metabolism. It is crucial to further investigate the regulation of choline transporters at the protein and RNA level and to clarify the role that protein kinase C may play, considering the fact that all choline transporters contain highly conserved potential PKC phosphorylation sites. The identification of choline binding sites and the precise mechanism of choline uptake of the different transporters, as well as the crystallization and identification of their tertiary structures, will present a further challenge in the future.

We apologize for the failure to cite many of the important and relevant papers in this field because of space limitations. We gratefully acknowledge the research and discussions contributed by members of our laboratory.

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