FEBS Letters 484 (2000) 92–97 FEBS 24239

Functional characterization of the human high-affinity choline transporter[1]

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Received 4 September 2000; revised 6 October 2000; accepted 9 October 2000

First published online 17 October 2000

Edited by Guido Tettamanti

Abstract **Na^+-dependent, high-affinity choline uptake in cholinergic neurons is the rate-limiting step in acetylcholine synthesis. Here we report the molecular cloning and functional characterization of the human high-affinity choline transporter (hCHT1). The hCHT1 exhibits significant homology with known members of the Na^+-dependent glucose transporter family, but not with members of the neurotransmitter transporter family. The human CHT1 gene is 25 kb in length with 9 exons and was assigned to chromosome II at position IIq11–12. Northern blot analysis showed that a 5.4 kb hCHT1 transcript was expressed exclusively in tissues containing cholinergic neurons. When expressed in *Xenopus* oocytes, the human clone induced Na^+- and Cl^--dependent, high-affinity choline uptake, which was sensitive to the specific inhibitor hemicholinium-3, with a K_i of 1.3 nM. The hCHT1-mediated choline uptake increased with increasing concentrations of choline, Na^+ and Cl^-, with EC_{50} values of 2.0 μM, 76 mM, and 48 mM, and with apparent Hill coefficients of 1, 2.5 and 2.3, respectively.**

Key words: High-affinity choline transporter; Acetylcholine

1. Introduction

Choline is the precursor of membrane lipids such as phosphatidylcholine and sphingomyelin, signaling lipids like platelet-activating factor and sphingosylphosphorylcholine, and the neurotransmitter acetylcholine. Choline is an essential nutrient and plays a critical role in the development and function of the brain [1–3]. Choline does not permeate cell membranes and therefore requires a carrier-mediated transport system to cross the plasma membrane.

The high-affinity choline transport system has been characterized in brain synaptosomes [4–9], and is thought to be unique to cholinergic neurons [9,10]. In cholinergic neurons, choline used in the acetylcholine synthesis is taken up by a high-affinity choline transporter (CHT) localized in the presynaptic terminals. This high-affinity choline uptake is Na^+-dependent, sensitive to hemicholinium-3 (HC3) and is thought to be the rate-limiting step in acetylcholine synthesis [4–9]. Studies using brain proteoliposomes have demonstrated that the membrane potential and Na^+ gradient provide the driving force for high-affinity choline uptake [8,11–13].

The CHT was not identified in molecular terms for a long time, in spite of the fact that cDNAs for most of the neurotransmitter transporters had been identified by homology or expression cloning. Very recently, we succeeded in isolating a cDNA encoding the CHT1 of the nematode *Caenorhabditis elegans* using information provided by the *C. elegans* Genome Project and used this cDNA to isolate the rat homolog [14]. The amino acid sequence of CHT1 tuned out to have significant homology with known members of the Na^+-dependent glucose transporter family [15], but no significant homology with neurotransmitter transporters [16]. The characteristics of CHT1-mediated choline uptake were essentially the same as those of the high-affinity choline uptake in rat brain synaptosomes, showing a high affinity for choline, Na^+- and Cl^--dependency, and high sensitivity to HC3.

Cholinergic neurons are known to be selectively degenerated in Alzheimer's disease, resulting in the loss of high-affinity choline uptake activity overall [17]. High-affinity choline uptake activity and HC3-binding activity, however, are reported to increase in spared nerve terminals in Alzheimer's disease patients [18,19]. It will be interesting to examine whether human CHT (hCHT) is overexpressed under these circumstances. However, no molecular information on the human transporter precludes the detailed examination of its regulation in cholinergic neurons.

In this study, we report the molecular cloning and functional characterization of a cDNA encoding the hCHT1. We have analyzed the hCHT1-mediated choline uptake to determine whether the hCHT1 shows choline uptake activity with the same characteristics as that found in cholinergic nerve terminals in human.

2. Materials and methods

2.1. Cloning of hCHT1 cDNA

Gene-specific primers were designed based on an entry (GenBank accession number AQ316435) that is homologous to *C. elegans* CHT1 (CHO-1) in the human genomic survey sequence (GSS). For primary 5′ and 3′ rapid amplification of cDNA ends (RACE), a gene-specific primer was paired with adapter primer to amplify human brain Marathon Ready™ cDNA (Clontech). This polymerase chain reaction product was used for secondary RACE reactions using a nested gene-specific primer. All RACE products were subcloned into pT7Blue T-Vector (Novagen) and sequenced. A human spinal cord

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[1] The sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB043997.

Abbreviations: CHT1, high-affinity choline transporter 1; CHO-1, high-affinity choline transporter 1 in *Caenorhabditis elegans*; HC3, hemicholinium-3; GSS, genomic survey sequence

0014-5793/00/$20.00
PII: S0014-5793(00)02134-7

A

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hCHT1  MAFHVEGLIAIIVFYLLILLVGIWAAWRTKNS----GSAEERSEAIIVGG   46
rCHT1  MPFHVEGLVAIILFYLLIFLVGIWAAWKTKNS----GNAEERSEAIIVGG   46
CHO-1  -MADLLGIVAIVFFYVLILVVGIWAGRKSKSSKELESEAGAATEEVMLAG   49
                    I
hCHT1  RDIGLLVGGFTMTATWVGGGYINGTAEAVYVPGYGLAWAQAPIGYSLSLI   96
rCHT1  RDIGLLVGGFTMTATWVGGGYINGTAEAVYGPGCGLAWAQAPIGYSLSLI   96
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                   II
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rCHT1  LGGLFFAKPMRSKGYVTMLDPFQQIYGKRMGGLLFIPALMGEMFWAAAIF  146
CHO-1  MGGLLFAKKMREEGYITMLDPFQHKYGQRIGGLMYVPALLGETFWTAAIL  147
       III                                    IV
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                        V
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rCHT1  IGLWISVPFALSHPAVTDIGFTAVHAKYQSPWLGTIES-VEVYTWLDNFL  245
CHO-1  VGLWVCVPAAMVHDGAKDISRNAG------DWIGEIGGFKETSLWIDCML  241
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rCHT1  LLMLGGIPWQAYFQRVLSSSSATYAQVLSFLAAFGCLVMALPAICIGAIG  295
CHO-1  LLVFGGIPWQVYFQRVLSSKTAHGAQTLSFVAGVGCILMAIPPALIGAIA  291
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                                         VIII
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CHO-1  VSAAVMSSADSSVLSAASMFAHNIWKLTIRPHASEKEVIIVMRIAIICVG  391
                                                  IX
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rCHT1  ASATAMALLTKTVYGLWYLSSDLVYIIIFPQLLCVLFIKGTNTYGAVAGY  439
CHO-1  IMATIMALTIQSIYGLWYLCADLVYVILFPQLLCVVYMPRSNTYGSLAGY  441
                              X
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rCHT1  IFGLFLRITGGEPYLYLQPLIFYPGYYPDKNGIYNQRFPFKTLSMVTSFF  489
CHO-1  AVGLVLRLIGGEPLVSLPAFFHYPMYT--DG---VQYFPFRTTAMLSSMA  486
          XI                                      
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rCHT1  TNICVSYLAKYLFESGTLPPKLDIFDAVVSR---HSEENMDKTILVRNEN  536
CHO-1  TIYIVSIQSEKLFKSGRLSPEWDVMGCVVNIPIDHVPLPSDVSFAVSSE-  535
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rCHT1  IKLNELAPVKPRQSLTLSSTFTNKEALLDVDSSPEGSGTEDNLQ        580
CHO-1  -TLNMKAPNGTPAPVHPNQQPSDENTLLHPYSDQSYYSTNSN--       576
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B

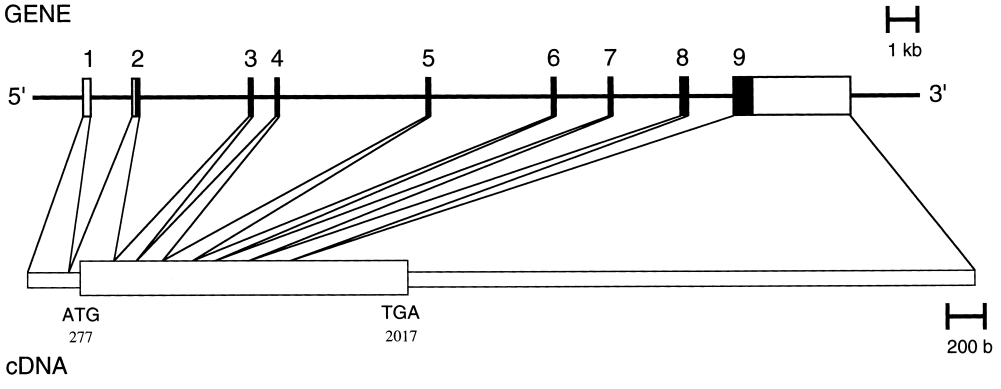

Fig. 1. Primary structure and genomic organization of hCHT1. A: Alignment of the predicted amino acid sequence of hCHT1 with rat CHT1 and *C. elegans* CHO-1. Numbers in the right column correspond to amino acid residues. Identical residues are in bold. The putative transmembrane domains I–XII are underlined. B: Structural organization of the hCHT1 gene and its organizational relationship to the hCHT1 cDNA. Alignment of the cDNA sequence with the genomic sequences (GenBank accession numbers AC009963 and AC023672) predicts nine exons. Black boxes in the gene represent the coding regions, and open boxes represent untranslated regions. The numbers in cDNA correspond to the position of the first nucleotide of each codon.

cDNA library was constructed using Superscript™ Plasmid System (Gibco BRL, Life Technologies). The hCHT1 cDNA was isolated from the cDNA library using the GeneTrapper cDNA Positive Selection System (Gibco BRL, Life Technologies), in which single strand cDNA clones hybridized with a biotinylated oligonucleotide probe are captured on streptavidin-coated paramagnetic beads, repaired and amplified. After characterization of the resulting cDNA clones, one positive clone was subcloned into the *Nco*I (filled-in) and *Not*I sites of modified pSPUTK (Stratagene).

2.2. Northern blotting analysis

Human multiple tissue Northern blots (Clontech) were used for Northern analysis. The *Sph*I–*Stu*I fragment of hCHT1 cDNA corresponding to 936–1958 bp was labeled with ^{32}P using *redi*prime™ II (Amersham Pharmacia Biotech). Filters were hybridized with the cDNA probe as described [14] and exposed to autoradiography film with an enhancing screen for 4 days at −80°C.

2.3. Expression in Xenopus oocytes

Xenopus laevis oocyte expression studies and uptake measurements were performed using [^{3}H]choline chloride (83 Ci/mmol) (Amersham Pharmacia Biotech) as described previously [14]. The hCHT1 cRNA (25 ng) was injected into manually defolliculated *Xenopus* oocytes. Two days after injection, choline uptake assays were performed by incubating 6–8 oocytes with [^{3}H]choline chloride in 0.75–1.0 ml of standard uptake buffer [14]. Because choline uptake mediated by hCHT1 was linearly dependent on the incubation time up to 60 min at the choline concentrations of 10 μM, uptake experiments were performed for 20 min in most cases. Uptake was terminated by washing oocytes with 3 ml of ice-cold uptake buffer five times. Non-specific choline uptake was determined from uptake in the presence of 1 μM HC3 (Sigma) or from uptake in water-injected oocytes. Each data for a single oocyte was represented by the mean ± S.E.M. (n = 6–8).

2.4. Ligand binding

A binding assay of [^{3}H]HC3 was carried out using intact *Xenopus* oocytes (HC3, 128 Ci/mmol, NEN Life Science Products). Two days after injection of cRNA, the [^{3}H]HC3 binding assay was performed using 3–5 oocytes in a final volume of 500 μl of standard uptake buffer containing indicated concentrations of [^{3}H]HC3. Binding was terminated after 1 h at room temperature by washing oocytes with 3 ml of ice-cold standard uptake buffer three times. Specific binding was calculated by subtracting non-specific binding, which was determined from the binding in the presence of 1 μM HC3, from total binding. Each data for a single oocyte was represented by the mean ± S.E.M. (n = 3–5). The turnover number for hCHT1 was calculated by dividing the V_{max} of hCHT1-mediated choline uptake by the B_{max} of HC3 binding sites.

3. Results and discussion

3.1. Cloning of hCHT1 cDNA

Recently, we isolated a cDNA encoding the CHT1 from the nematode *C. elegans* (CHO-1) [14]. A database search using the predicted amino acid sequence of *C. elegans* CHO-1 identified an entry (GenBank accession number AQ316435) in the human GSS. The nucleotide sequence of this GSS entry was used to isolate the corresponding cDNA from the human spinal cord. We constructed and screened a human spinal cord cDNA library and isolated positive clones, one of which was chosen for further characterization. This cDNA clone was designated hCHT1.

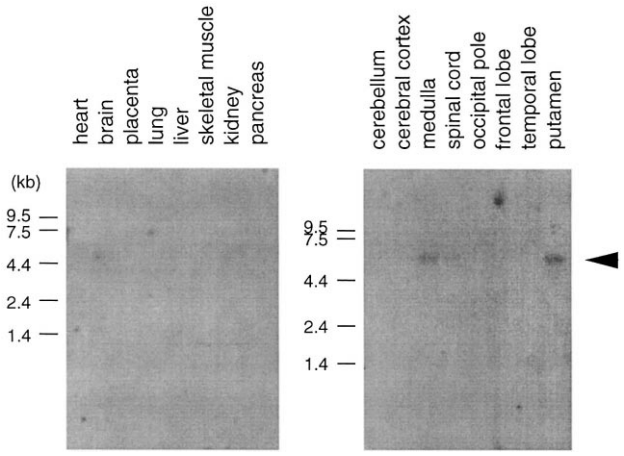

Fig. 2. Tissue distribution of hCHT1. Northern analysis of CHT1 mRNA transcripts in human tissues probed with ^{32}P-labeled CHT1 cDNA. The hybridization washes were done under high stringency conditions. The sizes of the molecular weight standards are indicated on the left. The arrowhead indicates positive signals.

The hCHT1 cDNA is 5144 bp long with an open reading frame of 1743 bp (including the termination codon), encoding a protein of 580 amino acids with a predicted molecular weight of 63 kDa, containing 12 putative transmembrane domains. The hCHT1 is 93% homologous to the rat CHT1 and 53% homologous to the *C. elegans* CHO-1 (Fig. 1A). The amino acid sequence of hCHT1 has significant homology (20–26%) with known members of the Na^+-dependent glucose transporter family [15], but not with neurotransmitter transporters [16].

A query of GenBank databases revealed human genomic sequences (GenBank accession numbers AC009963 and AC023672) that were identical to the hCHT1. Alignment of these sequences with the cloned cDNA predicts the hCHT1

Table 1
Ionic dependence of hCHT1-mediated choline uptake

Inorganic salts	hCHT1-mediated choline uptake (fmol/oocyte/30 min)	Percent control (fmol/oocyte/30 min)
NaCl	5.6 ± 0.2	100 ± 4.4
LiCl	< 0.1	< 1
KCl	< 0.1	< 1
RbCl	< 0.1	< 1
N-Methyl-D-glucamine Cl	< 0.1	< 1
NaBr	3.9 ± 0.1	69.3 ± 2.6
NaF	0.8 ± 0.1	14.3 ± 1.4
NaI	0.8 ± 0.1	14.4 ± 2.2
Na gluconate	0.4 ± 0.1	7.4 ± 1.6

Xenopus oocytes injected with either hCHT1 cRNA or water were incubated with 10 mM [^{3}H]choline for 30 min at room temperature either in standard buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM Tris, pH 7.4) or in buffers where NaCl was replaced with various inorganic salts. After incubation, the oocytes were washed with the respective buffer (ice cold), and the radioactivity associated with the oocytes was determined. Uptake measured in water-injected oocytes was subtracted to obtain hCHT1-mediated uptake. Values are means ± S.E.M.

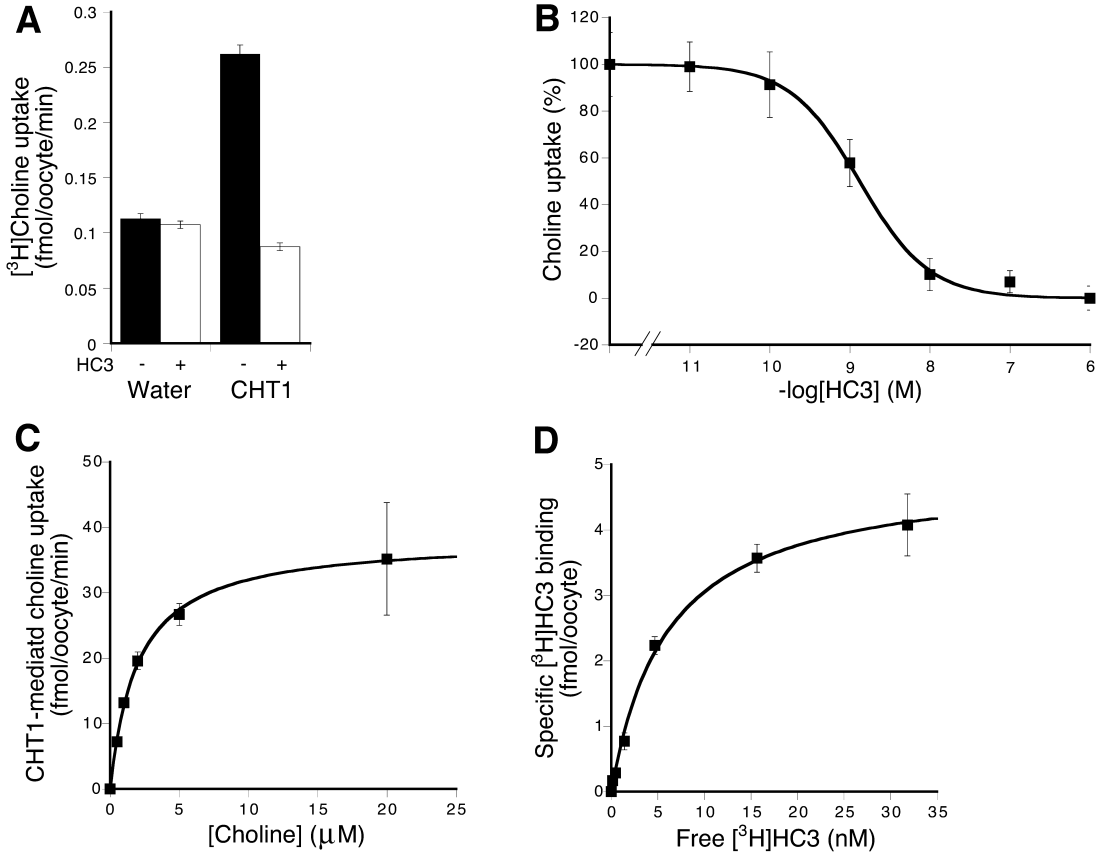

Fig. 3. Functional expression of hCHT1 in *Xenopus* oocytes. A: [^{3}H]Choline uptake into *Xenopus* oocytes injected with hCHT1 cRNA or water. Filled and open bars indicate choline uptake measured in the absence or presence of 1 μM HC3, respectively. Each bar represents the mean ± S.E.M. ($n = 6$–8 oocytes). Two days after injection of capped cRNA, choline uptake was measured by incubating 6–8 oocytes with [^{3}H]choline chloride for 20 min in 0.75 ml of standard uptake buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM Tris, pH 7.4). B: Inhibition by HC3 of choline uptake. Choline uptake was measured in the presence of the indicated concentrations of HC3. C: Effect of choline concentration on CHT1-mediated choline uptake. Choline uptake in the presence of 1 μM HC3 was subtracted from that in the absence of 1 μM HC3, yielding hCHT1-mediated choline uptake. The uptake was fitted to a Michaelis–Menten curve. D: Saturation analysis of specific [^{3}H]HC3 binding in intact oocytes. Specific binding was calculated by subtracting non-specific binding, which was determined from the binding in the presence of 1 μM HC3, from total binding.

gene structure. The hCHT1 gene is 25 kb in length and contains nine exons (Fig. 1B). The genomic sequences were assigned to chromosome II at position IIq11–12.

3.2. Tissue distribution of hCHT1 expression

Tissue distribution of hCHT1 mRNA was studied using commercially available membrane blots containing size fractionated poly(A)$^+$ RNA from human tissues. Northern blot analysis for hCHT1 mRNA showed the expression of a 5.4 kb transcript (Fig. 2). Expression of hCHT1 mRNA was detected in putamen, medulla and spinal cord. These regions are known to contain cholinergic neurons. On the other hand, no transcripts were detected in other areas of the brain or non-neuronal tissues examined. This tissue distribution of hCHT1 mRNA is virtually the same as that of rat CHT1 mRNA [14], indicating that hCHT1 mRNA is expressed exclusively in cholinergic neurons.

3.3. Functional characterization of hCHT1

Choline uptake mediated by hCHT1 was characterized by expressing it in *Xenopus* oocytes. The choline uptake in oocytes injected with hCHT1 cRNA was 2–4-fold greater than the background uptake in oocytes injected with water (Fig. 3A). The hCHT1-mediated choline uptake was inhibited by HC3 [22] with a K_i of 1.3 nM (Fig. 3B), which is similar to the value for rat CHT1 (K_i; 2–5 nM). These values are 10-fold lower than those in rat brain synaptosomes (5–100 nM) [6–8,20]. The ionic dependence of the hCHT1-mediated uptake was examined by measuring choline uptake in hCHT1 cRNA- and water-injected oocytes in the presence of various inorganic salts (Table 1). Control uptake was measured in the presence of 100 mM NaCl. The replacement of Na^+ with other cations such as Li^+, K^+ or Rb^+ almost completely abolished the hCHT1-mediated choline uptake, indicating that Na^+ is obligatory for the uptake function. Replacing Cl^- with other anions also reduced the CHT1-mediated uptake, although Br^- could partially substitute for Cl^- as the counter anion for Na^+ as previously observed for rat brain synaptosomes [21,22]. Thus, CHT1-mediated choline uptake is both Na^+- and Cl^--dependent. The CHT1-mediated choline uptake saturated with increasing concentrations of choline with a K_m of 2.0 μM, and V_{max} of 15–40 fmol/oocyte/min (Fig. 3C). The determined K_m value was similar to that for rat CHT1 (2.2 μM) and for rat brain synaptosomes (0.5–5 μM) [5–8]. We examined [^{3}H]HC3 binding in intact oocytes, using the same batch as one used for choline uptake. Specific

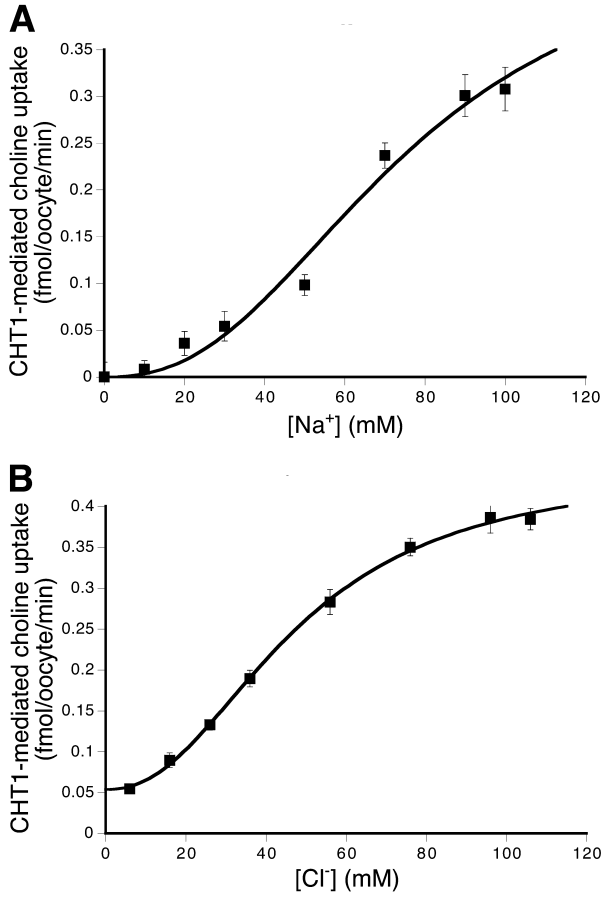

Fig. 4. Kinetic analysis of choline uptake mediated by hCHT1 in *Xenopus* oocytes. A: Effect of [Na^+] on CHT1-mediated choline uptake. The [Na^+] was varied between 0 and 100 mM, by replacing NaCl with LiCl. The data were fitted to the Hill equation. The choline uptake in oocytes injected with water was subtracted from that in oocytes injected with cRNA, yielding hCHT1-mediated choline uptake. B: Effect of [Cl^-] on CHT1-mediated choline uptake. The [Cl^-] was varied between 6 and 106 mM, by replacing NaCl with NaI. The data was fitted to the Hill equation. The choline uptake in oocytes injected with water was subtracted from that in oocytes injected with cRNA, yielding hCHT1-mediated choline uptake.

[^{3}H]HC3 binding was detected in oocytes injected with hCHT1 cRNA, but not in oocytes injected with water. The equilibrium dissociation constant (K_d) was estimated to be 6 nM and B_{max} was 1–5 fmol/oocyte (Fig. 3D). The calculated K_d value was similar to those obtained by the binding in human brain synaptosomes (5–10 nM) [23,24]. Assuming that one specific HC3 binding site corresponds to one functional CHT, we estimated the apparent turnover number for hCHT1 to be 0.1–0.2 choline molecule/s at room temperature. This value is lower than the one (3/s at 20°C) reported for synaptosomal membranes from *Torpedo* electric organ [25]. This difference may indicate that the hCHT1-mediated choline uptake in *Xenopus* oocytes does not completely duplicate the high-affinity choline uptake of brain synaptosomes.

As hCHT1-mediated choline uptake is both Na^+- and Cl^--dependent, the effects of Na^+ and Cl^- on the kinetics of hCHT1-mediated choline uptake were examined by measuring choline uptake in oocytes injected with hCHT1 cRNA in the presence of varying concentrations of extracellular Na^+ or Cl^-. The resulting choline uptake showed a sigmoidal dependence on Na^+ and Cl^- concentration. The dose–response curve fitted to the Hill equation with a Hill coefficient of 2.5, an EC_{50} of 76 mM, and a V_{max} of 0.48 fmol/oocyte/min (Fig. 4A). The Cl^- data yielded a Hill coefficient of 2.3, an EC_{50} of 48 mM, and a V_{max} of 0.39 fmol/oocyte/min (Fig. 4B). The apparent EC_{50} value for Na^+ of hCHT1 was similar to the values reported for rat brain synaptosomes (40–80 mM) [6,21]. A Hill coefficient of 2.5 for Na^+ and 2.3 for Cl^- suggest that the transport cycle involves the binding of 2–3 Na^+ ions and 2–3 Cl^- ions. As choline is a monovalent cation, our results suggest that the hCHT1-mediated choline uptake is electrogenic, and both the Na^+ gradient and the inside-negative membrane potential contribute to the driving force for the hCHT1-mediated choline uptake.

All the present results indicate that the characteristics of hCHT1-mediated choline uptake in *Xenopus* oocytes are essentially the same as those of the high-affinity choline uptake in human brain synaptosomes. This indicates that hCHT1 is sufficient to exhibit the high-affinity choline uptake activity in cholinergic nerve terminals. The expression level of hCHT1 in *Xenopus* oocytes, as assessed by the [^{3}H]HC3 binding activity, was 1–5 fmol/oocyte, which is comparable to those of membrane proteins in *Xenopus* oocytes in usual case (1 fmol/oocyte) [26], indicating that the low uptake activity of oocytes injected with hCHT1 cRNA is not due to the low expression level of hCHT1 but due to the low activity of expressed hCHT1. Actually the turnover rate of hCHT1, 0.1–0.2/s, was much lower than that of sodium/glucose cotransporter 1, 57/s [27]. The rate of 0.1–0.2/s was also low compared with the value of 3/s for synaptosomes derived from *Torpedo* electric organ [25]. It is interesting to know if there are other factor(s) which facilitate the uptake activity without changing the uptake characteristics or if CHT1 has an intrinsic low uptake activity.

In summary, we have cloned the hCHT1 that is responsible for high-affinity choline uptake in cholinergic neurons. We also described the kinetic analysis of hCHT1-mediated choline uptake in *Xenopus* oocytes.

Acknowledgements: We thank Ms. M. Suzuki and Ms. C. Kaitsuka for technical assistance. This work was supported by grants from Japan Science and Technology Corporation (CREST), and the Ministry of Japan Society for the Promotion of Science (Research for Future Program).

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