

*Full Paper***Expression and Functional Characterization of Choline Transporter in Human Keratinocytes**

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Abstract. Choline is essential for synthesis of the major membrane phospholipid phosphatidylcholine. Moreover, it serves as a precursor for synthesis of the neurotransmitter acetylcholine (ACh). Keratinocytes of the epidermis synthesize and release ACh. The uptake of choline is the rate-limiting step in both ACh synthesis and choline phospholipid metabolism, and it is a prerequisite for keratinocyte proliferation. However, the nature of the choline transport system in keratinocytes is poorly understood. In this study, we examined the molecular and functional characterization of choline uptake into cultured human keratinocytes. Choline uptake into keratinocytes was independent of extracellular Na⁺, saturable, and mediated by a single transport system with an apparent Michaelis-Menten constant of 12.3 μ M. Choline uptake was reduced when the keratinocyte membrane potential was depolarized by high K⁺. These results provide evidence that the choline transport activity is potential-sensitive. Various organic cations inhibit the choline transport system. RT-PCR demonstrated that keratinocytes expressed mRNA for choline transporter-like protein 1 (CTL1), mainly the CTL1a subtype. The present biochemical and pharmacological data suggest that CTL1a is functionally expressed in human keratinocytes and is responsible for the uptake of choline and organic cations in these cells.

Keywords: choline, acetylcholine, transporter, keratinocyte, cell proliferation

Introduction

Human epidermal keratinocytes synthesize, secrete, and degrade acetylcholine (ACh) and use their cell-surface nicotinic and muscarinic cholinergic receptors to mediate the autocrine and paracrine system of ACh. Endogenous ACh regulates keratinocyte functions such as cell proliferation, differentiation, migration, and adhesion (1–3). Thus, non-neuronal cholinergic systems are expressed in human keratinocytes and are involved in the regulation of their function, and cholinergic dysfunction is related to the pathophysiology of certain diseases such as atopic dermatitis and psoriasis (3). Choline acts as a precursor for synthesis of the neurotransmitter ACh. It is a precursor of phosphatidylcholine and sphingomyelin, which act as com-

ponents of biological membranes and precursors for intracellular messengers such as diacylglycerol or ceramide. Interestingly, choline is required by human epidermal keratinocytes for optimal proliferation and differentiation. This choline requirement is specific for keratinocytes since dermal fibroblasts and epidermal melanocytes were not affected by choline supplementation (4). Thus, intracellular choline accumulation through choline transporters is the rate-limiting step in ACh synthesis and choline phospholipid metabolism, and it is a prerequisite for keratinocyte proliferation. However, the uptake system of choline and the functional expression of choline transporters in human keratinocytes are poorly understood.

The choline transport system has been categorized into three transporter families according to their affinity for choline. A high-affinity choline transporter (CHT1) has recently been cloned and characterized and is thought to play an important role in cholinergic neurons (5, 6). CHT1 is a Cl⁻- and Na⁺-dependent co-transporter

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that is highly sensitive to the choline analogue HC-3, and it is thought to be part of the rate-limiting step in ACh synthesis. CHT1 is also expressed by subpopulations of non-neuronal cholinergic cells such as ciliated cells of the airway epithelium (7, 8) and keratinocytes (9). However, functional studies have shown that the airway epithelial cell line A549 expresses both a Na^+ -dependent CHT1 and a lower-affinity Na^+ -independent choline transporter (10). As an organic cation, choline is known to be a substrate for carriers of organic cation transporters (OCTs). To date, three different OCTs (OCT1-3) have been cloned and their function, which involves a Na^+ -independent uptake mechanism, has been characterized (11). These transporters recognize a multitude of endogenous and exogenous organic cations as substrates and exhibit considerable overlap in substrate specificity. Choline also interacts with these transporters with varying affinity. OCT1 and OCT2 accept choline as a substrate with comparatively low affinity. However, OCT3 does not recognize choline as a substrate (12–14). Recently, a distinct choline transporter called choline transporter-like (CTL) protein has been demonstrated in various human tissues (15). CTL1 has been cloned from *Torpedo marmorata* and was first cloned as a suppressor for a yeast choline transport mutation from a *Torpedo* electric lobe yeast expression library by functional complementation (15). Functional characterization studies with CTL1 in rats have shown that CTL1 is a Na^+ -independent, intermediate-affinity transporter of choline that can be completely inhibited by a high concentration of HC-3 (16, 17).

This study was performed to functionally characterize choline uptake and sought to identify the transporters that mediate choline uptake in human keratinocytes.

Materials and Methods

Materials

[Methyl- ^3H]choline chloride (specific activity: 3182 GBq/mmol) was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). Hemicholinium-3 (HC-3), choline chloride, acetylcholine, tetraethylammonium chloride (TEA), tetrabutylammonium chloride (TBA), tetrahexylammonium chloride (THA), clonidine, quinine, quinidine, desipramine, diphenhydramine, *p*-aminohippuric acid (PAH), vesamicol, BCH, MeAIB, DIDS, and *N*-methyl-D-glucamine (NMDG) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). ISOGEN and ethidium bromide were purchased from Nippon Gene (Tokyo). The SuperScriptTM III One-Step RT-PCR System with Platinum[®] Taq DNA Polymerase was purchased from Invitrogen (Carlsbad, CA, USA). High Capacity RNA-to-cDNA Kit, TaqMan[®] Fast

Universal PCR Master Mix, and TaqMan[®] Gene Expression Assays were purchased from Applied Biosystems (Foster City, CA, USA). Molecular ruler 100-bp DNA was purchased from Nippon Bio-Rad Laboratories (Tokyo). NuSieve agarose gel was purchased from Cambrex Bio Science Rockland, Inc. (Rockland, ME, USA). Human keratinocytes were purchased from DS Pharma Biomedical Co., Ltd. (Osaka). All other reagents were of analytical grade.

Cell culture

Human keratinocytes were pooled primary isolates from 250 individual neonatal donor foreskins. Cells were grown in the CS-2.0 complete serum-free medium containing CS-2.0 RocketFuelTM (which contains bovine pituitary extract and recombinant human epidermal growth factor) (Cell Systems, Kirkland, WA, USA) in non-coated flasks and 24-well plates (BD Biosciences, Bedford, MA, USA). Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C, and the medium was changed every 2–3 days.

[^3H]Choline uptake into human keratinocytes

Culture medium was removed from the 24-well culture plates by aspiration and the cells were washed twice with uptake buffer consisting of 125 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl_2 , 1.2 mM KH_2PO_4 , 5.6 mM glucose, 1.2 mM MgSO_4 , and 25 mM HEPES adjusted to pH 7.4 with Tris. Uptake was then started by the addition of 250 μl of uptake buffer containing [^3H]choline. After incubation, cells were washed twice with ice-cold uptake buffer and dissolved in 0.1 M NaOH and 0.1% Triton X-100, and aliquots were then taken for liquid scintillation counting and protein assay. Radioactivity was measured by a liquid scintillation counter (Tri-Carb[®] 2100TR; Packard Instrument Company, Meriden, CT, USA). The concentration of Na^+ in the uptake buffer was modified by replacing NaCl with an equimolar concentration of *N*-methyl-D-glucamine chloride. In experiments dealing with saturation kinetics, the concentration of [^3H]choline was kept constant at 10 nM and unlabeled choline was added to give the desired choline concentrations. The nonspecific component of uptake was determined from the radioactivity associated with the cells when incubation was performed with [^3H]choline in the presence of 30 mM unlabeled choline. Total uptake at each concentration of choline was adjusted for this nonspecific uptake to calculate the saturable component. Data were analyzed by nonlinear regression and confirmed by linear regression. K_i values were derived from IC_{50} values as described by Cheng and Prusoff (18). Inhibition data were plotted to a sigmoid curve with a variable slope

Table 1. Primer sequences used for transporters detection

cDNA	Primer sequence (5' to 3')	PCR product size (bp)
CTL1	Forward: 5'-TCA ACA GCA CCA ACT TCT GC-3'	246
	Reverse: 5'-ACA GGA AGC AAT GAG CGA CT-3'	
CTL1a	Forward: 5'-AAG CAG CCG TTT GCT AAT GT-3'	454
	Reverse: 5'-CTG TGC GGC TAT GAA ACA GA-3'	
CTL1b	Forward: 5'-AAA GCC GAT GCT GAA GAA AA-3'	206
	Reverse: 5'-TAG ACA GCC GCA CTG TAC CA-3'	
CTL1c	Forward: 5'-CAT TGA AAA ATT CCG GTG CT -3'	223
	Reverse: 5'-CAC CCC CAA AAAAA ACA AC-3'	
GAPDH	Forward: 5'-TAA AGG GCA TCC TGG GCT ACA CT-3'	200
	Reverse: 5'-TTA CTC CTT GGA GGC CAT GTA GG-3'	

and used to determine 50% inhibitory concentrations (IC_{50} s). K_i values were then obtained using the Cheng-Prusoff equation, $K_i = IC_{50} / [1 + (L / K_m)]$, where L is the concentration of radiolabeled ligand. Protein concentrations were determined using a DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Cells were washed with sterile Dulbecco's phosphate-buffered saline (D-PBS) and RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method. Briefly, cells were lysed in ISOGEN and stored for 5 min at room temperature. Following the addition of chloroform and vortex mixing, the solution was centrifuged at $12,000 \times g$ for 15 min at 4°C . The supernatant was collected, an equal volume of isopropanol was added, and the mixture was stored for 10 min at room temperature. The solution was centrifuged at $12,000 \times g$ for 10 min at 4°C . The supernatant was removed, and RNA was precipitated by the addition of 70% ethanol and pelleted by centrifugation at $12,000 \times g$ for 5 min at 4°C . Following a wash in 70% ethanol, the RNA pellet was air-dried and dissolved in RNase- and DNase-free water, and the optical density at 260 nm was measured to determine the amount of RNA.

Total RNA was reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions. The pairs of primers and the TaqMan probes for the target mRNAs (CHT1, OCT1-3, CTL1, and housekeeping gene GAPDH) were designed based on the human mRNA sequence using TaqMan® Gene Expression Assays (Applied Biosystems, Assay ID: CHT1, Hs00222367_m1; OCT1, Hs00427554_m1; OCT2, Hs01010723_m1; OCT3, Hs01009568_m1; CTL1, Hs00939357_m1; GAPDH, Hs99999905_m1). A master mix of TaqMan reagents was prepared and 10 ng of

each RT sample was used in the TaqMan PCR reaction. For real-time PCR, TaqMan® Fast Universal PCR Master Mix containing forward-, reverse-primer-, and TaqMan probe was used at $20 \mu\text{l}/\text{tube}$, and the reaction was performed using the Applied Biosystems StepOne Plus™ Real-Time PCR System.

The relative mRNA expression levels of the target genes in human keratinocytes were calculated using the comparative cycle time (Ct) method. The threshold Ct is the fractional PCR cycle number at which the fluorescent signal reaches the detection threshold. The target PCR Ct value is normalized to the GAPDH PCR Ct value by subtracting the GAPDH Ct value from the target PCR Ct value, which gives the ΔCt value. From this ΔCt value, the relative mRNA expression level to GAPDH for each target PCR can be calculated using the following equation: relative mRNA expression = $2^{-(\text{Ct}_{\text{target}} - \text{Ct}_{\text{GAPDH}})} \times 100 (\%)$.

The sequences of the specific primers for human CHT1, CTL1a, CTL1b, CTL1c, and GAPDH are summarized in Table 1. The PCR reaction was performed using a TaKaRa PCR Thermal Cycler MP (Model TP3000; TaKaRa Biomedicals, Tokyo). For RT-PCR amplification, $0.5 \mu\text{g}$ total RNA was added to a $50 \mu\text{l}$ master mixture containing $1 \times$ reaction buffer, 1.6 mM MgSO_4 , $1 \times$ enzyme mixture, and 200 nM primers by using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq. PCR thermocycling consisted of an RT step at 55°C for 30 min and hot-start activation at 94°C for 2 min, followed by 30 amplification cycles of 94°C for 15 s, 60°C for 30 s, and 68°C for 1 min, with a final extension cycle at 68°C for 5 min. The PCR products were loaded on a 2% NuSieve agarose gel that had been pre-stained with $0.5 \mu\text{g}/\text{ml}$ ethidium bromide for electrophoresis in parallel with an appropriate DNA size marker. PCR products were visualized by UV illumination using Chemi Doc (Bio-Rad Laboratories).

Results

Time-course and kinetics of [³H]choline uptake in human keratinocytes

We first examined the time-course of [³H]choline uptake at a concentration of 10 nM in the presence and absence of Na⁺ in keratinocytes for 60 min (Fig. 1a). [³H]Choline uptake in keratinocytes increased in a time-dependent manner; it was linear with time at up to 30 min. Based on these findings, subsequent experiments were performed using an uptake period of 10 min. When NaCl in the uptake buffer was replaced by NMDG, the uptake of [³H]choline under Na⁺-free conditions was similar to the control uptake under normal conditions. Thus, the uptake of [³H]choline was independent of extracellular Na⁺.

The characteristics of the kinetics of [³H]choline uptake by keratinocytes were determined (Fig. 1b). Cells were incubated for 10 min with [³H]choline at a concentration of from 1.56–200 μM. Kinetic analysis of [³H]choline uptake data (specific uptake), as computed by non-linear regression analysis, yielded a Michaelis–Menten constant (K_m) of $12.3 \pm 1.4 \mu\text{M}$ and a maximal velocity (V_{max}) of $2424 \pm 76.2 \text{ pmol} \cdot \text{mg protein}^{-1} \cdot 10 \text{ min}^{-1}$. The Eadie–Hofstee plot (Fig. 1b, inset) shows a single straight line ($r^2 = 0.9216$, $P = 0.0002$), suggesting that [³H]choline uptake by keratinocytes is mediated by a single transport system.

Effects of various compounds on [³H]choline uptake in human keratinocytes

An inhibition study was performed to determine the substrate-selectivity of the choline transport system in

keratinocytes (Fig. 2). [³H]Choline uptake was concentration-dependently inhibited by ACh, unlabeled choline, and HC-3 (Fig. 2a). The K_i values for the inhibition of [³H]choline uptake by ACh, unlabeled choline, and HC-3 were 14.5, 5.9, and 16.5 μM, respectively.

The inhibitory effects of organic cations, *p*-aminohippuric acid (PAH) and other uptake inhibitors on the uptake of [³H]choline in keratinocytes are shown in Fig. 2b. Various organic cations, such as quinine, quinidine, desipramine, diphenhydramine, and clonidine, markedly inhibited [³H]choline uptake. The prototypical organic cation TEA and PAH, which is an organic anion that is transported by organic anion transporters, did not cause any significant inhibition at up to 1 mM. However, other *n*-tetraalkylammonium compounds, such as TBA and THA, inhibited [³H]choline uptake. In addition, the selective inhibitors of L- and A-type amino acid transporters BHC (1 mM) and MeAIB (1 mM), the selective organic anion transport inhibitor DIDS (1 mM), and the vesicular monoamine transporter inhibitor vesamicol (10 μM) did not affect [³H]choline uptake.

Influence of membrane depolarization on [³H]choline uptake in human keratinocytes

We investigated the electrogenicity of choline transport by examining the influence of membrane potential on [³H]choline uptake in keratinocytes (Fig. 3). The membrane potential was depolarized by increasing the concentration of K⁺ in the uptake buffer from 6 to 106 mM. [³H]Choline uptake was significantly reduced when the keratinocyte cell membrane potential was depolarized by high K⁺.

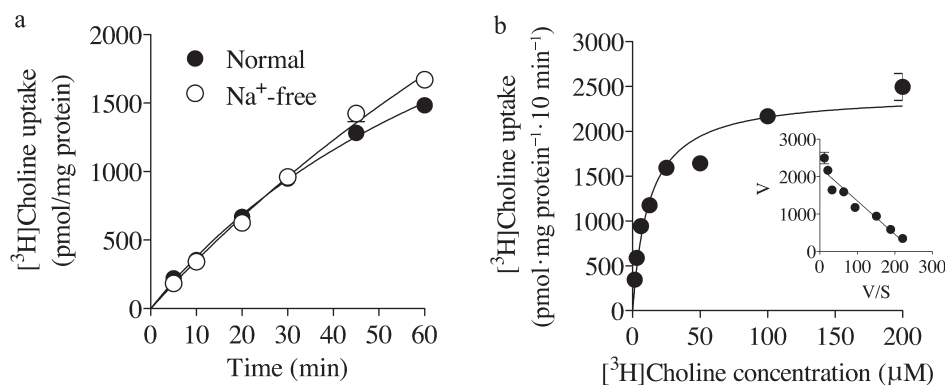


Fig. 1. Time-course and kinetics of [³H]choline uptake in human keratinocytes. a: Time-course of 10 nM [³H]choline uptake in the presence (closed circle) and absence (open circle) of Na⁺ in human keratinocytes for 60 min. Na⁺-free buffer was modified by replacing NaCl with an equimolar concentration of *N*-methyl-D-glucamine chloride. Each point represents the mean \pm S.D. ($n = 4$). The time-course of [³H]choline uptake was fitted to the experimental data by non-linear regression analysis. b: The kinetic characteristics of [³H]choline uptake in human keratinocytes. Keratinocyte cells were incubated for 10 min with [³H]choline at concentrations from 1.56–200 μM. Each point represents the mean \pm S.D. ($n = 4$). Specific [³H]choline uptake is saturable with a K_m of $12.3 \pm 1.4 \mu\text{M}$ and a V_{max} of $2424 \pm 76.2 \text{ pmol} \cdot \text{mg protein}^{-1} \cdot 10 \text{ min}^{-1}$. Inset: Eadie-Hofstee plots of [³H]choline uptake. The correlation is highly significant ($r^2 = 0.9216$, $P = 0.0002$).

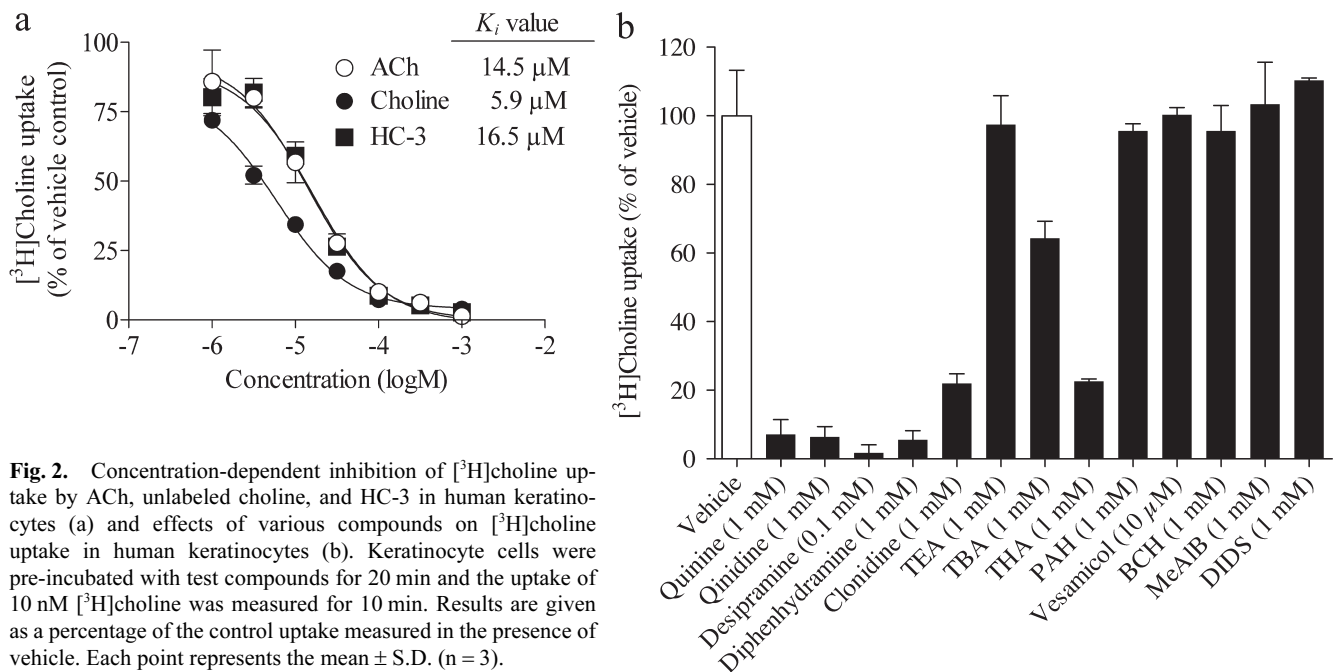


Fig. 2. Concentration-dependent inhibition of $[^3\text{H}]$ choline uptake by ACh, unlabeled choline, and HC-3 in human keratinocytes (a) and effects of various compounds on $[^3\text{H}]$ choline uptake in human keratinocytes (b). Keratinocyte cells were pre-incubated with test compounds for 20 min and the uptake of 10 nM $[^3\text{H}]$ choline was measured for 10 min. Results are given as a percentage of the control uptake measured in the presence of vehicle. Each point represents the mean \pm S.D. ($n = 3$).

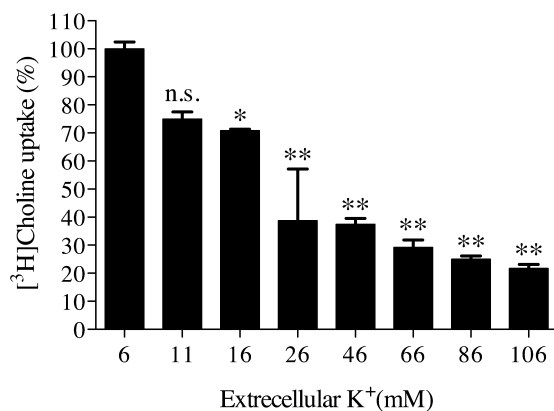


Fig. 3. Influence of membrane depolarization on $[^3\text{H}]$ choline uptake in human keratinocytes. Keratinocyte cells were pre-incubated with various concentrations of K^+ (6 – 106 mM) for 10 min, and the uptake of 10 nM $[^3\text{H}]$ choline was measured for 10 min. Results are given as a percentage of the control uptake measured in the presence of vehicle. Each point represents the mean \pm S.D. ($n = 3$). * $P < 0.05$ and ** $P < 0.01$, compared with $[^3\text{H}]$ choline uptake in 6 mM extracellular K^+ (physiological condition).

Expression of mRNA for *CHT1*, *OCT1-3*, and *CTL1* and its splice variants, *CTL1a*, *CTL1b*, and *CTL1c*, in human keratinocytes

The expression of mRNA for *CHT1*, *OCT1-3*, and *CTL1* in keratinocytes was investigated by real-time PCR analysis (Fig. 4a). *CTL1* mRNA was mainly expressed and *OCT1-3* mRNAs were expressed at low levels. However, under similar conditions, the expression of *CHT1* mRNA was not detectable in keratinocytes.

We investigated the expression of *CTL1* splice variants in keratinocytes (Fig. 4b). Expression of *CTL1a*, *CTL1b*, and *CTL1c* mRNA was investigated by RT-PCR in RNA samples extracted from human keratinocytes. *CTL1* and *CTL1a* mRNAs were expressed abundantly, and while *CTL1b* and *CTL1c* mRNAs were expressed at low levels, they were still detectable.

Discussion

It has been well established that human keratinocytes express receptors for ACh and that stimulation of these receptors promotes cell proliferation, differentiation, migration, and adhesion (3). Furthermore, keratinocytes can synthesize, secrete, and degrade ACh and this released ACh stimulates cell growth. Keratinocyte ACh acts in the epidermis as a local hormone (1). Choline is an essential nutrient for all cells because it plays a role in the synthesis of the membrane phospholipid components of cell membranes, as precursors for ceramide and ACh. The amount of ceramide is markedly reduced in the stratum corneum in lesional and non-lesional forearm skin of patients with atopic dermatitis (AD) (19). However, tissue levels of ACh are greatly (14-fold) enhanced in superficial skin in AD (20). Abnormal choline metabolism has been implicated in several cases of AD. Therefore, the study of choline transport and the characteristics of choline transporters are important for understanding the mechanisms that underlie membrane integrity and cell function in such disorders.

In this study, we found that human keratinocytes take

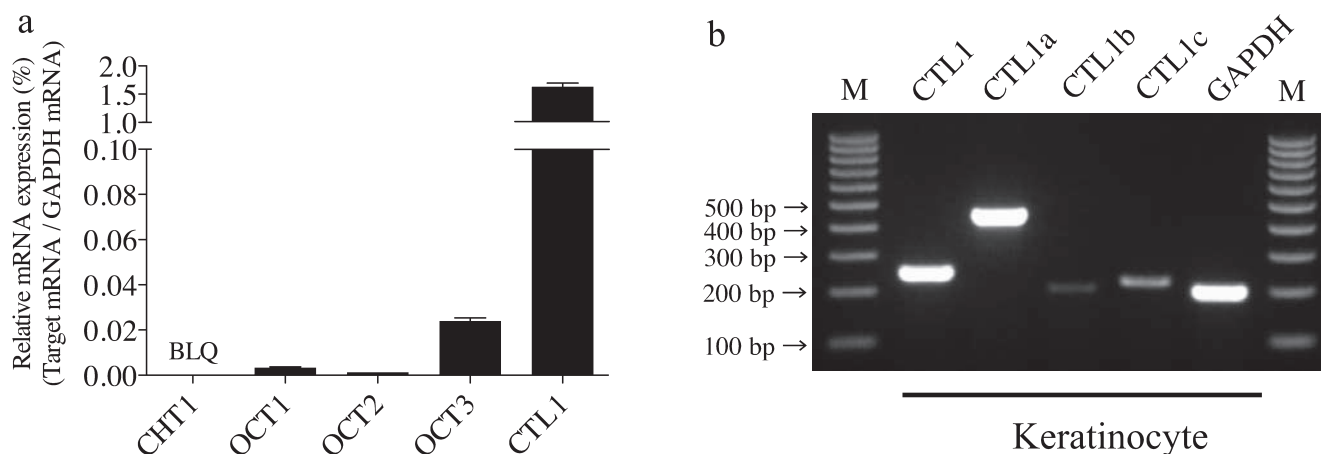


Fig. 4. Expression of mRNA for CHT1, OCTs, and CTLs in human keratinocytes. **a:** Real-time PCR analysis of the expression of CHT1, OCT1-3, and CTL1 mRNA in human keratinocytes. The percentage of relative mRNA expression is expressed as the ratio of target mRNA to GAPDH mRNA. Experiments were performed in duplicate. BLQ, below the limit of quantification. **b:** RT-PCR analysis of the expression of CTL1 and its splice variants (CTL1a, CTL1b, and CTL1c) in human keratinocytes. The reactions using specific primers were performed as described in Table 1. PCR products were separated by electrophoresis on 2% agarose gels and detected with ethidium bromide to visualize bands. M corresponds to a 100-bp DNA ladder.

up [^3H]choline by a saturable process that is mediated by a single transport system. The K_m value was $12.3 \mu\text{M}$, which is similar to the blood concentration of choline ($10 - 25 \mu\text{M}$). The kinetic parameters are very close to those of the choline transport system in endothelial cells of the blood-brain barrier (21), HaCaT cells (22), and astrocytes (16); and these reports have shown conclusively that choline is taken up into these cells by a specific transporter that is not OCT1-3 or CHT1. In astrocytes, the inhibition of CTL1 mRNA expression using siRNA inhibited Na^+ -independent choline uptake, suggesting that CTL1 is responsible for Na^+ -independent choline uptake in these cells. The K_m value for CTL1 was approximately $30 \mu\text{M}$ (17), which is very close to the K_m value of choline uptake in this study, in contrast to those for CHT1 ($K_m = 0.5 - 2 \mu\text{M}$) and the OCT family of transporters ($K_m = 100 - 450 \mu\text{M}$). The kinetics data indicate that choline transport in keratinocytes does not occur via CHT1 or the OCT family.

CHT1 is a high-affinity choline transporter that has been reported to be found in cholinergic neuronal membranes; it is Na^+ -dependent and can be blocked by HC-3 at a very low concentration ($K_i = 50 - 100 \text{ nM}$). Recently, CHT1 immunoreactivity has been identified in human keratinocytes and HaCaT cells (9) and in ciliated cells of the tracheal epithelium (7) by immunohistochemistry. These authors suggested that CHT1 is a novel component of the intrinsic non-neuronal cholinergic system of the skin. However, functional studies have shown that immortalized human keratinocyte HaCaT cells express a high-affinity Na^+ -independent choline transporter (22). We found that CHT1 mRNA

was not expressed in human keratinocytes. In addition, the K_i value of HC-3 ($K_i = 16.5 \mu\text{M}$) is much higher than the values in the literature, and [^3H]choline uptake was also observed under Na^+ -free conditions. These findings strongly suggest that CHT1 plays no role in the uptake of choline by human keratinocytes.

As an organic cation, choline is a substrate for the OCT family. To date, three different electrogenic organic cation transporters have been cloned and characterized with regard to functionally (23). These transporters, known as OCT1, OCT2, and OCT3, exhibit a broad and overlapping substrate-specificity toward organic cations (12 - 14). OCT1 and OCT2 accept choline as a substrate with comparatively low affinity. The transport process is Na^+ -independent, and the K_i values for OCT1 and OCT2 are 620 and $210 \mu\text{M}$, respectively. However, OCT3 does not recognize choline as a substrate (12, 14). Therefore, we examined whether choline uptake into human keratinocytes is mediated by OCTs. In the present study, there is very little expression of OCT1-3 mRNAs compared with the expression of CTL1 mRNA. The choline transport system in keratinocytes resembles OCTs with regard to some pharmacological and functional properties such as sensitivity of the organic cation compound, Na^+ -independence, and potential-sensitive. However, TEA, a prototypical OCT substrate, did not cause any inhibition at up to 1 mM . TEA is very often used as a high-affinity reference compound for OCTs and has K_i values of 46.6 and $52.2 \mu\text{M}$ for OCT1 and OCT2, respectively (24). These biochemical and pharmacological data show that choline uptake in keratinocytes does not occur via OCTs.

We investigated the electrogenicity of choline transport by examining the influence of membrane potential on [^3H]choline uptake in keratinocytes. [^3H]Choline uptake in keratinocytes was markedly reduced when the keratinocyte membrane potential was depolarized by increasing the concentration of K^+ in the uptake buffer. These findings support the notion of electrogenically-facilitated transport of choline in keratinocytes. Due to its positive charge at physiological pH, the rate of choline movement across cell membranes would be expected to be affected by changes in the membrane potential.

Next, we searched for a new transporter that could be part of a Na^+ -independent choline transport system. Recently, a novel family of choline transporters, called choline transporter-like protein 1 (CTL1), has been cloned from *Torpedo marmorata* (15, 17). Rat CTL1 was also cloned as a homologous rat gene of the CTL protein family and exhibited saturable, high concentration of HC-3-inhibitable (K_i value $>10\ \mu\text{M}$), and weak Na^+ -dependent uptake of choline (16, 17, 25). Recently, three major human splice variants of CTL1 (CTL1a, CTL1b, and CTL1c) were identified. However, the expression pattern and transporter function of these splice variants are not well known. We examined whether the CTL1 mediates choline uptake in human keratinocytes. In the present study, human keratinocytes expressed mRNA for CTL1, mainly the CTL1a subtype. Furthermore, kinetics data indicated that the K_m value of [^3H]choline uptake in keratinocytes was very close to the K_m value for CTL1. The Eadie-Hofstee plot shows a single straight line, suggesting that choline uptake is mediated by a single transport system. Together, these results suggest that CTL1a is functionally expressed in keratinocytes and is responsible for choline uptake in these cells.

In conclusion, the functional expression of the Na^+ -independent choline transporter CTL1, mainly the CTL1a subtype, by human keratinocytes is responsible for the uptake of choline and organic cations. Previous reports demonstrated that keratinocytes express specific receptors for ACh and that keratinocyte-derived ACh can affect physiological cell functions by an autocrine/paracrine system. Furthermore, changes in ACh metabolism have been found in skin lesions of various dermatologic patients (20, 26, 27). It is conceivable that perturbations of the keratinocyte choline transport system may be involved in skin diseases, and drugs that influence this system may be useful in treating them.

References

- 1 Grando SA, Kist DA, Qi M, Dahl MV. Human keratinocytes synthesize, secrete, and degrade acetylcholine. *J Invest Dermatol.* 1993;101:32–36.
- 2 Nguyen VT, Arredondo J, Chernyavsky AI, Kitajima Y, Grando SA. Keratinocyte acetylcholine receptors regulate cell adhesion. *Life Sci.* 2003;72:2081–2085.
- 3 Kurzen H, Wessler I, Kirkpatrick CJ, Kawashima K, Grando SA. The non-neuronal cholinergic system of human skin. *Horm Metab Res.* 2007;39:125–135.
- 4 Gordon PR, Gelman LK, Gilchrest BA. Demonstration of a choline requirement for optimal keratinocyte growth in a defined culture medium. *J Nutr.* 1988;118:1487–1494.
- 5 Apparsundaram S, Ferguson SM, George AL Jr, Blakely RD. Molecular cloning of a human, hemicholinium-3-sensitive choline transporter. *Biochem Biophys Res Commun.* 2000;276:862–867.
- 6 Okuda T, Haga T, Kanai Y, Endou H, Ishihara T, Katsura I. Identification and characterization of the high-affinity choline transporter. *Nat Neurosci.* 2000;3:120–125.
- 7 Pfeil U, Haberberger RV, Lips KS, Eberling L, Grau V, Kummer W. Expression of the high-affinity choline transporter CHT1 in epithelia. *Life Sci.* 2003;72:2087–2090.
- 8 Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, et al. Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. *Endocrinology.* 2004;145:2498–2506.
- 9 Haberberger RV, Pfeil U, Lips KS, Kummer W. Expression of the high-affinity choline transporter, CHT1, in the neuronal and non-neuronal cholinergic system of human and rat skin. *J Invest Dermatol.* 2002;119:943–948.
- 10 Kleinzeller A, Dodia C, Chander A, Fisher AB. Na^+ -dependent and Na^+ -independent systems of choline transport by plasma membrane vesicles of A549 cell line. *Am J Physiol.* 1994;267:C1279–C1287.
- 11 Koepsell H, Schmitt BM, Gorboulev V. Organic cation transporters. *Rev Physiol Biochem Pharmacol.* 2003;150:36–90.
- 12 Gründemann D, Schechinger B, Rappold GA, Schömig E. Molecular identification of the corticosterone-sensitive extra-neuronal catecholamine transporter. *Nat Neurosci.* 1998;1:349–351.
- 13 Gründemann D, Liebich G, Kiefer N, Köster S, Schömig E. Selective substrates for non-neuronal monoamine transporters. *Mol Pharmacol.* 1999;56:1–10.
- 14 Kekuda R, Prasad PD, Wu X, Wang H, Fei YJ, Leibach FH, et al. Cloning and functional characterization of a potential-sensitive, polyspecific organic cation transporter (OCT3) most abundantly expressed in placenta. *J Biol Chem.* 1998;273:15971–15979.
- 15 O'Regan S, Traiffort E, Ruat M, Cha N, Compaore D, Meunier FM. An electric lobe suppressor for a yeast choline transport mutation belongs to a new family of transporter-like proteins. *Proc Natl Acad Sci U S A.* 2000;97:1835–1840.
- 16 Inazu M, Takeda H, Matsumiya T. Molecular and functional characterization of a Na^+ -independent choline transporter in rat astrocytes. *J Neurochem.* 2005;94:1427–1437.
- 17 Traiffort E, Ruat M, O'Regan S, Meunier FM. Molecular characterization of the family of choline transporter-like proteins and their splice variants. *J Neurochem.* 2005;92:1116–1125.
- 18 Cheng Y, Prusoff WH. Relationship between the inhibition

- constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol.* 1973;22:3099–3108.
- 19 Imokawa G, Abe A, Jin K, Higaki Y, Kawashima M, Hidano A. Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *J Invest Dermatol.* 1991;96:523–526.
 - 20 Wessler I, Reinheimer T, Kilbinger H, Bittinger F, Kirkpatrick CJ, Saloga J, et al. Increased acetylcholine levels in skin biopsies of patients with atopic dermatitis. *Life Sci.* 2003;72:2169–2172.
 - 21 Friedrich A, George RL, Bridges CC, Prasad PD, Ganapathy V. Transport of choline and its relationship to the expression of the organic cation transporters in a rat brain microvessel endothelial cell line (RBE4). *Biochim Biophys Acta.* 2001;1512:299–307.
 - 22 Hoffmann K, Grafe F, Wohlrab W, Neubert RH, Brandsch M. Functional characterization of a high-affinity choline transport system in human keratinocytes. *J Invest Dermatol.* 2002;119:118–121.
 - 23 Koepsell H, Endou H. The SLC22 drug transporter family. *Pflugers Arch.* 2004;447:666–676.
 - 24 Urakami Y, Okuda M, Masuda S, Saito H, Inui KI. Functional characteristics and membrane localization of rat multispecific organic cation transporters, OCT1 and OCT2, mediating tubular secretion of cationic drugs. *J Pharmacol Exp Ther.* 1998;287:800–805.
 - 25 Fujita T, Shimada A, Okada N, Yamamoto A. Functional characterization of Na^+ -independent choline transport in primary cultures of neurons from mouse cerebral cortex. *Neurosci Lett.* 2006;393:216–221.
 - 26 Scott A. Acetylcholine in normal and diseased skin. *Br J Dermatol.* 1962;74:317–322.
 - 27 Shelly WB, Shelly ED, Ho AKS. Cholinergic urticaria: acetylcholine receptor-dependent immediate-type hypersensitivity reaction to copper. *Lancet.* 1983;I:843–846.