Neuroscience 252 (2013) 212–221

ORGANIC CATION TRANSPORTER 2 (SLC22A2), A LOW-AFFINITY AND HIGH-CAPACITY CHOLINE TRANSPORTER, IS PREFERENTIALLY ENRICHED ON SYNAPTIC VESICLES IN CHOLINERGIC NEURONS

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Abstract—Organic cation transporters (OCTs) are expressed mainly in the kidney and liver. OCTs transport intrinsic organic cations, including monoamine, dopamine, serotonine and choline, across the plasma membrane. Here, we demonstrate that OCT2 (SLC22A2) is expressed in cholinergic neurons, motoneurons in the anterior horn of the spinal cord, and is implicated in acetylcholine (Ach) recycling in presynaptic terminals. Application of rabbit anti-peptide antibody revealed that OCT2 was expressed in the anterior horn of the spinal cord. Double immunostaining of muscle sections with anti-OCT2 and alpha-bungarotoxin (BTX) revealed that OCT2 was localized in the neuromuscular junctions (NMJs). Immunoelectron microscopy revealed that OCT2 was localized both in synaptic vesicles (SVs) in presynaptic terminals around the motoneurons (C-terminals) and in SVs in nerve terminals in NMJs. The similarity in the distribution of OCT2 in cholinergic neurons and that of vesicular acetyl choline transporter (VAchT), and the fact that OCT2 can transport choline suggest that OCT2 could work as a low-affinity and high-capacity choline transporter at presynaptic terminals in cholinergic neurons in a firing-dependent manner.

Key words: organic cation transporter, OCT2, SLC22A2, cholinergic neuron, synaptic vesicle.

INTRODUCTION

Acetylcholine (Ach) is the neurotransmitter that cholinergic neurons release through their terminals (Cooper, 1994). Ach is synthesized from choline that is transported from the extracellular fluid, since neurons do not have the ability to synthesize choline molecules de novo (Michel et al., 2006). Choline in extracellular fluid has a physiological range of 5–10 μM (Ferguson and Blakely, 2004). A deficit in choline transport is reported to be associated with some diseases such as schizophrenia (Hyde and Crook, 2001), Alzheimer's disease, Parkinson's disease and brain ischemic events (Schliebs and Arendt, 2011).

On firing, cholinergic neurons release Ach molecules at synaptic clefts. Ach then binds to acetyl choline receptors (AchR) on postsynaptic membranes. After firing, Ach is hydrolyzed by choline esterase into two molecules: choline and acetate. About 50% of choline molecules released at the synaptic cleft are recycled for Ach synthesis in presynaptic terminals (Collier and Katz, 1974). At the presynaptic plasma membrane, cholinergic neurons have the Na^+-dependent, hemicholinium (HC-3)-sensitive choline transporter CHT1 that transports extracellular choline into the cytoplasm (Kobayashi et al., 2002; Ferguson et al., 2003, 2004). Incorporated choline molecules are acetylated by an enzyme, choline acetyltransferase (ChAT) in the cytoplasm using the acetyl moiety from acetyl-CoA. Newly synthesized Ach is packed into synaptic vesicles (SVs) by the H^+-driven, vesicular acetylcholine transporter (VAchT) on SVs (Parsons et al., 1993). In the Ach recycling steps, the transport of choline plays a pivotal role as a rate-limiting step (Haga and Noda, 1973; Lockman and Allen, 2002).

It has been demonstrated that there are three kinds of choline transport systems in cells: (1) low-affinity transport system (OCTs) by facilitated diffusion, whose apparent K_m for choline is 20–200 μM; (2) intermediate-affinity, Na^+-independent, HC-3-sensitive transport system (Choline Transporter-like Family), whose apparent K_m for choline is ~10 μM; and (3) high-affinity, Na^+-dependent, HC-3-sensitive transport system (CHT1), whose apparent K_m for choline is ~0.5–2 μM (Simon et al., 1976; Devés and Krupka, 1979; Porter et al., 1992; Grassl, 1994; van Rossum and Boyd, 1998;

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Abbreviations: Ach, acetylcholine; AchR, acetyl choline receptors; BTX, alpha-bungarotoxin; CA, central autonomic cells; CC, central canal; ChAT, choline acetyltransferase; CNS, central nervous system; Co, cortex; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; DPX, dibutyl phthalate xylene; ER, endoplasmic reticulum; FCS, fetal calf serum; HC-3, hemicholinium; HRP, horseradish peroxidase; Is, inner stripe of the outer medulla; KLH, keyhole limpet hemocyanin; MBS, m-maleimidobenzoyl-*N*-hydroxysuccinimide ester; NGS, normal goat serum; NMJs, neuromuscular junctions; OCTs, organic cation transporters; Os, outer stripe of the outer medulla; PB, phosphate buffer; PBS, phosphate-buffered saline; PC, partition cells; SVs, synaptic vesicles; TM, transmembrane; VAchT, vesicular acetyl choline transporter.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.08.011

Sweet et al., 2001; Michel et al., 2006). In those systems, intermediate-affinity systems are expressed ubiquitously for synthesizing lipid membrane components, such as phosphatidylcholine (Michel and Bakovic, 2012), while high-affinity systems are expressed exclusively in cholinergic neurons for Ach recycling at presynaptic terminals, as mentioned above (Ferguson et al., 2003).

On the contrary, organic cation transporters (OCTs) were mainly studied and cloned in the field of kidney biology related to the efflux of intrinsic metabolites and drug metabolism (Koepsell et al., 2003; Jonker and Schinkel, 2004). As expected, most of OCTs are expressed in liver cells and/or renal tubules. OCT2 is a Na^+, Cl^--independent transporter that has 12 transmembrane (TM) domains with intracellular N-terminal and C-terminal domains. OCT2, also called SLC22A2, has relatively wide substrate specificity and transport activity for a variety of intrinsic compounds such as monoamine, dopamine, serotonin, histamine, creatinine and choline (Lee et al., 2009). In addition to intrinsic substrates, OCT2 has a pivotal role in transport of medical drugs. For example, OCT2 transports extrinsic cationic drugs such as cisplatin, affecting the half-life of these drugs (Koepsell et al., 2003; Filipski et al., 2009).

OCT2 is broadly expressed in the central nervous system (CNS) (Busch et al., 1998; Amphoux et al., 2006; Bacq et al., 2012). However, the role of OCT2 in the control of synaptic transmission remains unclear. A recent study in OCT2-deficient mice demonstrated reduced concentrations of norepinephrine and serotonin in their brain and also a reduced uptake of these neurotransmitters in the presence of a norepinephrine–serotonin transport blocker (Bacq et al., 2012). These findings revealed that OCT2 has a role in monoamine uptake in the mouse brain as a complementary system to high-affinity monoamine transporters. Interestingly, OCT2 is also expressed in some neurons in the choroid plexus, where OCT2 is engaged in the reabsorption of choline from the cerebrospinal fluid (Busch et al., 1998; Sweet et al., 2001). Although OCT2 expression in the choroid plexus implies a function of OCT2 on the transmitter recycling in cholinergic pathways, precise localization of OCT2 in cholinergic neurons has not been determined. Here, we demonstrated that OCT2 is expressed in the SVs of cholinergic neurons. Taking into consideration that OCT2 could transport choline and there could be other choline transport systems besides CHT1, our results suggest that OCT2 has a pivotal role in Ach recycling in presynaptic terminals of cholinergic neurons.

EXPERIMENTAL PROCEDURES

Production of anti-OCT2 antibody

A synthetic peptide, NH_2-RDGASLSPPPKPTQTNC-COOH corresponding to the C-terminal peptide of rat OCT2 (residues 574–589) with cysteine was purchased from Hokkaido System Science Inc. (Sapporo, Japan). The synthetic peptide was conjugated to keyhole limpet hemocyanin (KLH) through the cysteine residue at the COOH terminus of the peptide via m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). Rabbits were normally immunized once in 2 weeks. All the immunization procedures were conducted by Keari Co., Ltd. (Osaka, Japan) using 1 mg of peptide conjugate (1 mg/ml) in total. About 12 weeks after the first injection, the rabbits were anesthetized and the blood was collected. The obtained antiserum was affinity purified by Epoxy-activated sepharose 6B column (GE Healthcare, Tokyo, Japan) conjugated with antigen peptides following the manufacturer's instructions. Sodium azide (0.1%) was added to the affinity-purified antibody in the final concentration. The antibody was stored at −80 °C in small aliquots until use.

Construction of expression vector

The full-length cDNA of rat OCT2 was isolated by screening the rat kidney cDNA library using the polymerase chain reaction (PCR) fragment of rat OCT2 cDNA (Okuda et al., 1996) (GenBank accession No. D83044) amplified by two primers (sense: 5′-TTC CTA GGA TTT ACC CCT GAC C-3′ and antisense: 5′-AGT TGG GAG AAA TCG CCA TGA G-3′) as a probe. It was then subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA), a mammalian expression vector. The obtained cDNA sequence was analyzed and confirmed to be the same as the one reported.

Expression of rat OCT2 in COS cells

Experiments were performed with COS cells exponentially growing in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin and streptomycin. Cells were maintained in 5% CO_2 at 37 °C. After spreading 1×10^6 of COS cells on a 100-mm dish, cells were incubated for 24 h. Thirty minutes before transfection, the medium was changed to Opti-MEM (22600-050, Invitrogen). After 3 μg of pcDNA3.1-rat OCT2 was mixed with 300 μl of Opti-MEM, 12 μl of 1 mg/ml PEI-Max (24765-2, Polysciences, Inc., Warrington, PA) was added to the mixture, thoroughly mixed, and incubated for 15 min at room temperature. After adding the final mixture to the medium, COS cells were incubated for 3 h at 37 °C in a CO_2 incubator. After 3 h, the medium was changed to DMEM containing 10% FCS, penicillin, and streptomycin. As a control, mock vector was used instead of pcDNA3.1-rat OCT2.

SDS–PAGE and western blotting

All the procedures described below were conducted at room temperature. Forty-eight hours after transfection, cells were collected for western blotting. After the cells were washed with 10 ml of phosphate-buffered saline (PBS) twice, cells were covered with 800 μl of lysis buffer (150 mM NaCl, 25 mM Tris–Cl, 0.5% NP-40, pH 7.5) with a protease inhibitor (Complete, Boehringer Mannheim, Mannheim, Germany) for 10 min on ice,