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Tight junction protein ZO-1 controls organic cation/carnitine transporter OCTN2 (SLC22A5) in a protein kinase C-dependent way

Dominika Jurkiewicz [a], Katarzyna Michalec [a], Krzysztof Skowronek [b,c], Katarzyna A. Nałęcz [a,*]

[a] Laboratory of Transport Through Biomembranes, Nencki Institute of Experimental Biology of Polish Academy of Sciences, 3 Pasteur Street, 02-093 Warsaw, Poland
[b] Core Facility, International Institute of Molecular and Cell Biology, 4 Ks. Trojdena Street, 02-109 Warsaw, Poland
[c] Laboratory of Biochemistry of Lipids, Nencki Institute of Experimental Biology of Polish Academy of Sciences, 3 Pasteur Street, 02-093 Warsaw, Poland

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ABSTRACT

OCTN2 (SLC22A5) is an organic cation/carnitine transporter belonging to the solute carrier transporters (SLC) family. OCTN2 is ubiquitously expressed and its presence was shown in various brain cells, including the endothelial cells forming blood-brain barrier, where it was mainly detected at abluminal membrane and in proximity of tight junctions (TJ). Since OCTN2 contains a PDZ-binding domain, the present study was focused on a possible role of transporter interaction with a TJ-associated protein ZO-1, containing PDZ domains and detected in rat Octn2 proteome. We showed previously that activation of protein kinase C (PKC) in rat astrocytes regulates Octn2 surface presence and activity. Regulation of a wild type Octn2 and its deletion mutant without a PDZ binding motif were studied in heterologous expression system in HEK293 cells. Plasma membrane presence of overexpressed Octn2 did not depend on either PKC activation or presence of PDZ-binding motif, anyhow, as assayed in proximity ligation assay, the truncation of PDZ binding motif resulted in a strongly diminished Octn2/ZO-1 interaction and in a decreased transporter activity. The same effects on Octn2 activity were detected upon PKC activation, what correlated with ZO-1 phosphorylation. It is postulated that ZO-1, when not phosphorylated by PKC, keeps Octn2 in an active state, while elimination of this binding in ΔPDZ mutant or after ZO-1 phosphorylation leads to diminution of Octn2 activity.

1. Introduction

Organic cation/carnitine transporter - OCTN2, coded by *SLC22A5* gene in humans, belongs to a solute carrier superfamily SLC22 comprising solute transporters for organic cations, organic anions, zwitterions, and urate and involved in transport of toxins, drugs, and endogenous metabolites [1,2]. OCTN2 (SLC22A5) transports in a Na-independent way several organic cations [3,4], including pharmacologically important compounds as quinidine [5] and β-lactam antibiotics [6]. OCTN2 is also known as anti-cancer agent transporter and takes part in transport of such drugs as imatinib [7], etoposide [8] or oxaliplatin [9]. However, the physiological substrate for this protein is L-carnitine [(3R)-3-hydroxy-4-(trimethylazaniumyl)butanoate)] [3], which is transported in a symport with Na^+ with 1:1 stoichiometry [10,11]. OCTN2 is considered to be the main high affinity carnitine transporter in plasma membrane, especially that other high affinity carnitine transporter – mouse Octn3 (Slc22a21) was reported to be localized intracellulary in peroxisomes [12,13]. The other member of OCTN family – OCTN1 (SLC22A4), described as ergothioneine-specific [14] has low affinity for carnitine and was shown to transport acetylcholine, as a physiological substrate [15–17]. In case of OCTN2, several mutations of its gene result in primary carnitine deficiency (On-line Mendelian Inheritance in Man, OMIM no. 212140) with such symptoms as skeletal and cardiac myopathies, cardiac arrhythmia, fatigability [18–21]. Mutations in *SLC22A5* are also associated with Crohn's disease and transporter's activity is postulated to play an important role in several pathologies, as diabetes, autism spectrum disorders and neurological diseases (for review, see [22]).

In mammals carnitine can be synthesized in liver and kidney, anyhow, it is mainly supplied with the diet [23], so has to be transported to cells in majority of tissues. Carnitine is necessary for transfer of fatty acids in form of their acyl esters through the inner mitochondrial membrane, a mechanism leading to β-oxidation inside mitochondria. The ability of carnitine to form its acyl derivatives is also considered to be an important step in controlling the pool of free CoASH in the cell [24], what affects several cellular processes, just to mention synthesis of cholesterol and isoprenoids.

Abbreviations: BSA, bovine serum albumin; DAPI, 2-(4-amidinophenyl)-1H -indole-6-carboxamidine; DMEM, Dulbecco's modified Eagle Medium; DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGEPAL CA-630, octylphenoxypolyethoxyethanol (nonidet P-40); MDCK, Madin-Darby Canine Kidney cells; OCTN, organic cation transporter novel family; PBS, phosphate buffered saline; PDZ, protein domain – the name derived from proteins: postsynaptic density 95/disc-large/ZO-1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SLC, solute carrier; ZO-1, *zonula occludens* protein 1.

* Corresponding author.
E-mail address: k.nalecz@nencki.gov.pl (K.A. Nałęcz).

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Surface presence of various plasma membrane transporters is strictly controlled. Many proteins coded by *SLC* genes have been observed to be present in the cytoplasm, while their trafficking to plasma membrane was reported to depend on post-translational modifications and interaction with other proteins. OCTN2 contains 12 transmembrane domains with N- and C-termini localized on the cytoplasmic side [3,25]. It is known to be glycosylated on several asparagine moieties in the first extracellular loop, and substitution of these residues results in transporter retention in the cytoplasm, although it seems to be a result of conformational change rather than elimination of glycosylation, since tunicamycin treatment did not affect OCTN2 maturation to plasma membrane [26]. Analysis of OCTN2 amino acid sequence indicates several possible protein kinase C (PKC) phosphorylation sites, although the role of this kinase in transporter activity has been excluded in choriocarcinoma trophoblasts [27]. On the other hand, our studies with rat astrocytes demonstrated an increased activity of Octn2 after PKC activation with phorbol ester, a phenomenon correlated with augmented amount of transporter in plasma membrane, anyhow, no phosphorylation of Octn2 could be detected [25,28]. PKC activation in astrocytes was shown to promote a direct interaction between Octn2 and caveolin-1 leading to a hypothesis that formation of a multiprotein complex containing both proteins is important in the process of transporter trafficking to the cell surface [25]. This hypothesis was supported by a recent observation that Octn2 can as well interact directly with protein phosphatase PP2A [28].

OCTN2 was described to interact with two proteins PDZK1 and PDZK2 [29,30], containing PDZ domains, named after proteins postsynaptic density 95/disc-large/ZO-1. This interaction seems to be particularly important in modulating OCTN2 activity in kidney, where OCTN2 localization was shown to be highly polarized [31].

One of the examples of strongly polarized cells is brain capillary endothelium, forming (in interaction with astrocytes and pericytes) blood-brain barrier [32]. This barrier is impermeable to solutes, due to presence of tight junctions and controls brain homeostasis through the activity of specialized transporters, very often localized asymmetrically [33]. Our earlier observations demonstrated presence of Octn2 in an *in vitro* model of the blood-brain barrier and showed transporter localization mainly at abluminal/basolateral membrane [34], although colocalization with an apical membrane marker P-glycoprotein was detected at cell borders, pointing to a possibility of Octn2 presence in the proximity of tight junctions. One of the proteins involved in formation of a tight junction complex is *zonula occludens* ZO-1 protein, containing three PDZ domains [35]. Mass spectrometry analysis of Octn2 proteome in rat astrocytes detected some PDZ domain-containing proteins, including ZO-1 [28]. Since the last four amino acids at OCTN2 C-terminus (STAF) were shown to be essential for interaction with PDZK1 and PDZK2 [29,30], this short motif could be a putative platform for OCTN2/ZO-1 interaction. Therefore, the present study was focused on a possible interaction of rat Octn2 with ZO-1 and the role of PDZ-binding motif in transporter localization and activity.

2. Materials and methods

2.1. Antibodies and chemicals

L-[Methyl-^{3}H]carnitine was from PerkinElmer (Kraków, Poland). Polyclonal anti-ZO-1 antibody, mouse anti-ZO-1 antibody, ProLong® Diamond Antifade Mountant with DAPI and pBluescript II KS (+) were from ThermoFisher Scientific (Life Technologies, Warsaw, Poland). Purified mouse anti-human ZO-1 Clone 1 (RUO) was from BD Transduction Laboratories (DIAG-MED, Warsaw, Poland). Polyclonal anti-AHNAK (NBP1-81058) antibody was from Novus Biological (Abingdon, UK). Phospho-(Ser) PKC Substrate antibody was from Cell Signaling Technology (Lab-JOT, Warsaw, Poland). Rabbit polyclonal antibody to Solute carrier family 22 member 5 was from Abcam (Prospecta, Warsaw, Poland). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Immobilon-P transfer membrane were purchased from Millipore (Merck, Poland). Alexa Fluor 488® labeled anti-mouse antibody and Alexa Fluor 568® labeled anti-rabbit antibody were from Molecular Probes (Invitrogen, Eugene, Oregon, USA). EZ-Link® Sulfo-NHS-LC-Biotin [Sulfosuccinimidyl-6-(biotinamido)hexanoate] and Pierce® Avidin Agarose Resin were from Pierce (SYMBIOS, Warsaw, Poland). Lipofectamine™ 2000, fetal bovine serum and Dulbecco's modified Eagle Medium (DMEM) were from Invitrogen (Life Technologies, Warsaw, Poland). Ampholine (Bio-Lyte, pH 3–10) and immobilized pH gradient (3–10) strips for first dimension protein separation were from BioRad (Warsaw, Poland). Proximity ligation assay kit, monoclonal anti-FLAG M2 antibody, p3xFLAG-CMV14, IGEPAL CA-630 (Roche), phosphatase inhibitor cocktail PhosSTOP (Roche), alkaline phosphatase and all other reagents were from Sigma-Aldrich (Poznań, Poland).

2.2. Vector construction

cDNA encoding rat Slc22a - Octn2 (accession no. NCBI AF110416) was cloned in pBluescript II KS(+) as given in [25]. The cDNA sequence coding for vector PDZ-domain binding site, encompassing amino acids 554–557 of rOctn2 was deleted from pBluescript II KS(+)/OCTN2 plasmid, by inside-out PCR with the primers 5′-ATCCACTAGTTCTAGAGGCGGCCG-CCAC-3′ and 5′-CCCTTTAGGACCGTTGGGCTTTCTCCACC-3′ followed by 5′ end phosphorylation with T4 polynucleotide kinase and blunt end ligation. Both full-length and truncated coding sequences were isolated as *Hin*dIII-*Bam*HI fragments and re-cloned to p3xFLAG-CMV14 (CMV) vector resulting in constructs p3xFLAG-CMV14/OCTN2 (OCTN2) and p3xFLAG-CMV14/OCTN2–Δ554–557 (ΔPDZ), respectively expressing C-terminally 3xFLAG tagged proteins.

2.3. Cell culture and treatment

Transfection of human embryonic kidney cells - HEK293 (ATTC®) with vectors indicated in the figure legends was performed with Lipofectamine™ 2000, according to supplier protocol. The cells were cultured in 10% fetal bovine serum (FBS), 90% Dulbecco's modified Eagle Medium (DMEM), supplemented with G418 (300 μg/ml), gentamycin (50 μg/ml) and fungizone (0.25 μg/ml) at 37 °C in a humid atmosphere of 5% CO_2. Before the experiment the cells were washed with PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/Ca/Mg) and treated either with 0.1% dimethylsulfoxide (DMSO) as vehicle or with 200 nM phorbol 12-myristate 13-acetate (PMA) in DMSO for 30 min in PBS/Ca/Mg. MDCK cells were kindly provided by prof. Krzysztof Zabłocki from the Nencki Institute of Experimental Biology in Warsaw (Poland) and were cultured in 10% FBS, 90% DMEM, supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml).

2.4. Western blot analysis

Cells were lysed in 150 mM NaCl, 50 mM Tris, pH 7.4 supplemented with 1% IGEPAL CA-630, protease inhibitor cocktail and phosphatase inhibitors. Phosphatase inhibitors were omitted in experiments with alkaline phosphatase. After estimation of protein content, the samples were subjected to electrophoresis and immunoblotting. Two-dimensional gel electrophoresis of protein extract was performed, as described in [28]. The blots were analyzed with antibodies indicated in Figure legends. The quantitative analysis of the bands intensity was performed with use of the gel analysis tools in Fiji [36].

2.5. Immunocytochemistry

Cells were seeded in a proportion 0.8×10^5 cells/well for HEK293 and 1×10^5 cells/well for MDCKII. They were washed three times with PBS/Ca/Mg and fixed with 4% paraformaldehyde, 5 mM $MgCl_2$, 10 mM EGTA, 116 mM sucrose in PBS for 10 min at 20 °C. After 3 consecutive washes with PBS, the cell membranes were permeabilized for 10 min