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Localization of Mitochondrial Carnitine/Acylcarnitine Translocase in Sensory Neurons from Rat Dorsal Root Ganglia

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Abstract The carnitine/acylcarnitine transporter is a transport system whose function is essential for the mitochondrial β-oxidation of fatty acids. Here, the presence of carnitine/acylcarnitine carrier (CACT) in nervous tissue and its sub-cellular localization in dorsal root ganglia (DRG) neurons have been investigated. Western blot analysis using a polyclonal anti-CACT antibody produced in our laboratory revealed the presence of CACT in all the nervous tissue extracts analyzed. Confocal microscopy experiments performed on fixed and permeabilized DRG neurons co-stained with the anti-CACT antibody and the mitochondrial marker MitoTracker Red clearly showed a mitochondrial localization for the carnitine/acylcarnitine transporter. The transport activity of CACT from DRG extracts reconstituted into liposomes was about 50 % in respect to liver extracts. The experimental data here reported represent the first direct evidence of the expression of the carnitine/acylcarnitine transporter in sensory neurons, thus supporting the existence of the β-oxidation pathway in these cells.

Keywords Sensory neurons · DRG · Mitochondria ·
Carnitine/acylcarnitine transporter ·
Immunohistochemistry · β-Oxidation

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Introduction

The mitochondrial carnitine/acylcarnitine carrier (CACT) is a transport system belonging to the mitochodnrial carrier protein family. It is widely acknowledged that this transporter is located in the inner mitochondrial membrane where it plays the role of providing mitochondria with fatty acids as fuel for the β-oxidation pathway, thus represents an essential component for energy metabolism [1–3]. CACT together with carnitine transferase enzymes (CPTs) constitute a cyclic pathway called "carnitine shuttle" which allows acyl groups translocation into the mitochondrial matrix in the form of acylcarnitines. CACT catalyses an antiport reaction responsible for acylcarnitines entry into mitochondria coupled to efflux of free carnitine. This process leaves unaltered the carnitine concentration in both cytosol and mitochondria [1, 2, 4]. The essential role of this function is demonstrated by the occurrence of a severe syndrome caused by defects of the CACT gene called secondary carnitine deficiency. This pathology is characterized by seizures and muscle weakness; patients

with CACT deficiency suffer from hypoketotic hypoglycaemia and hyperammonemia due to the deficient fatty acid oxidation, a condition which is generally not compatible with life [2, 5]. Fatty acids were firstly thought to be the primary energy source in muscles and liver [6] then transport of L-carnitine has also been evidenced in neurons of cerebral cortex [7, 8]. It was also reported that fatty acid metabolism within discrete hypothalamic region can function as a sensor of nutrient availability that can integrate multiple nutritional and hormonal signals [9]. Indeed, the enzyme of the carnitine shuttle system, carnitine palmitoyl transferase 1 (CPT1), localized in the outer mitochondrial membrane, has been revealed in neuronal cells. In these cells the enzyme CPT1 plays a relevant role in modulating neuronal signals [10]. Furthermore, the enzyme pathway of the carnitine biosynthesis has been revealed in neurons [11]. However, the "carnitine shuttle" also needs the CACT presence. There are some evidences of carnitine transport both in plasma and mitochondrial membranes of neurons [8, 12] and of the presence of low levels of CACT mRNA as assessed by Northern blot analysis in human brain [13]. Even though a mitochondrial carnitine transporter was reported to be present in neurons [8], at present, no "direct" evidence of the expression of CACT protein in the central nervous tissue at the single cell level and no data on CACT expression or transport in peripheral nervous tissues are available. Here, the presence and the subcellular localization of the CACT was investigated in sensory neurons purified from rat dorsal root ganglia (DRG).

Materials and Methods

Western Blotting

The animal experiments were performed according to the European Community guidelines for care and use of animals. Three adult male Sprague-Dawley rats (age of 6–8 months) were anaesthetized by isoflurane before decapitation and the DRG were gently pulled by their roots and harvested from all accessible levels using fine forceps. The DRG obtained from each rat were maintained separated and immersed in lysis buffer containing 100 mM PIPES, 5 mM MgCl, 20 % (v/v) glycerol, 0.5 % (v/v) Triton X-100, 5 mM EGTA, and the protease inhibitor Phenylmethanesulfonyl fluoride (Sigma–Aldrich). Liver, and selected nervous tissues from the same three rats were also collected and immersed in the lysis buffer. Each tissue was homogenized at 4 °C, using a 2 ml Potter–Elvehjem Tissue Grinders with PTFE Pestle, and then, after 15 min at 4 °C, centrifuged at $10,000 \times g$ for 10 min at 4 °C. The supernatants (lysates) were subjected to analysis of protein

content using a commercially available protein assay kit (Bio-Rad, UK). 10 μg of proteins were resolved on sodium dodecyl sulfate–polyacrylamide gel (SDS-page: 17 % acrylamide/0.23 % bis-acrylamide) and electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, PROTRAN BA 85 cellulosenitrat). Residual binding sites on the membrane were blocked by incubation with 3 % BSA in a buffer solution composed of NaCl 150 mM, 50 mM Tris–HCl, Tween 20 0.05 % pH 7, for 10 min and then incubated with a rabbit polyclonal anti-CACT (dilution 1:1,000) in 0.5 % BSA solution for 1 h at room temperature (RT). The anti-CACT antibody was produced in our laboratory injecting the purified recombinant CACT in rabbit and resulted specific for rat and human CACT [14, 15]. This antibody did not recognize neither protein extracts from rat kidney brush border membranes containing the carnitine transporter OCTN2 (organic cation transporter novel 2) nor the over-expressed OCTN2 (Indiveri et al. unpublished results; see also the Western Blot paragraph in the Results section). The blots were washed three times in washing buffer (150 mM NaCl, 50 mM Tris–HCl pH 7) and were then incubated with an anti-rabbit IgG-coupled horseradish peroxidise antibody, dilution 1:10,000 (cod. 31460; Pierce, Rockford, IL, USA), for 1 h at RT. A mouse monoclonal ß-actin antibody (Santa-Cruz,UK) was used for the loading control. The reactive bands were visualized using an enhanced chemiluminescence reagent kit (Amersham ECL system) according to the manufacturer's instructions. The amount of reaction was estimated by the Bio-Rad Chemi Doc and quantitative evaluations were carried out by densitometric analysis using the Bio-Rad Quantity One Analysis Software.

Immunohistochemistry

Dissociated sensory neurons, from one male Sprague–Dawley rat (age of 6–8 months) for each cellular assay, were prepared as previously reported [16]. The neurons were cultured on glass coverslips coated with laminin at 37 °C with 5 % CO_2 for 3 days with addition of NGF 1 μg/ml to promote neurites outgrowth. Neurons were stained with anti-βIII-tubulin to identify the neuronal soma, with anti-CACT to verify its cellular localization and with MitoTracker (MtT) Red (M7512, CMXRos, Invitrogen) to identify mitochondria [17]. Before cell fixation the culture medium was replaced with 500 μl of MitoTracker solution (40 nM MitoTracker Red CMXRos, diluted from a concentrated DMSO solution into PBS containing 2 % BSA) and incubated for 20 min at RT in the dark. After PBS washing, the cells were incubated with 4 % (w/v) PFA/PBS solution for 30 min at RT. After three further PBS washes, the cells were permeabilised in 0.02 % Triton

X-100/PBS for 20 min. The cells were then incubated with 5 % normal goat serum (NGS) or normal horse serum (NHS) (Vector Labs Inc.) for 1 h at RT to block non-specific binding sites. The blocking solution was replaced with PBS containing primary monoclonal mouse anti-βIII-tubulin antibody (Cell Signaling Technology) at 1:1,000 dilution or auto-produced primary polyclonal rabbit anti-CACT [14, 15] at 1:500 dilution for 24 h at 4 °C. The cells were finally incubated with secondary Fluorescein horse anti-mouse or with Fluorescein (Alexafluor 488) goat anti-rabbit antibodies at 1:100 dilution (Vector Lab Ltd, UK) for 1 h at RT. The cover slips were mounted with Vecta-shield on glass microscope slides. Confocal images were captured with a Leica TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) using 40X (N.A. = 1.25) or 63X (N.A. = 1.32) oil immersion objectives, a 100 mW Argon laser (488 and 543 nm lines) and a 50 mW diode (405 nm) as in [18, 19]. Confocal images were analyzed using the software Fiji [20].

Reconstitution into Liposomes and Transport Activity Measurement of CACT

The DRG obtained from one rat, as described in the western blotting section, were collected and placed in a Petri dish containing Ham's F12 medium at room temperature. The surrounding connective tissue and nerve roots of the DRG were removed under the microscope. The intact DRG were homogenized, using a 2 ml Potter–Elvehjem Tissue Grinders with PTFE Pestle in 250 mM sucrose/1 mM EGTA/20 mM Tris–HCl pH 7 at 4 °C. 200 μl of the obtained homogenates (20–30 mg protein/ml) were added with 100 μl of a buffer containing 9 % Triton X-100 (mass/vol) 150 mM NaCl/3 mM DTE/30 mM PIPES pH 7 (final concentrations: 3 % Triton X-100/50 mM NaCl/1 mM DTE/10 mM PIPES pH 7) and incubated on ice for 10 min to solubilize membranes. The suspension was then centrifuged at $10,000 \times g$ for 15 min at 4 °C and the supernatants (extracts) were collected. The same procedure was performed on rat liver. 100 μl of the DRG and liver extracts, containing about 15 mg protein/ml, were reconstituted into liposomes in the presence of cardiolipin (except where differently indicated) to obtain proteolipo-somes in which the membrane protein such as CACT is functionally inserted in the lipid bilayer as in [21]. The procedure of proteoliposome formation, i.e. the reconstitution, consists in slowly removing the detergent from mixed micelles of phospholipids, detergent and protein by repeated chromatography on hydrophobic resin [22]. To charge proteoliposomes with internal substrate, 15 mM carnitine was added during the reconstitution procedure and removed, after the proteoliposomes formation, from the external space by gel filtration on Sephadex G-75 resin.
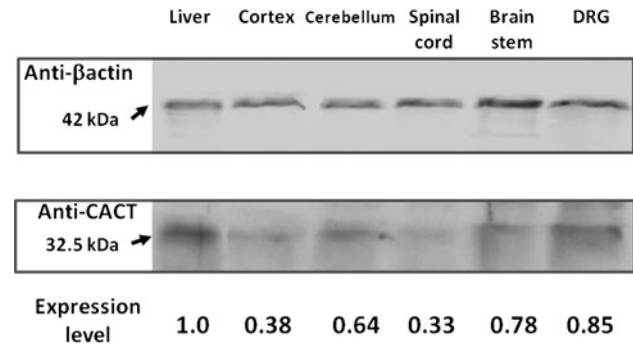
Fig. 1 Western blot analysis of CACT expression. Analysis of the level of CACT expression, normalized on β-actin, in liver, cortex, cerebellum, spinal cord, brain stem and DRG. Results are referred to the control, i.e., expression level of CACT in liver, which is taken as 1.0. Statistical data were obtained from three experiments from which the following mean ± SD were calculated: cortex 0.35 ± 0.035*; cerebellum 0.60 ± 0.057*; spinal cord 0.37 ± 0.050*; brain stem 0.84 ± 0.085; DRG 0.83 ± 0.028*. (*) Significantly different from the control (liver) as determined by Student's t test ($p < 0.05$)

The DRG pool from one rat was used for one reconstitution and transport assay. This is a suitable model for measuring the activity of CACT [23]. Transport activity was measured as [³H]carnitine$_{out}$/carnitine$_{in}$ homoexchange. Transport at 25 °C was started by adding, at time 0, 0.1 mM [³H] carnitine in a buffer containing 20 mM Tris–HCl pH 7.0 to proteoliposomes containing 10 mM internal unlabeled carnitine, and stopped by the addition of 1.5 mM N-eth-ylmaleimide at 10, 30 and 60 min. In controls, the inhibitor was added together with the labeled substrate at time 0, according to the inhibitor stop method [21]. Finally, the external labeled substrate was removed by chromatography on Sephadex G-75 columns and radioactivity into the proteoliposomes was measured. The experimental values were corrected by subtracting control values. The transport assay was performed three times to obtain statistical significant results.

Results

Western Blot

To evaluate the presence of the CACT polypeptide in the nervous system, and to compare the relative amount of protein in several brain areas and in the dorsal ganglia, western blot analyses were performed using a polyclonal anti-CACT antibody raised in rabbit, which is specific for the CACT of rat and human [15, 16]. Moreover, this protein was investigated in cortex, cerebellum, brain stem, spinal cord and DRG. A liver extract was used as a positive control.

As expected, the CACT was clearly detected in the liver extract (Fig. 1). The protein was also present in all the
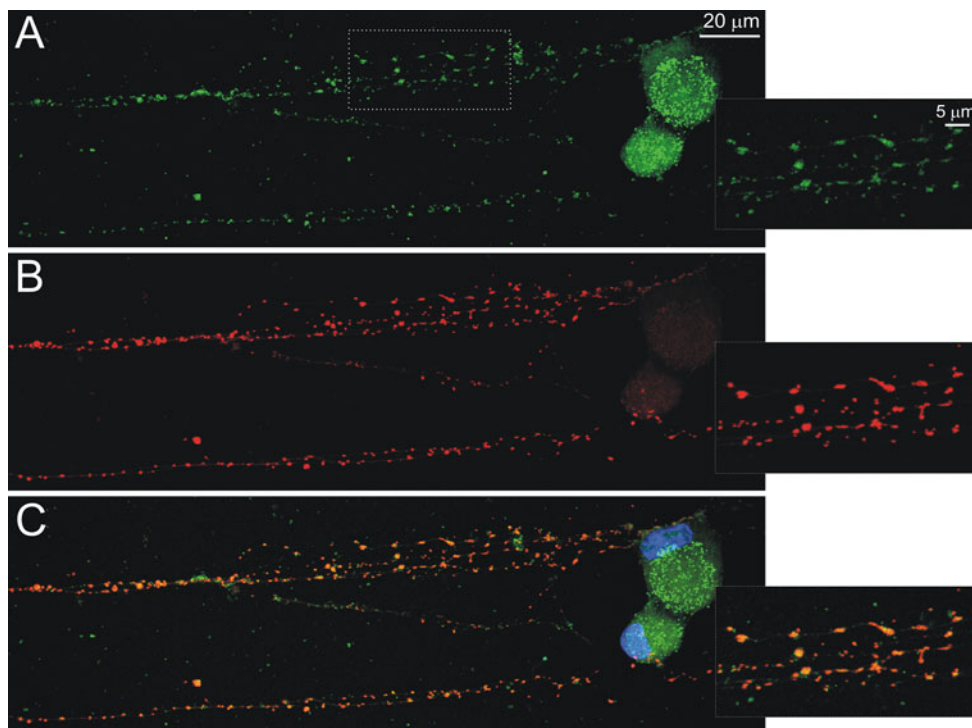
Fig. 2 Subcellular localization of CACT in sensory neurons. Maximum-intensity projections of confocal lasers scanning image stacks of two sensory neurons. CACT was labeled with a polyclonal antibody and visualized with an Alexa Fluor 488 conjugated anti-rabbit antibody (**a**). Mitochondria were stained with the specific dye MitoTracker Red

CMXRos (**b**). In **c** an overlay of the two images and counterstaining of nuclei with Hoechst is represented. The *orange/yellow* color denotes co-localization of *red* and *green* fluorescence signals. To allow a better appreciation of the mitochondrial structure, a higher magnification of the images is shown in the *insets* (Color figure online)

nervous tissue extracts analyzed. CACT expression was higher in liver compared to the nervous tissues analyzed and among these tissues the protein resulted to be more expressed in brain stem and DRG. By quantitative analysis of CACT expression in three different experiments, average amounts of 35, 60, 37, 84 and 83 % with respect to the control (liver), were found in cortex, cerebellum, spinal cord, brain stem and DRG, respectively. These findings clearly demonstrate the presence of CACT in the nervous tissue and, interestingly, evidence that in brain stem and DRG the protein amount was higher than in cerebral cortex and cerebellum. The CACT antibody used did not give any cross reaction with the OCTN2 protein (see Methods section), as expected from the very low sequence identity between the two proteins (~ 16 %), as calculated using the ClustalW software.

Immunohistochemistry

Immunostaining with βIII tubulin revealed that our DRG preparations contain sensory neurons with the morphology of healthy cells (not shown). Immunostaining with anti-CACT showed a robust expression of the protein in sensory neurons (Fig. 2a). Neurons were also stained with Mito-Tracker Red to reveal mitochondria localization. The

staining showed the presence of mitochondria in the soma and along the neurites (Fig. 2b). The merged images clearly showed co-localization of CACT with mitochondria (Fig. 2c), which appeared more evident in the neurites (inset 2c). To allow a better appreciation of CACT mitochondrial localization, a three dimensional reconstruction of the mitochondria localized in the neurites of another sensory neuron from a different DRG preparation is also reported (Supplementary Figure 1 and Supplementary Movie).

Reconstitution into Liposomes and Transport Activity Measurement of CACT

To evaluate the functionality of CACT in DRG, time courses of carnitine transport was measured in proteoliposomes reconstituted with either rat liver or DRG extracts. This method is suitable for measuring transport activity since it allows to drastically reduce interferences due to other transporters and enzymes. Furthermore, specific conditions were used to discriminate CACT from OCTN2 transport activity: (1) 0.5 mg/ml cardiolipin, which strongly stimulates CACT not OCTN2 [22, 23], was used; in this condition the activity of CACT in cell extracts is much higher than that of OCTN2; (2) transport was
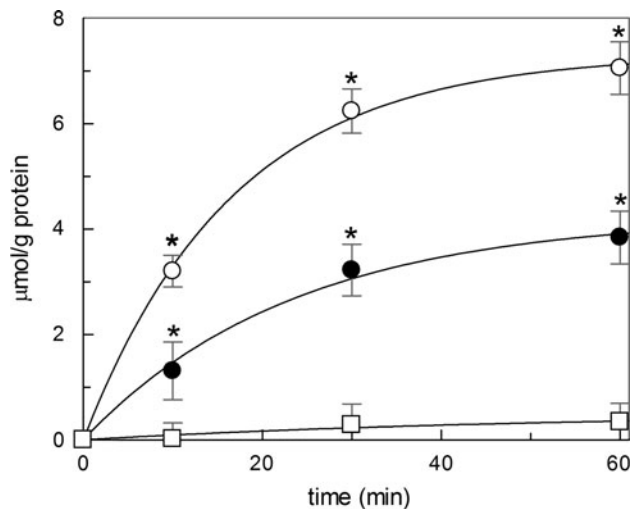
Fig. 3 Time course of [³H]carnitine uptake in liposomes reconstituted with liver and DRG extracts. Transport was started by the addition of 0.1 mM [³H]carnitine to proteoliposomes containing 15 mM carnitine and stopped at the indicated times. (*Open circle*) Liver extract; (*filled circle*) DRG extract; (*square*) DRG extract without cardiolipin (negative control). The data represent mean ± SD of three independent experiments. (*) Significantly different from the respective negative controls (*square*) as determined by Student's *t* test ($p < 0.05$)

measured in a solution buffered at pH 7.0 lacking NaCl, since OCTN2 has low activity at pH 7.0 and is Na⁺-dependent [23]; (3) *N*-ethylmaleimide was used as stopping reagent [24, 25] since it specifically inhibits CACT, and allows to subtract background radioactivity. As shown in Fig. 3 in proteoliposomes reconstituted with DRG extracts the accumulation of labeled substrate into the vesicles increased with the time, reaching the radioactivity equilibrium at 60 min. At the equilibrium 7.3 ± 0.15 and 4.2 ± 0.3 μmol/g protein, mean values from three independent experiments, for the liver and the DRG extracts respectively were measured. The overall exchange measured using the DRG extract was about 50 % in respect to the control (liver extract) at each time point. This result correlated well with the amount of protein detected with the antibody reaction in the western blot. Nearly no transport activity was detected in the absence of cardiolipin (Fig. 3) confirming that the transport activity above described was due to CACT and not to OCTN2.

Discussion

The nervous tissue, previously believed to rely mainly on glucose and ketone bodies for its energetic metabolism has also been shown to use fatty acids for energy supply and other functions [8, 26, 27]. The fatty acid oxidation pathway was postulated to be active in neurons, on the basis of

CPT1 localization [10, 28]. Moreover, acetylcarnitines have been proposed to have both energetic and neuroprotective roles in brain [27, 29]. According with these recent views, the present results directly demonstrate that CACT, which is an essential component of the biochemical pathway of the mitochondrial fatty acid β-oxidation [1, 3, 5, 30], is expressed in several nervous tissues, in particular in brain stem and DRG. The actual expression of the CACT in DRG has been demonstrated by immuno-fluorescence strategy which allowed to reveal the presence of the protein selectively in single neuronal cells. This finding correlates well with carnitine transport function assays performed in liposomes reconstituted in the presence of DRG extracts (Fig. 3), similarly to what was previously shown in brain tissues [31]. Indeed, the expression of CACT in CNS and PNS, is particularly relevant since its presence allows the completion of the carnitine cycle in these tissues in cooperation with CPT1 and CTP2 which expression was previously reported [27]. The presence of all the components of this cycle in neurons suggests that these cells could rely in part on metabolic energy coming from mitochondrial β-oxidation of acyl units, as previously suggested for specific cells such as astrocytes [25]. Moreover, as it has been previously described by us, CACT is essential for translocation of acylcarnitines with longer acyl chains into the mitochondrial matrix supplying substrates for β-oxidation, and also for translocation of shorter acylcarnitines such as acetylcarnitine, which could supply acetyl units to TCA cycle [32]. Some neuroprotective effects of acetyl-L-carnitine could be explained by energy boosts in neural cells provided by the activity of CACT in supplying acetyl units to mitochondria as acetylcarnitine, thus providing mitochondria with acetyl-CoA [8, 27]. The role of energy supply by the carnitine system and β-oxidation may be particularly relevant in sensory neurons for axonal transport during neuronal regeneration and neuronal outgrowth [33]. This may also explain the described effect of acetyl-L-carnitine in sensory neuron rescue [34] and neurodegeneration prevention [35, 36]. An additional important role of CACT may rely in eliminating intra-mitochondrial fatty acyl residues when working in the reverse mode [37]. Other effects mediated by acetyl-L-carnitine such as its anti-apoptotic action [38] may also be relevant in neurons, even though indirectly. Indeed, CACT has also been reported to be converted to a pore upon targeting of some Cys residues of the protein by chemical reagents, similarly to the mitochondrial Ornithine/citrulline transporter [39]. These functional modifications of mitochondrial transporters have been suggested to play some roles, in vivo, in cell death [40]. Thus, a contribution to these effects could not be excluded [41]. Also L-carnitine has shown neuroprotective effects on hippocampal slice cultures by preserving the key enzymes responsible for maintaining

carnitine homeostasis [42]. The presence of CACT in neurons is also in line with the finding that the acetyl moiety of acetyl-L-carnitine could be incorporated into glutamate [13, 14]. In conclusion, our results demonstrate the presence of CACT in CNS and PNS, and in particular its mitochondrial localization in sensory neurons. This supports the occurrence of the carnitine/acylcarnitine antiport and, hence, of the β-oxidation pathway in the nervous system.

Conflict of interest The authors declare that they have no conflict of interest.

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