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EXPRESSION AND TRAFFIC OF CELLULAR PROLYL OLIGOPEPTIDASE ARE REGULATED DURING CEREBELLAR GRANULE CELL DIFFERENTIATION, MATURATION, AND AGING

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Abstract—Prolyl oligopeptidase (POP) is an endopeptidase which cleaves short proline-containing neuropeptides, and it is involved in memory and learning. POP also has an intercellular function mediated through the inositol pathway, and has been involved in cell death. POP has been early considered as a housekeeping enzyme, but the recent research indicates that POP expression is regulated across tissues and intracellularly. In the brain, POP is exclusively expressed in neurons and most abundantly in pyramidal neurons of cerebral cortex, in the CA1 field neurons of hippocampus and in cerebellar Purkinje's cells. Intracellularly, POP is mainly present in the cytoplasm and some in intracellular membranes, like rough endoplasmic reticulum and Golgi apparatus. In this paper, we systematically studied the levels of expression of POP along the life of cerebellar granule cells (CGC) in culture and the distribution of POP within different intracellular compartments. We used the tight-binding inhibitor JTP-4819 covalently coupled with fluorescein (FJTP) as a tool to study the changes on expression and localization of POP protein. Our results indicate that POP activity levels are regulated during the life of the neurons. POP was found mainly in cytoplasm and neuronal projections, but at an early developmental phase significant amounts were found also in nuclei. Along the life of the neurons, POP activity fluctuated in 7-day cycles. In young neurons, the cytosolic POP activity was low but increased by maturation so that the activity peak coincided with full differentiation. Over aging, cytoplasmic POP was concentrated around nucleus, but the activity decreased with time. POP was also present in vesicles across the neuron. No major changes were seen in the nuclear or membrane bound POP over aging until activity disappeared upon neuronal death. This is the first time when POP was found in the nuclei of human neuronal cells.

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Abbreviations: AMC, amido-4-methylcoumarin; BME, basal Eagle medium; DAPI, 4′,6-diamidino-2-phenylindole; DIV, days *in vitro*; EDTA, ethylene-diamine tetraacetic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PBSA, phosphate-buffered saline supplemented with 0.5% albumin; PBSAT, phosphate-buffered saline supplemented with 0.5% albumin supplemented with 0.5% Triton X-100; PFA, paraformaldehyde; POP, prolyl oligopeptidase; ZTTA, N-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal.

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Prolyl oligopeptidase (POP; EC 3.4.21.26) is a prolyl specific peptidase, abundantly expressed in brain, where it cleaves several short neuropeptides involved in learning and memory (García-Horsman et al., 2007a). POP inhibitors are able to revert memory deficits in a number of drug- or lesion-induced amnesia models as well as in aged rodents (for a review, see Männistö et al., 2007). Although POP has been regarded as a housekeeping enzyme, confined to the cytoplasm (Polgar, 2002), experimental data indicate that the expression is neither exclusively cytoplasmic (Ohtsuki et al., 1997; Ishino et al., 1998; Agirregoitia et al., 2003; Myöhänen et al., 2008) nor homogeneous across the tissues (Irazusta et al., 2002; Bellemere et al., 2004; Myöhänen et al., 2007). POP has a very distinct expression pattern in the brain. It is amply present in cortical pyramidal neurons, in the CA1 field of hippocampus and in the cerebellar Purkinje cells (Bellemere et al., 2004; Myöhänen et al., 2007). Brain POP has been detected also in membranes (Agirregoitia et al., 2003; Myöhänen et al., 2008), and in tissue and neuronal cultures it has been localized perinuclearly (Schulz et al., 2005; Myöhänen et al., 2008). In peripheral tissues (Myöhänen, Venäläinen, García-Horsman & Männistö, unpublished observations) and non-neuronal cell cultures (Ishino et al., 1998), but not in the brain tissues (Myöhänen et al., 2007, 2008), POP protein has also been found in the nuclei.

We have synthesized a great number of potent and specific POP inhibitors (Wallen et al., 2002a,b, 2003a,b; Jarho et al., 2004, 2005, 2006, 2007; Venäläinen et al., 2004, 2005) and studied their inhibition kinetics and pharmacokinetics (Gynther et al., 2002; Venäläinen et al., 2002, 2006; Jalkanen et al., 2007). We recently synthesized a tightly bound POP inhibitor coupled to fluorescein (Venäläinen et al., 2005) which acts very similarly to the parent compound, JTP-4819. This fluorescent inhibitor, called here Fluo-JTP, binds tightly to POP with an enzyme-inhibitor complex half-life of several hours. We have now used this labeled compound to visualize the POP protein in neurons of cerebellar granule cells in culture, to study the intra-neuronal localization, the changes in the expression pattern and enzymatic activity of POP as a function of the age of the culture.

EXPERIMENTAL PROCEDURES

Cerebellar granule cell cultures

Primary cultures of cerebellar neurons were prepared as described in (Miñana et al., 1998) using cerebella from 7-day-old Wistar rats. Cerebella were dissected and incubated with 3 mg/ml dispase (grade II, Sigma-Aldrich, St. Louis, MO, USA) for 30 min in a 5% CO_2 incubator at 37 °C. The supernatant was removed and cells were incubated with basal Eagle medium (BME) containing 40 μg/ml DNase I (Sigma-Aldrich). The cellular suspension was filtered through a mesh with a pore size of 90 μm and centrifuged at 400×*g* for 5 min, and the cell bottom was rinsed twice with BME. Finally, the cells were resuspended in complete medium [BME containing 10% heat-inactivated fetal bovine serum (Gibco-Invitrogen, Grand Island, USA), 2 mM glutamine, 100 μg/ml gentamicin and 25 mM KCl]. Cells were plated onto polylysine-coated plates (312,000 cells/cm^2; 2 ml for 35-mm-diameter plates). After 20 min at 37 °C, medium containing unattached cells was removed and fresh medium was added. The cells were grown at 37 °C in a 5% CO_2 atmosphere. To prevent proliferation of non-neuronal cells, 10 μM cytosine arabinoside I (Sigma-Aldrich) was added 24 h after plating. Glucose (5.6 mM) was added to the culture medium every 3–4 days. Due to relative non-neuronal cell contamination at day 0, these cells were not used in our analysis.

Cytochemistry

Cerebellar granule cells were seeded on top of 10 mm coverslips. Fixation was performed as described in (Bell et al., 1987). Briefly, medium was removed and cells were rinsed twice with BME without serum. BME containing 0.1 mg/ml DTSP (3,3′-dithiodipropionic acid di(N-hydroxysuccinimide ester), Sigma-Aldrich) was added for 15 min. The cells were then rinsed twice with glycine–PBSA (0.1 M glycine in phosphate-buffered saline albumin; 137 mM NaCl, 3 mM KCl, 0.5 mM Na_2HPO_4, pH 7.4, 0.5% bovine serum albumin). Two more rinses were done with phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 0.5 mM Na_2HPO_4, pH 7.4) at 37 °C. Cells were incubated twice for 10 min in PBSAT (0.5 Triton X-100 in phosphate-buffered saline supplemented with 0.5% albumin) at 37 °C. Paraformaldehyde (PFA, Tousimis, Rockville, USA) was then added to a final concentration of 4% in PBSAT for 15 min at room temperature. PFA was removed and cells were washed twice with glycine–PBS and twice with PBS. Next, the cells were incubated with 10 μM Fluo-JTP (Department of Pharmaceutical Chemistry, University of Kuopio, Finland) in PBS for 20 min and washed again twice with PBS. Cells in coverslips were visualized in 85% glycerol containing 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) which stains DNA of all cells. Confocal microscopy was performed on a Leica TCS-SP2-AOBS exciting at 405 nm (blue) and at 485 nm (green). Detecting fluorescence emitted at 415–450 nm (blue) and at 500–575 nm (green).

Several controls were performed to ascertain FJTP specificity. Unspecific Fluo-JTP binding was determined by blocking specific sites with JTP-4819 or Z-Pro-Prolinal and was found minimal (not shown). Cells fixed as described were double labeled by immunocytochemistry using a primary anti-POP IgY as described before (Myöhänen et al., 2007, 2008) and FJTP as described here. No important qualitative differences were observed (see Supplementary data I Fig. S3). Additionally, cells were incubated with FJTP before fixation and fluorescence was detected by confocal microscopy after fixation. This control showed also qualitatively similar results compared with the labeling after fixation; however fluorescence intensity was decreased, and longer incubations with FJTP, before fixation, were needed to achieve same results when the label was added after. In particular, nuclei were less bright unless the fluorophore was added after fixing (see Supplementary data I Fig. S4).

Viability

Viability was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay. Ten microliters of 5 mg/ml stock of MTT in PBS was added to each plate, and they were incubated for 4 h in a humidified 5% CO_2 environment at 37 °C. Media were removed and formazan crystals were dissolved in dimethylsulfoxide. Cell viability was determined by absorbance measured on a plate reader (Victor2, PerkinElmer, USA) at a wavelength of 570 nm and with interference correction at 690 nm. Each measurement was done by triplicate.

Cell fractionation

Cells were first rinsed twice with PBS and then lysed with a hypotonic buffer (50 mM KH_2PO_4, pH 7.4; 1.5 mM $MgCl_2$; 10 mM NaCl; 1 mM EDTA; 1 mM dithiothreitol, DTT, AppliChem, Germany) and scratched from plates. Homogenates (total fraction) were centrifuged at 1000×*g*, the pellet (nuclear fraction) was set aside and the supernatant was centrifuged at 56,000×*g* for 30 min. The supernatant was labeled as cytoplasmic fraction and set aside. The pellet (membrane fraction) was washed with 1 M NaCl and resuspended in hypotonic buffer. Nuclear fraction pellet obtained in the first centrifugation step was washed twice with 50 mM potassium phosphate, pH 7, and resuspended in the minimal volume of hypotonic buffer plus 250 mM of NaCl and 0.1% Triton X-100. The samples were sonicated 2×5 s in a Branson Sonifier 150 (Danbury, USA) at amplitude of 3. All samples were aliquoted and stored at −70 °C. Protein concentration was assayed by the Bradford method kit (Bio-Rad, Hercules, USA).

POP activity assay

Standard enzyme activity assay was carried out in triplicate as published before (García-Horsman et al., 2007b). Briefly, a mixture of 12.5 μl of the enzyme preparation (from 2 to 10 μg of protein), 110 μl of 0.1 M sodium–potassium phosphate buffer, pH 7.0, and 2.5 μl of 1 mM Suc-Gly-Pro-7-amido-4-methylcoumarin (Suc-Gly-Pro-AMC, Bachem, Germany), was incubated at 30 °C for 90 min. The reaction was terminated by adding 500 μl of 1 M sodium acetate buffer, pH 4.2. Formation of AMC was determined fluorometrically with microplate fluorescence reader (Victor2, PerkinElmer, excitation at 360 nm and emission at 460 nm).

Western blotting

Protein samples for Western were acetone precipitated and resuspended in 1× loading buffer (50 mM Tris–HCl pH 6.8, 35% glycerol, 1% SDS, 0.002% Bromophenol Blue, 5 mM β-mercaptoethanol). Western blots were done as described before (Myöhänen et al., 2007) using a purified chicken anti-human POP (made in-house, see Myöhänen et al., 2007) as first antibody at 1:5000 dilution, and goat anti-chicken IgY-HRP conjugate (Sigma-Aldrich) as a secondary antibody at 1:10,000 dilution. Standard SDS-PAGE, transfer, blocking and blotting techniques were used. One to 5 femtomoles of purified recombinant porcine POP, prepared as described before (Myöhänen et al., 2007), was used as positive control.

Statistics

Statistical analyses were performed using GraphPad Prism (version 4.03, GraphPad Software, Inc., San Diego, CA, USA). One-way ANOVA followed by Tukey's test was performed to analyze differences activity peaks and valleys along the culture. Statistically significant differences were considered at $P<0.05$.