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cAMP stimulates the ubiquitin/proteasome pathway in rat spinal cord neurons

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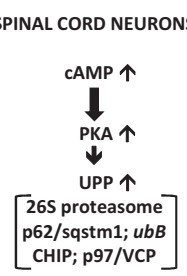
HIGHLIGHTS

- Elevating cAMP stimulates proteasomes in rat spinal cord neurons.
- Elevating cAMP raises the levels of components of the UPP.
- These include p62/sequestosome1, CHIP, p97 and *ubB*.
- Targeting cAMP/PKA to prevent neurodegeneration linked to Ub–protein aggregation.

GRAPHICAL ABSTRACT



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ABSTRACT

Proteasome impairment and accumulation of ubiquitinated proteins are implicated in neurodegeneration associated with different forms of spinal cord injury. We show herein that elevating cAMP in rat spinal cord neurons increases 26S proteasome activity in a protein kinase A-dependent manner. Treating spinal cord neurons with dibutyryl-cAMP (db-cAMP) also raised the levels of various components of the UPP including proteasome subunits Rpt6 and β5, polyubiquitin shuttling factor p62/sequestosome1, E3 ligase CHIP, AAA-ATPase p97 and the ubiquitin gene *ubB*. Finally, db-cAMP reduced the accumulation of ubiquitinated proteins, proteasome inhibition, and neurotoxicity triggered by the endogenous product of inflammation prostaglandin J2. We propose that optimizing the effects of cAMP/PKA-signaling on the UPP could offer an effective therapeutic approach to prevent UPP-related proteotoxicity in spinal cord neurons.

1. Introduction

The accumulation of ubiquitinated proteins is a pathological feature of spinal cord neurons detected upon injury [1] and in patients with amyotrophic lateral sclerosis (ALS) [5], implicating impairment of protein turnover by the ubiquitin/proteasome pathway (UPP). From a therapeutic point of view, the UPP is an ideal early target for preventing failures in protein degradation prior to formation of protein aggregates and the onset of cell death pathways (reviewed in [12]).

In the studies described herein, we demonstrate that elevating cAMP in rat spinal cord neurons stimulates different components of the UPP and diminishes neuronal damage induced by the endogenous product of inflammation prostaglandin J2. Enhancing protein turnover by the UPP via the cAMP/PKA pathway thus has potential as a neuroprotective strategy to prevent neuronal damage associated with proteasome impairment and accumulation/aggregation of ubiquitinated proteins in spinal cord neurons.

2. Materials and methods

Animal procedures were approved by the Hunter College IACUC.

Abbreviations: ALS, amyotrophic lateral sclerosis; CHIP, C-terminus of Hsc70 interacting protein; db-cAMP, dibutyryl-cAMP; DMSO, dimethyl sulfoxide; p62/sqstm1, sequestosome1; PKA, cAMP-dependent protein kinase; PGJ2, prostaglandin J2; Rp-cAMPS, adenosine 3′,5′-cyclic monophosphorothioate, Rp-Isomer, triethylammonium salt; Rpt, regulatory particle tripleA-ATPase; Suc-LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; UPP, ubiquitin/proteasome pathway.

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2.1. Reagents

Adenosine 3′,5′-cyclic monophosphate dibutyryl sodium salt (db-cAMP) from Calbiochem/EMD Bioscience. Rolipram and prostaglandin J2 (PGJ2) from Cayman Chemical. Forskolin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) from Sigma–Aldrich. Substrate Suc-LLVY-AMC from BACHEM Bioscience Inc.

Primary antibodies: anti-β5 (1:1000, #PW8895) and anti-Rpt6 (1:1000, #PW9265) from BIOMOL; anti-p62/sqstm1 (1:1000, #PM045, MBL International Corp.); anti-PKA C-α (1:1000, #4782), anti-CHIP (1:1000, #C386), and anti-Parkin (1:1000, #2132) from Cell Signaling; anti-p47 (1:1000, #365215, Santa Cruz Biotech), anti-p97/VCP (1:1000, #612182, BD Biosciences); anti-ubiquitinated proteins (1:1500, cat# Z0458, Dako North America); anti-βIII-tubulin (1:5000, #MMS-435P, Covance).

2.2. Spinal cord neurons

Dissociated cultures of Sprague Dawley rat embryonic (E18) spinal cords were prepared as in [11] with modifications. Isolated spinal cords free of meninges and dorsal root ganglia were digested with papain (2 mg/ml, Worthington Biochemical Corp.) in Hibernate E without calcium (BrainBits LLC.) at 37 °C for 30 min in a humidified atmosphere containing 5% CO_2. After removal of the enzymatic solution, tissues were gently dissociated in NbActiv4 media (BrainBits LLC.) and centrifuged at $300 \times g$ for 5 min. Pellets were resuspended in NBActiv4 media without antibiotics, and neurons were plated onto Lab-TekII-CC2 chamber slides (Nalgene Nunc International) pre-coated with 100 μg/μl poly-D-lysine (Sigma–Aldrich). Cultures were maintained in NbActiv4 medium and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Experiments were run upon 7DIV. According to manufacturer's specifications, NbActiv4 medium contains several proprietary factors that ensure a mostly pure (>95%) neuronal culture; glial growth is inhibited without a need for the anti-mitotic agent arabinofuranosyl cytidine [3,22].

2.3. Culture treatments

Neuronal cultures were treated for 24 h with water or DMSO (control) or with different drugs: db-cAMP in ultra pure filtered water, forskolin/rolipram or PGJ2 in DMSO, added directly to the NbActiv4 media. The final DMSO concentration in the medium was 0.5%.

2.4. Western blotting

Cells were plated (density of 1.5×10^6 cells) on 1-well chamber slides. After treatment, cells were rinsed twice with PBS and harvested by scraping into ice-cold lysis buffer [20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 1 mM EGTA, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% NP40, 1 mM Na_3VO_4, 1% glycerol and protease inhibitor cocktail (Sigma–Aldrich)]. Following lysis (at least 30 min, −80 °C) and centrifugation (19,000 × *g* for 10 min) at 4 °C, protein concentration was determined (BCA kit, Pierce). Normalized samples were boiled for 5 min in Laemmli buffer and loaded onto 10% gels (40 μg of protein/lane). Following electrophoresis, proteins were transferred onto Immobilon-P membranes (Millipore). After blocking for 30 min at 37 °C [10 mM Tris–HCl, pH 7.3, 5% (w/v) non-fat dry milk, 10 mM NaCl, and 0.1% (v/v) Tween 20], membranes were probed with primary antibodies followed by the respective secondary antibodies with HRP conjugate (1:10,000, Bio-Rad Laboratories). Antigens were visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent. Semi-quantitative analysis of protein detection was done by image analysis (ImageJ program, Rasband, W.S., ImageJ, U.S. NIH, MD).

2.5. In-gel proteasome activity and detection

Cells were plated (density of 1.5×10^6 cells) on 1-well chamber slides. Upon treatment cells were washed twice with PBS and harvested for the in-gel assay [20] with 80 μg protein/lane loaded for proteasome activity and 40 μg protein/lane loaded for western blotting. Proteasome activity was assessed by incubating the native gel for 10 min at 37 °C with 15 ml of 400 μM Suc-LLVY-AMC, followed by exposure to UV light (360 nm), and photographing with a NIKON Cool Pix 8700 camera with a 3-4219 fluorescent green filter (Peca Products, Inc.). Proteins on native gels were transferred onto PVDF membranes. Immunoblotting detected 20S and 26S proteasomes with anti-β5 and anti-Rpt6 antibodies. The anti-β5 antibody reacts with a core particle subunit detecting assembled 26S and 20S proteasomes. The anti-Rpt6 antibody reacts with a regulatory particle subunit detecting only assembled 26S proteasomes. The values reflect the semi-quantification obtained: with the Rtp6 antibody for 26S proteasome levels (single and double capped), as it generated the strongest signal; with the β5 antibody for 20S proteasomes. For loading control, aliquots of the samples were boiled for 5 min in Laemmli buffer and loaded onto 10% gels (40 μg of protein/lane) for immunoblotting with anti-βIII-tubulin.

2.6. Quantitative reverse transcription-PCR analysis

Cells were plated (density of 1.5×10^6) on 1-well chamber slides. Total RNA isolated with the RNAeasy Mini Kit from Qiagen, Inc., was evaluated for quantity and integrity (OD at 260/280 nm) and agarose gel electrophoresis. 10 ng of RNA was reverse transcribed with the DyNAmo cDNA Synthesis kit (Finnzymes Inc.). PCR primers (Applied Biosystems) to amplify rat cDNAs were: proteasome subunits Rpt6 [PSMC5 (Rn00579821_m1)] and β5 [PSMB5 (Rn01488741_m1)], PKA subunit Cα [PRKACA (Rn01432302_m1)], p62/sqstm1 [SQSTM1 (Rn00709977_m1)], ubiquitin B [UBB (Rn03062801_gH)] and ubiquitin C [UBC (Rn01789812_g1)], and GAPDH [GAPDH (Rn01775763_g1)] to normalize. Quantitative real-time PCR in 384-well plates: each well contained 5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 μl of each primer, 2.5 μl of RNase-free water and 2 μl of the reverse transcribed reaction in a LightCycler 480 (Roche Diagnostics Corp.). Thermal cycling conditions: initial denaturation step (10 min at 95 °C) followed by 45 cycles of: 10 s at 95 °C, 30 s at 60 °C and a cooling step of 10 s at 40 °C. Cycle threshold (C_T) values for each gene were obtained using a LightCycler 480 Software. Differences in C_T values between each gene and the reference gene (ΔC_T) were calculated: $2^{-[\Delta Ct(treated) - \Delta Ct(DMSO)]}$, where $\Delta C_T = C_T$ (gene of interest) − C_T (*gapdh*, reference gene as its mRNA level was not altered by db-cAMP or forskolin + rolipram).

2.7. PKA assay

Cells were plated (density of 1.5×10^6) on 1-well chamber slides. After treatment cells were rinsed twice with PBS and harvested as for western blotting including the centrifugation step, but without boiling. PKA activity was determined with 0.15 μg of protein per well. Absorbance (450 nm) was measured with a PowerWave HT Spectrophotometer. Relative kinase activity was determined as described in the nonradioactive assay kit (Assay Designs).

2.8. Cell viability

Cells were plated (density of 2.5×10^5 cells/well) on 4-well chamber slides. Cell viability was assessed with the