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GABA-shunt enzymes activity in GH3 cells with reduced level of PMCA2 or PMCA3 isoform

Antoni Kowalski, Ludmiła Żylińska, Tomasz Boczek, Elżbieta Rębas *

Department of Molecular Neurochemistry, Medical University of Lodz, 6/8 Mazowiecka Str., 92-215 Lodz, Poland

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ABSTRACT

GABA (γ-aminobutyric acid) is important neurotransmitter and regulator of endocrine functions. Its metabolism involves three enzymes: glutamate decarboxylase (GAD65 and GAD67), GABA aminotransferase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). As many cellular processes GABA turnover can depend on calcium homeostasis, which is maintained by plasma membrane calcium ATPases (PMCAs). In excitable cells PMCA2 and PMCA3 isoforms are particularly important. In this study we focused on GABA-metabolizing enzymes expression and activity in rat anterior pituitary GH3 cells with suppressed expression of PMCA2 or PMCA3. We observed that PMCA3-reduced cells have increased GAD65 expression. Suppression of PMCA2 caused a decrease in total GAD and GABA-T activity. These results indicate that PMCA2 and PMCA3 presence may be an important regulatory factor in GABA metabolism. Results suggest that PMCA2 and PMCA3 function is rather related to regulation of GABA synthesis and degradation than supplying cells with metabolites, which can be potentially energetic source.

1. Introduction

GABA and its precursor – glutamate are neurotransmitters found in all brain regions, but they play other roles in mammalian organism as well. Glutamate being the main precursor for glutamine, participates in transport of toxic ammonia between tissues. It is also an agonist of Ca^{2+}-permeable NMDA, AMPA and KA receptors, thus can be engaged in regulation of calcium transport across cellular membrane [1]. In endocrine cells, GABA and glutamate contribute to the regulation of anterior pituitary growth hormone (GH) and prolactin (PRL) secretion [2]. Both neurotransmitters can be metabolized to succinic acid in a short pathway, so-called GABA shunt [3]. At first, excitatory neurotransmitter glutamate is converted to γ-aminobutyric acid, the main inhibitory neurotransmitter. This rate-limiting step catalyzed by cytosolic glutamate decarboxylase keeps the balance between excitatory and inhibitory neurotransmitters in CNS, and can protect neurons against excess each of these signaling molecules [4]. GABA is next metabolized to succinate semialdehyde by GABA aminotransferase (GABA-T). This step determines the balance between GABA and glutamate, as glutamate is a second product of transamination [5]. Succinate semialdehyde is converted to succinate by NAD^+-dependent mitochondrial enzyme – semialdehyde succinate dehydrogenase (SSADH). In addition, GABA shunt can also play an important role in the cellular energetic status. As its final product – succinate – is a TCA cycle intermediate, GABA shunt can ensure additional ATP from glutamate. Inclusion of glutamate in the TCA cycle by this way appears to be cytoprotective, due to decreased production of toxic NH_3 by glutamate dehydrogenase. GABA shunt accounts for 17% of the tricarboxylic acid cycle activity in brain and might be a way to obtain energy from glutamate without production of ammonia [3,6]. It should be noted that GABA shunt is especially important for nervous tissue [3]. Disturbances in glutamate/GABA metabolism are linked with several neuronal disorders i.e. stiff person syndrome [7], chronic fatigue syndrome [8], anxiety disorders and seizures [9] Parkinson [10], Alzheimer and Huntington diseases [11].

Plasma membrane calcium ATPase (PMCA), a membrane carrier of calcium ions is responsible for the maintenance of low nM Ca^{2+} concentration in the cytoplasm of almost all cells, including brain. It possesses high affinity for calcium ions but low capacity, and slow rate of Ca^{2+} removal. Four main isoforms are encoded by four separate genes. PMCA1 and PMCA4 are ubiquitous, while PMCA2 and PMCA3 are tissue-specific and characteristic for excitable cells [12].

The role of plasma membrane calcium pump in GH3 cells has not been described so far. In the present study we have analyzed the effect of altered composition of PMCA isoforms on GABA metabolism in rat anterior pituitary somatotropic GH3 cell line. Experiments were performed using three cell lines: control GH3 (GH3_C) and lines with reduced expression of PMCA2 (GH3_2) or PMCA3 (GH3_3).

* Corresponding author. Fax: +48 42 678 24 65.
E-mail addresses: antoni.kowalski@umed.lodz.pl (A. Kowalski), ludmila.zylinska@umed.lodz.pl (L. Żylińska), tomasz.boczek@umed.lodz.pl (T. Boczek), elzbieta.rebas@umed.lodz.pl (E. Rębas).

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2. Materials and methods

2.1. Cell culture and transfection

GH3 cell lines was cultured with Ham's F-12K Medium (ATCC) supplemented with 15% horse serum (Biochrom), 2.5% FBS (Biochrom), penicillin and streptomycin (Sigma–Aldrich), at 37 °C in 5% CO_2 in humid atmosphere. Medium was changed every 48 h. Cells were harvested with 0.25 % trypsin/EDTA.

pcDNA3.1(+) plasmids with anti-PMCA2 or anti-PMCA3 cDNA sequences were obtained as described previously [13]. Plasmids were transfected into the cells using GeneJuice Transfection Reagent (Merck), according to manufacturer's instructions. To maintain stably transfected cell lines we used antibiotic G418 (Applichem) as selective agent. Cells transfected with a plasmid not containing anti-PMCA2 or anti-PMCA3 sequences, but expressing neomycin-resistance alone, was used as control.

2.2. Trypan blue staining

Cells were harvested and suspended in PBS, and then mixed in equal volumes with 0.4% Trypan Blue dye solution. After incubation (5 min, RT), cells were counted under inverted microscope using hemocytometer. Cells stained with dye were considered as dead.

2.3. WST-1 test

Cells were seeded on 96-well microplates at concentration of 10^4 cells/well and cultured in 100 μl/well of supplemented F12K medium. Test was performed for three days, starting from 24 h after seeding. After 4 h of incubation at 37 °C with 10 μl of Cell Proliferation Reagent WST-1 (Roche) reagent absorbance was measured at 450 nm on BioTek plate reader.

2.4. Measurement of intracellular $Ca^{2\pm}$ concentration

Experiments were performed after 7 days of cell culture. GH3 cells were incubated for 45 min at 37 °C with 1.2 μM Fura-2/AM indicator in a buffer containing (in mM): 125 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 6 glucose, 1.2 $CaCl_2$, 2.5 HEPES (pH 7.45), supplemented with 250 μM sulfinpyrazone (Sigma) . After loading, cells were washed twice and resuspended in a buffer without Fura-2/AM, to a density of 1.5×10^6 cells/ml. Measurements were made at 37 °C on Perkin–Elmer LS-50 spectrofluorimeter with continuous stiring. Changes in intracellular Ca^{2+} concentration were determined on the basis of fluorescence ratio of 340/380 nm excitation wavelength. Light emission was measured at 510 nm. Calibration curve was obtained by adding 0.1% Triton X-100 (maximum signal) and 10 mM EGTA (minimum signal).

2.5. Quantitative real-time PCR analysis

Total RNA was extracted from 7 days old cells using Total RNA isolation kit (A&A Biotechnology), according to manufacturer's protocol. Concentration and protein contamination of RNA were determined by spectrofluorimetric readings at 260 and 280 nm. One micro gram of total RNA was then used for cDNA synthesis with reverse transcriptase (Invitrogen, UK). Fifty nano grams of cDNA was amplified in triplicate by real-time PCR in ABI Prism 7000 system (Applied Biosystems) using Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas, Canada) and results were analyzed with accompanying software. The data are given as a relative expression level normalized to GAPDH housekeeping gene determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) method [14]. The following primers were used in the experiments:

PMCA1 [15]: F 5′-CCTGAGGTACCAGAGGCAATAAA-3′,
R 5′- TGGGTGTAAAAATACGCGCATTTG-3′;
PMCA2: F 5′- ACCGTGGTGCAGGCCTATGT-3′,
R 5′-GGCAATGGCGTTGACCAGCA-3′,
PMCA3: F 5′-AGCAGCGGGAAAGTGCCTCAT-3′,
R 5′-TCTGCAGTGCCTCAGCACCT-3′;
PMCA4: F 5-′ACGCGGTGTATCAGCTCGGA-3′,
R 5′-AGTGCTGGCTGGGTGGTGAA-3′;
GAD65: F 5′-TGAGAACCCGGGAACAGCGA-3′,
R 5′-TCTCCGTAGAGCAGAGCGCA-3′;
GAD67: F 5′-TGGCCTGCAACCTCCTCGAA3-′,
R 5′-TGGGCTACGCCACACCAAGT-3′;
GABA-T: F 5′-ACAGCCTCAAAACGCAGCA-3′,
R 5′-TTGGGCGCCACCGACATCAA-3′;
SSADH: F 5′-TGGCTCAACGGCAACGGGAA-3′,
R 5′-TGGTCCACGTTCCACTGTC-3′;
GAPDH [15]: F 5′-GGTTACCAGGGCTGCCTTCT-3′,
R 5′-CTTCCCATTCTCAGCCTTGACT-3′.

2.6. Western blotting

Cells were lysed in RIPA buffer containing protease inhibitors (1 mM PMSF, 1 mM pepstatin, and 10 mM leupeptin), incubated on ice for 30 min and centrifuged at 18 000*g* for 20 min at 4 °C. Supernatants were collected and stored for immunoassays at −80 °C. Protein concentration was measured using Protein Assay kit (BioRad).

Protein samples (40–100 μg) were separated on 8–10% SDS/PAGE and transferred to nitrocellulose. After blocking in TBS containing 5% BSA and 0.1% Tween-20 for 1 h, membranes were incubated with primary antibodies (anti-PMCA1, anti-PMCA2, anti-PMCA3, anti-PMCA4, anti-GAD65, anti-GAD67, anti-GABA-T or anti-SSADH, and GAPDH for normalization). For staining, appropriate secondary antibodies coupled with alkaline phosphatase were used. Blots were developed using Sigma Fast BCIP/NBT™ according to manufacturer's instructions. Densitometric analysis were performed with Quantity One software (Bio-Rad Laboratories, USA).

2.7. Enzyme activity assays

The activity of GABA-shunt enzymes was determined in postnuclear fraction. Cells were suspended in medium containing 0.32 M sucrose, 0.5 mM EDTA, 1 mM PMSF and 10 mM Tris–HCl, pH 7.4 and sonicated on ice for 2 s. After centrifugation (10 min, 900*g*, 4 °C) supernatant was used for determination of enzyme activity. Protein concentration was measured using Protein Assay Kit (BioRad).

GABA-transaminase and succinate semialdehyde dehydrogenase were assayed according to the modified coupled method with NAD^+ [16]. NADH formation was measured spectrophotometrically at 340 nm. GABA-T and SSADH activities are expressed as nmol NADH/mg protein/min. Glutamate decarboxylase assay based on fluorimetric measurement of GABA and ninhydrin condensation product [17]. Fluorescence of obtained product was recorded after 20 min at 470 nm, using 365 nm excitation wavelength. GAD activity are expressed as nmol GABA/mg protein/min.

2.8. Statistics

Data presented on figures are from at least three independent experiments. Statistical analysis was done using Statistica 9.1 (StatSoft, Inc.) program. A value of $p < 0.05$ was considered as statistically significant.