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Effects of gangliosides on the activity of the plasma membrane Ca^{2+}-ATPase

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ABSTRACT

Control of intracellular calcium concentrations ($[Ca^{2+}]_i$) is essential for neuronal function, and the plasma membrane Ca^{2+}-ATPase (PMCA) is crucial for the maintenance of low $[Ca^{2+}]_i$. We previously reported on loss of PMCA activity in brain synaptic membranes during aging. Gangliosides are known to modulate Ca^{2+} homeostasis and signal transduction in neurons. In the present study, we observed age-related changes in the ganglioside composition of synaptic plasma membranes. This led us to hypothesize that alterations in ganglioside species might contribute to the age-associated loss of PMCA activity. To probe the relationship between changes in endogenous ganglioside content or composition and PMCA activity in membranes of cortical neurons, we induced depletion of gangliosides by treating neurons with D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP). This caused a marked decrease in the activity of PMCA, which suggested a direct correlation between ganglioside content and PMCA activity. Neurons treated with neuraminidase exhibited an increase in GM1 content, a loss in poly-sialoganglioside content, and a decrease in PMCA activity that was greater than that produced by D-PDMP treatment. Thus, it appeared that poly-sialogangliosides had a stimulatory effect whereas mono-sialogangliosides had the opposite effect. Our observations add support to previous reports of PMCA regulation by gangliosides by demonstrating that manipulations of endogenous ganglioside content and species affect the activity of PMCA in neuronal membranes. Furthermore, our studies suggest that age-associated loss in PMCA activity may result in part from changes in the lipid environment of this Ca^{2+} transporter.

1. Introduction

Precise regulation of free intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in neurons is highly dynamic and serves as the basis for a vast array of signaling activities that are essential for neuronal viability and the integrity of the entire nervous system. Neurons express multiple systems that allow for rapid, transient Ca^{2+} pulses to initiate either short or long-term signaling events and to terminate equally rapidly the Ca^{2+}-mediated signals by returning $[Ca^{2+}]_i$ to normal resting levels. The extrusion of intracellular Ca^{2+} across the plasma membrane takes place against a 10,000-fold gradient of this ion [1,2]. Two integral membrane proteins mediate the extrusion of Ca^{2+}, the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (NCX). The PMCA is a high affinity but low capacity system for pumping Ca^{2+} to the extracellular medium [3–5], while the NCX is a low affinity, high capacity antiporter. The PMCA is responsible for the fine-tuning of $[Ca^{2+}]_i$ and the maintenance of low resting $[Ca^{2+}]_i$ [3–5].

The aging process in the brain is accompanied by increases in overall Ca^{2+} content in brain neurons and synaptic terminals and a slower clearance of intracellular Ca^{2+} from nerve endings following a depolarizing stimulus [6,7]. Previously, we reported that the activity of PMCA in synaptic plasma membranes (SPMs) progressively decreases with age [8,9] and suggested that this loss in activity may contribute to the aberrant cellular Ca^{2+} overload observed in aging neurons.

The PMCA is an integral membrane protein with ten transmembrane domains [10]. The activity of this enzyme is regulated by multiple factors, including calmodulin (CaM) binding, proteolysis, protein kinases, and acidic phospholipids in the membrane environment of the protein [10,11]. In addition to the modulation of the enzyme by acidic phospholipids residing in the inner leaflet of the plasma membrane, the activity

Abbreviations: CaM, calmodulin; CTXB, cholera toxin subunit B; D/L-PDMP, D/L-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol·HCl; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; LC–MS, liquid chromatography–mass spectrometry; MS, mass spectrometry; NCX, Na^+/Ca^{2+} exchanger; PMCA, plasma membrane Ca^{2+}-ATPase; $[Ca^{2+}]_i$, intracellular calcium concentration

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of purified and reconstituted PMCA is also sensitive to exogenously introduced gangliosides, a class of glycosphingolipids that reside in the outer leaflet of the plasma membrane [12,13]. However, the influence of endogenous gangliosides on PMCA activity has not been explored.

Gangliosides are highly enriched in the central nervous system of vertebrates. This is particularly true in the brain where they constitute 20–25% of the outer leaflet of neuronal membranes or 10–15% of the total lipids in nerve ending (synaptosomal) membrane fractions [14]. Gangliosides participate in a variety of vertebrate cellular processes such as toxin uptake and cell adhesion, growth, mobility, and differentiation [15]. In the nervous system, gangliosides play critical roles in neuritogenesis [16,17], synaptic transmission [18,19], axonal regeneration, myelin stability [20], and signal transduction [21–24].

Due to their amphiphilic properties and their tendency to complex with Ca^{2+} ions, gangliosides are implicated in the modulation of Ca^{2+} signaling and the maintenance of cellular Ca^{2+} homeostasis during normal neuronal functioning [25]. Null mutant mice for GM2/GD2 synthase are deficient in GM2, GD2, and all gangliotetraose gangliosides and exhibit an impaired capacity for Ca^{2+} regulation, which supports the hypothesis that the maintenance of Ca^{2+} homeostasis is a key function of complex gangliosides [26]. In rabbit skeletal muscles, both GM3 and GM1 can induce a conformational change in the sarco/endoplasmic reticulum Ca^{2+}-ATPase and alter the activity of this Ca^{2+}-sequestering enzyme [27]. At the plasma membrane, GM1 activates indirectly a non-voltage-gated channel, the transient receptor potential C5 channel, by cross-linking with $\alpha5\beta1$ integrin, thereby activating the integrin signaling cascade and promoting Ca^{2+} influx and the initiation of neurite outgrowth [28,29]. However, unlike the information that exists with regard to regulation of Ca^{2+} influx into neurons by endogenous gangliosides, there is only limited information on the effects of endogenous gangliosides on Ca^{2+} efflux across the plasma membrane [25]. This is particularly true with regard to the regulation of PMCA by endogenous gangliosides.

In our studies of the PMCA activity in synaptic membranes, we found that the highest activity was present in the raft domains, i.e., membrane microdomains that are highly enriched in cholesterol and gangliosides [30]. This observation led us to hypothesize that the content or composition of gangliosides in brain synaptic plasma membranes (SPMs) may affect the activity of PMCA and that age-dependent alterations in endogenous ganglioside content or composition may contribute to the age-associated loss of PMCA activity. Therefore, our goals in these studies were: (1) to determine if endogenous SPM gangliosides change with age; (2) to assess whether decreases in neuronal membrane content of endogenous gangliosides lead to changes in PMCA activity; and, (3) whether alterations in certain ganglioside species can selectively increase or decrease the PMCA activity associated with neuronal membranes. Our results indicated that: (1) the composition of gangliosides in rat brain SPMs was altered during aging, and (2) the activity of PMCA was highly sensitive to alterations in both content and composition of endogenous gangliosides in membranes of primary cortical neurons. The findings from this study indicate a novel, CaM-independent mechanism for the regulation of PMCA activity and the modulation of Ca^{2+} signaling in the CNS.

2. Materials and methods

2.1. Reagents, antibodies and cell culture media

The following reagents were purchased from Matreya LLC (Pleasant Gap, PA): D- and L-threo-1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol·HCl (D-PDMP and L-PDMP, respectively), purified bovine ganglioside mixture, and purified preparations of the ammonium salts of GD1a, GD1b, and GT1b. Purified preparations of the ammonium salt of GM1, all solvents (HPLC-grade only) used in lipid extraction or HPLC, neuraminidase (*Clostridium perfringens*, type V), ouabain, thapsigargin, oligomycin, ATP, horseradish peroxidase-coupled cholera toxin subunit B (CTXB), secondary anti-mouse and anti-rabbit antibodies, and Phosphatase Inhibitor Cocktail 3, were obtained from Sigma-Aldrich (St. Louis, MO). The bicinchoninic acid protein assay kit was from Pierce-Thermo Scientific (Rockford, IL), the Protease Inhibitor Cocktail 3 and the high performance thin layer chromatography (HPTLC) plates (Silica gel 60, 10 × 20 cm, Merck) were from Calbiochem-EMD (San Diego, CA), and the purified sodium salt of GQ1b and anti-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Tris-Glycine gradient gels (8–16%) and the serum-free Neurobasal™ cell media were purchased from Invitrogen (Carlsbad, CA), pan-PMCA antibodies (monoclonal) and antibodies to isoform-specific PMCA1–4 were obtained from Affinity Bioreagents (Golden, CO). The polyclonal rabbit pan-PMCA antibody, raised against a 21 amino acid sequence in the middle of the PMCA, was generated by our laboratory. All aqueous HPLC solvents were prepared in 18 MΩ resistivity water.

2.2. Animals and isolation of brain SPMs

Fisher 344/Brown Norway hybrid (F344/BNF1) male rats at 6, 23, and 34 months of age were obtained from the National Institute on Aging colony maintained by Harlan Industries. All protocols were implemented in accordance with NIH guidelines and approved by the University of Kansas Institutional Animal Care and Use Committee. The rats were quarantined for 2 weeks prior to their use in the studies. The rats were anesthetized by CO_2 inhalation, decapitated using a guillotine, and the brains recovered quickly. The brains from individual rats at each of the 3 ages were processed in parallel. Argon was bubbled through all solutions immediately preceding their use. Each whole brain was homogenized and processed for the isolation of synaptosomes by discontinuous ficoll density gradient centrifugation [30,31]. The synaptosomes were lysed in 3 mM Tris–HCl, at pH 8.5, buffer that contained a cocktail of protease inhibitors, were centrifuged at 64,200 ×*g* for 15 min, and the SPM pellet resuspended in 10 mM Tris–HCl, 50 μM $MgCl_2$, and 0.32 M sucrose, at pH 7.4.

2.3. Capillary HPLC mass spectrometric analysis (LC–MS) of gangliosides

Gangliosides were extracted from SPM samples using the Folch method [32]. Briefly, SPM samples (50 μl of each with an average protein concentration of 14.8 mg/ml) were made up to a volume of 250 μl in methanol:water (1:1, v/v) and mixed with 4 ml of chloroform:methanol (2:1, v/v). After the 25 min incubation, the solutions were centrifuged (1000 ×*g*, 5 min). The aqueous (upper) phase was collected while the chloroform layer was re-extracted three more times with 800 μl methanol:water (1:1, v/v) each time. The aqueous extracts were combined, dried under vacuum, and stored at −80 °C until use. Prior to LC–MS analysis, samples were resuspended in 500 μl methanol:water (1:1, v/v). HPLC analysis was performed on a nanoAcquity HPLC system (Waters, Milford, MA). SPM samples and the mixture of bovine brain gangliosides used as an external standard (5 μl of each) were initially loaded (45 μl/min, 99% acetonitrile, 1% of 50 mM ammonium acetate buffer, pH 5.6) onto a self-packed reversed phase C8 trap column (Zorbax RX-SB/C8, 5 μm, Agilent Technologies, Santa Clara, CA) for pre-concentration/desalting. After 3 min, the trap was switched in-line with an Acquity UPLC HILIC column (BEH-Amide column, 1.7 μm particle size, 1.0 × 50 mm, Waters). The gangliosides were resolved with an acetonitrile/ammonium acetate buffer gradient. The total column effluent was introduced into a SYNAPT G2 hybrid quadrupole time of flight mass spectrometer (Waters). The instrument was operated in sensitivity mode with a cone voltage of 45 eV and helium was admitted to the collision (trap) cell. Spectra were acquired at a mass range of 250–3000 m/z. Time to mass calibration was made with NaI cluster ions acquired under the same conditions, and lock mass corrected with an auxiliary sprayer presenting the doubly charged ion (m/z = 554.2614) from leucine enkephalin (YGGFL) peptide. Lock mass spectra were acquired every 30 s. Quantification of each class of