Specific Proteolysis of Neuronal Protein GAP-43 by Calpain: Characterization, Regulation, and Physiological Role

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Abstract—The mechanism of specific proteolysis of the neuronal protein GAP-43 in axonal terminals has been investigated. In synaptic terminals in vivo and in synaptosomes in vitro GAP-43 is cleaved only at the single peptide bond formed by Ser41; this is within the main effector domain of GAP-43. Proteolysis at this site involves the cysteine calcium-dependent neutral protease calpain. The following experimental evidences support this conclusion: 1) calcium-dependent proteolysis of GAP-43 in synaptosomes is insensitive to selective inhibitor of µ-calpain (PD151746), but it is completely blocked by µ- and m-calpain inhibitor PD150606; 2) GAP-43 proteolysis in the calcium ionophore A23187-treated synaptosomes is activated by millimolar concentration of calcium ions; 3) the pattern of fragmentation of purified GAP-43 by m-calpain (but not by µ-calpain) is identical to that observed in synaptic terminals in vivo. GAP-43 phosphorylated at Ser41 by protein kinase C (PKC) is resistant to the cleavage by calpain. In addition, calmodulin binding to GAP-43 decreases the rate of calpain-mediated GAP-43 proteolysis. Our results indicate that m-calpain-mediated GAP-43 proteolysis regulated by PKC and calmodulin is of physiological relevance, particularly in axonal growth cone guidance. We suggest that the function of the N-terminal fragment of GAP-43 (residues 1-40) formed during cleavage by m-calpain consists in activation of neuronal heterotrimeric GTP-binding protein Gq, this results in growth cone turning in response to repulsive signals.

Key words: neuronal protein GAP-43, calpain, protein kinase C, calmodulin, synaptic terminals, growth cone guidance, proteolysis
occurs under the action of some attractive extracellular signals: nerve growth factor (NGF), basic fibroblast growth factor (bFGF), and neural cell adhesion molecule (NCAM) [16-18].

Another important domain of GAP-43, the N-terminal domain, consists of amino acid residues 1-10. This is required for transport of GAP-43 to the plasma membrane of axon terminals [19, 20]. Residues Cys3 and Cys4, which can be in palmitoylated form, are especially important [21]. The N-terminal domain can directly (inside the cell) activate heterotrimer GTP-binding protein Go [22, 23]. This process also involves Cys3 and Cys4 (in the non-palmitoylated state) and a cluster of basic residues—Arg6, Arg7, and Lys9—mimicking a cytoplasmic tail of G-protein coupled receptors [24, 25]. G, protein is the dominant G protein in neurons; it mediates effects of some repulsive signals to growth cones [26]. Its activation in neurons after the treatment with N-terminal decapeptide of GAP-43 (1-10) results in collapse of growth cones and inhibition of neurite growth [27, 28].

Certain evidence exists that GAP-43 is involved in reception of some repulsive molecules, e.g., semaphorin-3, by growth cones [29]. Moreover, increased expression of GAP-43 in neurons can induce apoptosis [30]. Transgenic mice with overexpression of GAP-43 were characterized by reduction in the total number of neurons in various brain regions due to apoptosis [31]. In these mice, neurons were prone to cell death when axon damaged [32]. On the contrary, GAP-43 knockout mice had increased number of brain neurons at the stage E18 [29].

Thus, GAP-43 plays a dual role in growth cone guidance: it is involved in realization of the response to both attractive signals, stimulating growth cone movement towards attractive signals, and repulsive signals leading to collapse of growth cone or its certain site (and in some cases to cell death). In spite of the evident importance of this problem, the molecular mechanism underlying the involvement of GAP-43 into these processes remains poorly understood.

We previously demonstrated the presence of two shorter forms of GAP-43 protein, lacking 4 and 40 N-terminal residues (GAP-43-2 and GAP-43-3, respectively) in neurons [33, 34]. It was also shown that these forms are products of calcium-dependent proteolysis of GAP-43 [35]. In the present study, we demonstrate that in synaptic endings cleavage of GAP-43 occurs at the single peptide bond formed by Ser41 within the main effector domain. This cleavage is catalyzed by a cysteine calcium-dependent protease, calpain, namely m-calpain. We also demonstrate here that GAP-43 proteolysis by calpain is controlled by other signaling proteins of axon terminals—PKC and calmodulin. Based on results of this study putative mechanism of involvement of GAP-43 proteolysis by calpain in the reaction of growth cones to repulsive extracellular signals is proposed.

MATERIALS AND METHODS

Materials. The following reagents and materials were used in this study: 80K subunit of rabbit muscle m-calpain, MG132 (ZLLLa), leupeptin, A23187, dioleoyl glycerol, arachidonic acid, dimethylated bovine milk casein, N,N′-diallyltartardiamide (Sigma, USA); pig erythrocyte μ-calpain, bovine brain calmodulin, PD150606, PD151746 (Calbiochem, USA); Cil, EDTA-containing cocktail of protease inhibitors CompleteTM (Boehringer Mannheim, Germany); phenylmethylsulfonyl fluoride (PMSF) (Merck, Germany); [γ-32P]ATP, specific activity 3000 Ci/mole (Amersham, USA); Staphylococcus aureus protein A conjugated with horseradish peroxidase (Pasteur Institute of Epidemiology and Microbiology, St. Petersburg, Russia); secondary antibodies conjugated with horseradish peroxidase (Dako, Denmark); kits for chemiluminescence detection of immunoblots (Pierce, USA); nitrocellulose membrane filters Pragopor, pore size of 0.4 µm (Pragochema, Czech Republic); goat polyclonal antibodies to GAP-43 phosphorylated at the Ser41 residue (Santa-Cruz Biotechnology, USA). Polyclonal antibodies to GAP-43 were obtained by rabbit immunization with subcutaneous injection of electrophoretically pure bovine GAP-43 protein [36]. Rat brain tissue (P60-P80) and bovine brain tissues (P180) immediately frozen in liquid nitrogen after isolation from animals were used.

Synaptosome isolation. Synaptosomes were isolated by the method described in [37]. Rat brain (5 g) was quickly thawed, minced with a scalpel, and homogenized in 30 ml of buffer A containing 0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl buffer, pH 7.4, in Dounce homogenizer. All steps of synaptosome isolation were carried out at 0-4°C. The homogenate was centrifuged for 10 min at 1000g. The supernatant was aspirated, and the pellet was homogenized again in 20 ml of buffer A and centrifuged for 10 min at 1000g. Supernatants (S1) were pooled and centrifuged for 30 min at 12,000g. The resulting supernatant (S2) containing all soluble and microcorpuscle material of neuron bodies was considered as the cytosol fraction. The upper (lighter) part of pellet (P2) was aspirated, washed in buffer A, and considered as “crude” synaptosome fraction.

Endogenous proteolysis of synaptosomal proteins. Synaptosomal fraction was washed in Krebs–Ringer buffer (124 mM NaCl, 5 mM KCl, 1.3 mM MgCl2, 1.2 mM NaH2PO4, 26 mM NaHCO3, 10 mM D(-)-glucose, 20 mM Hesper-NaOH, pH 7.4) saturated with oxygen–carbon dioxide mixture (95 : 5), and centrifuged for 20 min at 12,000g. The final pellet was resuspended in the same buffer at protein concentration 5-6 mg/ml. Synaptosomes were divided into aliquots containing 10-20 µg of endogenous GAP-43 protein. After addition of 10 µM of calcium ionophore A23187, they were incubated with various additions (2 mM EGTA or 0.2 µM-10 mM CaCl2, in the presence of various protease