Biochemistry (Moscow), Vol. 70, No. 8, 2005, pp. 897-907. Translated from Biokhimiya, Vol. 70, No. 8, 2005, pp. 1086-1098.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-207, December 19, 2004.

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

V. V. Zakharov*, M. N. Bogdanova, and M. I. Mosevitsky

Molecular and Radiation Biophysics Division, Petersburg Nuclear Physics Institute, Russian Academy of Sciences, 188300 Gatchina, Leningrad Region, Russia; fax: (7-813) 713-2303; E-mail: v.zakharov@vz5518.spb.edu

Received July 22, 2004
Revision received August 13, 2004

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 G_o; 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

Neuron specific protein GAP-43 (B-50, neuromodulin) is one of the important constituents involved in basic nervous system specific processes (see for review [1, 2]). GAP-43 is an acidic protein (p*I* 4.5) of molecular mass 25 kD located on the inner surface of axon terminal plasma membrane. In the developing nervous system, GAP-43 is required for axonal growth cone guidance (selection of correct route to the target cell). In mice GAP-43 knockout results in 90-95% lethality within the first two weeks after birth due to strong deviations in topography of inter-neuronal connections [3-5]. In mature neurons, GAP-43 localized in the presynaptic region is involved in synaptic plasticity; in particular, it controls neurotransmitter release [6, 7]. Overexpression of GAP-43 protein in the nervous system of adult transgenic mice is accompanied by increased learning capacity [8].

A positively charged calmodulin-binding site (residues 39-55), which contains a phosphorylation site for protein kinase C (PKC), Ser41, is suggested to be the main effector domain of GAP-43 [9, 10]. However, GAP-43 binding to calmodulin occurs in non-canonic mode because it takes place in the absence of calcium ions [11]. Ser41 phosphorylation by PKC prevents GAP-43 binding to calmodulin; moreover, it induces calmodulin release from its complex with GAP-43 [12]. GAP-43 can interact with F-actin, and phosphorylation of Ser41 critically influences this interaction. The phosphorylated form of GAP-43 promotes formation of long actin filaments, whereas the non-phosphorylated calmodulin-bound form of GAP-43 blocks actin polymerization [13]. Phosphorylation of GAP-43 by PKC plays an important role in reorganization of actin membrane skeleton underlying growth cone motility and its adhesion to substrate [13-15]. GAP-43 phosphorylation in growth cones

Abbreviations: BASP1) brain acid soluble protein 1; CiI) calpain inhibitor I; bFGF) basic fibroblast growth factor; GAP-43-2) GAP-43 fragment (5-226 residues); GAP-43-3) GAP-43 fragment (residues 41-226); NCAM) neural cell adhesion molecule; NGF) nerve growth factor; PKC) protein kinase C; PMSF) phenylmethylsulfonyl fluoride.

* To whom correspondence should be addressed.

0006-2979/05/7008-0897

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 G_o [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_o 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 (ZLLLal), 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); CiI, EDTA-containing cocktail of protease inhibitors Complete™ (Boehringer Mannheim, Germany); phenylmethylsulfonyl fluoride (PMSF) (Merck, Germany); [γ-^{32}P]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 chemiluminescent 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 1000*g*. The supernatant was aspirated, and the pellet was homogenized again in 20 ml of buffer A and centrifuged for 10 min at 1000*g*. Supernatants (S_1) were pooled and centrifuged for 30 min at 12,000*g*. The resulting supernatant (S_2) containing all soluble and microcorpuscle material of neuron bodies was considered as the cytosol fraction. The upper (lighter) part of pellet (P_2) 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 $MgCl_2$, 1.2 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM D(+)-glucose, 20 mM Hepes-NaOH, pH 7.4) saturated with oxygen–carbon dioxide mixture (95 : 5), and centrifuged for 20 min at 12,000*g*. 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 $CaCl_2$, in the presence of various protease

inhibitors) for 2 h at 37°C. Incubation was terminated by rapid freezing of the synaptosomes. Synaptosomal proteins were isolated using extraction by 1% Triton X-100 and 5% perchloric acid [35], and proteins were then analyzed by electrophoresis and immunoblotting.

Isolation and purification of proteins. Proteins GAP-43, GAP-43-3, and BASP1 were extracted from bovine brain using chloroform and 3% perchloric acid by the following method. Brain (30-50 g) was minced with a scalpel and homogenized in three volumes of 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, 0.5 mM PMSF (buffer B) at 0-4°C. After addition of an equal volume of chloroform and intensive shake for 10 min in a round bottom retort, the mixture was centrifuged for 15 min at 8000*g*. The supernatant (aqueous phase) was aspirated, and the chloroform phase was discarded. Protein sediment was suspended in two volumes of buffer B, and after addition of an equal volume of chloroform, the mixture was shaken and centrifuged as above. The supernatants were pooled, and after careful drop-wise addition of 40% perchloric acid up to final concentration 3%, they were incubated for 15 min and then centrifuged for 10 min at 8000*g*. The resulting supernatant was treated with 15% trichloroacetic acid (TCA, final concentration) for protein sedimentation during overnight incubation at 0°C. The proteins were then sedimented by centrifugation for 15 min at 8000*g*, washed with acetone, centrifuged again, and dried under vacuum at room temperature. Subsequent protein purification was carried out using preparative electrophoresis in 12% polyacrylamide gel cross-linked with N,N′-diallyltartardiamide (0.15%) in an acetic acid/urea system with addition of 0.3% (w/v) of Triton X-100 for effective separation of bovine GAP-43 and BASP1 proteins [38].

Electrophoresis and immunoblotting. Content of GAP-43 and BASP1 proteins was analyzed by electrophoresis in 12% polyacrylamide gel in the system 0.9 M acetic acid/2.5 M urea [39]. During electrophoresis of bovine GAP-43 and BASP1, 0.3% Triton X-100 (w/v) was added to the gel [33, 36]. Electrophoresis in polyacrylamide gel in the presence of SDS was carried out by the method of Laemmli [40]. Gels were stained with 0.1% Coomassie R250 in solution containing 25% isopropanol and 10% acetic acid. Proteins were transferred from gel onto nitrocellulose membrane filters by diffusion (exposure of wet filter on the gel for 15 min). Immunoblots were detected by: 1) antibodies to GAP-43 (antiserum dilution (1 : 100)-(1 : 200)) and protein A conjugated with horseradish peroxidase (using a standard method); 2) antibodies specifically reacting with Ser41 phosphorylated form of GAP-43 (dilution 1 : 1000) using secondary antibodies conjugated with horseradish peroxidase and kit for chemiluminescent detection of protein bands. Gels stained with Coomassie R250 and immunoblots were subjected to densitometry followed by analysis using ScanDens software.

Phosphorylation of GAP-43 by protein kinase C. Preparation enriched with PKC was isolated from 15 g of bovine brain as described in [41] (but without the final purification stage employing threonine-Sepharose). Activity of PKC in the chromatographic fractions obtained at various purification stages was detected using GAP-43 as substrate and [γ-^{32}P]ATP by the method of dot-blots and subsequent autoradiography [42]. Isolated PKC was kept in 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EGTA, 0.02% Triton X-100 (w/v), 50% glycerol at −20°C. Phosphorylation of GAP-43 was carried out in reaction mixture (final volume 15 μl) containing 5 μg GAP-43, 0.01 μg PKC, 7.5 μM ATP, 1 μCi [γ-^{32}P]ATP, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 5 μM dioleoyl glycerol, 10 μg/ml arachidonic acid, 20 mM Tris-HCl, pH 7.5, for 15 min at 37°C. The reaction was stopped by heating for 5 min at 100°C. Effectiveness of GAP-43 phosphorylation by PKC was verified by electrophoresis in the acetic acid/urea system followed by subsequent autoradiography or using antibodies specific to Ser41 phosphorylated GAP-43 (by immunoblotting).

Proteolysis of GAP-43 by calpain *in vitro*. Purified bovine GAP-43 was subjected to proteolysis by μ- or m-calpain *in vitro*. Calpain preparations were stored in 20 mM imidazole-HCl, pH 6.8, 5 mM dithiothreitol, 1 mM EGTA, 50% glycerol at −20°C. Proteolysis was carried out in 10 mM Tris-HCl, pH 7.5 (final volume 15 μl), for 20-90 min at 37°C. The reaction mixture contained: 5 μg of GAP-43, 0.01 U of calpain, 0.1 or 1 mM $CaCl_2$ (in the case of μ- or m-calpain, respectively). The presence of 0.15 M KCl did not influence proteolysis. In some experiments, calmodulin (1-7 μg) was added to the reaction mixture. If protein GAP-43 was phosphorylated by PKC, protein was dialyzed against 10 mM Tris-HCl, pH 7.5, for 1 h before proteolytic treatment.

RESULTS

GAP-43 fragments in synaptic endings *in vivo*. Previously detected fragments of GAP-43 (GAP-43-2 and GAP-43-3) [33, 34] are differently distributed in various parts of the neuron (Fig. 1). Cytosol fraction S_2 (neuron bodies) contains both fragments, whereas synaptosomal fraction (isolated synaptic endings) P_2 contains only GAP-43-3. In synaptosomes, GAP-43-3 represents about 40-50% of intact GAP-43. Since brain was homogenized very quickly in ice bath in the presence of protease inhibitor cocktail and subsequent protein isolation employed 5% perchloric acid, artificial proteolysis of GAP-43 during isolation of this protein can be ruled out. Consequently, data of Fig. 1 reflect *in vivo* distribution of GAP-43 in neurons. Thus, synaptic endings contain GAP-43 and its fragments GAP-43-3 and GAP-43 (1-40), which are products of specific proteolysis of GAP-43 near Ser41.

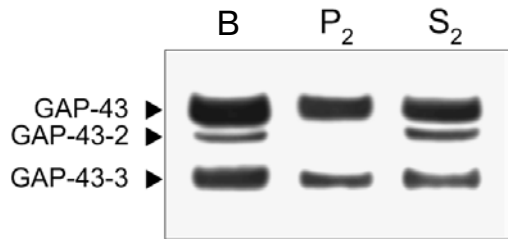

Fig. 1. Distribution of fragments of GAP-43 protein (GAP-43-2 and GAP-43-3) in various subcellular fractions of rat brain. Immunoblotting was carried out using polyclonal antibodies to GAP-43. Proteins from various fractions isolated in the presence of protease inhibitor cocktail using 1% Triton X-100 and 5% perchloric acid were analyzed by electrophoresis in 12% polyacrylamide gel in the acetic acid/urea system. Under the experimental conditions used, the fragment of GAP-43 (1-40) leaves the gel (and therefore it is not shown here) [35]. B is initial brain homogenate, P_2 is pellet P_2 (crude synaptosomal fraction), S_2 is supernatant S_2 (cytosol).

Calcium-dependent proteolysis of GAP-43 in synaptosomes. Previously we demonstrated that formation of GAP-43-2 and GAP-43-3 due to GAP-43 proteolysis can be reproduced *in vitro* after long-term incubation of synaptosomes at 37°C in the presence of calcium ions [35]. Use of calcium ionophore A23187 increasing calcium permeability of membranes accelerated GAP-43 proteolysis within synaptosomes *in vitro*. Figure 2a shows the result of incubation of synaptosomes treated with A23187 for 2 h with various additions. In the presence of 5 mM $CaCl_2$, a significant proportion of the GAP-43 (more than 60%) was converted into GAP-43-3 fragment. Formation of GAP-43-2 fragment after 2 h incubation of synaptosomes in the presence of calcium ions was not detected. Thus, GAP-43 proteolysis in synaptosomes resulted in formation of GAP-43-3 fragment, which was also detected during GAP-43 proteolysis in synaptic endings *in vivo*. Addition of EGTA (2 mM) completely prevented GAP-43 proteolysis (Fig. 2). This indicates that GAP-43 proteolysis in synaptosomes is a calcium-dependent process; the latter suggests possible involvement of the calpain protease in proteolysis of this protein [43-45]. Results of study of the effect of various protease inhibitors on GAP-43 proteolysis in synaptosomes support this suggestion. Proteolysis of GAP-43 in synaptosomes was insensitive to serine protease inhibitor PMSF (0.5 mM), whereas peptide inhibitors of calpain (20 μM leupeptin, 10 μM calpain inhibitor I (CiI), and 10 μM MG132) blocked this process (Fig. 2b). However, these are rather low specificity inhibitors because besides calpain they can also inhibit some other proteases [44, 45]. However, among proteases sensitive to these inhibitors, calpain is the only calcium-dependent protease, and so the conclusion on its involvement in GAP-43 proteolysis is quite reasonable.

To determine the type of calpain responsible for GAP-43 cleavage in synaptosomes, we tested two new specific non-peptide inhibitors of calpain: a) PD150606 causing equipotent inhibition of both μ- and m-calpains, and b) PD151746 inhibiting μ-calpain more selectively [46]. The presence of high concentration of PD151746 (100 μM) did not influence GAP-43 proteolysis in synaptosomes, whereas 10 μM PD150606 significantly reduced this process (Fig. 2b). The absence of total inhibition of GAP-43 by PD150606 even at 100-μM concentration may be attributed to non-competitive mode of the inhibitory effect of this compound blocking the calcium-dependent domain of calpain [46]. This indicates that m-calpain is involved in specific GAP-43 proteolysis in synaptosomes.

Calcium dependence of GAP-43 proteolysis in synaptosomes *in vitro*. For determination of calcium dependence of endogenous proteolysis of GAP-43 in synaptosomes treated with calcium ionophore A23187, synaptosomes were incubated with various concentrations of calcium ions from 0.2 μM to 10 mM. Figure 3 shows the

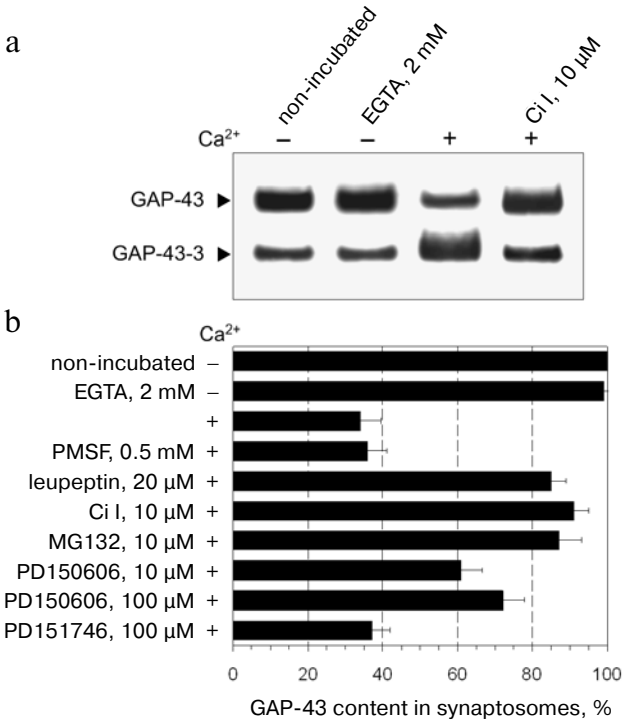

Fig. 2. Proteolysis of GAP-43 protein in synaptosomes. Synaptosomes were incubated for 2 h at 37°C in the presence of EGTA (2 mM) or calcium (5 mM) and the indicated inhibitors. Proteins isolated using 1% Triton X-100 and 5% perchloric acid were analyzed by electrophoresis in 12% polyacrylamide gel in the acetic acid/urea system. a) Immunoblot developed using polyclonal antibodies to GAP-43 protein; b) effect of various inhibitors preincubated with synaptosomes on content of intact GAP-43 protein. The amount of GAP-43 (mean ± SEM of three experiments) was determined by immunoblot densitometry.

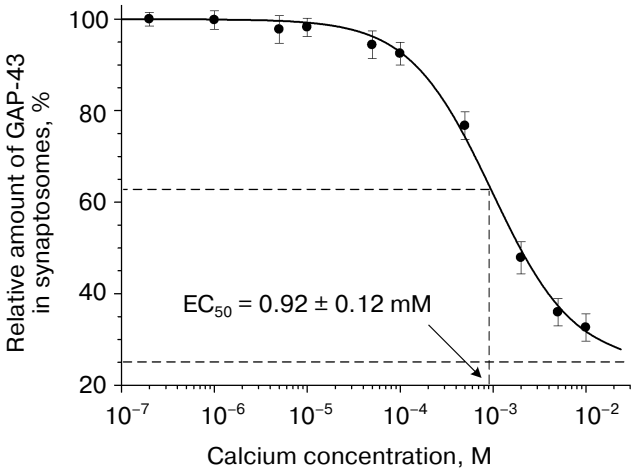

Fig. 3. Calcium dependence of GAP-43 proteolysis in synaptosomes. Synaptosomes pretreated with calcium ionophore A23187 were incubated in the presence of various calcium concentrations for 2 h at 37°C. The amount of GAP-43 in synaptosomes (mean ± SEM, $n = 3$) was evaluated by immunoblot densitometry. Results were approximated to a logistic curve using Microcal Origin 5.0 software. This determined the calcium ion concentration required for 50% activation of calpain cleaving GAP-43 (EC_{50}). Horizontal broken lines correspond to the level of 50 and 100% calpain activity.

dependence of intact GAP-43 protein content in synaptosomes incubated in the presence of various calcium concentrations. Marked proteolysis of GAP-43 was observed only in the presence of calcium concentrations ≥0.5 mM. Approximation of experimental data shown at Fig. 3 to a logistic curve allowed determining of calcium ion concentration required for 50% activation of GAP-43 cleaving protease; $EC_{50} = 0.92 \pm 0.12$ mM. These results demonstrate that total activation of this protease occurs in the millimolar range of calcium concentrations. Such high requirement of calcium concentrations is typical for m-calpain (EC_{50} = 0.4-0.8 mM), rather than for μ-calpain (EC_{50} = 3-50 μM) *in vitro* [44]. Thus, study of calcium dependence of GAP-43 in synaptosomes and insensitivity of proteolysis to μ-calpain inhibitor has provided convincing evidence that m-calpain is the only protease responsible for cleavage of GAP-43 at Ser41.

Proteolysis of GAP-43 by μ- and m-calpain *in vitro*. Use of purified preparations of μ- and m-calpain revealed that *in vitro* GAP-43 is a substrate for both types of calpain, but the proteolytic behavior of these enzymes differs (Figs. 4a and 4b). Cleavage of GAP-43 by μ-calpain *in vitro* resulted in formation of several large proteolytic fragments of GAP-43 (Fig. 4a). Fragment GAP-43-3 was detected among these, but it was not the main product of

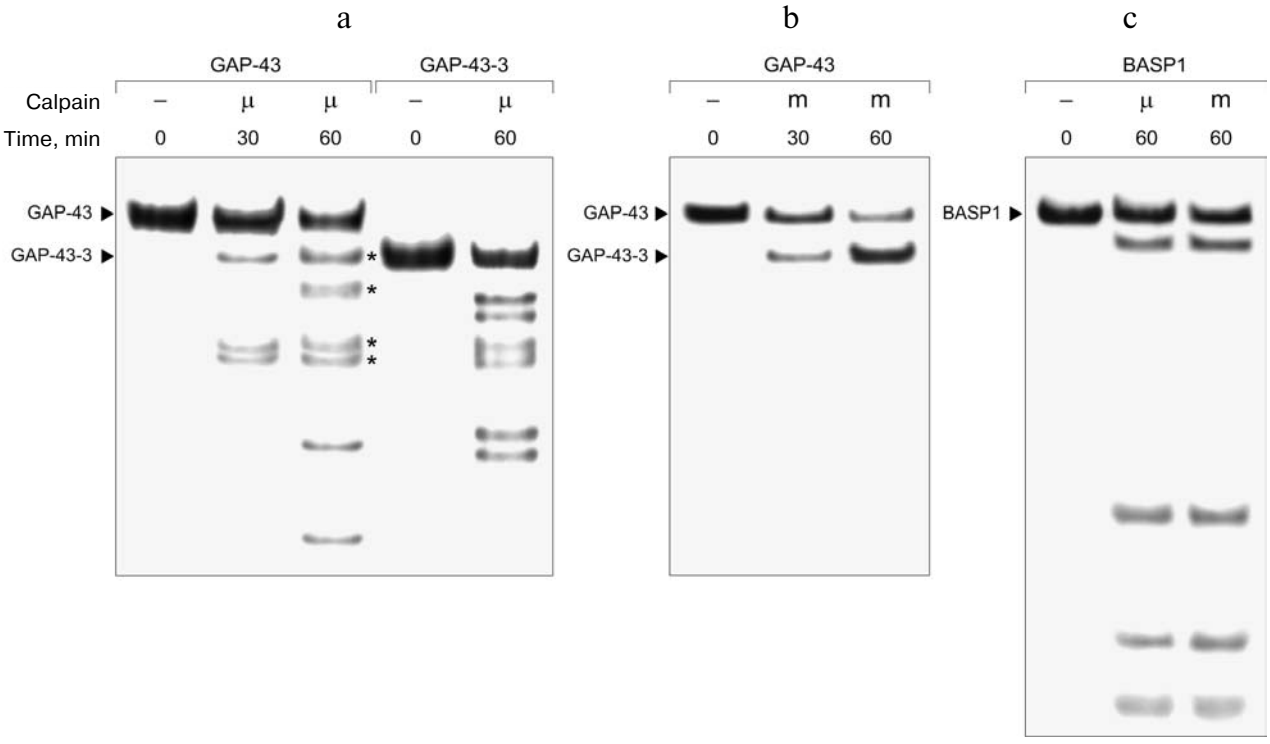

Fig. 4. Proteolysis of purified proteins GAP-43, GAP-43-3, and BASP1 by m- and μ-calpain *in vitro*. a) Proteolysis of GAP-43 and GAP-43-3 by μ-calpain; b) proteolysis of GAP-43 by m-calpain; c) proteolysis of BASP1 by μ- and m-calpain. Proteolysis was carried out at 37°C for the indicated time intervals. Proteins were analyzed by electrophoresis in 12% polyacrylamide gel in the acetic acid/urea system. Gels were stained with Coomassie. Asterisks mark GAP-43 fragments that can be phosphorylated by PKC *in vitro*.

GAP-43 proteolysis. A similar pattern was observed during various time intervals of GAP-43 proteolysis (Fig. 4a, lanes *2* and *3*). Moreover, GAP-43-3 is a substrate for μ-calpain (Fig. 4a). This suggests that the peptide bond at Ser41 is not the only cleavable site for μ-calpain during its action on GAP-43. Such mode of GAP-43 proteolysis by μ-calpain (formation of GAP-43-3 and other fragments of lower molecular mass) is consistent with results obtained earlier by another laboratory [47]. Fragments of GAP-43 formed during proteolysis of this protein by μ-calpain *in vitro* (which are marked with asterisks in Fig. 4a) contain Ser41 because they can be labeled with ^{32}P by PKC (data not shown). This suggests that cleavage sites for proteolysis of GAP-43 by μ-calpain are located in the C-terminal part of the molecule.

In contrast to treatment of GAP-43 with μ-calpain, the treatment of this protein with m-calpain *in vitro* resulted in reduction of GAP-43 content with simultaneous accumulation of the only large protein fragment, GAP-43-3 (Fig. 4b). Prolonged incubation of GAP-43 with m-calpain did not result in subsequent proteolysis of GAP-43-3 and formation of additional fragments. Thus, the mode of *in vitro* cleavage of GAP-43 by m-calpain coincides with the mode of calcium-dependent proteolysis of GAP-43 observed in both synaptosomes *in situ* and synaptic endings *in vivo*.

We also tested another GAP-43 related protein of synaptic endings, BASP1 [36, 38]. In contrast to GAP-43, the treatment of BASP1 with μ- and m-calpains resulted in identical cleavage of this protein (Fig. 4c) as in the case of other calpain substrates [44].

Effect of GAP-43 phosphorylation by PKC on proteolysis of the protein by calpain. According to literature data, the fraction of GAP-43 molecules phosphorylated by PKC at Ser41 is about 10-15% of the total GAP-43 content in the brain [48]. However, use of antibodies reacting with GAP-43 phosphorylated at Ser41 revealed that purified GAP-43 was not phosphorylated at this residue (data not shown). It is possible that GAP-43 was dephosphorylated during its isolation and purification. Thus, the resulting preparation of this protein did not require phosphatase treatment for yielding GAP-43 totally dephosphorylated at Ser41. Incubation of GAP-43 with PKC isolated from bovine brain caused rapid phosphorylation of GAP-43 at Ser41 detected by ^{32}P incorporation into the protein (Fig. 5, lane *3*) using antibodies specifically reacting with GAP-43 phosphorylated at Ser41 (data not shown). The fragment GAP-43-3 can also be phosphorylated by PKC (Fig. 5, lane *5*); however, in agreement with previous data, its phosphorylation was less effective [9].

To evaluate the effect of GAP-43 phosphorylation by PKC on its proteolysis by calpain, phosphorylated and intact GAP-43 preparations were treated with m-calpain *in vitro*. Figure 5 shows that phosphorylation of GAP-43 by PKC blocked proteolysis of the protein by m-calpain.

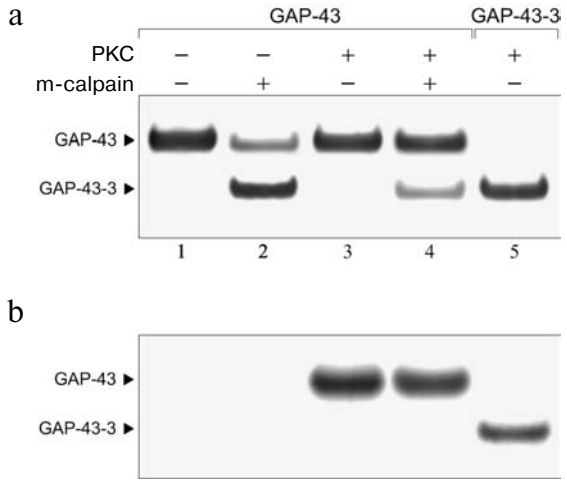

Fig. 5. Effect of GAP-43 protein phosphorylation by PKC on its sensitivity to proteolysis by m-calpain. a) Initial non-phosphorylated (PKC–) or phosphorylated (PKC+) GAP-43 was treated with m-calpain at 37°C for 1 h. Proteins were analyzed by electrophoresis in 12% polyacrylamide gel in the acetic acid/urea system. The gel was stained with Coomassie. Lane *5* shows phosphorylated GAP-43-3. b) Autoradiograph of the gel (a).

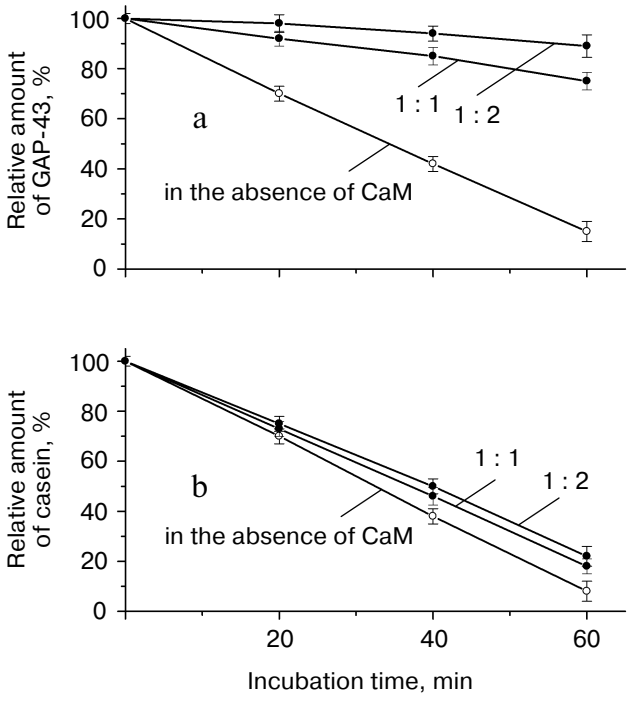

Fig. 6. Effect of calmodulin (CaM) on proteolysis of GAP-43 (a) and casein (b) by m-calpain. Proteolysis was carried out at 37°C for the indicated time intervals in the absence of calmodulin (*1*), in the presence of an equimolar amount of calmodulin (1 : 1) (*2*), or in the presence of twofold excess of calmodulin (1 : 2) (*3*). Proteins were analyzed by electrophoresis in 12% polyacrylamide gel in the presence of SDS. The amount of GAP-43 and casein (mean ± SEM, $n = 4$) was determined by densitometry of gels stained with Coomassie.

Formation of small quantities of GAP-43-3 during treatment of phosphorylated GAP-43 by calpain can be attributed to incomplete GAP-43 phosphorylation *in vitro*, because GAP-43-3 did not contain the radioactive label (Fig. 5, lane *4*) and therefore it is the proteolytic product of non-phosphorylated GAP-43 cleavage. Thus, GAP-43 phosphorylated at Ser41 is resistant to m-calpain. The inhibitory effect of GAP-43 phosphorylation by PKC was also found in the case of GAP-43 proteolysis by μ-calpain (data not shown).

Effect of calmodulin on GAP-43 proteolysis by calpain. Figure 6a shows that the presence of calmodulin in the reaction mixture attenuated GAP-43 proteolysis by m-calpain. Significant reduction in the rate of GAP-43 proteolysis was observed at equimolar ratio of calmodulin and GAP-43. A similar result was observed during GAP-43 treatment with μ-calpain (data not shown). To exclude possible direct inhibition of calpain by calmodulin (observed in [49]), we compared the effect of calpain in the presence of calmodulin on GAP-43 and casein (Figs. 6a and 6b). Casein is a model substrate for calpain and it does not interact with calmodulin. Our experiments showed that calmodulin significantly reduced only GAP-43 proteolysis by calpain (Fig. 6a) without any influence on proteolysis of casein (Fig. 6b). This suggests that the mechanism of the calmodulin effect on proteolysis of GAP-43 by calpain involves direct binding of calmodulin to GAP-43, which protects the latter against proteolysis by calpain.

DISCUSSION

Specific proteolysis of GAP-43 by calpain in synaptic endings and synaptosomes *in vitro*. Calpain, the cysteine calcium-dependent protease, is involved in numerous cell signal cascades as the enzyme responsible for limited proteolysis of signal cascade proteins; therefore, calpain is an important factor modulating specific activity of signal proteins [43-45]. Calpain plays an important role in processes typical for nervous system such as regulation of neurite growth, growth cone motility and adhesion, and synaptic plasticity [50-52]. However, the endogenous calpain substrates *in vivo* remain unknown. Our data of the present study on cleavage of the neuronal protein, GAP-43, by calpain are important for understanding the molecular mechanisms of functioning of both GAP-43 and calpain.

Study of specific proteolysis of GAP-43 in synaptic endings *in vivo* (using analysis of initial content of GAP-43 fragments) and also in synaptosomes revealed that proteolytic cleavage of GAP-43 in synaptic endings occurs at a single site, Ser41. Analysis of GAP-43 proteolysis in synaptosomes demonstrated that this process depends on the presence of calcium ions and involves calpain, because calpain-specific inhibitors block the proteolysis. This conclusion can be extrapolated to GAP-43 proteolysis in synaptic endings *in vivo*, because in both cases the same proteolytic product, GAP-43-3, has been found. Evidence on the involvement of calpain in GAP-43 proteolysis has been obtained for the first time.

Calpains μ and m are ubiquitous types of calpains that exhibit different calcium dependence *in vitro* [43-45]. Three types of experiments including study of the effects of calpain inhibitors PD150606 and PD151746 on proteolysis of GAP-43 in synaptosomes (Fig. 2b), calcium dependence of GAP-43 in A23187-treated synaptosomes (Fig. 3), and direct comparison of effects of μ- and m-calpain on GAP-43 *in vitro* (Fig. 4) provide convincing evidence that m-calpain is the protease cleaving GAP-43 in synaptic endings. Different site-specificity of μ- and m-calpain with respect to GAP-43 found *in vitro* is rather unexpected, because results from other laboratories indicate that the mode of cleavage of a protein substrate by these proteases *in vitro* is very similar if not identical [44]. In this study, we also found identical mode of cleavage of BASP1 by μ- and m-calpain (Fig. 4c). In the literature, we found only one case of different cleavage of a substrate by μ- and m-calpain; this was proteolysis of PKC [53, 54]. Different site specificity for proteolysis by μ- and m-calpain found in the cases of PKC and GAP-43 may represent a basis for different physiological functions of these proteases.

We can suggest two plausible explanations of GAP-43 resistance to μ-calpain, which is present in synaptic endings [55]. First of all, GAP-43 and μ-calpain may have different compartmentalization in the synaptic endings. Second, our pilot results show that treatment of GAP-43 with calpain *in vitro* is accompanied by substrate inhibition (in preparation for publication). The latter can lead to blocking of μ-calpain activity due to its rather low cellular concentration as compared to that of GAP-43.

Regulation of GAP-43 proteolysis by PKC and calmodulin. The effect of PKC and calmodulin, playing a major role in GAP-43 functioning, on proteolysis of this protein is most interesting. Since the Ser41 residue and the domain responsible for calmodulin binding neighbor the cleavage site of GAP-43 by calpain, blockade of proteolysis by PKC-dependent phosphorylation and calmodulin binding has evident structural relevance.

We have shown here that phosphorylation of Ser41 by PKC completely blocked GAP-43 proteolysis by calpain (Fig. 5). The crucial role of Ser41 phosphorylation in the regulation of GAP-43 interaction with calpain is probably determined by introduction of negative charge into a positively charged calmodulin binding domain [35]. GAP-43 phosphorylation by PKC blocks its proteolysis by calpain near Ser41 specifically, because the rate of GAP-43 hydrolysis by α-chymotrypsin cleaving peptide bond Phe42–Arg43 remained unchanged [56]. Interestingly, resistance of a phosphorylated form of a

protein to cleavage by calpain was also observed with some other protein substrates such as PKC-phosphorylated connexin-32 [57] and PKA (cAMP-dependent protein kinase A) phosphorylated platelet filamin and protein τ [58, 59].

Our data suggest that calmodulin attenuates GAP-43 proteolysis by calpain due to direct interaction with GAP-43; this interaction sterically prevents the attack of GAP-43 by calpain (Fig. 6a). Decrease in GAP-43 proteolysis by calpain in the presence of calmodulin is a rather unexpected finding because GAP-43 binding with calmodulin in the presence of calcium ions (required for calpain activation) is suggested to be extremely low [11]. Nevertheless, our data clearly indicate that there is GAP-43 binding with calmodulin even in the presence of calcium ions. This is also supported by rather close K_d values for dissociation of GAP-43/calmodulin complex with and without calcium ions of 1 and 0.23 μM, respectively [12]. It is also possible that activation of m-calpain in the cell can occur via phosphorylation by protein kinase ERK even in the absence of calcium ions [60]. This suggests that regulation of GAP-43 proteolysis by calmodulin might be physiologically important at various calcium concentrations. Regulation of calpain-dependent proteolysis by calmodulin was also found for some other calpain substrates. In these cases, calmodulin either accelerated proteolysis by calpain (of brain spectrin, calponin, and calcineurin) or attenuated it (e.g., plasma membrane Ca^{2+}-ATPase); in some cases changes in cleavage pattern (e.g., myosin light chain kinase) was also observed (see for review [44, 47, 49]). In the case of Ca^{2+}-ATPase as in the case of GAP-43, calpain cleavage sites were located inside the calmodulin binding domain [61]. This explains the nature of calmodulin-dependent decrease in proteolysis of these proteins by calpain.

Physiological role of GAP-43 proteolysis by calpain. The high degree of GAP-43 proteolysis in the brain resulting in formation of significant amounts of GAP-43-3 fragment *in vivo* (Fig. 1) suggests physiological importance of proteolysis of this protein by m-calpain. The content of GAP-43-3 in rat brain during embryonal and early postnatal development (up to 5-10 days after birth) is significantly higher than in adult brain and even exceeds the content of intact GAP-43 [62, 63]. Thus, GAP-43 proteolysis by calpain in the growth cones of the developing nervous system is more intensive than in adult synaptic endings. Regulation of proteolysis of GAP-43 by PKC and calmodulin suggests a tight interrelationship with other signaling pathways in axon terminals. Taking into consideration involvement of GAP-43 into growth cone guidance towards extracellular signals (see the introductory part), we believe that GAP-43 proteolysis by calpain also plays an important role in growth cone guidance; we also believe that proteolysis of this protein is specifically involved into response of growth cone to repulsive signals (see Fig. 7). This response consists of local impairment of adhesion contacts with substrate at the site of repulsive signal action and dissociation of actin skeleton from plasma membrane followed by growth cone turning to the direct opposite to the signal effect. This hypothesis is based on the following data.

1. Recently it was demonstrated that local calpain activation in growth cone by calcium pulses results in repulsive growth cone turning to the opposite direction [52]. Studies on non-neuronal migrating cells revealed that m-calpain was activated at the back side of the cell; it was involved in disruption of intracellular part of adhesion contacts preventing cell movement that is required for retraction of the tail part of the cell (see for review [60]). Thus, GAP-43 proteolysis in the growth cone is probably related to calpain activation induced by the effects of inhibitory extracellular signals.

2. We have demonstrated here that phosphorylation of Ser41 of GAP-43 prevents proteolysis of this protein by calpain at this residue. It is known that GAP-43 phosphorylation by PKC occurs in growth cones in response to extracellular attractive signals (NGF, bFGF, NCAM); this stimulates actin skeleton mobilization and formation of growth cone adhesion contacts with substrate [13-15]. This underlines a possible role of GAP-43 proteolysis by calpain (in contrast to the role of GAP-43 phosphorylation) in the reaction of growth cones to an inhibitory environment. Involvement of GAP-43 in realization of repulsive signals and induction of apoptosis was demonstrated in a number of studies, but a putative mechanism of this phenomenon was not proposed [29-32].

3. We do believe that the N-terminal GAP-43 (1-40) fragment formed due to GAP-43 proteolysis by calpain plays a certain role in growth cone response to repulsive signals. This fragment contains a domain for G_o protein activation [22, 23]. Activation of G_o in neurons results in collapse of growth cones and inhibition of neurite growth [26-28]. G_o and GAP-43 are predominating proteins of growth cone plasma membrane [22] and growth cone rafts (detergent resistant membrane domains) [64]. However, it has recently been demonstrated that G_o and GAP-43 are located in various types of rafts and so they are spatially separated on the growth cone membrane [65]. Thus, in the growth cone GAP-43 cannot activate G_o directly; otherwise, G_o would be constitutively active. We suggest that GAP-43 (1-40) fragment formed during GAP-43 proteolysis by calpain might function as G_o activator. In non-palmitoylated form [24] this fragments is not bound to the membrane; diffusing along the membrane, this fragment can potentially activate G_o and trigger cascade of reaction resulting in local collapse of the growth cone (Fig. 7).

The function of the GAP-43-3 fragment remains unknown. It cannot bind calmodulin and is a poor substrate for PKC [9, 66]. Nevertheless, GAP-43-3 might still bind F-actin (see Fig. 5 of [13]). It is possible that GAP-43-3 can directly block growth of actin filaments (Fig. 7).

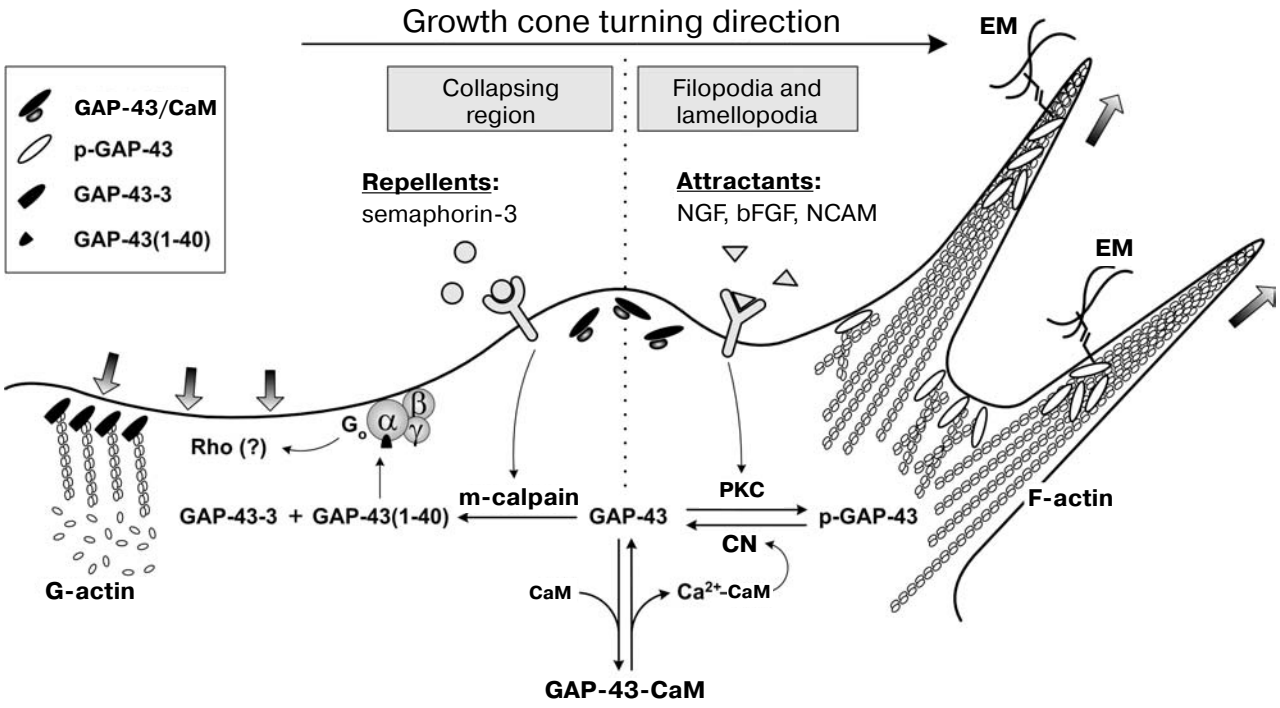

Fig. 7. Putative dual mechanism of involvement of GAP-43 in growth cone guidance by attractive and repulsive extracellular signals. The attractive signals (the right part of this scheme) cause local activation of PKC, which activates GAP-43 phosphorylation at Ser41 [16-18]. Phosphorylated GAP-43 participates in stabilization of adhesive contacts and F-actin filaments; this results in growth of filopodia and lamellopodia towards attractive signal [13-15]. GAP-43 phosphorylation at Ser41 also protects this protein against cleavage by calpain. Repulsive signals (the left part of this scheme) cause local increase in calcium ion concentrations inside the growth cone [52]; this results in dissociation of GAP-43/calmodulin complex. Ca^{2+}/calmodulin released from this complex activates calcium-dependent phosphatase (calcineurin) dephosphorylating Ser41 of GAP-43. m-Calpain activated by calcium ions cleaves dephosphorylated GAP-43; (this yields GAP-43 (1-40) and GAP-43-3 fragments). Activation of G_o protein by GAP-43 (1-40) causes local collapse followed by growth cone turning opposite to the repulsive signal direction [27, 28]. Small Rho GTPase [67] mediating repulsive signals in growth cones is one of the putative effectors of G_o protein [68]. GAP-43-3, presumably, blocks F-actin polymerization. CaM, calmodulin; CN, calcineurin; EM, extracellular matrix; p-GAP-43, GAP-43 protein phosphorylated at Ser41.

According to the proposed scheme, hyperactivation of calpain under conditions of potent inhibitory effect of the environment results in over accumulation of GAP-43 fragments. This results in total growth cone collapse followed by neurite retraction and induction of neuronal apoptosis. Thus, this scheme explains the molecular mechanism underlying involvement of GAP-43 in realization of both attractive and repulsive signals including those leading to development of apoptosis. This scheme explains increased apoptosis in brain of transgenic mice overexpressing wild type GAP-43 or GAP-43 with mutation Ser42→Ala (Ser42 is the phosphorylation site for PKC in chicken GAP-43 transgenic protein) but not with mutation Ser42→Asp modeling constitutively PKC-phosphorylated GAP-43 [31]. This is because mutation Ser42→Asp evidently induces GAP-43 resistance to calpain.

The dual mechanism of GAP-43 functioning proposed in the present study requires careful experimental verification. However, it is clear that results of the present study on GAP-43 proteolysis by calpain and modes of regulation of this process extend out knowledge of regulatory mechanisms responsible for control of physiological functions of GAP-43.

This study was supported by grants from the Russian Foundation for Basic Research (02-04-48521), INTAS (2001-0064), President of the Russian Federation (MK-689.2003.04), Government of St. Petersburg (PD04-1.4-244), and a fellowship of the Governor of Leningrad Region.

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