Effects of Semax and Its Pro-Gly-Pro Fragment on Calcium Homeostasis of Neurons and Their Survival under Conditions of Glutamate Toxicity

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Semax (100 μM) and its Pro-Gly-Pro fragment (20 and 100 μM) delayed the development of calcium dysregulation and reduction of the mitochondrial potential in cultured cerebellar granule cells under conditions of glutamate neurotoxicity. Incubation with these peptides improved neuronal survival by on average 30%. The neuroprotective effect of semax in cerebral ischemia/hypoxia can be due to improvement of mitochondrial resistance to "calcium" stress.

Key Words: *glutamate; semax; intracellular calcium; mitochondria*

Semax, an ACTH-like peptide devoid of hormonal activity and possessing an intrinsic neurotrophic effect, attract great attention of scientists in recent years [8]. Semax is a synthetic analog of ACTH(4-10) with Met-Glu-His-Phe-Pro-Gly-Pro (MEHFPGP) amino acid sequence synthesized at Institute of Molecular Genetics. The neuroprotective effects of semax were detected in experiments on animals exposed to hypobaric hypoxia and in clinical practice in the treatment of patients with brain stroke [1,3]. Semax improved survival of PC12 pheochromocytoma cells under conditions of oxidative stress induced by H_2O_2 [4]. A potent trophic effect of the preparation (comparable to the effect of nerve growth factor) on cholinergic neurons under conditions of glucose and oxygen deprivation was shown [2]. Semax proteolysis products also exhibited neurotropic activity. The mechanisms of the neuroprotective effects of semax and its analogs are little known. It was shown that semax specifically binds with membranes of rat brain cells [2], but no sites binding its Pro-Gly-Pro (PGP) fragment were detected on the plasma membrane. Semax in a concentration of 100 μM 5-7-fold increased the expression of mRNA of trophic factors BDNF and NGF in primary culture of glial cells [13]. We studied the mechanisms of the neuroprotective action of semax and PGP on the cell model of glutamate neurotoxicity. Glutamate is the main stimulatory neurotransmitter in the brain. However, during anoxic depolarization caused by acute cerebrovascular disorders glutamate concentration in the extracellular space can increase from about 1 μM to 1 mM [6] and cause neuronal damage and death [9,10, 12]. The neurodestructive effect of glutamate is explained by irreversible increase in cytosol concentration of Ca^{2+} ($[Ca^{2+}]_i$) and depolarization of mitochondria [9,12]. Glutamate causes a biphasic increase of $[Ca^{2+}]_i$ in cultured cerebellar, hippocampal, and cerebrocortical cells, phase 2 being linked with a drop of the mitochondrial potential ($\Delta\Psi_m$) [5,7,14]. After $\Delta\Psi_m$ collapse the cells do not restore the basal level of $[Ca^{2+}]_i$ and $\Delta\Psi_m$ after glutamate discontinuation and die (the so-called de-

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layed calcium dysregulation, DCD) [7,9,12]. We studied to what measure the neuroprotective effects of semax and its PGP fragment are determined by their effects on $[Ca^{2+}]_i$ homeostasis and mitochondrial potential during long-term exposure to glutamate.

MATERIALS AND METHODS

The study was carried out on 7-9-day primary culture of cerebellar granule cells from the brain of one-week-old Wistar rats (the method of culture preparation was described previously [5]). The levels of $[Ca^{2+}]_j$ and $\Delta\Psi_m$ were measured by fluorescent microscopy using a system for image analysis consisting of Axiovert-200 inverted-stage microscope (Zeiss), photofilter rings, SnapCool-fx digital camera, and Metafluor software. The cells were washed from the culture medium, suspended in buffered saline, and loaded with fluorescent dye Fura-2FF for measuring $[Ca^{2+}]_i$ (5 μM, 40 min) and Rh123 for $\Delta\Psi_m$ registration (3 μM, 15 min). The composition of the buffer was as follows: 130 mM NaCl, 5.6 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20.0 mM HEPES, and 5.0 mM glucose; pH 7.4. Slides with cells were then placed into a 0.2-ml perfusion chamber mounted on the microscope stage. Fluorescence was excited using photofilters

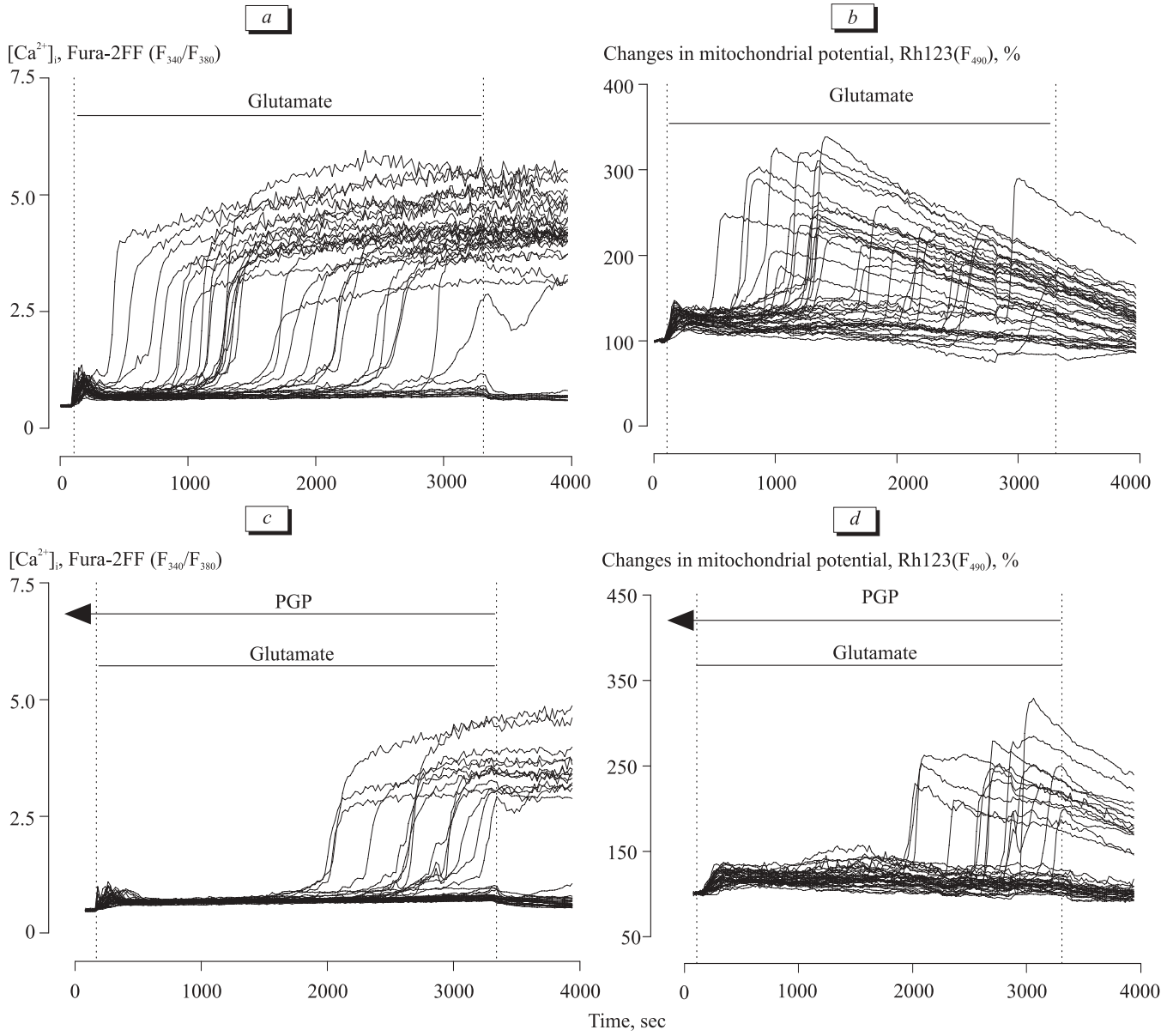

Fig. 1. Changes in $[Ca^{2+}]_i$ and $\Delta\Psi_m$ in cerebellar granule cells during and after glutamate treatment in control (*a*, *b*) and after incubation with 100 μM PGP (*c*, *d*).

with transmission wavelengths of 340 and 380 nm (Fura-2FF) and 490 nm (Rh123). Emission filter transmitted the spectrum of 505±15 nm for Fura-2FF and 535±15 nm for Rh123. The F_{340}/F_{380} fluorescence ratio in individual neurons reflected the level of $[Ca^{2+}]_i$. Changes in $\Delta\Psi_m$ were expressed in percents of initial Rh123 fluorescence taken for 100%. Neuronal survival was evaluated by the MTT test based on the capacity of dehydrogenases (active only in live cells) to reduce MTT (3-(4,5-dimethylthiasolyl-2)-2,5-diphenyltetrasolium bromide) to insoluble stained formazan. The cells were inoculated in 96-well plates, incubated with the corresponding peptides and glutamate for 1 h, washed, and after 3 h MTT was added (10% volume); the resultant formazan precipitate was dissolved in dimethylsulfoxide. Formazan absorption was measured spectrophotometrically (λ=492 nm). The data were processed using Student's *t* test. Probes and reagents from Sigma, Molecular Probe, and Fluka were used.

RESULTS

The levels of $[Ca^{2+}]_j$ and $\Delta\Psi_m$ changed in cerebellar granule cells exposed to 100 μM glutamate and 10 μM glycine (free from Mg^{2+}) for 50 min (Fig. 1, *a*, *b*). The primary elevation of $[Ca^{2+}]_i$ was followed by its minor decrease in the majority of neurons, after which (at different time) a second $[Ca^{2+}]_i$ rise developed. Phase 1 of $[Ca^{2+}]_i$ elevation was paralleled by slight depolarization of mitochondria, phase 2 was associated with a significant reduction of $\Delta\Psi_m$. Neurons with second elevation of $[Ca^{2+}]_i$ did not restore the basal $[Ca^{2+}]_i$ level and $\Delta\Psi_m$ after discontinuation of glutamate exposure, which attested to the development of DCD [9,12]. The effects of glutamate on $[Ca^{2+}]_i$ and $\Delta\Psi_m$ in the control and in the presence of semax (100 μM) or PGP (20 and 100 μM) were then compared. The main criteria for quantitative analysis of the effects were two parameters: number of cells responding to glutamate by second elevation of $[Ca^{2+}]_i$ and $\Delta\Psi_m$ drop (DCD development) and the time of DCD manifestation (t, sec). The cells in these experiments were incubated with peptides in saline 1 h before glutamate application, after which glutamate was added. The effect of PGP in a concentration of 100 μM on glutamate-induced shifts in $[Ca^{2+}]_i$ and $\Delta\Psi_m$ was noted: phase 2 of $[Ca^{2+}]_i$ rise and collapse of $\Delta\Psi_m$ were observed in the majority of cells much later than in the control (Fig. 1, *c*, *d*). Similar results were obtained in 4 other independent cultures.

Statistical analysis showed that semax and PGP significantly inhibited the development of DCD under the effect of glutamate. A relationship between the number of cells with DCD and duration of glutamate treatment in the control and after co-incubation with semax (100 μM) or PGP (20 and 100 μM; Fig. 2) was detected. By the 50th min of glutamate treatment the number of neurons with DCD reached 82.3±6.5%, while after incubation with PGP (100 3.7% cells ($p<0.05$); the effect of PGP in concentration of 20 μM was not inferior to that of 100 μM (Fig. 2). Semax (100 μM) was less effective than PGP (Fig. 2) and exhibited no effect in concentrations below 100 μM. The mean delay of phase 2 of $[Ca^{2+}]_i$ elevation during incubation with glu-

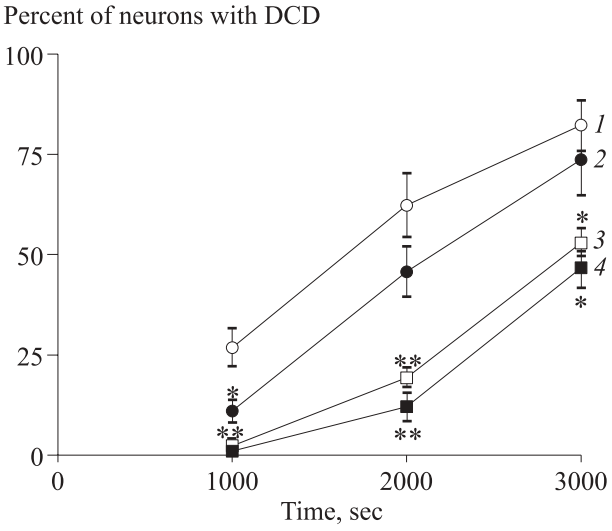

Fig. 2. Effects of semax (100 μM) and PGP (20 and 100 μM) on the relationship between neurons with DCD and duration of incubation with glutamate ($n\leq900$ cells per point). *1*) glutamate (control); *2*) glutamate+100 μM semax; *3*) glutamate+20 μM PGP; *4*) glutamate+100 μM PGP.

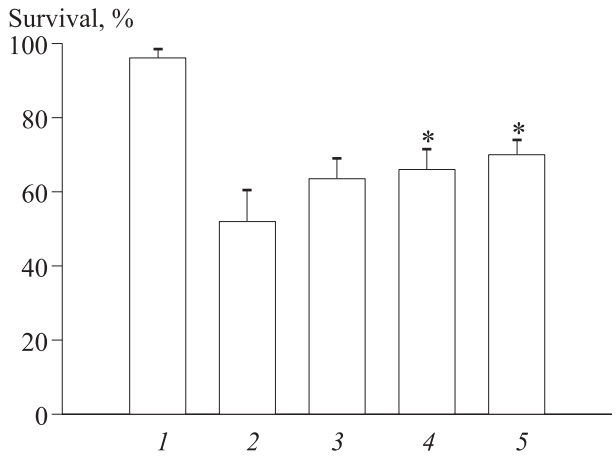

Fig. 3. Neuronal survival under conditions of glutamate toxicity. MTT test. Survival is directly proportional to formazan absorption and is expressed in % of control (100%). *1*) control; *2*) glutamate; *3*) glutamate+100 μM semax; *4*) glutamate+20 μM PGP; *5*) glutamate+100 μM PGP. *$p<0.05$ compared to incubation with glutamate (*2*).

tamate was 1470.0±13.6 sec. Addition of semax or PGP significantly prolonged this time (almost 2-fold; Fig. 2). It is noteworthy that mitochondrial depolarization similarly as phase 2 of $[Ca^{2+}]_i$ elevation, was observed later in these cells (after prolonged latency).

Neuronal death under conditions of toxic glutamate exposure are in line with the findings of fluorometry. Preincubation of cultures with semax or PGP improved neuronal survival after glutamate treatment by on average 30% ($p<0.05$; Fig. 3). PGP in a concentration of 100 μM was most effective.

Summing up the results, we conclude that semax and PGP prolonged the latency of DCD development. Since the development of mitochondrial depolarization, similarly as DCD, was delayed in neurons treated with semax and PGP, we hypothesized that mitochondria in these neurons were more resistant to the destructive effect of glutamate. These results suggest that the neuroprotective effects of the studied peptides are to a certain measure due to their effects on calcium homeostasis and mitochondrial function. The molecular mechanisms of the protective effect of these peptides remain not quite understood. Ca^{2+} entry into the cells via glutamate receptor channels causes receptor-mediated appearance of factors promoting accumulation of Ca^{2+} in mitochondria and collapse of the mitochondrial potential, such as reduced activity of protein kinase C phosphorylating many proteins, reduced content of ATP, activation of phospholipase, formation of free-radical compounds, *etc*. Oxidative stress can increase the probability of induction of nonselective pores in neuronal mitochondria with the release of cytochrome C and other apoptosis-provoking factors from mitochondria [12]. Semax stimulates the synthesis of neurotrophins, *e.g.* NGF and BDNF [13], and hence, its neuroprotective effect can be explained by enhanced expression of antioxidant defense proteins and increased resistance of mitochondria to oxidative and "calcium" stress [11]. Further studies of the effects of semax and its analogs can include measurements of the production of free-radical compounds in neurons exposed to glutamate in parallel with these peptides. By the present time we cannot rule out possible effects of semax and PGP on the efficiency of glutamate receptor activation. This problem deserves further investigation.

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