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Effect of Enteropeptidase on Survival of Cultured Hippocampal Neurons under Conditions of Glutamate Toxicity

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Abstract—The effects of full-size bovine enteropeptidase (BEK) and of human recombinant light chain enteropeptidase (L-HEP) on survival of cultured hippocampal neurons were studied under conditions of glutamate excitotoxicity. Low concentrations of L-HEP or BEK (0.1-1 and 0.1-0.5 nM, respectively) protected hippocampal neurons against the death caused by 100 μM glutamate. Using the PAR1 (proteinase-activated receptor) antagonist SCH 79797, we revealed a PAR1-dependent mechanism of neuroprotective action of low concentrations of enteropeptidase. The protective effect of full-size enteropeptidase was not observed at the concentrations of 1 and 10 nM; moreover, 10 nM of BEK caused death of 88.9% of the neurons, which significantly exceeded the cell death caused by glutamate (31.9%). Under conditions of glutamate cytotoxicity the survival of neurons was 26.8% higher even in the presence of 10 nM of L-HEP than in the presence of 10 nM BEK. Pretreatment of cells with 10 nM of either form of enteropeptidase abolished the protective effect of 10 nM thrombin under glutamate cytotoxicity. High concentrations of BEK and L-HEP caused the death of neurons mainly through necrosis.

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Key words: enteropeptidase, glutamate toxicity, apoptosis, hippocampal neurons, proteinase activated receptor

Enteropeptidase (enterokinase) (EC 3.4.21.9), a highly specific proteinase of processing, is synthesized by enterocytes of the duodenum and is localized in mucosa of duodenum and small intestine [1]. Activating trypsinogen during digestion, enteropeptidase very effectively catalyzes hydrolysis of the polypeptide chain detaching the *N*-terminal activating peptide after the sequence of -Asp-Asp-Asp-Asp-Lys- [2, 3]. This highly conserved sequence is present in trypsinogens of all vertebrates [4]. The resulting trypsin activates in turn some other zymogens including chymotrypsinogen, proelastase, procarboxypeptidase, prolipase, and prophospholipase [1]. Enteropeptidase mRNA is expressed in the stomach, large intestine, and in the brain [5] where trypsin-like proteinases have been found [6]. In some diseases (e.g. in acute pancreatitis) enteropeptidase is detected in the bloodstream [7, 8]. Trypsin and enteropeptidase are expressed in the cells of stomach carcinoma [9].

Human keratinocytes were recently shown to synthesize enteropeptidase and multiple forms of trypsinogens [10] including trypsinogen IV, which was initially identified in human brain [6]. By now both trypsin and trypsin-like serine proteinases are shown to play an important role not only in digestion, but also in the development of neurons, brain plasticity, and in neurodegeneration and neuroregeneration of brain [11, 12]. Therefore, it is rather important to study the effect of enteropeptidase on brain neurodegeneration.

Amino acid sequences of mammalian enteropeptidases [5, 13-17] are highly homologous, their identities being up to 82-85%. The enteropeptidase molecule consists of two polypeptide chains, a heavy one (120-140 kDa) and a light one (35-62 kDa) bound by a disul-

Abbreviations: APC, activated protein C; BEK, full-size bovine enteropeptidase; FXa, activated factor X of blood coagulation; L-HEP, recombinant light chain of human enteropeptidase; MTT, 3-(4,5-dimethylthiasolyl-2)-2,5-diphenyltetrasolium bromide; PAR, proteinase-activated receptor.

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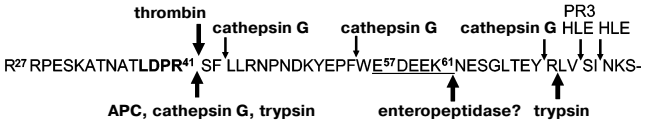

Proteolysis of *N*-terminal peptide of human PAR1 resulting in activation of the receptor (thrombin, activated protein C (APC), trypsin, cathepsin G (CG)) and in inactivation of the receptor (trypsin, CG, proteinase 3 (PR3), elastase (HLE), and hypothetically enteropeptidase)

fide bond. The light chain of enteropeptidase has a sequence which is conservative for serine proteinases: it contains the *N*-terminal Ile16 (IVGG), the typical catalytic triad Asp102-His57-Ser195, an oxyanionic cavity formed by atoms of major chain of residues 193 and 194, and also a typical for enzymes with primary trypsin specificity "binding pocket" with residue Asp189 on the bottom (S1-site of the substrate binding) [18]. In addition to the S1-site, the major determinant of recognition by enteropeptidase of the substrate DDDDK-sequence is residue Lys99, which is a fragment of the sequence of four positively charged enteropeptidase residues (96-99) [18]. Bovine enteropeptidase contains the sequence -Lys-Arg-Arg-Lys-, and porcine and human enteropeptidases contain the sequence -Arg-Arg-Arg-Lys- [17]. It is thought that in artificial substrates of enteropeptidase asparagine acid residues are equivalent to glutamic acid residues and the lysine residue is equivalent to the arginine residue [18, 19].

Recombinant light chains of bovine enteropeptidase (L-BEK) [19] and of human enteropeptidase (L-HEP) [20] retain high specificity of the natural enzyme in hydrolysis of the substrate polypeptide chain after the sequence DDDDK. Also, the natural full-size and short recombinant enzymes are markedly different in the efficiency of hydrolysis and some of their features in the case of both L-HEP and L-BEK [21].

Until recently, the sequence -Asp-Asp-Asp-Lys- was thought to be conservative and to occur in natural proteins only in the structure of the *N*-terminal activating peptides of different trypsinogens [4]. But recently it has been found that proteinase-activated receptors (PAR1) contain sequences similar to the activating peptide of trypsinogen: -Glu-Asp-Glu-Glu-Lys- (PAR1 of human and monkey), -Asp-Glu-Glu-Glu-Glu-Lys- (PAR1 of mouse), -Asp-Glu-Glu-Glu-Lys- (PAR1 of hamster and rat). Considering this and also data on the role of trypsin-like serine proteinases (thrombin, activated protein C, and trypsin IV) in degeneration of brain neurons [6, 22-25], it was interesting to study the effect of enteropeptidase on the membrane proteinase-activated receptor of thrombin (PAR1), which seems to be another natural substrate of the highly specific enzyme enteropeptidase. Thrombin interacts with PAR1 and provides effective proteolysis of the peptide bond Arg41–Ser42 in the extracellular domain of PAR and thus exposes a new *N*-terminus of the receptor, the so-called tethered ligand (SFLLRN), which acts as an agonist of this receptor [26, 27] (Scheme). Some proteinases cleave PAR at sites other than the activation sites with generation of receptors unable for further proteolytic activation. Thus, cathepsin G and trypsin not only cleave PAR1 at the site Arg41–Ser42, but also nonspecifically cleave other bonds within the receptor structure [28, 29]. This is associated with removal of the tethered ligand, which deprives the receptor of its sensitivity to thrombin. During hydrolysis of PAR1 by enteropeptidase at site Lys61–Asn62, the receptor can also be inactivated with the resulting termination of the signal (Scheme) [30]. This inactivation can be a regulatory mechanism for terminating PAR activation by extracellular proteinases and proteinases related with the cell surface [31] and serving as their inactivators.

PAR1 is widely distributed in the gastrointestinal tract and is found, in particular, on apical membranes of enterocytes [32]. Prothrombin mRNA and PARs are expressed in regions of the brain (cortex, striatum, hypothalamus, hippocampus, cerebellum, etc.) that are the most vulnerable to ischemic damage [11]. However, the functional role of enteropeptidase in the brain remains unclear.

In our experiments we studied the effect of full-size bovine enteropeptidase (BEK) and of the light chain of recombinant human enteropeptidase (L-HEP) on survival of cultured hippocampal neurons under conditions of glutamate cytotoxicity, which models neurodestructive processes in the brain.

MATERIALS AND METHODS

Materials used were as follows: bovine thrombin, ARAC, poly-D-lysine, geldanamycin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and BPTI (Sigma, USA); neurobasal medium A containing 2% Supplement B-27 and L-glutamine (Gibco, USA); the PAR1 antagonist SCH 79797 (TOCRIS, Great Britain).

Production of enteropeptidase. Enteropeptidase was isolated from mucosa of bovine duodenum and purified as described in [33]. Activities of the enzyme preparations were determined by rates of activation of trypsinogen and of hydrolysis of Z-Lys-S-Bzl [34].

L-HEP [20] was kindly presented by M. E. Gasparyan (Institute of Biological Chemistry, Russian Academy of Sciences).

Culture of hippocampal neurons. The studies were performed using primary cultures of hippocampal neurons (9-10-day-old) isolated from the brain of 1-3-day-old Wistar rats. The cell suspension (10^6 cells/ml) was prepared as described earlier [35] and transferred onto

cover slips (200 μl per slip) covered with poly-D-lysine (10 mg/ml). Upon incubation for 1 h at 37°C in the presence of 5% CO_2, unattached cells were removed and 1.5 ml of culture medium was added (neurobasal medium A containing 2% Supplement B-27 and 0.5 mM L-glutamine). On the third day arabinoside (ARAC, 10^{-5} M) was added to suppress the growth of glial cells.

Assessment of death of neurons. The death of neurons in cultures was assessed 24 h after exposure for 30 min to 100 μM glutamate and substances under study, which were added to the cells on replacing the culture medium by Hepes-saline buffer (HBSS). The HBSS composition was as follows (mM): NaCl, 145; KCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 1.0 (without $MgCl_2$ when the cells were exposed to glutamate); Gly, 0.01; Hepes, 20; glucose, 5 (pH 7.4). The studied compounds were added 15 min before the subsequent addition of glutamate or other substances.

The death of neurons was assessed using MTT [36]. A solution of MTT was added into the culture medium to the final concentration of 1 mg/ml, and the cells were incubated for 1.5 h at 37°C. Then the medium was removed, and dimethylsulfoxide was added to dissolve formazans. Absorption was measured spectrophotometrically at 590 nm with an Anthos Lucy-1 universal microplate spectrophotometer. The results were evaluated in percent with respect to control.

Morphological assessment of the death of neurons included the determination of fragmentation and condensation of nuclei with the fluorescent DNA-tropic dye Hoechst 33342 (absorption 360 nm, emission 460 nm), which easily penetrates into living cells and is bound with damaged fragmented DNA in regions of A-G pairs [37], and with the vital dye SYTO-13 (absorption 488 nm, emission 590 nm) [38]. Cell death was determined by the apoptotic morphology of the nucleus. The stained cells were examined and counted using an Axiovert 200 fluorescence microscope (Zeiss, Germany). Cells with highly condensed chromatin and lacking structural heterogeneity were considered as apoptotic. The collapsed chromatin acquired the shape of a ring, half-moon, sphere, or a bunch of grapes. The number of necrotic neurons was determined using ethidium homodimer (EthD-1), which stains cells with a disturbed integrity of the plasma membrane, the primary morphological sign of cell death. Using the dye SYTO-13 the number of living cells with normal nucleus was determined, and to a lesser degree the staining of cytoplasm that allowed us to visualize the soma of cells with outgrowths.

In each series of experiments the sample included three or four independent experiments, each of which included two or three coverslips of sister cultures. On each coverslip 500 cells were counted in eight-to-ten fields. The numbers of apoptotic, necrotic, and viable neurons were expressed in percent relative to the total number of cells.

Statistical processing of data. The data were processed statistically in paired samples using the Student's *t*-test or the Kruskal–Wallis test with *n* as the number of independent experiments. The findings are presented as the mean and the error of the mean.

RESULTS AND DISCUSSION

Effects of two enteropeptidase forms on survival of rat hippocampal neurons in culture. To study the effect of enteropeptidase on neurons, we chose cultured hippocampal neurons of rat brain. The primary culture of neurons is a good experimental model for studies on neurodestructive processes caused by glutamate because cytological and biochemical characteristics of cultured neurons are similar to those observed for neurons *in situ* [39].

Incubation of cells for 45 min in the presence of increasing concentrations of enteropeptidase resulted 24 h later in a dose-dependent decrease in the survival of the neurons. Low concentrations (0.1 nM) of BEK and L-HEP did not cause significant changes in the number of living neurons (Fig. 1). Increase in the BEK concentration to 1 nM resulted in significant death of some hippocampal neurons and in about 30% decrease in the fraction of living cells. Incubation of the cells with 10 nM L-HEP or BEK caused significant death of neurons (up to 60 and 85%, respectively) (Fig. 1). Toxic doses of L-HEP were higher (≥10 nM) than those of BEK (≥1 nM) (Fig. 1).

Thus, the toxic effect of 10 nM BEK was much more pronounced than the effect of 10 nM L-HEP.

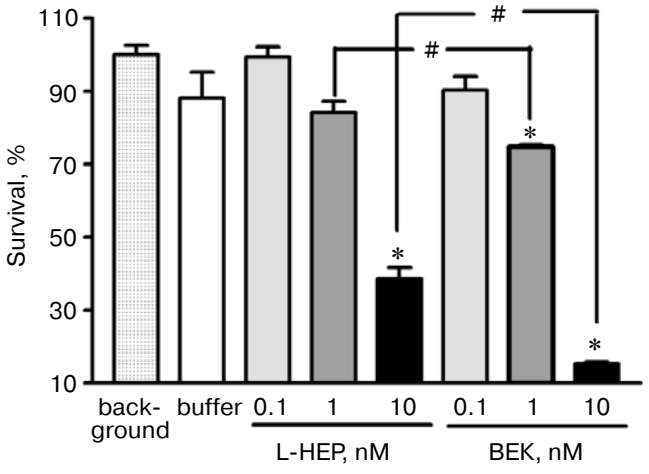

Fig. 1. Changes in survival of hippocampal neurons depending on the concentration of L-HEP and BEK. The survival of neurons was assessed using MTT 24 h later after 45-min incubation of the cells with the enteropeptidase. Results are presented in percent relative to control (background) as the mean and error of the mean. Here and in Figs. 2-5 differences are significant at $p < 0.05$; $n = 3$-4 independent experiments; *, compared to buffer; #, comparing the groups.

Effect of enteropeptidase on survival of hippocampal neurons under conditions of glutamate cytotoxicity. Hyperstimulation of glutamate receptors is known to cause the death of neurons and to be a universal triggering mechanism for development of neurodegeneration under conditions of excitotoxicity. We found earlier that *in vitro* hyperstimulation of glutamate receptors of rat hippocampal neurons caused the death of 31-34% of the cells 24 h after incubation with 100 μM glutamate [24, 25].

In the present work the effects of BEK and L-HEP on survival of hippocampal neurons were studied under conditions of glutamate cytotoxicity. Exposure of cultures for 30 min in medium containing 100 μM glutamate caused 24 h later a decrease in the number of living cells to 68.8% (Fig. 2). We assessed the survival of neurons in the presence of different concentrations (0.1-10 nM) of each enteropeptidase form under conditions of glutamate toxicity. At low concentrations, both L-HEP (0.1-1 nM) and BEK (0.1-0.5 nM) protected the hippocampal neurons against the death caused by glutamate (Fig. 2). The preincubation of cultures with bovine enteropeptidase (1 nM) decreased the survival of neurons treated with glutamate, whereas an increase in the enteropeptidase concentration to 10 nM significantly decreased the number of living neurons in the culture (Fig. 2). Note that the toxic effect was more pronounced in the presence of 10 nM BEK, when the fraction of living cells decreased to 22.4% (Fig. 2). L-HEP at the concentration of 10 nM increased the glutamate-induced death of neurons and decreased the number of living neutrons to 37.9%, this being 15.5% higher than the survival in the presence of 10 nM full-size BEK. This difference is significant (Fig. 2).

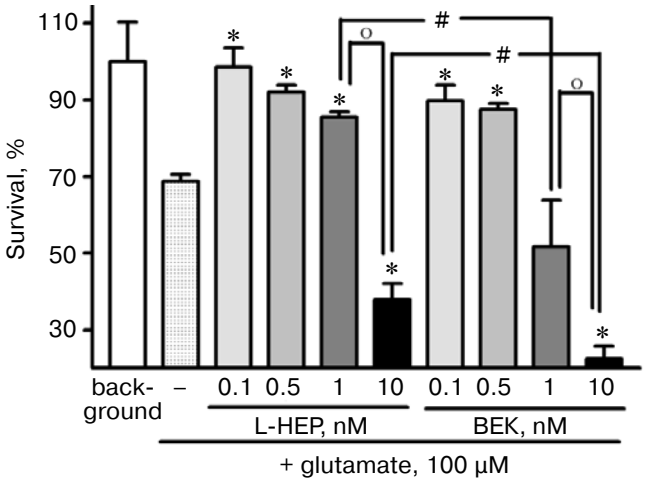

Fig. 2. Changes in survival of hippocampal neurons depending on concentration of L-HEP and BEK upon incubation of cultures with 100 μM glutamate for 30 min. The survival of neurons was assessed using MTT 24 h after the exposure to glutamate. Results are presented in percent relative to control (background). *, comparison to glutamate; # and °, comparing the groups.

Thus, bovine enteropeptidase at very low concentrations (0.1-0.5 nM) protected hippocampal neurons against glutamate excitotoxicity, and at concentrations of 1 and 10 nM did not protect them. Moreover, at the concentration of 10 nM this proteinase caused threefold higher death of neurons (22.4% survival) than glutamate (68.8% survival). As differentiated from BEK, human recombinant enteropeptidase at the concentration of 1 nM protected hippocampal neurons against the glutamate cytotoxicity. Although the survival of neurons decreased in the presence of 10 nM L-HEP, it remained twofold higher than the survival in the presence of 10 nM BEK under conditions of glutamate toxicity (Fig. 2). In this connection, we then studied the possibility of enteropeptidase as a terminator of neuroprotective effects of low concentrations of thrombin, which is an agonist of PAR1.

Influence of enteropeptidase on neuroprotective effect of thrombin under conditions of glutamate toxicity. Low concentrations of thrombin (0.1-10 nM) were earlier shown to protect hippocampal neurons of rats against glutamate cytotoxicity [24, 25]. In our experiments 10 nM thrombin also protected hippocampal neurons against glutamate cytotoxicity, increasing the fraction of living neurons by nearly 25% (92.3 as compared to 68.8% in the presence of glutamate alone). Pretreatment of cells with 10 nM enteropeptidase (BEK and L-HEP) abolished the protective effect of 10 nM thrombin against the glutamate cytotoxicity, decreasing the number of living cells to 42.6 and 34.6%, respectively (Fig. 3). At the concentrations of 0.1 and 0.5 nM, BEK did not abolish the protective effect of thrombin, and the survival was not significantly different from the effect of thrombin alone under conditions of excitotoxicity: 88.8 and 87.5% for 0.1 and 0.5 nM BEK, respectively, as compared to 92.3% living neurons under the combined influence of thrombin and glutamate (Fig. 3) (data for 0.5 nM BEK not presented).

The toxicity of high concentrations of BEK and L-HEP can be explained by inactivation of PAR1 as a result of hydrolysis by the Lys61–Asn62 bond [30]. We found that the 15-min preincubation of cultures with the PAR1-specific antagonist SCH 79797 at the concentration of 0.2 μM did not abolish the influence of either enteropeptidase form at the concentration of 10 nM on the survival of neurons under conditions of glutamate cytotoxicity in the presence of thrombin (Fig. 3). The neuroprotective effect of low concentrations of L-HEP and BEK is still unexplained, although the PAR1 antagonist SCH 79797 significantly decreased the protective effect of 0.1 nM enteropeptidases in the presence of thrombin (Fig. 3).

The death of neurons under toxic influences can occur through necrotic or apoptotic pathways. By morphologic studies, we revealed that glutamate caused the death of neurons mainly through apoptosis, whereas the pretreatment of the cells with thrombin decreased the

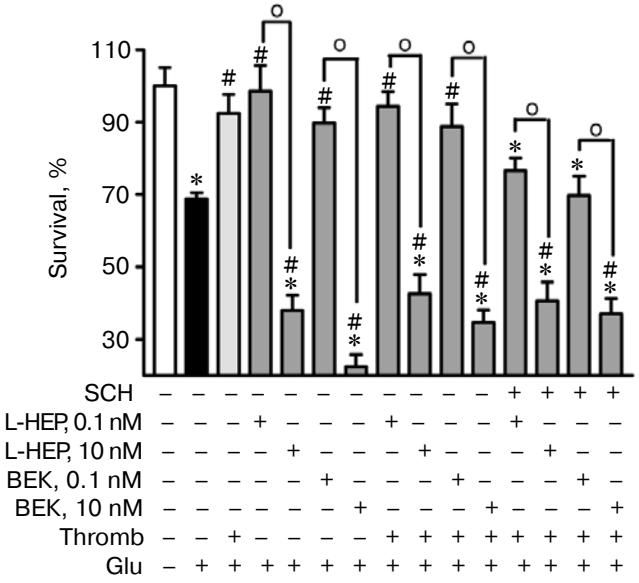

Fig. 3. Effects of L-HEP and BEK on survival of hippocampal neurons under glutamate toxicity (Glu) or under the action on the cells of 10 nM thrombin (Thromb) and 100 μM glutamate. The cell cultures were pretreated for 15 min in the presence of the PAR1 antagonist (SCH) and/or incubated for 15 min in the presence of enteropeptidase, and then 10 nM thrombin and 100 μM glutamate were added (incubation for 30 min). The death of neurons was assessed using MTT 24 h later after the exposure to glutamate. Results are presented in percent relative to control (the survival in the absence of the studied substances). * and #, compared to the effect of thrombin under conditions of glutamate toxicity and relative to glutamate, respectively; °, comparing the groups.

fraction of apoptotic neurons from 33.1 to 13.1% (Fig. 4). Enteropeptidase (10 nM) in the presence of glutamate significantly increased the fraction of necrotic neurons (from 15.8% under the influence of glutamate alone to 32.5 and 41.17% in the presence of human and bovine enteropeptidase, respectively) (Fig. 4) and increased the number of dead neurons compared to glutamate alone (Figs. 2 and 3). High concentration of enteropeptidase (10 nM) abolished the neuroprotective effect of 10 nM thrombin, increasing the number both of apoptotic and even more markedly of necrotic neurons (Fig. 4).

Thus, low concentrations of either enteropeptidase form displayed neuroprotective effect under conditions of glutamate toxicity, acting either alone or in the presence of thrombin. But increasing the enteropeptidase concentration to 10 nM significantly decreased the survival of hippocampal neurons under glutamate cytotoxicity. Moreover, the high concentration of enteropeptidase abolished the neuroprotective effect of thrombin under these conditions.

Involvement of PAR1 in effect of enteropeptidase on survival of hippocampal neurons under glutamate toxicity. To elucidate the role of PAR1 in the effect of enteropeptidase on hippocampal neurons under glutamate cytotoxicity, we used the specific nonpeptide PAR1 antagonist SCH 79797 (SCH) [40] and confirmed the earlier data on the involvement of PAR1 in the protective effect of thrombin (Fig. 5). SCH at the concentration used did not change the survival of neurons either alone or in the presence of glutamate (Fig. 5). A 15-min preincubation of cultures with 0.2 μM SCH 79797 did not abolish the effect of either form of enteropeptidase at the concentration of 10 nM under glutamate cytotoxicity either in the presence or in the absence of thrombin (Figs. 3 and 5). The antagonist of PAR1 also did not abolish the death of hippocampal neurons caused by high concentrations of enteropeptidase (data not presented). However, the antagonist of PAR1 abolished the neuroprotective effect of low concentrations of both forms of enteropeptidase (Fig. 5); therefore, PAR1 is supposed to be involved in the protective effect of this proteinase.

The directed effect of L-HEP and BEK at low concentrations was confirmed by the finding that 15-min preincubation of cultures with SCH 79797 abolished the effect of the enteropeptidases under glutamate cytotoxicity both in the presence and in the absence of thrombin (Figs. 3 and 5). This supported our hypothesis that the effect of enteropeptidase was mediated by PAR1 similarly the effects of thrombin and of a peptide agonist of PAR1.

By analogy with a similar protective effect of thrombin (0.1-10 nM), which is explained by a specific activation of PAR1 on the cleavage of the Arg41–Ser42 bond in

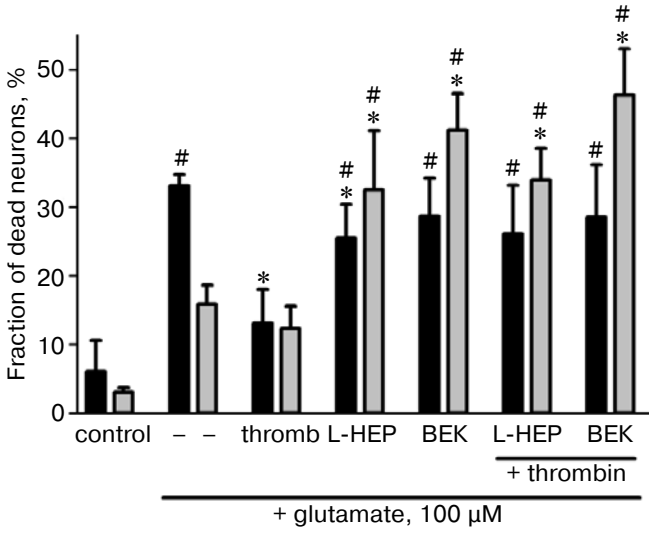

Fig. 4. Effect of thrombin (10 nM) and of L-HEP (10 nM) or BEK (10 nM) on the level of apoptosis (black columns) and necrosis (gray columns) under conditions of glutamate toxicity. The death of neurons was assessed morphologically 24 h later using the DNA-tropic fluorescent dye Hoechst 33342 for identification of apoptotic neurons and ethidium homodimer (EthD-1) for determination of number of necrotic cells. *, compared to glutamate; #, compared to the group of thrombin + glutamate.

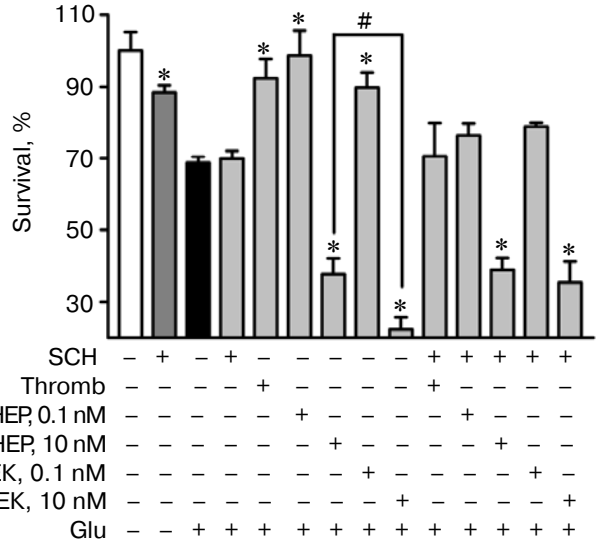

Fig. 5. Effect of SCH 79797 (SCH, 0.2 μM) on the effects of peptidases (thrombin, L-HEP, and BEK) on the survival of neurons under conditions of glutamate toxicity (Glu). The survival of neurons was assessed using MTT 24 h after the exposure to glutamate. Results are presented in percent relative to control (survival in the absence of the studied substances). *, compared to glutamate; #, comparing the groups.

the PAR1 exodomain [25], it seems reasonable to suppose that enteropeptidase can also activate this receptor. However, results obtained on model peptides indicate that enteropeptidase preferentially hydrolyzes the polypeptide chain in the region of the -EDEEK61-N62E- amino acid sequence, but not in the region of LDPR41-S42F- [21, 31].

Thus, our findings suggest that low concentrations of both enteropeptidase forms can protect hippocampal neurons against glutamate toxicity. The range of protective concentrations of L-HEP is wider than the range of BEK concentrations. Using the antagonist SCH 79797 allowed us to reveal a PAR1-dependent mechanism of the neuroprotective effect of low concentrations of the neuropeptidase, similarly to that earlier found for low concentrations of thrombin. These findings confirm earlier data that bovine enteropeptidase determines the PAR1-dependent calcium response of thrombin in hippocampal neurons [30]. High concentrations of both forms of enteropeptidase are toxic for cultured hippocampal neurons and sharply decrease the cell survival of intact neurons and neurons under glutamate toxicity. It seems that enteropeptidase at high concentrations, similarly to cathepsin G and trypsin, can cleave certain peptide bonds in the receptor, which results in removal of the tethered ligand and inactivation of the receptor.

Our findings suggest that the effect of low concentrations of enteropeptidase should be realized through activation of PAR1 (hydrolysis of the Arg41–Ser42 bond) similarly to the action of thrombin, whereas the effect of its high concentrations should be mediated through inactivation of this receptor (hydrolysis of the Lys61–Asn62 bond). In previous works [23, 24] we have reported the differently directed action of low and high concentrations of PAR1-specific proteinases: thrombin and activated protein C. Further studies are needed for explanation of the differently directed actions of low and high concentrations of enteropeptidase.

The concentration-dependent properties of enteropeptidase found by us enlarge the range of potential activators and terminators of PAR1. Thus, the data on both the protective and toxic effects of enteropeptidase are promising for detecting new pathways regulating cell functions mediated by these proteinases in some physiological and pathophysiological processes.

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