Proteinase-Activated Type 1 Receptors are Involved in the Mechanism of Protection of Rat Hippocampal Neurons from Glutamate Toxicity

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Survival of cultured rat hippocampal neurons was estimated 4, 24, and 48 h after 15-min exposure to the toxic effect of glutamate under conditions of pre- or coincubation with 10 nM thrombin. Thrombin inhibited glutamate-induced apoptosis in neurons 24 and 48 h after treatment, but had no effect on necrosis. Selective peptide agonist of proteinase-activated type 1 receptors simulated, but receptor antagonist suppressed the neuroprotective effect of thrombin. Our results suggest that peptide antagonist of type 1 receptors play a role in the mechanisms of neuronal protection from glutamate toxicity.

Key Words: *brain; neurotoxicity; glutamate; thrombin; proteinase-activated type 1 receptors*

Hyperstimulation of glutamate receptors underlies death of brain neurons during ischemic and hemorrhagic strokes, brain traumas, and some neurodegenerative diseases [3,4,7]. These disorders are associated with increased permeability of the blood-brain barrier, which results in the appearance of serine thrombin proteinase in the nervous tissue. Thrombin causes cerebral edema, neuronal death of during hypoglycemia, and apoptosis in cultured bird hippocampal neurons [5,13,14]. Recent studies showed that thrombin in very low concentrations serves as a neuroprotector. For example, intracerebral administration of thrombin in low doses (thrombin preconditioning) alleviates symptoms of brain injury produced by treatment with thrombin in high doses, intracerebral hemorrhages, and cerebral ischemia [9,15]. Expression of prothrombin was detected in the brain [5,12]. Probably, thrombin is formed in the brain and activates specific receptors without impairment of the blood-brain barrier. Expression of receptors for serine proteinases (type 1-4 PAR) was found in neurons and glial cells in various regions of the central nervous system (CNS) [11,15]. The intracellular mechanisms mediated by brain PAR attract much attention. Previous studies showed that binding of thrombin to PAR on hippocampal neurons induced a short-term release of Ca^{2+} from intracellular stores and Ca^{2+} entry through the plasma membrane.

Here we studied the influence of thrombin on survival of hippocampal neurons exposed to the toxic effect of glutamate. Synthetic peptides of PAR agonists and antagonists were used to evaluate the type of receptors mediating this effect.

MATERIALS AND METHODS

Experiments were performed on a 9-14-day-old primary culture of hippocampal neurons from Wistar rats. Cell suspension for culturing was obtained as described elsewhere [3]. This suspension was put on cover glasses (1 hippocampus, 200 ml per 1 glass) coated with poly-D-lysine (10 mg/ml). The cells were

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allowed to adhere at 37°C and 5% CO_2 for 2 h. Nonadherent cells were removed and 1 ml culture medium (neurobasal medium A containing 2% Supplement B-27 and 0.5 mM L-glutamine) was added. Arabinoside (10^{-5} M) was added on days 3-4 to suppress growth of glial cells. Neuronal survival was estimated by morphological and biochemical methods. Nuclear fragmentation was studied by the morphological method with fluorescent dyes. Hoechst-33342 (Sigma) and ethidium bromide (Sigma) were used to study apoptosis and necrosis, respectively. Examination was performed 4, 24, and 48 h after 15-min exposure to glutamate alone or in combination with thrombin or synthetic peptides. We used a selective PAR1 agonist (TFLLRN, Biosyntan) and PAR antagonist (Mpr(Cha), mercaptopropionyl-F(Cha-Cha)RKPNDK-NH_2; Kawabata, Japan). The cells were washed 2 times with a buffer (145 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM HEPES, and 5 mM glucose; pH 7.4), incubated with Hoechst-33342 (1 mg/ml) dissolved in buffer of 1% bovine serum albumin (37°C, 20 min), treated with 1 mg/ml ethidium bromide (37°C, 10 min), washed 3 times with buffer, and fixed with 4% formaldehyde. Stained cells were examined under an Axiovert 200 fluorescence microscope (Zeiss). Fluorescence of Hoechst-33342 and ethidium bromide was recorded at excitation wavelengths of 360 and 550 nm, respectively. The results were analyzed using Meta Fluor software (Universal Imaging).

In series II neuronal survival was estimated by activity of lactate dehydrogenase (LDH) released into the culture medium. LDH activity was measured spectrophotometrically on an Anthos Lucy1 microplate luminometer (Anthos labtec Instruments, Salzburg) at 340 nm using LDH-L reagent (Diagnostic Chemicals Ltd., Charlottetown). LDH activity in cultures was assayed 24 h after 15-min exposure. The percentage of dead neurons was calculated as the ratio between LDH activity in the medium and total LDH activity multiplied by 100%. Total LDH activity was estimated as LDH activity in the culture medium after 15-min incubation of cultured cells in the solution of 0.2% triton X-100 at 37°C. The results were analyzed by pairwise Student's *t* test.

RESULTS

In series I survival of hippocampal neurons was studied 4, 24, and 48 h after 15-min exposure to glutamate (100 μM glutamate, 10 μM glycine, and 0 Mg^{2+}), thrombin (10 nM), or selective synthetic agonist of PAR1 (PAR1-AR, 100 μM, Fig. 1). Apoptosis in 12.8, 33.7, and 41.8% neurons was revealed 4, 24, and 48 h after incubation of cells with glutamate (Fig. 1). Thrombin was less potent in causing cell death (6.6, 11.0, and 23.8% cells after 4, 24, and 48 h, respectively). The effect of peptide PAR1-AR was similar to that of thrombin. The count of necrotic cells remained unchanged with increasing the period between treatment and staining (25, 10, and 12% cells after exposure to glutamate, thrombin, and PAR1-AR, respectively). In the next series neuronal survival was studied 24 h after treatment.

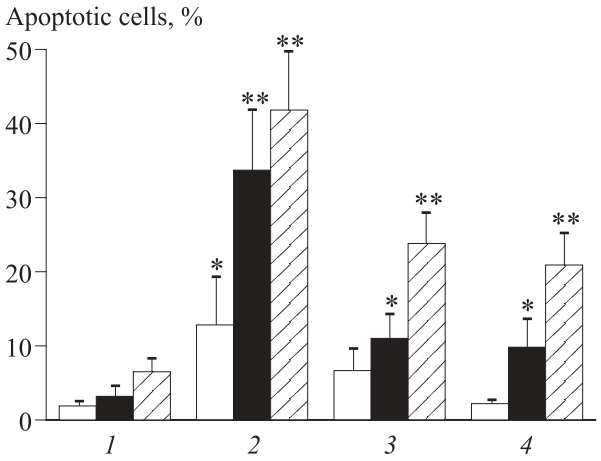

Fig 1. Count of apoptotic hippocampal neurons 4, 24, and 48 h after 15-min incubation of cells with glutamate, thrombin, or peptide PAR1 agonist (spectrofluorometric study). Control (*1*); glutamate (*2*); thrombin (*3*); and peptide agonist (*4*). Light bars, 4 h; dark bars, 24 h; shaded bars, 48 h. **p*<0.05 and ***p*<0.01 compared to the control; *n*=5.

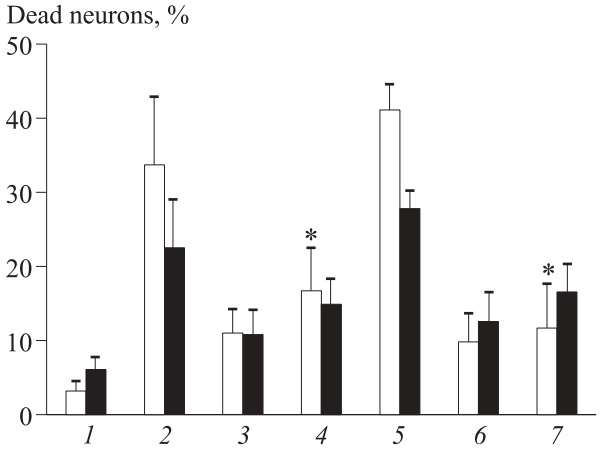

Fig. 2. Effect of thrombin, peptide PAR1 agonist, and PAR1 antagonist on glutamate-induced death of hippocampal neurons (spectrofluorometric study). Control (*1*); glutamate (*2*); thrombin (*3*); thrombin and glutamate (*4*); PAR1 antagonist and glutamate (*5*); peptide PAR1 agonist (*6*); and PAR1 agonist and glutamate (*7*). Light bars: apoptosis; dark bars, necrosis. **p*<0.01 compared to glutamate; *n*=6.

We studied the effect of a PAR1 antagonist Mrp(Cha) on survival of neurons. Mrp(Cha) in a concentration of 100 μM did not cause death of neurons. The degree of PAR1-AR-induced apoptosis in neurons decreased to the control level after combined treatment with this compound and Mrp(Cha). These data

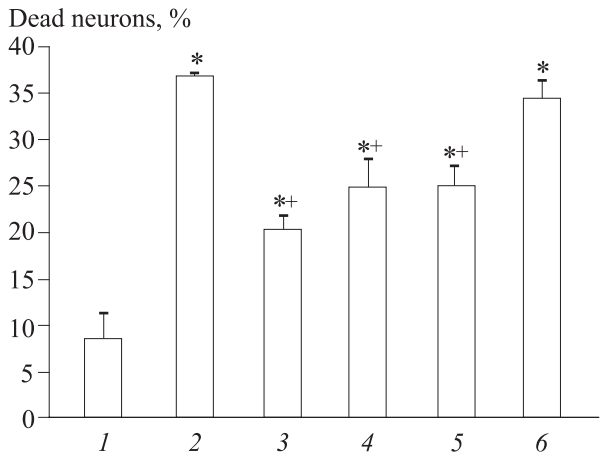

Fig. 3. Effect of thrombin, PAR1 agonist, and PAR1 antagonist on the percentage of dead hippocampal neurons 24 h after 15-min exposure to glutamate (by lactate dehydrogenase activity). Control (*1*); glutamate (*2*); thrombin (*3*); thrombin and glutamate (*4*); peptide PAR1 agonist and glutamate (*5*); and PAR1 antagonist, thrombin, and glutamate (*6*). $p<0.05$: *compared to the control; +compared to glutamate; $n=5$.

suggest that thrombin causes neuronal death by activating PAR1.

We studied the effects of thrombin and PAR1-AR on survival of hippocampal neurons after 15-min exposure to glutamate (Fig. 2). After 30-min preincubation of cells with thrombin (or coincubation with thrombin and glutamate) the count of apoptotic neurons decreased by 2 times compared to that observed upon glutamate treatment (16.7±6.9 and 33.7±9.3%, respectively, $p<0.01$, Fig. 2). Peptide PAR1-AR had a similar protective effect. After treatment with this peptide the number of apoptotic neurons decreased to the level observed in experiments with PAR1-AR (11.7±3.9% for PAR1-AR and glutamate; 9.8±5.9% for PAR1-AR). A PAR1 antagonist Mrp(Cha) abolished the protective effect of thrombin (Fig. 2). Our findings suggest that PAR1 mediates the neuroprotective effect of thrombin. Thrombin and PAR1-AR did not modulate glutamate-induced necrosis of cells, including secondary necrosis of primarily apoptotic cells (Fig. 2).

In series II we studied survival of hippocampal neurons exposed to 15-min incubation with glutamate after preincubation with thrombin or PAR1-AR. LDH activity was measured (Fig. 3). Death of 37% cultured cells was observed 24 h after exposure to 100 μM glutamate (Fig. 3). Preincubation with thrombin decreased the percentage of dead cells to 24% ($p<0.05$). Preincubation of cells with 100 μM PAR1-AR improved neuronal survival rate after glutamate treatment (Fig. 3). PAR1-AR antagonist Mrp(Cha) (100 μM) abolished the protective effect of thrombin. Quantitative differences in the results of spectrofluorometry are probably related to the fact that LDH serves as a marker of cell membrane destruction (necrosis, including secondary necrosis of primarily apoptotic cells).

Thrombin caused death of up to 20% hippocampal neurons (Figs. 1 and 3). Under conditions of combined treatment, thrombin in the low dose protected these cells from apoptosis observed in the delayed period after glutamate administration. The mechanism of neuroprotection is poorly understood. Activation of PAR1 coupled to $G\alpha_{q11}$ proteins results in hydrolysis of phosphoinositides with the formation of protein kinase C activator diacylglycerol [2]. Phosphorylation of plasma membrane Na^+/K^+ ATPase with protein kinase C decreases enzyme activity and reduces ATP consumption during treatment with glutamate [8]. These changes probably contribute to the release of Ca^{2+} excess from neurons by the membrane ATP-dependent Ca^{2+} pump and recovery of intracellular Ca^{2+} homeostasis during the postglutamate period. It cannot be excluded that intracellular signal transduction upon activation of PAR1 and excitation of metabotropic glutamate receptors coupled to G proteins are mediated by the same mechanisms. Preactivation of these receptors decreases the toxic effect of postexposure to glutamate [10]. Initiation and prevention of apoptosis via activation of PAR1 are mediated by various signal pathways. They include JAK/STAT, RhoA, MLCK (myosin light chain kinase), ERK1/2, and members of the Bcl-2 family [2]. The increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) produced by activation of PAR1 [1] modulates function of several intracellular factors in the nucleus and cytoplasm (transcriptional factors NF-kB, FosB/JunD AR-1, *etc*.) and stimulates/inhibits expression of proteins (Bax/Bad of the Bcl family, AIF, MAP kinase, and caspases 3, 7, and 9). Activity of these factors determines the degree of apoptosis upon exposure to the toxic effect of glutamate [6]. Study of the mechanism of interaction between glutamate receptors and PAR would allow us to develop new methods for the protection of neurons during diseases of CNS.

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