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Activated Protein C via PAR1 Receptor Regulates Survival of Neurons under Conditions of Glutamate Excitotoxicity

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Abstract—The effect of an anticoagulant and cytoprotector blood serine proteinase—activated protein C (APC)—on survival of cultured hippocampal and cortical neurons under conditions of glutamate-induced excitotoxicity has been studied. Low concentrations of APC (0.01-10 nM) did not cause neuron death, but in the narrow range of low concentrations APC twofold and stronger decreased cell death caused by glutamate toxicity. High concentrations of APC (>50 nM) induced the death of hippocampal neurons similarly to the toxic action of glutamate. The neuroprotective effect of APC on the neurons was mediated by type 1 proteinase-activated receptor (PAR1), because the inactivation of the enzyme with phenylmethylsulfonyl fluoride or PAR1 blockade by a PAR1 peptide antagonist ((Tyr[1])-TRAP-7) prevented the protective effect of APC. Moreover, APC inhibited the proapoptotic effect of 10 nM thrombin on the neurons. Geldanamycin, a specific inhibitor of heat shock protein Hsp90, completely abolished the antiapoptotic effect of 0.1 nM APC on glutamate-induced cytotoxicity in the hippocampal neurons. Thus, APC at low concentrations, activating PAR1, prevents the death of hippocampal and cortical neurons under conditions of glutamate excitotoxicity.

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Key words: activated protein C, glutamate toxicity, apoptosis, hippocampal and cortical neurons, proteinase-activated receptor, geldanamycin

Activated protein C (APC) is a narrow specificity serine proteinase of the trypsin family which is generated during the initiation phase of blood coagulation as a result of limited proteolysis of blood protein C by thrombin bound with endothelial thrombomodulin [1, 2]. Activation of protein C (PC) by the thrombin–thrombomodulin complex is considerably accelerated on the binding of PC with an endothelial protein C receptor (EPCR) [3, 4]. APC inhibits the generation of thrombin during the early stage of blood coagulation by a negative feedback mechanism due to inactivation of the blood coagulation cofactors via hydrolysis of three peptide bonds in factors Va and VIIIa [5-7].

APC is a multifunctional enzyme involved in the regulation of blood coagulation and of inflammation and apoptosis. This enzyme manifests antiinflammatory and cytoprotective features [8-10]. A recombinant form of APC (drotrecogin α) lowers the death of patients with severe sepsis [11-13]. APC inhibits apoptosis and suppresses inflammation by changing the gene expression profile [14, 15] and regulates endothelium dysfunction by decreasing the production of proinflammatory cytokines by activated monocytes [16-18]. APC prevents the death of cortical neurons as a result of NMDA- and staurosporine-induced apoptosis [19].

Molecular mechanisms of the antiinflammatory and antiapoptotic effects of APC are not clear in detail. APC induces protective genes in the endothelial cells either through activation of EPCR [20-22] or through the receptor cascade EPCR-PAR1 [23]. Some data indicate that the PAR1 and PAR3 subtype receptors are involved in neuroprotection in NMDA- and staurosporine-induced apoptosis of neurons [19].

Studies on the expression of PAR (proteinase-activated receptor) by neurons and its contribution to the effect of APC on the cells seem to be very promising for

Abbreviations: APC) activated protein C; EPCR) endothelial protein C receptor; Hsp90) heat shock protein 90; PAR) proteinase-activated receptor; PAR1-AP) PAR1 peptide agonist; PC) protein C; PMSF) phenylmethylsulfonyl fluoride.

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elucidation of mechanisms of the influence of the enzyme on the survival/death of neurons under conditions of excitotoxicity. It is known that hyperstimulation of glutamate receptors leads to the death of neurons and is a universal mechanism for triggering degeneration of neurons in excitotoxicity. Primary culture of neurons is a suitable experimental model for studies on neurodegeneration induced by glutamate (cytological and biochemical characteristics of neurons in culture are similar to those of neurons *in situ*) [24]. There are still insufficient data on the role of APC in neuron survival under glutamate-induced death. Under conditions of glutamate excitotoxicity, thrombin at the concentration of 1-10 nM was earlier shown to protect hippocampal neurons due to PAR1 activation, although the higher concentration of thrombin (more than 10 nM) could cause the death of neurons [25]. Geldanamycin, a specific inhibitor of the heat shock protein 90 (Hsp90), prevents the thrombin-caused changes in the astrocyte skeleton but does not influence the cell morphology in the absence of the enzyme [26]. The PAR1 molecule C-end specifically interacts with the cytosolic form of Hsp90 in yeast [26, 27].

In the present work the APC effect was studied on cultured hippocampal neurons [28] and on brain cortex neurons under normal and glutamate excitotoxicity conditions, and the antiapoptotic effect of APC on the neurons was shown to be mediated by PAR1 and Hsp90.

MATERIALS AND METHODS

Cultures of hippocampal and cortical neurons. The studies were performed on primary cultures of hippocampal (9-10 days) or cortical neurons (7-8 days) isolated from the brain of 1-3-day-old Wistar rats. The cell suspension (10^6 cell/ml) was prepared as described in [29] and placed onto cover slips (200 µl per slip) covered with poly-D-lysine (10 mg/ml). After incubation for 1 h at 37°C in the presence of 5% CO_2, the unattached cells were removed and 1.5 ml of the culture medium was added (neurobasal medium A containing 2% Supplement B-27 and 0.5 mM L-glutamine). On the 3rd-4th day, the cultures were treated with 10^{-5} M arabinoside (ARAC) for 24 h to suppress growth of glial cells.

Assessment of neuron death. The death of neurons in the culture was determined 24 h after exposure to 100 µM glutamate for 30 min and to substances under study, which were added to the cells placed during this treatment into Hepes-saline buffer instead of the culture medium. The buffer composition was as follows (in mM): NaCl, 145; KCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 1.0 (without $MgCl_2$ during the exposure to glutamate); glycine, 0.01; Hepes, 20; glucose, 5 (pH 7.4). Cell death was determined biochemically with MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) [30] and by a morphological approach. Aqueous solution of MTT was added into the culture medium to the final concentration of 1 mg/ml, and the cells were incubated for 3 h at 37°C. Then the medium was removed, and DMSO was added to dissolve formazans. The absorbance was measured at 590 nm with a universal Anthos Lucy-1 microplate spectrophotometer. The results were expressed in percent with respect to the control. The morphological determination of neuron death included the assessment of nuclear fragmentation and chromatin condensation with a DNA-specific fluorescent dye Hoechst 33342 (excitation at 360 nm, emission at 460 nm) which could easily penetrate into living cells and bind with damaged fragmented DNA [31] and with the vital dye SYTO-13 (excitation at 488 nm, emission at 590 nm) [32]. In the cells stained with Hoechst 33342 the nucleus state was assessed visually using an Axiovert 200 microscope (Zeiss, Germany). The cells with pycnotic and incorrectly shape nuclei were considered as apoptotic, and their number was expressed in percent of the total cell number.

In every series of experiments, a sample included 5-7 independent experiments with 2-3 specimens of sister cultures. On each slip, 200-500 cells were counted in 8-10 fields. The number of apoptotic neurons was expressed in percent of the total cell number.

Recording of the intracellular calcium concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was determined by fluorescence microscopy with high affinity Fura-2 fluorescent indicator. The cells were loaded with membrane-penetrating Fura-2/AM (5 µM, 40 min, at 26°C) mixed with 0.02% nonionic detergent Pluronic F-127 (Molecular Probes, USA), and then the cultures were washed in Hepes-saline buffer. The cell-carrying slip was placed into a 0.2-ml perfusion chamber mounted on the table of the Axiovert-200 inverted microscope, and the cultures were washed in Hepes-saline buffer. Fluorescence of Fura-2 was excited by alternating illumination of the cells at 340 and 380 nm within 100-200 msec with an interval of 10 sec; the emission was recorded in the range of 505-535 nm. The cell images were obtained using a cooled CCD-chamber (Roper Scientific, CoolSnap-fx, USA). The digital record of the experiment and data processing were performed with the Metafluor 6.1 computer program (Universal Imaging Corp., USA). Absolute values of $[Ca^{2+}]_i$ were determined using calibration solutions as described in [33]. All measurements were performed at room temperature (22-26°C).

Reverse transcription (RT) and polymerase chain reaction (PCR). Total RNA was isolated with an SV Total RNA Isolation System Kit (Promega, USA), and $4 \cdot 10^6$ neurons per sample were lysed. The sample was treated with DNase on a column during the isolation. The reverse transcription was performed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania) according to the producer's protocol.

PCR was performed in the final volume of 25 µl containing 2.5 µl of the reaction buffer (×10) (670 mM Tris-

HCl, pH 8.8, 166 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 25 mM $MgCl_2$), 0.5 μM of each primer, 0.5 μl of 5 mM dNTP, 0.5 μl of Hot-rescue DNA polymerase with enzyme-inhibiting antibodies (5 U/μl) (Russian Academy of Agricultural Sciences), and 2 μl of DNA template. Similar samples with adequate amounts of RNA not subjected to reverse transcription were used as negative controls. PCR was performed as follows: initial denaturation of template, 95°C, 15 min; denaturation, 94°C, 30 sec; annealing of primers, 64°C, 30 sec; elongation, 72°C, 30 sec; final stage of reaction product completion, 72°C, 10 min. The reaction was conducted through 40 cycles.

Two standard control genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) and hypoxantine-guanine-phosphoribosyl transferase (*HPRT*)) with different expression levels were used as positive controls. The primers for PCR were chosen based on the GenBank data. Sequences of the primers (direct/reverse) used and the product lengths in base pairs were as follows. GAPD: TGCCATCAACGACCCCTTCA/ACTCAGCACCAGC ATCACCC (188 bp); HPRT: CAGACTTTGCTTT-CCTTGGTC/ACTTGGCTTTTCCACTTTCGCT (217 bp); PAR1 (F2r): GAAAGACAAACACAGCG-ATGG/GGGCAGGGCAGTCTACTTA (185 bp); PAR2 (F2rl1): GCTGCTGGGAGGTATCAC/ATTCATCAA-CGGAAAAGCCTGG (167 bp); PAR3 (F2rl2): TGCT-GTTTCTGCCGACCACTGTT/GTTGTAAT-GCTTTCTTCGGGACA (187 bp); PAR4 (F2rl3): AGACGCCCAGCATCTACGAC/GGACCCACCCCA-GAAGCAG (198 bp). The PCR product was visualized by electrophoresis in 2% agarose gel in the presence of ethidium bromide at the field voltage of 10 V/cm.

Materials. Human APC, bovine thrombin, NaCl, KCl, $CaCl_2$, $MgCl_2$, KH_2PO_4, Hepes, glucose, glutamate, phenylmethylsulfonyl fluoride (PMSF), ARAC, poly-D-lysine, geldanamycin, and MTT were from Sigma (USA); fluorescent dyes Hoechst 33342 and SYTO-13 were from Molecular Probes (USA); synthetic PAR1 peptide agonist (PAR1-AP, TFLLRN) and PAR1 antagonist (YFLLRN, (Tyr^1)-TRAP-7) were from Bachem Biochemica (Germany); neurobasal medium A containing 2% Supplement B-27 and L-glutamine were from Gibco (USA).

Data processing. The findings were analyzed by the data distribution in the groups, and the resulting distribution was qualified as normal using the Prizma statistical program. The data were processed in paired samples using Student's *t*-test, *n* being the number of independent experiments. The results are presented as the mean and the error of the mean.

RESULTS

Effect of APC on survival of cultured neurons under normal conditions and exposed to glutamate. We have earlier shown that the *in vitro* hyperstimulation of glutamate receptors of hippocampal neurons causes the death of 34% of the cells 24 h after the exposure [25, 34]. In the present work, we assessed effects of high concentrations of glutamate and the influence of APC over a wide range of concentrations on hippocampal and cortical neurons by a biochemical MTT method (Fig. 1) and by morphological approaches (Hoechst 33342 and SYTO-13) (Figs. 2 and 3; see color insert). Twenty-four hours after the exposure of the culture for 30 min in the presence of 100 μM glutamate resulted in the death of 33.4% of hippocampal and of 29.9% of cortical neurons (Figs. 1-3). Incubation of the cultures for 45 min in the presence of low APC concentrations (<50 nM) did not cause death of the hippocampal and cortical neurons (Figs. 1 (a and b) and 2). However, APC at concentrations >50 nM caused the death of 26.9% of hippocampal neurons, which was comparable to the glutamate-induced death. As differentiated from the hippocampal neurons, a significant death of cortical neurons (21.2% of cells) was observed when the APC concentration was increased to 100 nM, which suggested the lower sensitivity of cortical neurons to the enzyme.

In a narrow range of low doses (over 50 pM), APC dose-dependently protected the neurons against death caused by the toxic effect of glutamate (Figs. 1-3). The range of effective APC concentrations was narrower for the hippocampal neurons (0.05-1 nM) (Figs. 1a and 2) than for the cortical neurons (0.05-10 nM) (Figs. 1b and 3). APC at the concentration of 0.01 nM did not protect either type of neurons. APC at the concentration of 100 nM manifested a proapoptotic effect and did not protect the hippocampal and cortical neurons against glutamate-induced excitotoxicity (Fig. 1).

APC deprived of the proteolytic activity because of inactivation with PMSF (APCi) did not protect neurons (Fig. 1); therefore, it was suggested that the proteinase-activated receptor (PAR) should be involved in this function of the enzyme. PAR1 seems to be required for the protection by APC of neurons, as occurs in the case of endothelial cells [23]. To test this hypothesis, we studied: first, the effect of APC on neurons under normal conditions and under glutamate-induced excitotoxicity when PAR1 was inhibited by the peptide antagonist of this PAR subtype ((Tyr^1)-TRAP-7, YFLLRN); second, the effect of APC on neuron death induced by thrombin, which is a specific PAR1 agonist; and third, the effect of APC on neurons under Hsp90 inhibition with specific inhibitor geldanamycin, which seems to compete with PAR1 for Hsp90 [28].

Effect of PAR1 peptide antagonist on neuroprotective effect of APC. To elucidate the contribution of PAR1 to the neuroprotective effect of APC, we preliminarily showed the expression of all four PAR subtypes (PAR1, PAR2, PAR3, and PAR4) with domination of the PAR1 subtype in the culture of hippocampal neurons under normal conditions (Fig. 4).

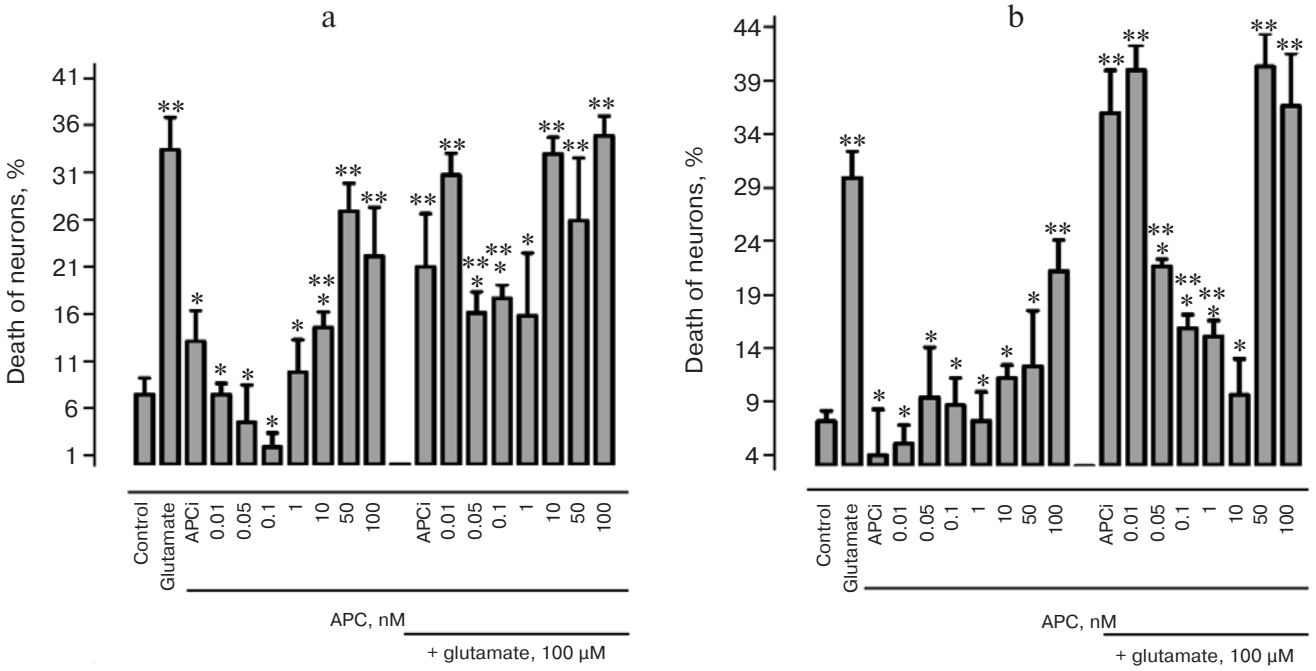

Fig. 1. Effects of varied concentrations (from 0.01 to 100 nM) of activated protein C (APC) and of the PMSF-inactivated enzyme APCi (1 nM) on the death of hippocampal (a) and cortical (b) neurons under normal conditions and upon 30-min incubation of the cultures with 100 μM glutamate. The death of neurons was assessed by the MTT method 24 h after the exposure to glutamate. Results are presented in percent with respect to control. * $p < 0.05$ compared with glutamate, ** $p < 0.05$ compared to control; here and further $n = 5$-7 independent experiments.

The peptide (Tyr[1])-TRAP-7 (YFLLRN) was earlier shown to inhibit PAR1 during the activation of platelets with thrombin [35]. We studied the effect of 20 μM (Tyr[1])-TRAP-7 on the ability of thrombin at concentrations ⩾0.1 nM to cause a transient increase in $[Ca^{2+}]_i$ in neurons. Tyr[1]-TRAP-7 completely abolished the increase in $[Ca^{2+}]_i$ induced by 10 nM thrombin in the hippocampal neurons (Fig. 5, inset), and this confirmed the effect of the peptide as a PAR1 antagonist in the cultured neurons. The APC concentrations used by us (1-50 nM) did not cause changes in the $[Ca^{2+}]_i$ in 9-day-old cultures of the hippocampal neurons and in 7-day-old cultures of the cortical neurons (data not presented). Therefore, in the next series of experiments we studied the influence of the PAR1 peptide antagonist (Tyr[1]-TRAP-7) and of APC on the neuron survival in the presence of glutamate. At 20 μM, Tyr[1]-TRAP-7 did not directly influence neuron death but abolished the protective antiapoptotic effect of low (0.1 nM) concentrations of APC under conditions of glutamate toxicity (Fig. 5). Thus, it was concluded that a direct neuroprotective effect of APC on the hippocampal neurons under conditions of excitotoxicity can be realized through receptors of the PAR1 subtype.

Influence of APC on survival of thrombin-treated neurons. Even low concentrations of thrombin (1-10 nM) activating PAR1 could cause the death of neurons in the control [25], and this was confirmed in the present work (Fig. 6). But if PAR1 was involved in the protective effect of APC, then preincubation of the cells with APC would desensitize the receptor and cancel the response to following exposures to the PAR1 agonist—thrombin. In fact, preincubation of hippocampal neurons with 1.8 nM APC before the treatment with 10 nM thrombin decreased 9-fold (to 2.0%) the percent of neurons killed as a result of the treatment with thrombin (Fig. 6). This was comparable with the control values of cell death in the population (4.6%). These data confirmed the contribution of PAR1 to the effect of APC on neurons.

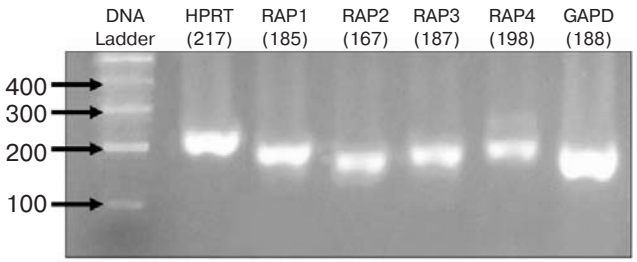

Fig. 4. Expression of PARs in 9-day-old cultures of hippocampal neurons. Genes *GAPD* (glyceraldehyde-3-phosphate dehydrogenase) and *HPRT* (hypoxantine-guanine-phosphoribosyl transferase) were used as controls. Results of RT-PCR were visualized by electrophoresis in 2% agarose gel.

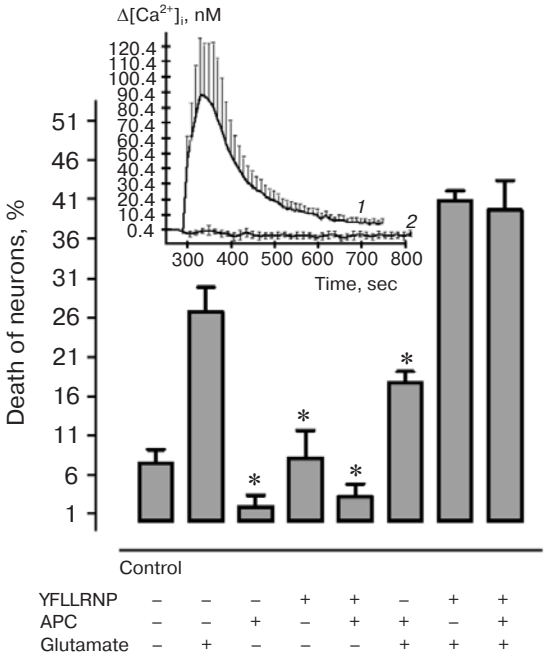

Fig. 5. Effect of 0.1 nM APC and PAR1 peptide antagonist—YFLLRN ((Tyr[1])-TRAP-7, 20 μM)—on the death of hippocampal neurons caused by incubation of cultures with 100 μM glutamate for 30 min. Neuron death was assessed by the MTT method 24 h after the treatment with glutamate. The results are presented in percent with respect to the control. * $p < 0.05$ compared to glutamate. Top: changes in $[Ca^{2+}]_i$ (nM) in hippocampal neurons ($n = 16$) in response to 10 nM thrombin (*1*) and to 10 nM thrombin after addition of the PAR1 peptide antagonist—YFLLRN ((Tyr[1])-TRAP-7, 20 μM) (*2*).

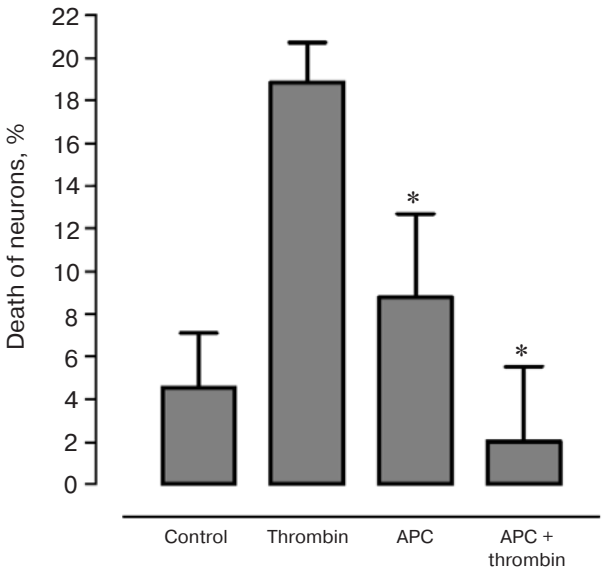

Fig. 6. Effect of 1.8 nM APC on the death of hippocampal neurons induced by 15-min incubation of cultures with 10 nM thrombin. Death was assessed by the MTT method. * $p < 0.05$ compared to thrombin.

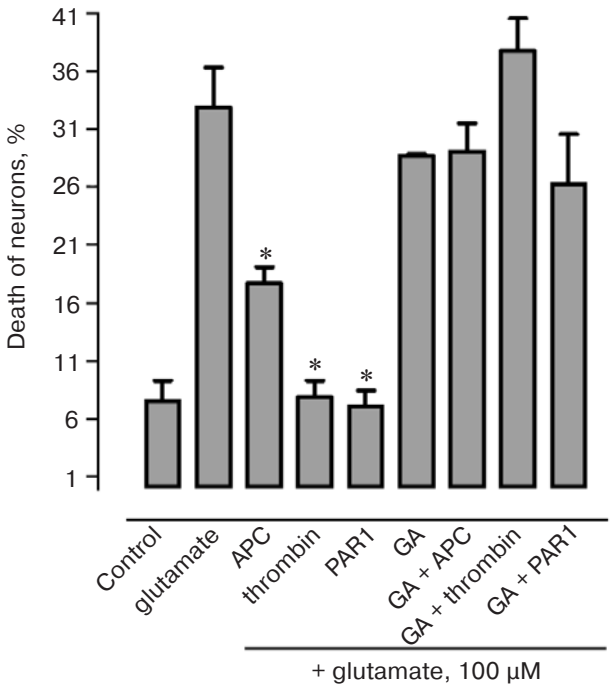

Fig. 7. Effects of 0.1 nM APC, 10 nM thrombin, and 200 nM geldanamycin (GA) added 2 h before the experiment on the death of hippocampal neurons caused by 30-min incubation of the cultures with 100 μM glutamate. Death was assessed by the MTT method. * $p < 0.05$ compared to glutamate.

Effect of heat shock protein Hsp90 inhibitor—geldanamycin—on neuroprotective effect of APC, thrombin, and PAR1 peptide agonist. To elucidate the role of Hsp90 in the neuroprotective effects of thrombin, the PAR1 peptide agonist (TFLLRN), and APC we used the specific Hsp90 inhibitor geldanamycin (GA), which binds the N-terminal domain of Hsp90, inhibits ATPase activity, and prevents interaction of cytosolic Hsp90 with client proteins [36, 37] including the thrombin receptor PAR1 [27, 28]. We found that 2-h incubation of neurons with 200 nM geldanamycin abolished the neuroprotective effect of 10 nM thrombin, 100 μM PAR1 agonist, and 0.1 nM APC under conditions of glutamate excitotoxicity (Fig. 7). This supported our hypothesis that the antiapoptotic effect of APC is mediated through PAR1 by an Hsp90-dependent mechanism, similarly to the effects of thrombin and PAR1 peptide agonist. Geldanamycin at the concentration used did not influence the glutamate-caused death of neurons (Fig. 7). The findings indicated the involvement of cytosolic Hsp90 in the PAR1-mediated protective effect of APC.

DISCUSSION

Hyperstimulation of glutamate receptors (the excitotoxicity phenomenon) is the most important mechanism

of neurodegeneration in brain stroke, brain trauma, and in Alzheimer's, Parkinson's, Huntington's, and other diseases [38]. Detection of endogenous modulators of cell death under conditions of excitotoxicity and studies of their action mechanisms are urgent and necessary for optimization of therapy of neurological diseases.

An increase in the permeability of the blood–brain barrier in critical states leads to appearance in the nervous tissue of blood coagulation system serine proteinases, which display features of cell regulators or stimulators of tissue inflammation and repair, including neurorepair and neurodegeneration [14, 39-44]. Data on functions of hemostatic proteinases and their PARs in the central nervous system are contradictory [14, 23, 39, 43, 45]. Thus, the effect of the principal PAR1 agonist—thrombin—on brain cells depends on its concentration [46-49]. High concentrations of thrombin induce apoptosis of dopaminergic neurons [50], decrease cognitive functions in rats [51], and increase the sensitivity of hippocampal neurons to glucose deprivation [46]. Nevertheless, thrombin prevents the death of astrocytes and hippocampal neurons caused by hypoglycemia and oxidative stress [52, 53]. Intracerebral injections of low doses of thrombin (preconditioning) reduced the brain damage caused by subsequent injections of high doses of thrombin or by cerebral ischemia [54]. But mechanisms of the action of thrombin on neurons are still unclear.

Even low concentrations of thrombin activate the production of an anticoagulant APC from blood protein C due to binding its high affinity receptor, endothelial thrombomodulin [6, 55]. In addition to regulation of thrombin generation and blood coagulation, APC also displays cytoprotective and antiinflammatory activities [10, 19, 56, 57]. APC reduces the infarction area and edema in mice with experimental occlusion of brain arteries and inhibits hypoxia-induced apoptosis, possibly via a signaling pathway induced by activation of endothelial protein C receptor (EPCR) and of thrombin receptor PAR1 [19, 56, 57]. There are considerable differences in the regulation of gene expression by thrombin and APC in endothelial cells stimulated by a proinflammatory cytokine [15]. But mechanisms of the action of APC under conditions of excitotoxicity caused by high glutamate concentrations are unclear.

We have found that low concentrations of APC (0.05-1 nM) prevent the glutamate-caused death of hippocampal and cortical neurons (Figs. 1 and 2). The effective concentration of APC corresponds to the concentration (0.04 nM) of the endogenous enzyme generated on activation of blood coagulation [58]. Note that the effects of APC on cortical and hippocampal neurons are different. The minimal APC concentration (0.05 nM) resulting in a noticeable neuroprotective effect under conditions of excitotoxicity reduced the death of hippocampal neurons by 16.8% and the death of cortical neurons by 8.2% (Fig. 1). The maximal APC concentration protecting hippocampal neurons was 1 nM, whereas for cortical neurons the corresponding concentration was 10 nM, i.e. one order higher (Fig. 1). Our data obtained on glutamate-untreated cell cultures revealed different sensitivities of hippocampal and cortical neurons to APC. Low concentrations of APC did not cause the death of neurons (Fig. 1), but an increase in the enzyme concentration to 50 or 100 nM resulted in cell death, similarly to the action of toxic concentrations of glutamate. The resistance of neurons to death caused by high APC concentrations was different. The percent of dead cortical neurons was significantly (to 21.2%) increased only upon the action of 100 nM APC (glutamate caused the death of 29.9%), whereas 50 nM APC led to the death of 26.9% of hippocampal neurons (glutamate caused the death of 33.4%) (Fig. 1). The elevation of APC concentrations to 50 and 100 nM did not protect the cells against the glutamate-induced death (Fig. 1).

Earlier, 10 nM APC was shown to prevent the development of apoptosis induced in cortical neurons by NMDA and staurosporine [19]. We were the first to show that even 50 pM APC could protect hippocampal and cortical neurons against death under conditions of excitotoxicity. The hippocampal neurons were more sensitive to both damaging and protective effect of APC than the cortical neurons. The regulatory action of APC on neurons recorded by us depended on its proteolytic activity, and the inactivation of the proteinase by PMSF cancelled the cytoprotective properties of the enzyme (Fig. 1). These data confirmed the involvement of the activated thrombin receptor, possibly PAR1, in the neuroprotective action of APC.

There are data in the literature on the action of APC on cells not only through the PAR1 subtype, but also through other subtypes—PAR2 and PAR3 [19, 59]. Thus, APC was shown to activate the expression of protective genes (including MCP-1) through PAR1 and PAR2 of human endothelial cells via a mechanism dependent on the non-activated EPCR [59]. The antiapoptotic action of APC on cortical neurons was mediated through PAR1 and PAR3 [19]. In the present work we studied the involvement of PAR1 in the cytoprotective action of APC using a PAR1 peptide antagonist—(Tyr^1)-TRAP-7, which completely inhibited the thrombin-induced and PAR1-dependent increase in $[Ca^{2+}]_i$ in the hippocampal neurons (Fig. 5). We found earlier that thrombin manifested a neuroprotective effect and increased $[Ca^{2+}]_i$ via PAR1 [25]. We found that APC, as differentiated from thrombin, did not induce changes in $[Ca^{2+}]_i$ in 9-day-old hippocampal neurons. Therefore, we studied the effect of the PAR1 peptide antagonist—(Tyr^1)-TRAP-7—on the neuron survival under the influence of APC and glutamate. We found that this peptide inhibited the neuroprotective effect of APC on the hippocampal (Fig. 5) and cortical neurons (data not presented). Moreover, preincubation of the cells with APC in the effective concentration of 0.1 nM significantly (9-fold) reduced cell death caused by 10 nM thrombin (Fig. 6), which also suggested that the effect of APC should be mediated through PAR1 [60, 61].

Thus, the antiapoptotic effect of APC under glutamate-induced death of hippocampal and cortical neurons, as well as the action of thrombin, is realized through PAR1, although other receptors or their regulators may also contribute. Elucidation of the role of PAR1 regulators in realization of the effect of APC on neurons is a subject of our further studies.

Some heat shock proteins, in particular Hsp90, capable of preventing cell death, play a special role in the mechanism of cell death induced by hyperactivation of glutamate receptors [62-65]. Hsp90 was earlier shown to be involved in the changing of astrocyte shape caused by thrombin through activation of PAR1 [26] and to specifically interact with the C-end of PAR1 in yeast [27].

Because PAR1 could interact with cytosolic Hsp90 [27, 37], it was suggested that inhibition of the Hsp90 activity by geldanamycin [66] would suppress the protective effect of APC and other PAR1 agonists. We have shown that the neuroprotective effects of APC and of low concentrations of thrombin and PAR1 peptide agonist are abolished by geldanamycin (Fig. 7). These findings confirmed the PAR1-mediated pathway of the neuroprotective action of APC on the cultured neurons under glutamate toxicity and suggest a possible involvement of Hsp90 in this process.

The colocation of EPCR, PAR1, and thrombomodulin in lipid rafts of endothelial cell membranes was recently shown by Bae et al. [67]. These data suggest a possibility of a rapid binding by thrombomodulin of thrombin, which converts the EPCR-bound protein C into APC. On interacting with PAR1, APC seems to trigger the cytoprotective pathways in activated endothelial cells [68]. Further studies on the receptor-mediated action of APC are promising for detecting the involvement of receptors colocated in lipid rafts of neuronal membranes in the cytoprotective effect of APC under conditions of excitotoxicity.

Thus, the neuroprotective function of APC in cultured hippocampal and cortical neurons is supposed to be mediated through PAR1 with involvement of Hsp90. APC can be considered to be an endogenous cellular regulator capable of modulating the survival of cells, including neurons, in diseases.

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