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Erk1/2 and Akt kinases are involved in the protective effect of aniracetam in astrocytes subjected to simulated ischemia in vitro

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Abstract

The present study focused on the mechanism of cytoprotective effect of aniracetam on the primary rat astrocyte cultures exposed to simulated ischemia conditions in vitro. To study these mechanisms, the aniracetam-mediated modulation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K)/Akt kinase pathways was determined. Simulated in vitro ischemia caused death of ~ 35% of astrocytes via apoptosis and decreased cell viability about 50% at 8 h. Exposure to aniracetam at concentrations of 0.1–10 μM in these conditions significantly decreased the number of apoptotic cells. Moreover, the intensification of 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolinum bromide (MTT) conversion and the decrease of lactate dehydrogenase (LDH) release after 1 and 10 μM aniracetam treatment were observed indicating a significant increase in cell viability. When cultured astrocytes were incubated during 8 h simulated ischemia with [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene] (U0126), an extracellular regulated kinase 1 and 2 (Erk1/2) inhibitor or wortmannin, a phosphatidylinositol 3-kinase (PI3 kinase)/Akt inhibitor, the cell apoptosis was accelerated. These effects of used kinase inhibitors (both U0126 and wortmannin) were antagonized by adding 1 and 10 μM aniracetam to the culture medium. In addition, aniracetam significantly stimulated of phospho-Erk1/2 kinase and phospho-Akt expression. Maximum levels of Erk1/2 and Akt activation were observed as a result of treatment with 10 μM aniracetam. U0126 and wortmannin markedly attenuated the effects of aniracetam on expression of activated kinases. Results of the present study indicate that both Erk1/2 and PI 3-K/Akt kinase pathways are vital for cytoprotective effect of aniracetam.

Keywords: Aniracetam; Astrocyte; Ischemia; Apoptosis; Erk1/2; Akt

1. Introduction

Aniracetam (1-anisoyl-2-pyrrolidinone) is a dual positive allosteric modulator of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and mGlu receptors (Martin and Haevely, 1993). Aniracetam is clinically used as a cognition enhancer for treating neuropsychiatric complications occurring after cerebral infarction. Drug given to patients with cerebrovascular diseases, Parkinson's and Alzheimer's diseases reduced emotional disturbances, cognition deficits, sleep disorders and behavioral abnormalities (Otomo et al., 1991). It has been suggested that aniracetam could potentially ameliorate ischemia- or drug-induced memory impairment and might have a neuroprotective activity against excitotoxin-induced cell death in vitro (Himori and Mishima, 1994; Cumin et al., 1982). However, the detailed molecular mechanisms of drug action are still unknown.

We have showed previously that experimental model of simulated in vitro ischemia induced apoptotic death of astrocytes (Gabryel et al., 2001, 2002). In this experimental paradigm, we have found that aniracetam exerted protective effect on astrocytes in primary cell culture through elevation of intracellular ATP concentration, and through decrease of caspase-3 activity, reactive oxygen species production and c-*fos* and c-*jun* genes expression (Gabryel et al., 2002). The current study was conducted to understand further the molecular mechanisms involved in the protective effect of aniracetam against ischemic injury in astrocytes. We planned to examine if antiapoptotic effect of drug is mediated through activation of extracellular signal regulated kinases 1 and 2 (Erk1/2)

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and/or phosphatidylinositol 3-kinase (PI3-K)/Akt pathways. Additionally, we estimated the effect of aniracetam on astrocyte apoptosis by determining the number of apoptotic nuclei and cell viability. We demonstrate that exposure of astrocytes to aniracetam in ischemic conditions in vitro significantly and specifically activate the Erk1/2 and Akt kinase signaling and that these pathways are involved in protection against ischemia-induced apoptosis of glial cells.

2. Methods

2.1. Cell culture

Astrocytes were isolated from 1-day-old Wistar rat pups and cultured essentially according to the method of Hertz et al. (1985). The study was approved by the Ethics Committee of the Silesian Medical University. Briefly, hemispheres of newborn Wistar rats were removed aseptically from the skulls, freed of the meninges, minced and mechanically disrupted by vortexing in Dulbecco's modified Eagle medium (DMEM) containing penicillin (100 U/ml) and streptomycin (100 μg/ml). The suspension was filtered through sterile nylon screening cloth with pore sizes 70 μm (first sieving) and 10 μm (second sieving). Subsequently, cultures were incubated at 37 °C in 95% air and 5% CO_2 with 95% relative humidity (CO_2-Incubator, Kebo-Assab, Sweden). The cells were counted in a Coulter Z1 counter (Coulter Counter, UK). The concentration of cells in suspension was adjusted to 1×10^6 cells/ml. For 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolinum bromide (MTT) conversion and lactate dehydrogenase (LDH) release assays 0.1 ml was poured into each well of 96-well Nunc tissue culture plate. The cells destined for Western blot analysis were sieved onto plastic dishes of 100 mm in diameter at the density of 1×10^6/dish. For Hoechst 33342 staining, astrocytes were grown on coverslips covered with poly-D-lysine (100 μg/ml) at a density 3×10^5 cell/dish. The culture medium initially contained 20% of fetal bovine serum (FBS) and after 4 days was replaced with medium containing 10% FBS. The total volume of culture medium was changed twice a week. The cells were cultured for 2 weeks until confluence. On 14th day in vitro (DIV), astrocyte cultures were deprived of microglia by shaking for 5 h and incubating with 5 mM L-leucine methyl ester (Simmons and Murphy, 1992). To identify astrocytes, cultures were stained immunocytochemically for glial fibrillary acidic protein (GFAP) (Sigma, USA), a specific marker for astrocytes. Analysis of the cultures has shown that 90–95% of cells were GFAP-positive. About 1–2% of cells in cultures reacted with Ricinus Communis Agglutinin-1, a lectin that binds to surface glycoproteins on microglia (Vector, Burlingame, USA). No neurons, as confirmed by an immunocytochemical staining method using monoclonal antibodies against microtubule associating protein-2 (MAP-2) (Promega, USA), were detected. All experiments were performed on 21-day cultures.

2.2. Treatment of astrocyte cultures

Prior to the experiment, the cells were incubated overnight with fresh medium. At the 21st DIV, cultures of astrocytes were placed in the medium deprived of glucose and serum, and incubated for 8 h in the ischemia simulating conditions: 92% N_2, 5% CO_2 and 3% O_2 at 37 °C (CO_2 incubator, Heraeus, Germany). Osmolarity of the medium was measured and adjusted to 319 mOsm with mannitol. Cells were treated with aniracetam (0.1, 1 and 10 μM) for 8 h in normoxia and for 8 h of simulated ischemia. Aniracetam was purchased from Hoffmann-La Roche (Switzerland). Drug was dissolved in dimethyl sulphoxide (DMSO) at an initial concentration of 10 mM. Further dilutions were performed in the appropriate medium. Control astrocyte cultures, exposed only to normoxic or ischemic conditions for 8 h, were treated with appropriate DMSO concentration. xKinase inhibitors (Sigma): [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenyl-thio)butadiene] (U0126) was prepared as DMSO stocks at 10 mM and added to the culture medium at final concentration of 10 μM; wortmannin was added to the culture medium at 0.1 μM. During the normoxic and ischemic incubation, the inhibitors remained in the media. The final DMSO concentration in medium did not exceed 0.05% and as previously checked, did not show any effect on astrocyte cell cultures. The doses of U0126 and wortmannin were chosen according to the IC_{50} value provided on the data sheet accompanying each compound.

2.3. Hoechst 33342 staining

Apoptosis of astrocytes was determined by Hoechst 33342 (Sigma) staining, which allows determination and quantification of cells with fragmented and condensed chromatin. After washing with phosphate-buffered saline (PBS), astrocytes cultured on coverslips were fixed for 10 min with a 4% paraformaldehyde at room temperature. Subsequently, after being washed twice with PBS the samples were dehydrated first in 70% ethanol and then in absolute ethanol. The samples were kept in −20 °C until they were stained with Hoechst 33342 (5 μg/ml in PBS) for 5 min at room temperature. Then the cells were washed again with PBS. Cell nuclei analysis was conducted with the fluorescence imaging MiraCal Pro III workstation (Life Science Resources, UK) comprising of inverted microscope Eclipse TE200 (Nikon, Japan) (ex/em 340/510 nm). A 20× objective was used. The number of apoptotic nuclei was determined on at least six randomly selected areas from three coverslips of every experimental group, each containing approximately 200 cells. The results were expressed as a percentage of apoptotic cells relative to the total number of cells.

2.4. MTT conversion

Cell viability of astrocytes treated with aniracetam was evaluated with 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolinum bromide (MTT) conversion method (Mosman, 1983). The cells ability to convert MTT indicates mitochondrial integrity and activity, which might in turn indicate cell viability. The cleavage of tetrazoline ring in MTT takes place mainly with the participation of the mitochondrial succinate dehydrogenase and depends on the activity of the respiratory chain and the redox state of the mitochondria (Mosman, 1983; Shearman et al., 1995).

MTT (final concentration—0.25 mg/ml) was added to the medium 3 h before the scheduled end of the experiment and then the cultures were incubated at 37 °C in proper conditions. At the end of the experiment, after being washed twice with PBS, cells were lysed in 100 μl dimethyl sulphoxide which enabled the release of the blue reaction product—formasan. Absorbance at the wavelength of 570 nm was read on a microplate reader and results were expressed as a percentage of absorbance measured in control cells.

2.5. Lactate dehydrogenase (LDH) release

LDH activity was estimated by measuring the decrease in absorbance at 340 nm due to the conversion of enzyme co-factor NADH to NAD^+ (one enzyme activity unit = 0.001 ΔA/min). Briefly, 100-μl aliquots of culture media were collected and dissolved in 0.1 M pyruvate buffer (0.25 mg sodium pyruvate in PBS). NADH solution (0.25 mg in PBS) was then added to the samples. Cells were lysed by repeated freezing and thawing and total released LDH was measured. Absorbance was measured immediately after the addition of NADH and 60 s after the initial reading. The data are presented as the percentage of the total releasable LDH.

2.6. Western blot

Astrocyte cell cultures were washed with ice-cold PBS and the proteins were extracted with 150 μl lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Igepal, 0.1% sodium dodecyl sulfate (SDS), 10 μg/ml phenylmethysulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml of heat activated sodium ortovanadate]. After 20 min on ice, the cell lysates were centrifuged at 12 000 rpm for 15 min at 4 °C. The protein concentrations in the samples were determined according to Bradford (1976) with serum albumin as a standard. Samples containing equal amounts of protein (100 μg) were boiled in protein loading buffer for 3 min, separated on a 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Nonspecific binding was inhibited by incubation in TBST [20 mM Tris-buffered saline (pH 7.5) with 0.1% Tween 20] contain-

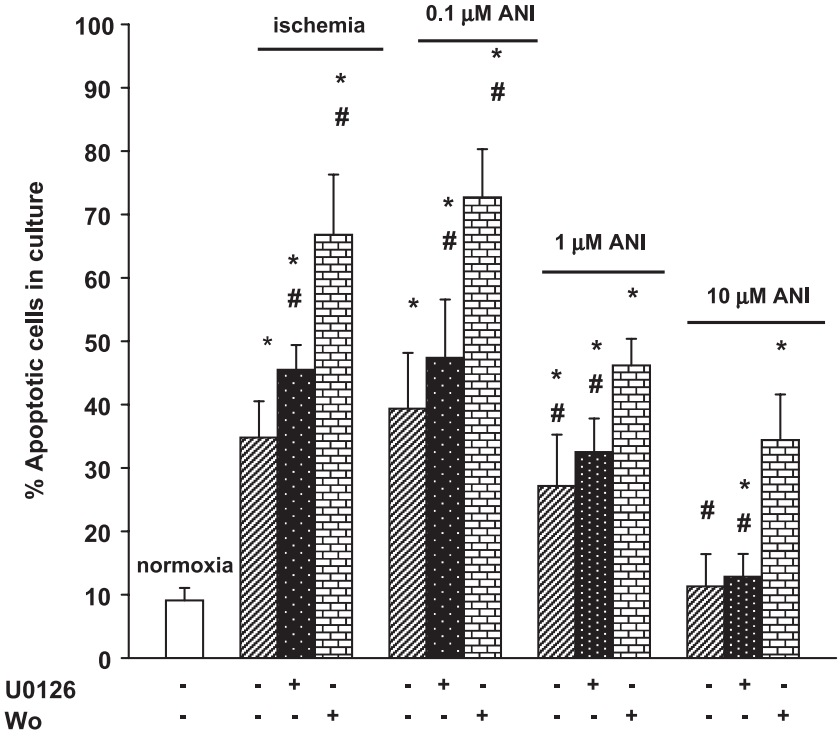

Fig. 1. Effect of aniracetam (ANI) on apoptosis of cultured rat astrocytes exposed to normoxia or ischemia and treated with aniracetam (0.1, 1 and 10 μM) in the absence or presence of 10 μM U0126 or 0.1 μM wortmannin (Wo). The nuclei were stained with Hoechst 33342 and then visualized with a fluorescent microscope. Astrocytes with fragmented or condensed DNA and apparently normal DNA were counted. The results are shown as a percentage relation of the apoptotic nuclei to the total amount of nuclei in the field and are a mean ± S.D. of the six randomly selected areas from three culture dishes; *$P < 0.05$ vs. normoxia; $^{\#}P < 0.05$ vs. ischemia.

ing 5% nonfat dried milk for 1 h at room temperature. Polyclonal antibodies against: phospho-Erk1/2, Erk1/2, phosho-Akt (Ser 473) and Akt (Santa Cruz Biotechnology, USA) were diluted (1:1000) in TBST containing 5% milk. Membranes were incubated with antibodies overnight at 4 °C, washed with TBST and incubated at room temperature for 60 min with a horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) and washed three times for 10 min with TBST. The chemiluminescence emitted from luminol oxidized by horseradish peroxidase (ECL Western blotting detection system; Amersham Biosciences, Piscataway, USA) was detected using Kodak XAR-5 film for autoradiography. Densitometric values of the bands corresponding to phosphorylated Erk1 (pErk1), Erk2 (pErk2) and Akt (pAkt) were determined using a specialized Image Pro Plus software. The values of relative phosphorylation of kinases were shown as the ratios of phosphorylated kinases to the total kinase. Experiments were repeated three times and the values of relative optical density were subjected to statistical analysis.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare mean responses among the treatments. For each endpoint, the treatment means were compared using Bonferroni least significant difference procedure. In all analyses, P value <0.05 was considered as statistically significant. All data were expressed as mean ± S.D.

3. Results

3.1. Effect of aniracetam on apoptosis

In the initial experiment, we investigated the effect of aniracetam on the apoptotic death of astrocytes deprived of serum and glucose. A quantitative results were obtained by counting the number of apoptotic cells stained with the Hoechst 33342. The cells were maintained for 8 h in simulated ischemic conditions in the absence or presence of aniracetam. We have found that simulated ischemia significantly increased the number of apoptotic nuclei in comparison with the normoxic conditions. Aniracetam in a dose-dependent manner significantly rescued cells from ischemia-induced damage. The protective effect of aniracetam reached a maximum with 10 μM aniracetam, i.e. ~11% apoptosis of control in cells exposed to ischemia. To confirm the hypothesis that activation of Erk1/2 and Akt contribute to antiapoptic effect of aniracetam, we examined the effects of the specific PI3-K/Akt kinase inhibitor wortmannin (0.1 μM) and MAPK/ Erk1/2 inhibitor U0126 (10 μM). The apoptosis rate after treatment of the cells with U0126 (10 μM) and wortmannin (0.1 μM) during ischemia

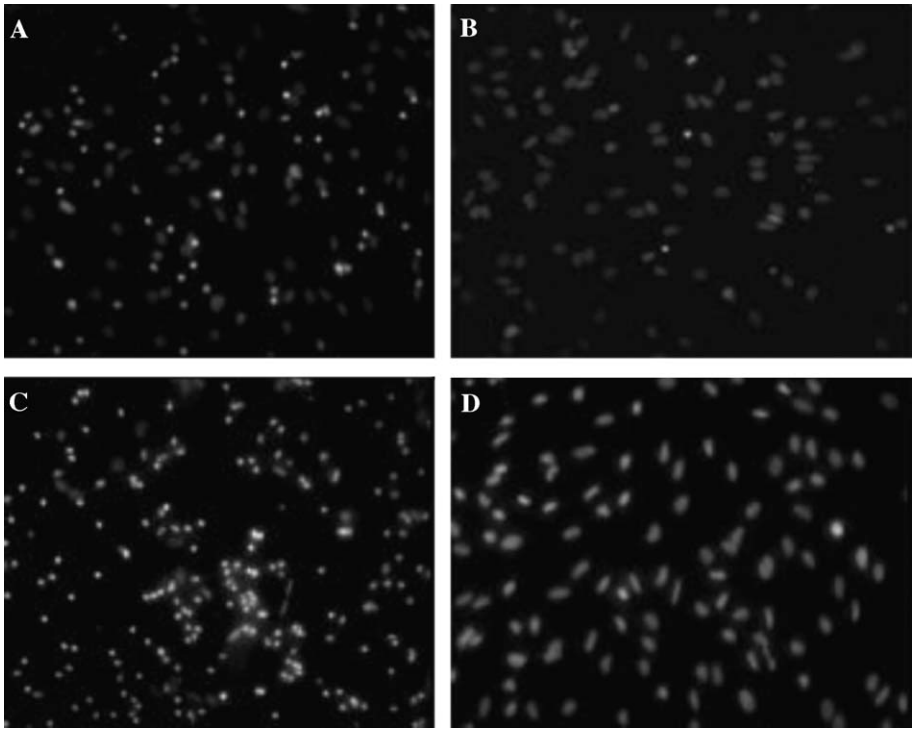

Fig. 2. Effect of aniracetam on astrocyte apoptosis induced by simulated ischemia and U0126 or wortmannin exposure. Cells were incubated with 10 μM U0126 (A) and 0.1 μM wortmannin (C) in the absence or presence of 10 μM aniracetam (B, D, respectively) for 8 h ischemia. Then, the cells were fixed and stained with the Hoechst 33342 dye. Microphotographs were taken with the use of fluorescent microscope and 20× objective.

were 45.5% and 66.8%, respectively (Fig. 1). Simultaneous use of 10 μM aniracetam and U0126 or wortmannin decreased the number of apoptotic nuclei to 3.9 + 1.3% and 11.8 + 2.9%, respectively, providing the evidence of potent drug effect on MEK and PI 3-kinase pathways (Fig. 1). Nuclear staining of ischemic astrocytes treated with kinase inhibitors showed condensation of chromatin and fragmentation of the nucleus (Fig. 2A and B). Treatment of astrocytes with 10 μM aniracetam and U0126 (Fig. 2C) or wortmannin (Fig. 2D) during ischemia prevented the process of apoptosis.

3.2. Effect of aniracetam on MTT conversion

Fig. 3 presents the effect of aniracetam on MTT conversion into formazan dye in the cultures of rat astrocytes. The 8-h exposure of astrocytes to simulated ischemia in vitro resulted in 57% attenuation of MTT conversion. The significant decrease in cell viability was observed after an additional exposure of astrocytes to kinase inhibitors in ischemic conditions. Treatment of cultures with U0126 or wortmannin during simulated ischemia inhibited the cell viability to 45% and 37%, respectively. A substantial increase of MTT conversion in ischemia stimulated both by 1 and 10 μM of aniracetam indicated a significant restoration of mitochondrial activity to 77% and 115% of values observed in control cultures. Co-treatment of cells with aniracetam (1 or 10 μM) and U0126 attenuated the effect of aniracetam, still the viability of cells was evaluated in comparison with the untreated cells in ischemia. Simultaneous addition of aniracetam and wortmannin did not change cells viability in comparison with cultures exposed to ischemia conditions.

3.3. Effect of aniracetam on LDH release

Fig. 4 shows the aniracetam effect on LDH release into the culture medium. The LDH level in the medium of normoxic culture was very low. The increase in LDH activity in the medium occurred after 8 h simulated ischemia as a result of cell membrane integrity loss and an enzyme leakage. The released LDH activity increased in cultures exposed simultaneously to ischemia as well as to U0126 or wortmannin. Treatment of ischemic astrocytes with tested concentrations of aniracetam reduced the LDH release in a dose-dependent manner. No changes were observed in percentage of LDH release when the cells were exposed to kinase inhibitors and 0.1 μM aniracetam. At the same time, simultaneous co-administration of 1 or 10 μM aniracetam with U0126 or wortmannin caused a significant reduction of LDH activity in the medium.

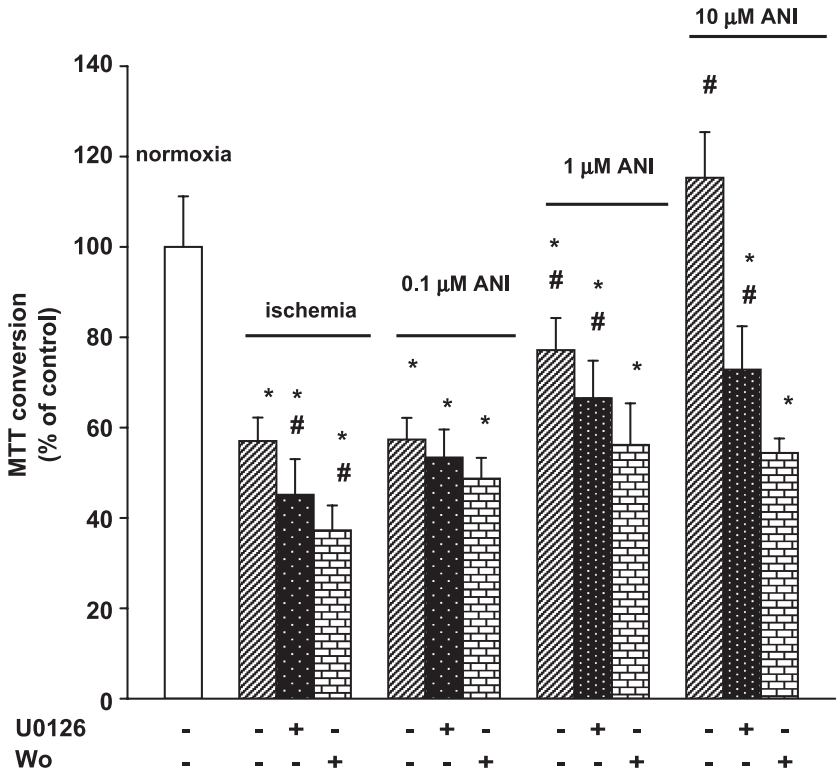

Fig. 3. Effect of aniracetam (ANI) on cell viability cultured rat astrocytes exposed to normoxia or ischemia and treated with aniracetam (0.1, 1 and 10 μM) in the absence or presence of 10 μM U0126 or 0.1 μM wortmannin (Wo) measured by MTT conversion assay. The results are presented as a percentage relation of the control value in normoxia. Each value is means ± S.D. of 12 wells in three separate experiments ($n = 12$); $^{*}P < 0.05$ vs. normoxia; $^{\#}P < 0.05$ vs. ischemia.

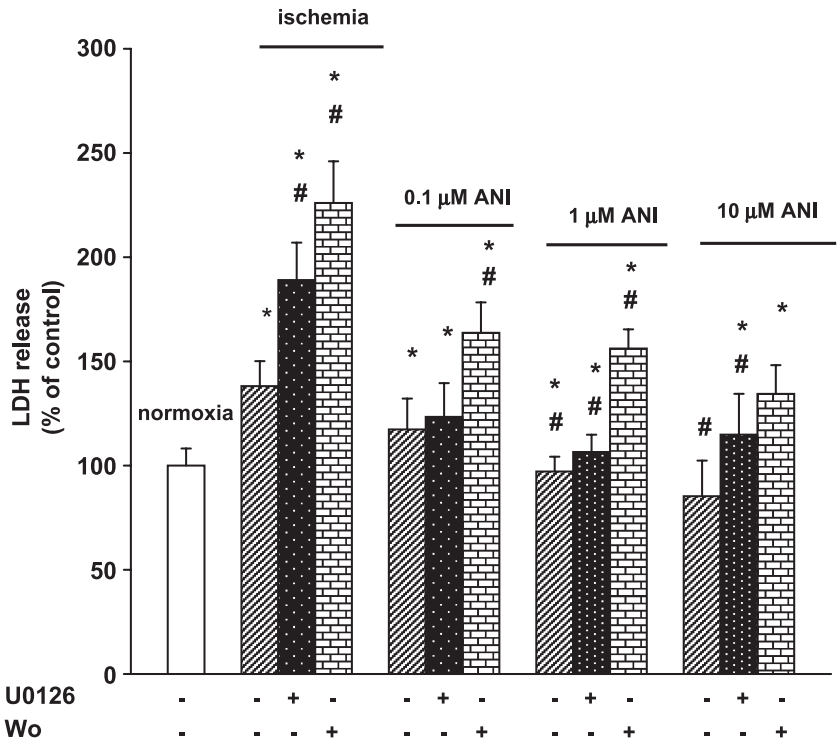

Fig. 4. Effect of aniracetam (ANI) on LDH release in cultured rat astrocytes exposed to normoxia or ischemia and treated with aniracetam (0.1, 1 and 10 μM) in the absence or presence of 10 μM U0126 or 0.1 μM wortmannin (Wo). The results are presented as a percentage relation of the control value in normoxia. Each value is means ± S.D. of 12 wells in three separate experiments ($n = 12$); *$P < 0.05$ vs. normoxia; $^{\#}P < 0.05$ vs. ischemia.

3.4. Effect of aniracetam on the expression of Erk1/2 kinases

Fig. 5 illustrates the dose-dependent effect of aniracetam on the expression of Erk1/2 kinases determined by Western blot method. Immunoblotting was performed with an antibody recognizing the MAPKs Erk1 and Erk2 double phosphorylated on Thr 202 and Tyr 204 residues within TEY motif (Fig. 5A—upper panel) and with a polyclonal antibody recognizing Erk1/2 regardless of its phosphorylation state (Fig. 5A—lower panel). These experiments were repeated three times, the bands were scanned and their optical densities were compared and statistically analyzed (Fig. 5B). Phosphorylations of Erk1 and Erk2 were markedly attenuated in astrocytes' cultures exposed to simulated ischemia in vitro. Aniracetam increased the phosho-Erk1 and phospho-Erk2 expression in a concentration-dependent manner. The expression of p-Erk1 kinase after 10 μM aniracetam treatment was about 6-fold higher than after the exposure to ischemia (Fig. 5B). In the same experimental conditions, exposure to 10 μM aniracetam resulted in five times increased p-Erk2 expression (Fig. 5C). We also examined the effect of drug administered concomitantly with specific inhibitor of these kinases (U0126). Incubation of astrocytes with U0126 effectively blocked Erk1/2 phosphorylation in each experimental group.

3.5. Effect of aniracetam on the expression of Akt kinase

The dose-dependent effect of aniracetam on the expression of Akt kinase in cultured astrocytes determined by Western blot method is shown in Fig. 6. Immunoblotting was done with an antibody against Akt phosphorylated on Ser 473 residue (Fig. 6A—upper panel) and with a polyclonal antibody recognizing Akt regardless of its phosphorylation state (Fig. 6A—lower panel). These experiments were repeated three times, and the bands optical density was statistically analyzed (Fig. 6B). As illustrated (Fig. 6), under ischemic condition phosphorylation of Akt was markedly attenuated in comparison with normoxia. The phosphorylated Akt (p-Akt) level increased after aniracetam treatment in a dose-dependent manner. The expression was elevated to 180% of control by 10 μM of aniracetam. The values of relative phosphorylation of Akt kinase were shown on Fig. 6. B. In each case, we also examined the effect of drug with combination with wortmannin-specific inhibitor of Akt kinase. Incubation of astrocytes with wortmannin in each experimental group effectively blocked Akt phosphorylation.

4. Discussion

In the present study, we examined the cellular mechanisms of the protective effect of aniracetam against ische-

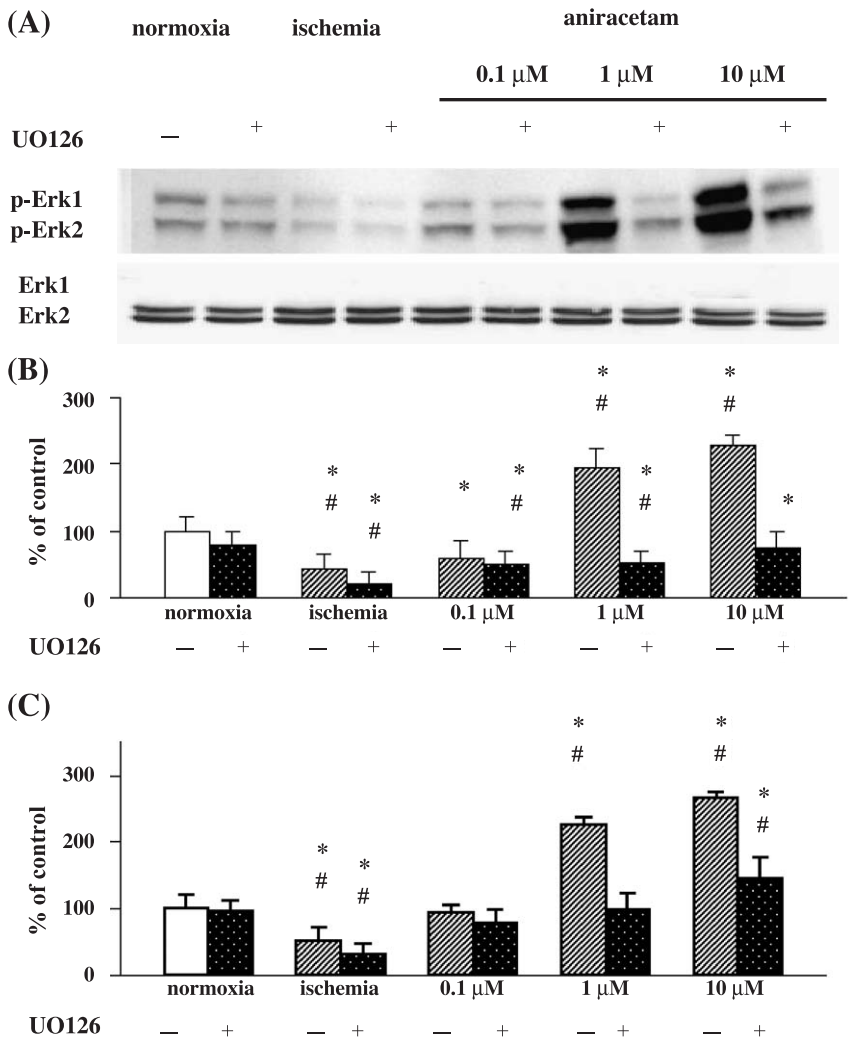

Fig. 5. (A) Western blot analysis of phosphorylated Erk1/2 (upper panel) and total Erk (lower panel) after 8 h simulated ischemia and treatment with aniracetam (0.1, 1 and 10 μM) in the absence or presence of 10 μM U0126. Cell lysates (20 μg of protein) from experimental groups were analyzed by immunoblotting using antibodies against phospho-Erk1/2 (p44/p42 MAPK) and total Erk. Blots shown are each representative of three independent experiments. (B) The relative phosphorylation of Erk1 during ischemia. The results are presented as means ± S.D. from three independent experiments; $^{*}P<0.05$ vs. normoxia; $^{\#}P<0.05$ vs. ischemia. (C) The relative phosphorylation of Erk2 during ischemia. The results are presented as means ± S.D. from three independent experiments; $^{*}P<0.05$ vs. normoxia; $^{\#}P<0.05$ vs. ischemia.

mia-induced apoptosis in cultured rat astrocytes. We used the experimental model of simulated in vitro ischemia developed in our laboratory, which may contribute to clarification of the mechanisms of drugs that ameliorate ischemia-induced brain dysfunction (Gabryel et al., 2001, 2002). Previously, in this model we indicated that aniracetam was able to protect cultures of astrocytes obtained from rat cerebral cortex (Gabryel et al., 2002). In the present study, we provide evidence that aniracetam treatment stimulate both MAPK and Akt kinases in cultured astrocytes, and Erk1/2 kinase pathway is vital for cytoprotective effect of aniracetam.

Aniracetam is an allosteric stimulator of AMPA receptors that preferentially affects flop splice variants. The modulation mechanism is based on inhibiting the desensitization of the GluR2 subunit which is characterized by the low Ca^{2+} permeability (Tsuzuki et al., 1992). A decrease in expression of the GluR2 subunit of the AMPA receptor is observed in brain ischemia (Gu et al., 1996). This may lead to the increase in the sensitivity and number of the neuronal AMPA receptors permeable for Ca^{2+} which in turn may results in cell damage and changes attributed to the late phase of neurodegeneration (Gu et al., 1996). Increase in Ca^{2+} concentration may stimulate also phospholipase C (PLC). In this known schema, diacylglycerol (DAG) activates protein kinase C (PKC) which, via phoshorylation, initiates mitogen-activated protein (MAP) kinase cascade. The changes in phosphoinositide turnover which seem to play an important role in neuroprotective effect of aniracetam are seen at ca. 500–1000 times lower concentrations than those inhibiting AMPA receptor. However, inhibition of AMPA receptor desensitisation has been observed at ca. 2

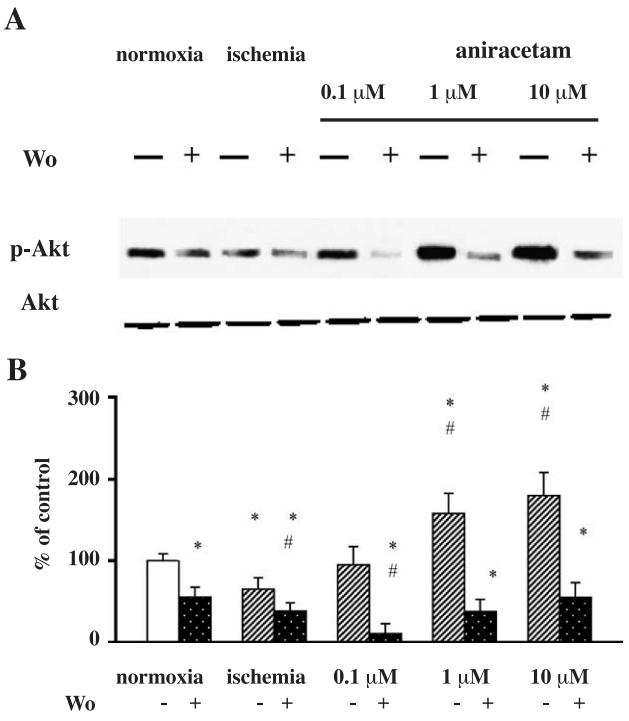

Fig. 6. (A) Western blot analysis of phosphorylated Akt (upper panel) and total Akt (lower panel) after 8 h simulated ischemia and treatment with aniracetam (0.1, 1 and 10 μM) in the absence or presence 0.1 μM wortmannin. Cell lysates (20 μg of protein) from experimental groups were analyzed by immunoblotting using antibodies against phospho-Akt and total Akt. Blots shown are each representative of three independent experiments. (B) The relative phosphorylation of Akt during ischemia. The results are presented as means ± S.D. from three independent experiments. *$P<0.05$ vs. normoxia; #$P<0.05$ vs. ischemia.

mM concentration of this drug (Zajączkowski and Danysz, 1997).

In the cultures subjected to 8-h-long simulated ischemia, we have observed a significant increase in the number of apoptotic astrocytes in comparison with the control. Increasing concentrations of aniracetam significantly attenuated ischemia-induced apoptosis. The doses of aniracetam (1 and 10 μM) that protected astrocytes in this study were inside the clinically useful range. The brain concentration of aniracetam itself never reaches higher level than 10 μM after systemic administration of pharmacologically effective doses to animals and humans, since the drug is rapidly converted to active metabolites (e.g. *N*-anisoyl-γ-aminobutyric acid and *p*-anisic acid) (Shirane and Nakamura, 2001). The decrease in apoptosis rate of astrocytes may eventually lead to increased reactive astrogliosis observed after nervous tissue injury. Such astrogliosis may have deleterious effects on the restoration of central nervous system functions and, possibly on nervous tissue regeneration processes (Hatten et al., 1991). We cannot exclude, that short-term protective effect of astrocytes on neurons observed during and after injury might be for some extent, counterbalanced in the long term by the negative results of reactive astrogliosis.

The results of our previously published data strongly indicated the involvement of MAP and Akt kinase pathways in the protective effects of aniracetam (Gabryel et al., 2002). To investigate the role of PI3-K/Akt and MAPK/Erk1/2 kinase signal pathways in antiapoptotic effect of aniracetam, the effects of the PI3-K/Akt kinase inhibitor wortmannin (0.1 μM) and MAPK/Erk1/2 inhibitor U0126 (10 μM) were examined. The addition of both inhibitors into the culture medium of the cells exposed to 8-h ischemia significantly increased the number of apoptotic cells. At the same time, it has been observed that simultaneous administration of kinase inhibitors and 10 μM aniracetam prevented the apoptosis development, and the number of apoptotic cells in cultures treated with drug and U0126 was similar to their number in normoxia conditions. Consistently with data on MTT conversion and LDH release, aniracetam treatment significantly protected astrocytes from damage caused by ischemic conditions in vitro.

This study showed a significant intensification of phospho-Erk1/2 expression after treatment with aniracetam. To confirm the role of this finding in protective effect of aniracetam, it was demonstrated that this drug also significantly enhanced cell viability in simulated in vitro ischemia. Evidences indicate that AMPA receptor agonists stimulate Ca^{2+}-dependent phosphorylation of cyclic AMP responsive element binding protein (CREB), a common glutamate-receptor-mediated signal transduction pathway involving Ras and MAP kinase in different glial cell types (Pende et al., 1997). Mitogen-activated protein kinases (MAPKs) are a widely conserved intracellular family of serine/threonine protein kinases. Within the central nervous system, MAPK pathways have been implicated in many cellular functions such as synaptic plasticity, gene expression and ion-channel activation (Sweat, 2001). Signaling through Erk1/2 (p44/42 MAP kinase) involves the receptor-mediated activation of MAPK kinase kinases (MKKK), which activate MAP/Erk kinase (MEK1/2); MEK1/2 in turn, activated Erk1/2 by phosphorylation at threonine and tyrosine residues (Widmann et al., 1999; Chang and Karin, 2001). Activated p44/42 MAP kinase translocates to the nucleus and activates transcription by phosphorylation of 90-kDa ribosomal protein S6 kinase (p90 RSK), transcription factors (Elk1, Myc), and pro-apoptotic Bad protein from the Bcl-2 family (Schaeffer and Weber, 1999). Activated transcription factors bind to the corresponding response elements in their promoters, leading to a subsequent MAPK-dependent gene activation (e.g. c-*jun*, c-*fos*, MKP-1). Our previously published results fully support the importance of gene activation, such as c-*fos* and c-*jun* in the cytoprotective effects of aniracetam (Gabryel et al., 2002). We observed the intensification of c-*fos* and c-*jun* genes expression after exposition of astrocytes to ischemia and decrease of gene expressions proportionately to the aniracetam concentration used (Gabryel et al., 2002).

Erk1 and Erk2 have been implicated in regulating cell survival and death after ischemia, but the role of this pathway seems to depend on the cell type. Jiang et al. (2002) showed the implication of MAPK/Erk1/2 signaling in the protection of cultured cerebral cortical astrocytes against ischemic injury. It was also shown that Erk2 increased expression of *bcl*-2 and inhibited cellular apoptosis (Heusch and Maneckjee, 2001). Because the MAP Erk kinase (MEK) signaling pathway is proposed to play a role in cell survival, it is possible that it may constitute a target for the drugs ameliorating ischemia/reperfusion injury (Yujiri et al., 1998).

The serine/threonine kinase Akt, also referred to as protein kinase B (PKB), plays a critical role in controlling the balance between survival and apoptosis. This protein kinase is activated by various growth and survival factors and it has a role in a wortmannin-sensitive pathway involving PI 3-kinase (Burgering and Coffer, 1995; Franke et al., 1997). Recently, it was suggested that PI-3K/Akt kinase pathway is critical for the survival of several cell types including neurons, fibroblasts, and oligodendrocytes (Vemuri and McMorris, 1996). Akt opposes apoptosis by inactivation of proapoptotic factor Bad through its phosphorylation on a serine residue. This process lead to its dissociation from Bcl-x_L, which is then capable of suppressing death pathways (Cardone et al., 1998). Moreover, Akt inactivates caspase-9 (Yuan et al., 1993). This suggests a particularly important regulatory role for caspase-9 in apoptosis, since there is currently no evidence for similar Akt-mediated inactivation of other caspases. Inhibition of caspase-9, together with cytochrome *c* and Apaf-1 disables activation of caspase-3. Recently, Noshita et al. (2001) showed a protective effect of Akt against neuronal ischemic insults. However, on the other hand, Jiang et al. (2002) suggested that the PI3-K/Akt pathway in ischemic astrocytes did not play a protective role but promotes cell injury. We demonstrated significant increase of the apoptotic nuclei after adding wortmannin as well as aniracetam ability to reverse this pro-apoptotic effect. Indeed, in the present study we indicated that activation of PI 3-kinase/Akt pathway might be a result of the involvement in the antiapoptotic effect of drug.

Summing up, the study implies that the Akt and MEK kinase pathways have therapeutic significance in brain ischemia associated with accelerated astrocyte apoptosis. These results directly indicate that exposure of glial cells to ischemic conditions in vitro in the presence of aniracetam enhance both Akt and Erk1/2 kinase expression. Specifically, inhibition of the MAPK and Akt kinase pathways completely counteracted aniracetam-mediated protection against ischemia-induced apoptosis. These effects appear to have clinical importance on survival of cortical astrocytes. It seems that our experiments bring the hypothesis of the new cytoprotective mechanisms of aniracetam indicating its potential protective influence towards astrocytes in ischemia conditions.

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