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Short communication

Effect of aniracetam on phospatidylinositol transfer protein alpha in cytosolic and plasma membrane fractions of astrocytes subjected to simulated ischemia *in vitro*

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Abstract:
Brain ischemia affects phosphoinositide metabolism and the level of lipid-derived second messengers. Phosphatidylinositol transfer proteins (PI-PTs) are responsible for the transport of phosphatidylinositol (PI) and other phospholipids through membranes. Isoform of PI-TPs (PI-TPα) is an essential component in ensuring substrate supply for phospholipase C (PLC). The current study was conducted to examine potential effect of aniracetam on PI-TPα expression and to characterize the PI-TPα isoform distribution between membrane and cytosol fractions of astrocytes exposed to simulated ischemia *in vitro*. After 8 h period of ischemia, the level of PI-TPα was significantly higher in cytosol (by about 28%) as well as in membrane fraction (by about 80%) in comparison with control. We have found that aniracetam treatment of astrocytes in normoxia significantly increased the level of PI-TPα in membrane fraction with a maximal effect at 0.1 μM concentration of aniracetam (by about 195% of control). In membrane fractions of ischemic cells, aniracetam increased PI-TPα expression in a concentration-dependent manner. In ischemic cells, aniracetam (10 μM) has elevated PI-TPα expression up to 155% and 428% in cytosolic and membrane fractions in comparison with ischemic untreated cells, respectively. The study has shown that aniracetam significantly activates PI-TPα in cell membrane fraction and this effect might be connected with previously described activation of MAP kinase cascade.

Key words:
aniracetam; astrocytes; ischemia; phosphatidylinositol transfer protein-α

Abbreviations: AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, DAG – diacylglycerol, DIV – day *in vitro*, FBS – fetal bovine serum, IP_3 – inositol trisphosphate, MAPK – mitogen-activated protein kinase, PI – phosphatidylinositol, PIP_2 – phosphatidylinositol- 4,5-biphosphate, PI-TPs – phosphatidylinositol transfer proteins, PITPα – isoform α of phosphatidylinositol transfer protein, PKC – protein kinase C, PLA_2 – phospholipase A_2, PLC – phospholipase C

Introduction

Aniracetam (1-anisoyl-2-pyrrolidinone) is a nootropic drug acting as a modulator of ionotropic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and metabotropic glutamate (mGlu) receptors [12]. Aniracetam shows positive clinical effects in pa-

tients with cerebrovascular diseases, Parkinson's and Alzheimer's diseases, sleep disorders and behavioral abnormalities [13]. Moreover, the drug ameliorates ischemia-induced memory impairment and has a neuroprotective activity against excitotoxin-induced cell death *in vitro* [14]. Beneficial effects of aniracetam on the function of the central nervous system are probably related to its influence on multiple cellular mechanisms.

Results of our previous studies allowed us to establish hypothesis of the new cytoprotective mechanisms of aniracetam indicating its potential protective influence on astrocytes in ischemia conditions. We have shown that aniracetam in ischemic conditions *in vitro* significantly and specifically stimulated the mitogen-activated protein kinase (MAPK) and Akt kinase signaling in astrocytes and that these signaling pathways are involved in protection against ischemia-induced apoptosis of glial cells [6]. Data of Pizzi et al. [14] indicated that aniracetam at low concentrations (about 5 μM) *in vitro* could stimulate phospholipase C (PLC) activity and enhance quisqualate and *trans*-1-aminocyclopentane-1,3-dicarboxylate (tACPD)-mediated neuroprotective effect. Therefore, it was suggested that the changes in phosphoinositide turnover might play an important role in neuroprotective effect of aniracetam.

Numerous data indicate that brain ischemia affects phosphoinositides metabolism and the level of lipid-derived second messengers. Analysis of lipid products accumulated during ischemia indicated that both phospholipase A_2 and PLC are involved in phosphoinositide degradation [16].

Proteins called phosphatidylinositol transfer proteins (PI-TPs) are responsible for the transport of phosphatidylinositol (PI) and other phospholipids between membranes. PI-TPs play a role in delivery of PI to PI4-kinase, which synthesize PI 4,5-biphosphate (PIP_2). Because of the high affinity of PI-TPs for PIP and PIP_2, these lipids remain bound to PI-TPs which may also deliver these substances for PLC [17].

Kauffmann et al. [9] have identified the α isoform of PI-TPs (PI-TPα) as an essential component in ensuring substrate supply to PLC. This enzyme hydrolyzes PIP_2 to generate the second messengers: diacylglycerol (DAG) and inositol trisphosphate (IP_3). In the next step DAG activates protein kinase C (PKC) which, *via* phoshorylation, initiates MAP kinase cascade. The current study was conducted to examine potential effect of aniracetam on PI-TPα expression and to characterize the distribution of this isoform of PI-TPs between membrane and cytosol fraction of astrocytes exposed to simulated ischemia *in vitro*. We have shown that the drug significantly activated PI-TPα in membrane fraction of the cells and that this effect might be connected with previously described activation of MAP kinase cascade.

Materials and Methods

Cell culture

Astrocytes were isolated from one day old Wistar rat pups and cultured essentially according to the method of Hertz et al. [7]. The study was approved by the Local Ethics Committee for the Animal Experimentation. Before the material was plated onto the plastic dishes, 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 and then the cells were sieved onto plastic dishes of 100 mm in diameter at the density of 1×10^6/dish. The culture medium initially contained 20% of fetal bovine serum (FBS) (Gibco, Grand Island, USA) 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 two 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. To identify astrocytes, cultures were stained immunocytochemically for glial fibrillary acidic protein (GFAP) (Sigma, St. Louis, 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 MAP-2 (Promega, Madison, USA), were detected. Experiments were performed on 21-day cultures.

Treatment of astrocyte cultures

Prior to the experiment, the cells were incubated overnight with fresh medium. On 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

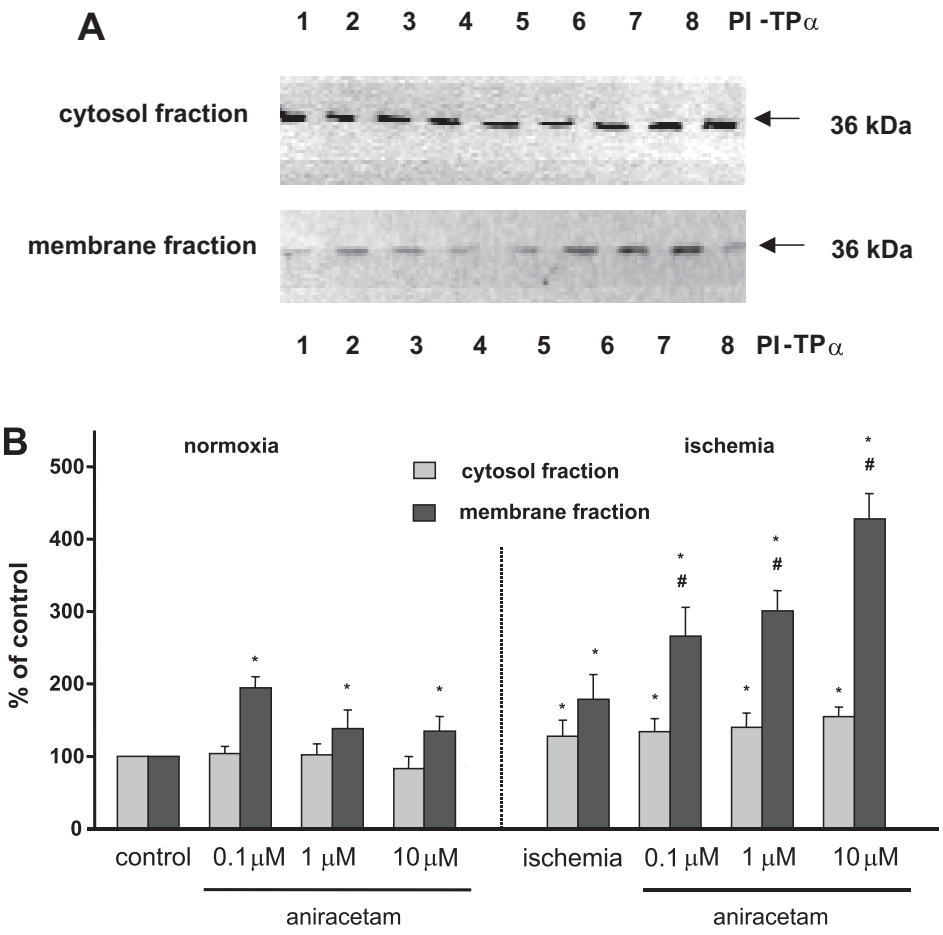

Fig. 1. (**A**) Effect of aniracetam on PI-TPα in cytosolic and membrane fractions of astrocytes exposed to normoxia (lane 1–4) or simulated ischemia (lane 5–8). Lane 1 – normoxia (control); lane 2 – 0.1 μM aniracetam; lane 3 – 1 μM aniracetam; lane 4 – 10 μM aniracetam; lane 5 – ischemia; lane 6 – 0.1 μM aniracetam; lane 7 – 1 μM aniracetam; lane 8 – 10 μM aniracetam. 15 μg of protein of cytosolic and membrane fractions was analyzed as described in Materials and Methods. (**B**) Densitometric analysis of the intensity of the 36 kDa bands determined on Western blots that correspond to PI-TPα in cytosolic and membrane fractions of astrocytes exposed to normoxia or simulated ischemia and treated with the indicated doses of aniracetam. Data are the mean ± SD of 3 experiments. The statistical analysis was performed by two-way ANOVA and Newman-Keuls *post-hoc* test. The results are shown as a percentage relation of the control value measured under normoxia conditions; * $p < 0.05$ *vs.* normoxia; # $p < 0.05$ *vs.* ischemia

37°C (CO_2 incubator, Heraeus, Germany) [5]. 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 (Hoffman-La Roche, Switzerland), was dissolved in dimethyl sulfoxide (DMSO) at an initial concentration of 10 mM. Further dilutions were performed in the appropriate medium. The final DMSO concentration in medium did not exceed 0.05% and as previously checked, did not show any effect on astrocyte cell cultures. Control astrocyte cultures were exposed only to normoxic or ischemic conditions for 8 h.

Preparation of membrane and cytosolic fraction

Treated astrocyte cell cultures were washed twice with ice-cold phosphate-buffered saline (PBS) and cells were harvested from culture dishes in 150 μl of lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Igepal, 0.1% sodium dodecyl sulfate (SDS), 10 μg/ml of phenylmethysulfonyl fluoride, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin and 10 μg/ml of heat activated sodium orthovanadate (all from Sigma, St. Louis, USA)]. Cell lysates were additionally homogenized by a brief sonification. Then the homogenate was centrifuged at 10 000 × g for 10 min to

obtain membrane fractions and supernatant, crude cytosolic fraction. The protein content of each subcellular fraction was determined by the method of Bradford with serum albumin as a standard [3].

Gel electrophoresis and Western blotting

The membrane and cytosolic fractions (25 μg of protein) were mixed with an equal volume of sample buffer (62.5 mM Tris-HCl, 2% SDS, 100 mM DTT, 0.2 mM 2-mercaptoethanol, 20% glycerol and 0.2% bromophenol blue, pH 6.8). The samples were heated for 5 min at 95°C. 10% polyacrylamide gel was used for protein electrophoresis [10]. Proteins were electrophoretically transferred from the SDS polyacrylamide gel to nitrocellulose membranes (Bio-Rad, USA). The membrane was blocked in 5% milk powder (non fat dry-milk) in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) for 1 h at 37°C. Then, the blot was incubated with rabbit anti-PI-TPα antibody [raised against recombinant PI-TPα, diluted at 1:100 in TBS-T containing 0.2% (w/v) of non-fat milk] overnight at room temperature. The PI-TP antibody complex was identified with goat-anti rabbit IgG conjugated with alkaline phosphatase [GAR-AP, 1:5000 diluted in TBS-T containing 0.2% (w/v) non fat milk]. The GAR-AP was visualized with 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and p-nitro blue tetrazolium chloride (BCIP/NBT) (Sigma, St. Louis, USA) as color development substrate for alkaline phosphatase. The optical densities of the PI-TPα bands on the immunoblot were quantified using NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

Experiments were repeated three times and the values of relative optical density were subjected to statistical analysis by two-way ANOVA and Newman-Keuls *post-hoc* test.

Results

The levels of α isoform of PI-TP were analyzed by Western blot method in cytosol and membrane fractions of rat brain cortex astrocyte cultures treated with aniracetam in normoxic and ischemic conditions (Fig. 1).

After 8 h period of ischemia, the level of PI-TPα was significantly higher in cytosol (by about 28%) as well as in membrane fraction (by about 80%) in comparison with normoxic control. We have found that treatment of astrocytes with aniracetam in normoxia significantly increased the PI-TPα level in membrane fraction comparing to control, with a maximal effect at concentration of 0.1 μM aniracetam, i.e. ~ 195% of control. On the other hand, we have not observed significant changes in the level of PI-TP in the cytosolic fraction of astrocytes treated with the drug under normoxic conditions.

We have observed that both in cytosolic and in membrane fraction derived from cells exposed to simulated ischemia *in vitro*, aniracetam increased PI-TPα expression.

The expression of PI-TPα at 10 μM aniracetam was as high as 155 and 428% in cytosolic and membrane fraction, respectively, in comparison with ischemic untreated cells.

Discussion

Using this experimental model, we previously indicated that aniracetam treatment stimulated both MAPK and Akt kinases in cultured astrocytes, and that Erk1/2 kinase pathway was vital for cytoprotective effect of aniracetam [6]. On the basis of these data, it was possible to suggest that aniracetam could affect PI-TPα and that activation of these proteins was involved in escalation of MAP kinase cascade.

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 [18]. Furthermore, aniracetam has been reported to counteract glutamate-induced neurotoxicity in rat cerebellar granule cells through potentiation of the signaling via mGluR coupled to phospholipase C (PLC) [14]. Consequently, in a well known schema: diacylglycerol (DAG) activates protein kinase C (PKC) which, finally, via phoshorylation, initiates MAP kinase cascade. Jiang et al. [8] showed that MAPK/Erk1/2 signaling was involved in the protection of cultured cerebral cortical astrocytes against ischemic injury. It has been also shown that Erk2 caused an increase in *bcl-2* expression and inhibition of cellular apoptosis. Counteraction of glutamate-induced neurotoxicity occured

at concentration of aniracetam two- or three-fold lower than those potentiating AMPA receptor function [14]. The data of Lucci et al. [11] support the hypothesis that PKC activation might be a common mechanism amongst nootropic drugs from different chemical classes. The changes in phosphoinositide turnover seem to play an important role in neuroprotective effect of aniracetam [17]. Moreover, experimental data from numerous studies performed *in vivo* and *in vitr*o have shown that significant activation of membrane-bound PKC isoforms by aniracetam is involved in its pharmacological action [17]. It might indirectly explain the stimulating effect of the drug on PI-TPα expression in membrane fraction of astrocytes under normoxic conditions, which we observed in this study.

The role of PI-TPs in physiology and pathology of the central nervous system is still not clear. Isoform α of PI-TPs has been suggested to be involved in phospholipid degradation, phospholipid transport as well as in dynamic cytoskeleton changes and in Golgi system functioning [16]. Recently, experimental results described by Alb et al. [1] indicated that ablation of PI-TPα function caused aponecrotic spinocerebellar disease suggesting a novel and unanticipated role for this protein. These data were preceded by studies in knock-out mice, showing that mice lacking PI-TPα die soon after birth [2]. Moreover, it was shown that the increase in PI-TPs expression in glial cells might be beneficial because of facilitation of antioxidants influx (e.g. α-tocopherol). An increased PI-TPs-mediated lipid transfer might also contribute to the repair of the damaged ischemic neurons affecting lipoprotein delivery of cholesterol and phospholipids from glial cells to neurons, a process necessary for repair to occur [19].

In this study, we have observed a significant increase in the PI-TPα expression in membrane fraction of astrocytes subjected to 8 h long simulated ischemia in comparison with the control. These results indicate that ischemia affects the concentrations of PI-TPα in astrocytes (especially in membrane fraction).

Increasing concentrations of aniracetam significantly intensify ischemia-induced alternation in PI-TPα expression in membrane fraction. Fallarino et al. [4] have described [^{3}H]aniracetam binding to specific and saturable recognition sites in membranes prepared from discrete regions of rat brain. High levels of bound [^{3}H]aniracetam were detected in hippocampal, cortical, or cerebellar membranes, containing a high density of excitatory amino acid receptors. We observed the augmentation of PI-TPα expression proportional to aniracetam concentration. The doses of aniracetam (1 and 10 μM) used in this study are inside the clinically useful range. The brain concentration of aniracetam itself never exceeds 10 μM after systemic administration of therapeutically effective doses to animals and humans, since the drug is rapidly converted to active metabolites (e.g. N-anisoyl-GABA and *p*-anisic acid) [15].

Summing up, the results prompt us to speculate that some of the pharmacological effects of aniracetam may be related to its effect on PI-TPα proteins. However, significance of the drug-induced changes in PI-TPs expression in ischemic glial cells should be investigated in more detail.

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