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EFFECT OF NEBRACETAM ON CONTENT OF HIGH–ENERGY PHOSPHATES AND MORPHOMETRY OF RAT ASTROCYTES *IN VITRO*. COMPARISON WITH PIRACETAM.

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Abstract: The present study was initiated to examine the effect of nebracetam, a nootropic drug, on various biochemical and morphometric parameters in order to gain some insight into the mechanism of this agent action. The content of adenosine triphosphate (ATP) and phosphocreatine (PCr) and ^{3}H–valine incorporation into proteins was measured and the morphometry was performed after nebracetam and piracetam treatment of rat astrocytes cultured in vitro with or without dibutyryl 3',5'–cyclic adenosine monophosphate (dBcAMP). Nootropics were added into the culture medium for 2 weeks at the final concentration of 10^{-7} M. Cultured astrocytes treated with either nebracetam or piracetam showed decreased intracellular ATP and PCr levels. The addition of nebracetam and dBcAMP to cultures caused an increase of PCr content in astrocytes. The astrocytes treated with nebracetam showed a decrease in ^{3}H–valine incorporation. The increase of ^{3}H–valine incorporation into astrocytes after piracetam with dBcAMP treatment was found. Nootropic drugs change morphometric parameters (cell area, perimeter and form factor) of cultured astrocytes. It can be concluded that nootropics have differentiated influence on both the energetic metabolism and morphology of rat astrocytes *in vitro*.

Keywords: nebracetam, piracetam, astrocytes, ATP, phosphocreatine, morphometry.

Nebracetam (4–amino–methyl–1–benzyl–pyrrolidine–2–one–fumarate, NEB) is pyrrolidinone derivative endowed with nootropic, cytoprotective and cholinomimetic activity (1). Several investigators have reported that treatment with NEB exerts an agonistic action on cerebral M1 muscarinic receptors in rats (2), increases acetylcholine (Ach) release from the isolated cardiac sympathetic ganglion in dogs (3) and improves the impairment of the rat working memory induced with scopolamine (4). It has also been shown that treatment with NEB reduces the mortality in mice exposed to hypoxia, and attenuates the cerebral ischemia–induced reduction in ATP and PCr contents in the rabbit brain (1). The mechanism through which these effects are brought about is not yet fully understood.

Measurements of the availability of energy in the form of ATP and PCr are the most commonly used parameters to describe cellular energetic, because retention of high levels usually means that the ATP –producing machinery is functionally intact (5). In cases of the increased demand for ATP, the phosphatic group is transferred from PCr to ADP with the use of creatine kinase (PK) (6). Moreover, cerebral high–energy phosphates play an important role in the metabolism of neurotransmitters such an ACh, catecholamines and amino acids.

Astrocytes are extremely versatile in their metabolic requirements and can metabolise not only glucose and pyruvate but also, e.g., fatty acids and glutamate (7). Astrocytes subjected *in vivo* to such pathological factors as hypoxia, bleedings and demyelinating diseases show morphological changes and start to proliferate to form glial scars (8). The effects simulating pathological states of the astrocytes can be achieved by culturing astrocytes in the presence of cyclic adenosine monophosphate (cAMP) derivatives e.g. dBcAMP (9, 10). The flat astrocytes adopt a star–shaped morphology resembling their in vivo appearance (11, 12). Stellation is characterised by the formation of thin processes, cytoplasmatic retraction and rounding of the cell body (13). The morphological changes are accompanied by the significant biochemical changes, e.g. increased glycogen breakdown and changes in the levels of ATP and PCr (14). For this reason, primary culture of rat astrocytes has been used for biochemical, physiological and pharmacological studies in order to demonstrate protective effects of several drugs on these cells (15).

It was decided to examine the influence of NEB on the content of ATP and PCr in rat astrocytes cultured *in vitro* with and without the presence of dBcAMP. In experiments, as point of reference, the other nootropic drugs, piracetam (2–oxo–1–pyrrolidine–acetamide, PIR) was included for comparison. In order to evaluate objectively changes in the intracellular content of ATP and PCr, the intensity of protein metabolism was determinated by measuring the amount of ^{3}H–valine incorporated into the astrocyte proteins. In addition, a morphometric analysis of the astrocyte cells also cultured in two models and treated with nootropic drugs was performed.

EXPERIMENTAL

Astrocytes were isolated and cultured essentially by the method of Hertz et al. (16). Briefly, hemispheres from one–day old Wistar rat pups were removed aseptically from skulls, freed of the meninges, minced and mechanically disrupted by vortexing in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing penicillin (100 U/ml, Polfa) and streptomycin (100 μg/ml, Polfa). This suspension was filtered through sterile nylon screening cloth with pore sizes 70 μm (first sieving) and 10 μm (second sieving). The cells were plated at 3×10^5 cells/dish (35 mm in diameter, Becton Dickinson) in culture medium containing 20% foetal calf serum FCS (GIBCO). Subsequently, cultures were incubated at 37°C in 95% air and 5% CO_2 with relative humidity 95% (CO_2–Incubator, Kebo–Assab). The culture medium was changed after 4 days of seeding and replaced with medium containing 10% FCS twice a week. Some cultures were also incubated with fresh medium containing 10% FCS and 0.25 mM N^6,O^2–dibutyryl–adenosine–3',5'–phosphate cyclic Na–salt (dBcAMP, Serva) at 14th– day after plating to induce morphological differentiation with pronounced extension of cell processes (10). After 14–days of incubation, the cultures were transferred into fresh medium containing 10% FCS with or without dBcAMP and were grown for 2 weeks with addition of NEB (1×10^{-7} M, Boehringer Ingelheim) and PIR (1×10^{-7} M, Polfa). The concentrations of drugs were chosen based on the data obtained from preliminary experiments. The cells treated for 2 weeks with higher concentration of the drugs were not able to survive. At the same time, the lower dose of drugs had no effect on the cultured cells (data not shown). Control cultures with or without dBcAMP were placed in the same media for 28 days. To identify astrocytes, cultures were stained immunocytochemically for the astrocyte marker by DAKO PAP kit system glial fibrillary acidic protein (GFAP) test, (DAKO Co.). Analysis of such cultures has shown that 90–95% of the cells were astrocytes.

The ATP and PCr concentrations were determined by high specific, firefly luciferin–luciferase bioluminescence assay system using a 1250–luminometer (Bio–Orbit). The determination of ATP and PCr was performed according to the procedure described by Bessho et al. (17) with slight modifications. Then the conversion of PCr into ATP catalysed by creatine kinase was performed according to the method of Lowry and Passonneau (18). ATP concentrations were measured by the bioluminescence method of Lust et al. (19). Briefly, 100 μl samples of disrupted cells were added to equal volumes of ice–cold 5% trichloracetic acid solution (TCA, Sigma) with 2 mM etylenediaminetetraacetic acid disodium salt (EDTA 2Na, Sigma) vortexed rapidly for 5 s and incubated for 30 min at room temperature. Then the samples were diluted 20–fold with Tris–acetate buffer pH 7.75 and 10 μl solution of known ATP concentration was added to each cuvette as internal standard and the total amount of ATP was determinated. The results were calculated as nM of ATP and PCr per mg of protein (nM/mg).

The amount of the incorporated ^{3}H–valine was measured according to the method of Perez–Polo (20). After incubation with nootropic drugs, the cells were incubated with L–[3,4–^{3}H]–valine (57.3 Ci/mM, NEN) 3μCi/dish (4 ml) for 3 h in serum–free DMEM. The incubation was terminated by aspiration of the medium and rinsing twice with ice–cold Gey's balanced salt solution. Cells were scraped from the dishes with a plastic policeman (Costar Co.) and added to a tube with 0.5 ml of ice–cold 1 mM Tris–HCl buffer at pH 7.2 (containing 0.05 M glucose and 0.03 M NaCl). Later, these cells were digested in 1 ml of 0.1 N NaOH for 30 min 0.5 ml of 50% TCA was then added to each tube and samples were incubated overnight at 4°C. Next day the samples were filtered through GF/B filters (Whatmann). The filters were washed with 15 ml of ice–cold 5% TCA, dried under a heat lamp and transferred to a scintillation vials, each with 8 ml of Bray's scintillating solution. Radioactivity was determined in a Beckman LS 6000 IC counter (Beckman Instruments Inc.). The results were calculated in dpm per mg of protein (dpm/mg).

Cultures of astrocytes after the treatment with nootropic drugs were stained with 1% toluidine blue solution (Sigma) and used for morphometric studies. The cytoplasmic areas of 80 consecutive

Table 1. Effect of dBcAMP on intracellular ATP and PCr concentration, ^{3}H–valine incorporation and morphometric parameters of astrocytes in primary cell culture

Condition	ATP nM/mg protein	PCr Nm/mg protein	^{3}H–valine dpm/mg protein	Area μm^2	Perimeter μm	Form factor
without dBcAMP	25.7±0.69 (12)[a]	99.5±2.79 (12)[a]	15135±152 (12)[a]	1805±48 (80)[b]	244±4.5 (80)[b]	0.3855±0.009 (80)[b]
with dBcAMP	15.4±0.62** (16)[a]	73.9±2.9** (16)[a]	3809±184** (16)[a]	3122±86** (80)[b]	386±9.5** (80)[b]	0.27±0.007** (80)[b]

The concentration of dBcAMP was 0.25 mM. The shown data indicate means ± SEM for number of dishes[a] or cells[b] given in parentheses. **p<0.001, statistically significant differences from the group the astrocytes cultured without or with dBcAMP followed by Student's t–test.

single, flat cells were outlined for each treatment group using a cursor–guided digitising tablet attached to computerised video imaging system VIDS IV (Analytical Measuring Systems, Cambridge) connected to an OPTI 200M microscope (Nicon Inc.). The system was calibrated and the cells outlined at 20x objective. For each cell, perimeter (μm), area (μm^2) and „form factor" defined as (4π) (area/perimeter^2) were determined. Results were reported as the mean ± SEM.

Protein content of astrocytes was measured according to the method of Lowry et al. (21).

The unpaired data were analysed with Student's t–test. The results were statistically significant when $p<0.01$.

RESULTS

Addition of dBcAMP resulted in the changes both in biochemical and morphometric astrocytes' parameters in vitro. Table 1 presents the intracellular ATP and PCr content as well as the amounts of ^{3}H–valine incorporated into proteins and morphometric parameters of astrocytes treated and untreated in vitro with dBcAMP at a concentration of 0.25 mM.

The amount of ATP in a 28–day astrocyte culture was 25.7±0.69 nM/mg of protein and was close to the results obtained by other investigators (22, 23). It is close to the amount of ATP present in the brain of an adult rat, which approximates 30 nM/mg of protein (24). It is known that intracellular PCr concentration is greater than ATP (25, 26). The extractability of PCr from biological materials is dependent on the method used (17, 27, 28). In our study the mean PCr concentration after the direct perchloric acid extraction was 99.5±2.8 nM/ mg of protein in the culture untreated with dBcAMP. Significant decreases in intracellular ATP and PCr content by 40% and 26%, respectively, compared with the contents in the cells cultured in the medium devoid of dBcAMP were found.

The intensity of ^{3}H–valine incorporation into protein was measured to confirm an effect of PIR and NEB on astrocyte energy metabolism. The initial incorporation of ^{3}H–valine into astrocyte proteins was 5135±152 dpm/mg of protein. Measurement performed on astrocytes treated with dBcAMP revealed the 26% decrease in the amino acid incorporated into proteins compared with the untreated cultures.

Morphometric analysis of culture plates stained with 1% toluidine blue solution confirmed the essential influence of dBcAMP on the shapes of astrocytes. It obtained the following average results of the morphometric parameters for the astrocyte cells, which were not treated with dBcAMP: area 1805±48 μm^2, perimeter 244.5±4.5 μm, form factor 0.38±0.009. These results of morphometric analysis of the astrocytes in primary culture are similar to values obtained by Stiene–Martin et al. (29). In the culture with dBcAMP, it was observed significant changes in the size and form of astrocytes. The amount of processes was also increased which is indicated by the form factor value. The area and the perimeter were larger than 72% and 58%, respectively, compared with the control values without dBcAMP. The increase in astrocyte branching was confirmed by form factor smaller than 29%.

The results obtained in the studies conducted on astrocytes cultured in the medium without dBcAMP are control values in studies of NEB and PIR effects on astrocytes cultured *in vitro* without dBcAMP addition. Cells cultured with dBcAMP at

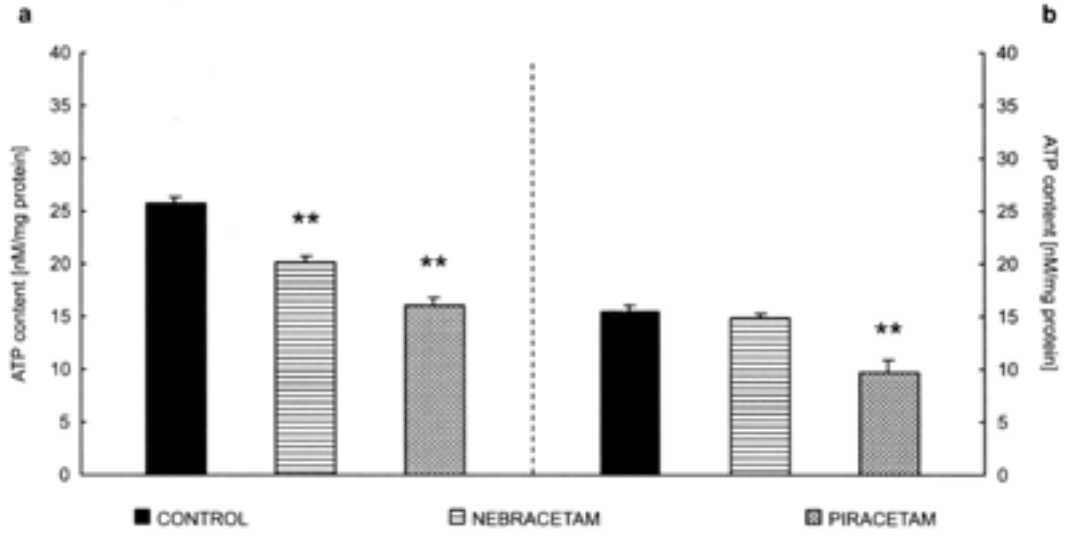

Figure 1. Effect of nebracetam and piracetam on ATP levels in astrocytes cultured *in vitro* without (**a**) or with dBcAMP (**b**). The concentration of nebracetam and piracetam was 1×10^{-7} M. Each value is mean of eight dishes ± SEM (n=8); **p<0.001.

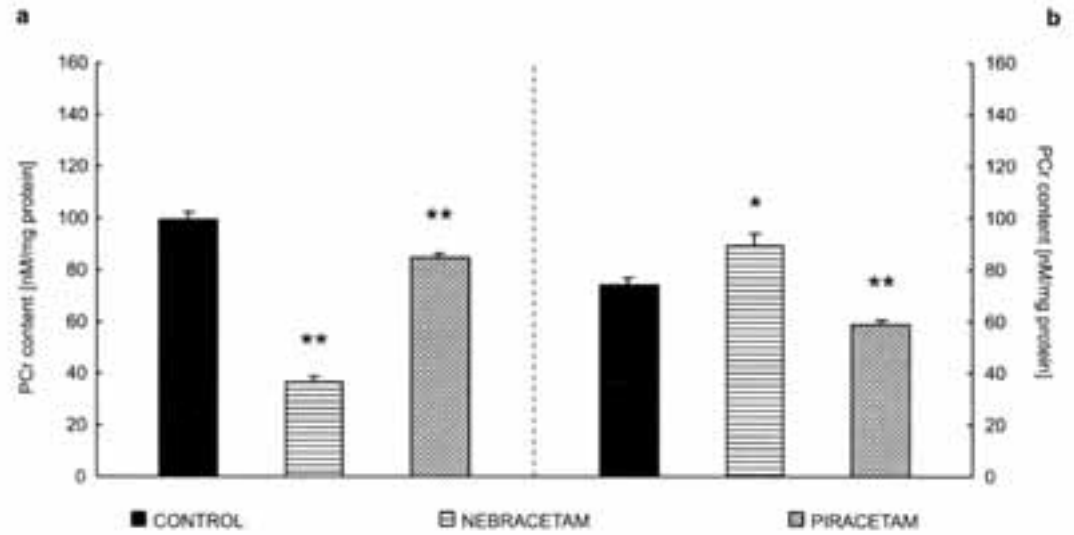

Figure 2. Effect of nebracetam and piracetam on PCr levels in astrocytes cultured *in vitro* without (**a**) or with dBcAMP (**b**). The concentration of nebracetam and piracetam was 1×10^{-7} M. Each value is mean of eight dishes ± SEM (n=8); ** p<0.001.

a concentration of 0.25 mM for 2 weeks served as a control for astrocytes treated simultaneously with dBcAMP and nootropic drugs.

NEB, in the cultures performed without the addition of dBcAMP, had caused statistically significant decrease in ATP in astrocytes by 26% in comparison with the control (Figure 1a). It was shown that in both cultures PIR had reduced the concentration of intracellular ATP by 37% and 40%, respectively, in comparison with the control values (Figure 1). NEB did not cause any changes in this parameter when was added together with

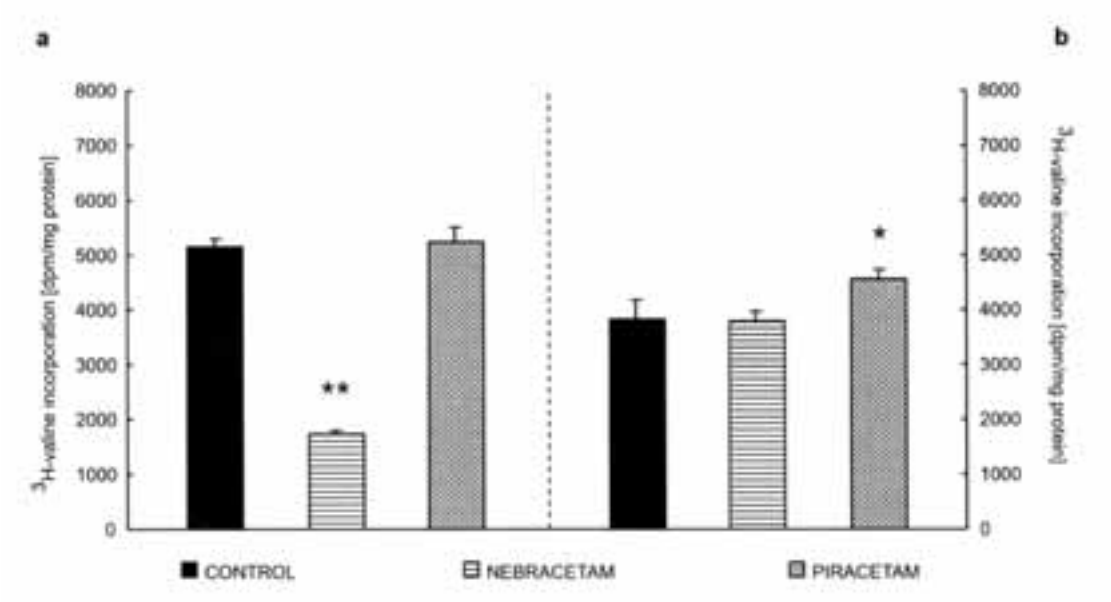

Figure 3. Effect of nebracetam and piracetam on ^{3}H–valine incorporation into astrocytes cultured *in vitro* without **(a)** or with dBcAMP **(b)**. The concentration of nebracetam and piracetam was 1×10^{-7} M. Each value is mean of eight dishes ± SEM (n=8): *p<0.01; **p<0.001.

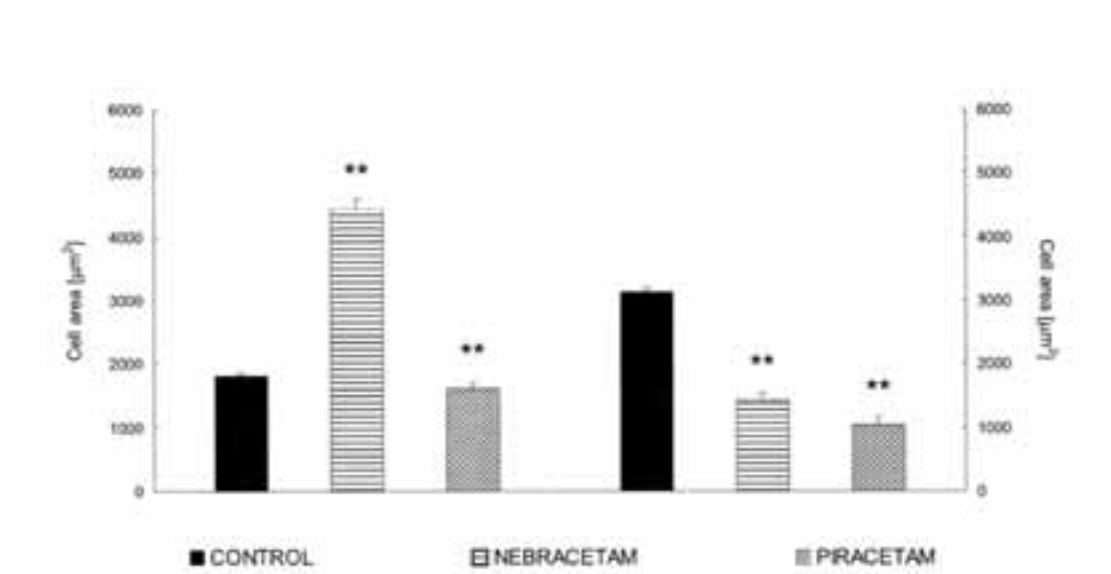

Figure 4. Changes in the cell area of astrocytes cultured *in vitro* without **(a)** or with dBcAMP **(b)** and treated with nebracetam and piracetam at concentration 1×10^{-7} M. A total of 80 astrocytes were measured for each drug. Values are mean ± SEM; **p<0.001.

dBcAMP to the astrocyte culture (Figure 1b). As shown in Figure 1b, NEB decreased the amount PCr in culture untreated with dBcAMP. In the culture to which NEB and dBcAMP were added, an increase in the intracellular amount of PCr by 22% was observed in comparison with the control values (Figure 2b). The reference agent, PIR decreased the amount of PCr in astrocyte culture performed either with or without dBcAMP by 15% and 20% respectively, in comparison with the control (Figure 1).

As shown in Figure 3, the intensity of ^{3}H–valine incorporation was decreased by 68% in

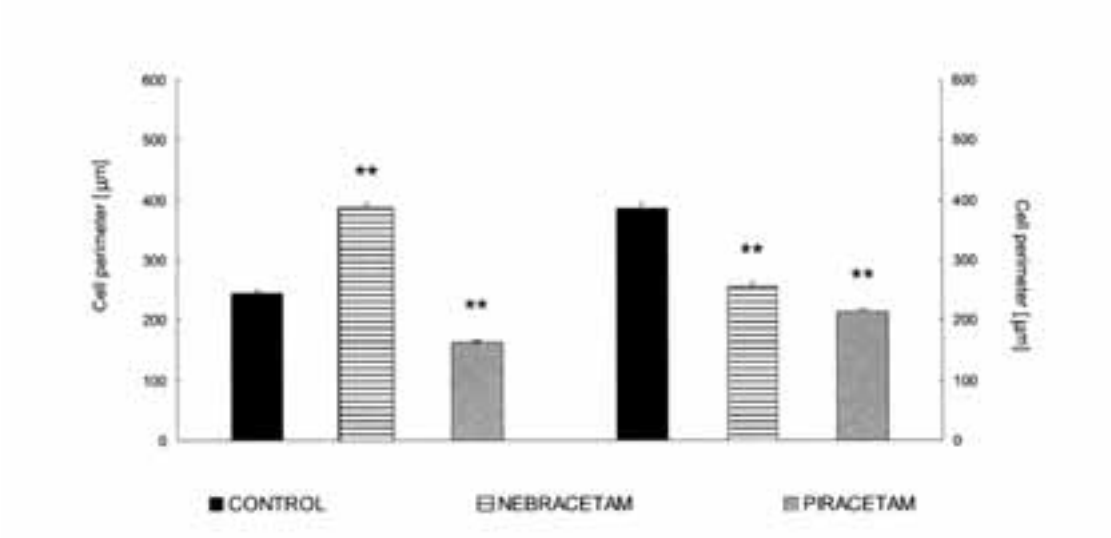

Figure 5. Changes in the perimeter of astrocytes cultured *in vitro* without **(a)** or with dBcAMP **(b)** and treated with nebracetam and piracetam at concentration 1×10^{-7} M. A total of 80 astrocytes were measured for each drug. Values are mean ± SEM; **p<0.001.

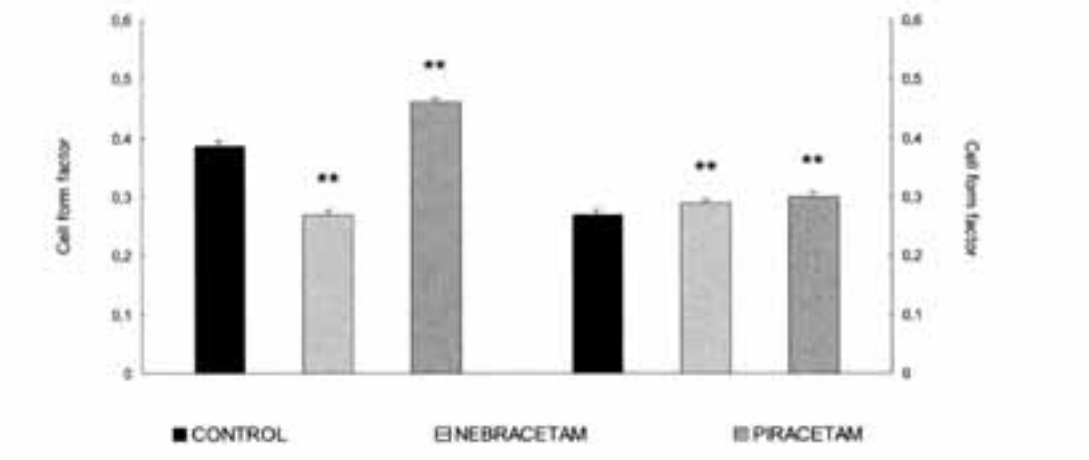

Figure 6. Changes in the form factor of astrocytes cultured *in vitro* without **(a)** or with dBcAMP **(b)** and treated with nebracetam and piracetam at concentration 1×10^{-7} M. A total of 80 astrocytes were measured for each drug. Values are mean ± SEM; **p<0.001.

astrocytes treated with NEB, but did not change after the simultaneous of NEB and dBcAMP. The absence dBcAMP in the medium is consistent with no change PIR in the amount of the ^{3}H–valine incorporated into astrocyte proteins. On the contrary, after adding PIR together with dBcAMP 20% increase in the ^{3}H–valine incorporated into protein was observed (Figure 3).

Nootropic drugs influences on energy metabolism and protein synthesis might also be expected to induce compensatory changes in the cells' shape. In the culture untreated with dBcAMP PIR reduced

the astrocytes' area by 11% and perimeter by 23% in comparison with the control (Figure 4a, 5a). However, the form factor value was increased by 20% (Figure 6a). NEB increased the cell area and perimeter by 165% and 31% respectively, in this culture. Nootropic drugs altered the values of parameters measured in the culture with dBcAMP. Simultaneous adding of NEB and dBcAMP caused statistically significant decrease of cells' area by 54%, the perimeter by 34% and increase the form factor by 11%. On the contrary, PIR reduced the area by 11% and the perimeter by 44%, but no changes in form factor was observed (Figure 4b, 5b, 6b).

DISCUSSION

Pathogenesis of cognitive dysfunction may result from a cholinergic deficit (30, 31). However, ACh and choline uptake is energy dependent mechanisms, which are directly linked to glucose metabolism (32, 33). Moreover, the brain tries to maintain its energy–rich phosphates under hypoxia and other deteriorating conditions. What incited us to carry out the hereby research was one of the hypotheses assuming that the effectiveness of the treatment with nootropic drugs is connected with their influence on the energetic metabolism of the brain and cytoprotective effect on the brain cells (34, 35, 36). The findings with the reference compound PIR are controversial. Some authors were unable to find effects of PIR on the amount of high–energy compounds in the brain, while others reported some improvement (36, 37). Nickolson and Wolthuis (36) did not find any changes in the amount of ATP in homogenates of a rat brain after a single PIR dose of 150 mg/kg. However, they showed the increased activity of adenyl kinase, catalysing the transformation of ADP into ATP and AMP. Qian et al. (38) showed that, as a result of long–term treatment rats with PIR at the dose of 600 mg/day for 30 day, the activity of Na^+/K^+ ATP–ase in the animals' brain is reduced. Thus, there were attempts to explain the nootropic activity of PIR through its influence on the activity of the enzymes catalysing the synthesis or decomposition of ATP by the decreased consumption of glucose. Unexpectedly, the present results indicate that neither of the two drugs has any positive effect on the level of high–energy phosphates in astrocytes in primary cell culture. Adding NEB at the concentration of 1×10^{-7} M to the astrocyte culture caused the decrease in the amount of high–energy compounds (Figure 1a, 2a). The direct measurement of the intracellular amount of ATP and PCr in undamaged astrocytes, which we performed, did not show the NEB ability to increase the high–energetic phosphates *in vitro*. The results of the hereby research suggest either decrease in synthesis or increase in metabolism of ATP in the astrocytes treated with PIR. Some investigators assign to NEB the protective qualities especially on the brain cells *in vitro* damaged in the result of energy deficiency (39, 40).

Alternations in a number of biochemical properties have been observed following dBcAMP treatment. (14, 41). The changes cause, among others, intensification of aerobic metabolism through the increase in glucose and glycogen utilization (10). Results of cell culture with dBcAMP indicate possible protective activity of the drugs against the changes caused by cAMP. We observed a decrease in ATP and PCr concentrations in the control cultures performed with dBcAMP, suggesting that sustained impairment of energy metabolism occurred. Reduction of high–energy phosphates, particularly a decrease of ATP is considered to cause of disturbances in the transmembrane fluxes of ions, which are important factors in the regulation of intracellular ion concentration (41). Just like in the experiment with astrocytes, which were not treated dBcAMP, also in the research model with dBcAMP, PIR reduced the amount of high–energy compounds in the astrocyte cells (Figure 1b, 2b). So the qualities assigned to PIR, like preventing the reduction of ATP and PCr concentrations in the brain in harmful conditions (e.g. ischemia), cannot be confirmed (36). NEB, in a culture with dBcAMP, increased in the amount of PCr by 22%, without changing in intracellular amount of ATP (Figures 1b, 2b). The enhancement of the adenylate kinase activity might offer an explanation for the protective effect of NEB against damage of astrocytes by dBcAMP. When damage set in, oxidative phosphorylation stops and the ATP content of the brain starts to decrease, whereas the AMP and initially, the ADP content increase (36). In such a situation the decrease of the ADP content is slowed down by formation of ATP, catalysed by adenylate kinase. An increase of the adenylate kinase by NEB would accelerate this compensatory process and could thereby offer some protection against effects of dBcAMP. The results seem to confirm Kuhn's et al. hypothesis (1), that NEB can effectively prevent the decrease in ATP and PCr in the brain cells in cases of oxygen and glucose deficiency in the extracellular environment. Also, Takeo et al. (42) showed that treatment of microsphere–embolized with NEB exerted even after induction of cerebral ischemia

a beneficial effect on the brain energy metabolism. Ohno et al. (4) suggested that the mechanism, underlying an improvement of the rat working memory, might be due to the beneficial effect of NEB on the energy metabolism and to its cholinergic activation of the central nervous system.

Not only the high–energy phosphates contents of astrocytes *in vitro* were measurement, but also the amount of incorporated ^{3}H–valine was determinated, a parameter, which appears to be the important determinant of state of energy metabolism. The results obtained indirectly provide information about the cell energy resources and are a simple indicator of the examined compound toxicity. Measurement of protein synthesis *in vitro* has been used as a description of toxicity of several drugs. Greene and Magasanic (43) first used this criterion to estimation of levallorphan on the growth of the HeLa cells. They showed that reduction of the amount of ATP was connected with the increase in activity of Na^+/K^+ ATP–ase and the decrease in protein synthesis.

There are reports available concerning the stimulating effect of PIR on protein synthesis in brains of both young and old animals (44). However, in culture without dBcAMP NEB as well as PIR significantly reduced the amount ATP. This resulted in turn by inhibited protein synthesis. The mechanism involved in the depression of astrocytes protein synthesis appears to be depression in the activity of the ribosomal fraction (45). In turn, the mechanism behind this NEB and PIR effect might be a general depression of metabolism of astrocytes (46). The ATP level and intensity incorporation ^{3}H–valine into protein tended to return toward control levels after simultaneous influence of NEB and dBcAMP on astrocytes in vitro.

Astrocytes undergo morphological transformation as a response to the changes in extracellular environment. This phenomenon is called „astrocyte plasticity" (47). O'Callaghan et al. (48) suggest using the morphometric analysis for the measurement of qualitative changes in the cell cultures taking place under the influence of drugs and xenobiotics, stressing at the same time the rare occurrence of this sort of research. It has been shown that the changes in the intracellular biochemical processes, particularly in the protein biosynthesis are closely connected with the morphological changes. For this reason, we decided to measure three morphometric parameters: area, perimeter and form factor. The last one determines the degree of cell branching and as such indirectly reflects changes taking place in the astrocyte cytoskeleton. Goldman and Chiu (49) in their research of the astrocytes *in vitro* proved that the increase of cell branching is connected with the increase in the intracellular amount of cAMP. Under the influence of cAMP, there appear long, thin cytoplasmic processes and the amount of GFAP in astrocytes increases. The rate of cell division is reduced. Similar results were also obtained by Kimelberg et al. (50). The addition of dBcAMP to the culture medium has dramatic effects upon glial morphology. It has rather modest effects upon most biochemical and physiological function examined, e.g. moderate increases in the activity of Na^+/K^+–ATPase which may be a reflection of increased membrane area associated with process formation, activation of the protein kinase C and lactate dehydrogenase (9, 16, 50, 51). In the culture with dBcAMP, we observed significant changes in the size and form of the astrocytes. The area and the perimeter were larger compared with the control values without dBcAMP. The increase in astrocyte branching is confirmed by smaller form factor.

The influence of nootropic drugs on the astrocyte form transformation processes remains unknown. It was shown that nootropic drugs changed in a different way the morphometric parameters measured in culture of astrocytes with and without dBcAMP. PIR reduced area and perimeter cells untreated with dBcAMP. However, the form factor value was increased.

The astrocytes reacted in a different way to the presence of NEB in the culture medium that increased the area, the perimeter and the form factor. Nootropics also altered the values of parameters in the culture with dBcAMP. NEB and PIR reduced the cell area and perimeter. The form factor was increased under the influence of NEB and dBcAMP. We cannot exclude the possibility that different response of astrocytes to the examined nootropic drugs is connected with their different influence in the intracellular cAMP.

The experiment showed differentiated influence of NEB and PIR on both the energetic metabolism and the morphology of astrocytes *in vitro*. In this study, NEB was in most respects decidedly superior in comparison to PIR. These results suggest that NEB is effective agent for the restoration of astrocytes energy metabolism against dBcAMP–induced, sustained astrocyte damage. It seems that the presented research model enables the estimation, in *in vitro* conditions, of the effectiveness of NEB in prevention of the energetic deficiency of brain. It will be necessary to examine the influence of NEB and PIR and other pyrrolidinone derivatives on carbohydrate metabolism.

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