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Different Mechanisms of Protective and Differentiative Activities of Homological Peptides TGENHR and TQVEHR

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Abstract—Previously we identified a six-membered fragment ^{354}TQVEHR^{359} of the C-terminal part of the PEDF (Pigment Epithelium-Derived Factor) differentiation factor molecule that shares homology with fragment ^{41}TGENHR^{46} of the HLDF (Human Leukemia Differentiation Factor) differentiation factor molecule, which is responsible for its differentiation activity. HLDF has been isolated from the culture medium of human promyelocytic leukemia cell line HL-60. Hexapeptides HLDF-6 (TGENHR) and PEDF-6 (TQVEHR) corresponding to these HLDF and PEDF molecule fragments, which were previously shown to induce cell differentiation (Kostanyan et al. (2000) *Russian Journal of Bioorganic Chemistry*, **26**, 505-511), also have neuroprotective properties. Both peptides prevent degeneration of Purkinje cells of rat cerebellar vermis upon chemical hypoxia induced by sodium azide *in vivo*; this effect is also observed on a behavioral level. Peptide HLDF-6 but not PEDF-6 promotes survival of HL-60 cells upon chemical hypoxia. Peptides HLDF-6 and PEDF-6 affect different second messenger biosynthesis systems in HL-60 cells. HLDF-6 diminishes cyclic AMP level in those cells due to adenylate cyclase inhibition, while PEDF-6 inhibits phosphatidylinositol-specific phospholipase C stimulated by aluminum tetrafluoride anions.

Key words: HLDF, PEDF, peptide, protective activity, chemical hypoxia, cerebellum, HL-60 cell line, adenylate cyclase, phosphatidylinositol-specific phospholipase C

Neuroprotector research and development is presently of great importance due to a dramatic increase in neurodegenerative diseases. Neuroprotectors are the substances, which prevent degeneration of neurons occurring spontaneously or induced by some damaging factors.

PEDF (Pigment Epithelium-Derived Factor) is an endogenous neuroprotector first isolated in 1989 from the culture medium of eye retinal pigment epithelium cells of the human embryo [1, 2]. Its neuroprotective effect has been shown on different types of neurons, such as spinal cord neurons, hippocampal neurons, retinal photoreceptor cells, and cerebellar granule cells [3-7]. In addition, PEDF induces differentiation of neuroblasts, retinoblasts, and embryonic motor neural precursors: these cells change their phenotype to mature, functionally active neurons [2, 8, 9]. More recently, anti-angiogenic properties of PEDF were discovered: it inhibits formation of new blood vessels, particularly in some tissues and cell-free mediums which normally do not contain blood vessels, such as eye retina and vitreous humor [10].

PEDF is a single polypeptide chain protein containing 418 amino acid residues. Its molecular mass is 50.1 kD. Based on typical features of primary and spatial structure, it was ascribed to the SERPIN (SERine Protease INhibitor) superfamily, but its inhibitory effect has not been observed on any known protease [11]. PEDF receptor was identified in the surface of retinoblastoma cells and spinal cord neurons. PEDF-receptor binding was shown to be associated with the PEDF molecule fragment ^{78}V-^{121}T which is located in its N-terminal region [12, 13]. However, it is not clear yet whether the C-terminal region of PEDF plays a role in its neuroprotective activity.

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Previously we have shown a structural homology between the PEDF molecule fragment ^{349}K-^{366}E, which is located in the C-terminal part of PEDF, and the C-terminal fragment ^{37}K-^{54}K of the HLDF (Human Leukemia Differentiation Factor) molecule [14]. HLDF differentiation factor (54 amino acid residues polypeptide chain, molecular mass 8.2 kD) was first isolated in our laboratory from the culture medium of human promyelocytic leukemia cell line HL-60 stimulated to differentiation by all-*trans*-retinoic acid [15]. HLDF induces differentiation of HL-60 cell line by the granulocyte/neutrophilic pathway. It also protects this cell line from several damaging factors, e.g., cold shock. These effects of HLDF were shown to be associated with the six-membered molecule fragment ^{41}TGENHR^{46} [16, 17]. It should be noted that the hexapeptide TGENHR, which was named HLDF-6, also induces HL-60 cells differentiation. Earlier investigations of molecular mechanisms of interaction between HLDF-6 peptide and HL-60 cells showed the absence of specific receptors on the cell membrane surface. The peptide interacts with cell membrane lipids, increasing their mobility in the bilayer structure. This was confirmed by experiments on artificially made phosphatidylcholine liposomes [16].

We first showed that PEDF induces differentiation of HL-60 cells; this effect is associated with the six-membered molecule fragment ^{354}TQVEHR^{359}, which shares homology with the HLDF molecule fragment ^{41}TGENHR^{46}; the latter is responsible for HLDF differentiation activity. Hexapeptide TQVEHR (which was named PEDF-6) reproduces the differentiation effect of PEDF on HL-60 cells [14]. Taking into account the structural homology between HLDF-6 and PEDF-6 peptides, we suggested that the latter can act not only as an inducer of cell differentiation, but also as a cell protector.

One of the models for testing of neuroprotective activity is the application of an agent being tested to the vermis of rat cerebellum in the presence of some factor causing neurodegenerative processes. The vermis of the cerebellum is its middle part, which is located between the hemispheres. Vermal neurons are involved in some behavioral reactions such as conditional fear formation, taste aversion, neophobia, and long-term habituation of acoustic startle reaction (ASR), i.e., attenuation of response to repetitive stimulation by intensive sound impulses. Lesions of the vermis lead to failure of those reactions [18, 19].

In the present study, protective effects of HLDF-6 and PEDF-6 peptides have been tested on Purkinje cells of the vermis of rat cerebellum (*in vivo*) and HL-60 cell culture (*in vitro*) in chemically induced hypoxia conditions. The peptides were shown to mediate their effects on HL-60 cells by different pathways: HLDF-6 inhibits adenylate cyclase activity, while PEDF-6 decreases phosphatidylinositol-specific phospholipase C activity upon its stimulation by aluminum tetrafluoride anions.

MATERIALS AND METHODS

Materials. RPMI-1640 cell culture medium and fetal calf serum (FCS) were purchased from Gibco (USA); 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazoliumbromide (MTT reagent) and 3-(3-cholamidopropyl)-dimethylammonium-3-propansulfate (CHAPS) were from Sigma (USA); guanosine-5′-[β,γ-imido]-trisphosphate (β,γ-imido-GTP) was from Serva (Germany), and forskolin was from Calbiochem (Great Britain).

Salts used in buffer solutions were produced in Russia, chemically pure grade.

HLDF-6 and PEDF-6 peptides were synthesized by the solid-phase method using the Boc/Bzl methodology as described previously [14].

HL-60 cell line cultivation. HL-60 cell line was kindly provided by Dr. R. G. Vasilov (Institute of Biotechnology, Moscow). Cells were cultivated in RPMI-1640 medium containing 7% fetal calf serum at 37°C in an atmosphere of 5% CO_2.

Animals. Male Wistar rats (80-100-day-old, 250-300 g) were used. The rats were obtained from the Biological Testing Laboratory, Branch of Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry (Pushchino, Moscow Region). Animals were kept three in a cage at constant temperature 22 ± 1°C, 14/10 h light/dark cycle, food and water *ad libitum*.

Quantification of hyperchromic Purkinje cells in the vermis of rat cerebellum. Behavioral testing. Four groups of rats were tested, each containing eight animals. Animals of the first group were once injected intracranially with 1 mM sodium azide in saline (0.15 M NaCl). The second and third groups were injected with 1 mM sodium azide in saline containing 100 μM HLDF-6 or PEDF-6, respectively. The fourth (control) group was injected with pure saline. Injections were made by a microsyringe through the cranial hole according to stereotaxic coordinates: 3 mm posterior to lambda; L – 0; DV – 3 mm. Before injections, animals were anesthetized with sodium thiopental (40 mg/kg).

Primary testing of behavioral reactions was performed 5 days after injections, and secondary testing was performed 24 h later. Both testings were performed between 11 and 15 o'clock. Animals were placed into a chamber with wide-band background noise (intensity 72 dB). Chamber structure as well as testing protocol were described in detail by Pletnicov et al. [20]. The following parameters were registered: 1) duration of full immobility ("freezing") period as a result of the conditional fear caused by sudden animal replacement from home cage to the chamber; 2) maximal pressure on the chamber platform made by sudden paw extension as a reaction to the first sound impulse in series (impulses are wide band, intensity 110 dB).

The data were statistically analyzed using Statistica software. Pressures on the chamber platform were recalculated to reduce to a standard animal mass (250 g).

In 48 h after behavioral testing animals were anesthetized with sodium thiopental (50 mg/kg of weight) and decapitated. The cerebellum was taken out and treated by a standard histological procedure including formaldehyde fixation and embedding in paraffin [21]. Histological slices (6 μm thick) were deparaffinized and stained with hematoxylin and eosin B. Slices were then studied using light microscopy. Normal and hyperchromic Purkinje cells in the cerebellar vermis were counted visually. Hyperchromic cells were characterized by darkened vacuolized cytoplasm. Five or more slices were analyzed for each animal, and 100 or more Purkinje cells were counted on each slice.

MTT test. HL-60 cells were incubated for 24 h with sodium azide and peptides in RPMI-1640 medium containing 7% fetal calf serum. Then the cells were washed with phosphate-buffered saline. Aliquots of cell suspension (200 μl) were then transferred to 96-well microplates previously treated with poly-L-lysine (10 mg/liter). MTT reagent solution (20 μl) in serum-free RPMI-1640 medium (4 mg/ml) was added to cells followed by 4 h incubation at 37°C, then the medium was removed. Viable cells containing formazan granules were lysed with 0.04 M HCl in isopropanol (100 μl per well) for 15 min at room temperature and continuous shaking. Extinction at 540 nm was measured using a Multiskan MCC/340 spectrophotometer (Labsystem, Finland). The data were obtained as assay/control ratio (%).

HL-60 cell membrane preparation. HL-60 cells ($5 \cdot 10^7$) were precipitated by centrifugation (5 min, 1000*g*), resuspended in 5 ml buffer A (0.04 M Tris-HCl, 0.8 M sucrose, 5 mM $Mg(CH_3COO)_2$, 1 mM PMSF, pH 7.4) and homogenized using a tissue grinder for 5 min at 4°C followed by centrifugation (20 min, 100,000*g*, 4°C). The precipitate was resuspended in 1.5 ml buffer B (0.04 M Tris-HCl, 0.25 M sucrose, 5 mM $Mg(CH_3COO)_2$, 1 mM PMSF, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, pH 7.4), incubated with stirring for 5 min at 4°C, and centrifuged under the same conditions as before. The precipitate consisting of membranes was resuspended in 0.5 ml buffer B and stored at −70°C.

For solubilizing with CHAPS, precipitated membranes were resuspended in 1 ml buffer B containing 10 mM CHAPS followed by 1 h incubation at 4°C and continuous stirring. Solubilized membrane fraction was then separated from the insoluble residue by centrifugation (20 min, 100,000*g*, 4°C).

Adenylate cyclase assay. HL-60 cells (10^7) in 0.5 ml RPMI-1640 medium containing 7% fetal calf serum or 0.5 ml serum-free RPMI-1640 medium were stimulated with 5 μl of HLDF-6 or PEDF-6 aqueous solution (10^{-4} M). The final peptide concentration in the cell suspension was thus 10^{-6} M. Cells were incubated with peptides at 37°C for the determined time period, then the reaction was stopped by adding of 1 ml 95% ethanol. After that, cells were homogenized by 30-time forcing through an insulin syringe U-40 with a thin needle (Becton Dickinson, Ireland). cAMP was extracted by ethanol extraction procedure: homogenates were shaken for 2 min followed by incubation for 10 min at 4°C and centrifugation (15 min, 10,000*g*, 4°C). cAMP-containing supernatants were then dried.

Adenylate cyclase assay using HL-60 cell membranes was performed in a special medium: 80 mM Tris-HCl, 1.2 M sucrose, 4 mM ATP, 4 mM DTT, 8 mM $MnCl_2$, 4 mM caffeine, 0.1% (w/v) BSA and, if necessary, 1 μM forskolin or 10 μM β,γ-imido-GTP. The medium (0.1 ml) was mixed with 0.1 ml of peptide solution with 4-fold concentration and added to 0.2 ml of membrane suspension or solubilized membrane fraction. The reaction was performed for 20 min at 37°C and then stopped by adding of 95% ethanol (0.8 ml). Extraction of cAMP was performed as described above.

cAMP was quantified using Cyclic AMP (^{3}H) Assay System (Amersham Biosciences, UK) according to the supplier's manual.

Phosphatidylinositol-specific phospholipase C assay. HL-60 cells (10^6/ml) were cultivated with myo-[^{3}H]-Ins (10 μCi/ml of culture medium) for 24 h. Then cells were washed twice with a buffer containing 10 mM Hepes-NaOH, 5.6 mM glucose, 154 mM NaCl, 5.6 mM KCl, 1.3 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, pH 7.4, followed by resuspension in the same buffer containing 20 mM LiCl. This buffer (0.3 ml) containing peptide in twofold concentration and, if necessary, 20 μM $AlCl_3$ and 20 mM NaF was added to 0.3 ml of cell suspension. Cells were incubated for 1 h at 37°C in a CO_2 incubator. After removal of incubation mixture, cells were lysed with 0.1 M HCl for 20 min at room temperature. Inositol mono- and bisphosphates were separated from other cell components by ion-exchange chromatography using Dowex AG 1×8 resin (Serva, Germany) as described by Stutchfield and Cockcroft [22]. ^{3}H-Labeled InsP and $InsP_2$ were eluted from columns with 1 M ammonium formate/0.1 M formic acid buffer.

RESULTS AND DISCUSSION

HLDF-6 and PEDF-6 peptides protect Purkinje cells of rat cerebellar vermis from degeneration caused by chemical hypoxia *in vivo*. In the vermis of normal rat cerebellum, some of the Purkinje cells represented as "hyperchromic cells" under light microscopic investigations. They correspond to "darkened" or osmiophilic neurons under electron microscopic investigations. According to the majority of the data in the literature, hyperchromic cells are regarded as neurons with diminished functional activity [23]. The ratio of hyperchromic and normochromic (i.e., normal) Purkinje cells in the vermis of cerebellum is an integrative index characterizing the functional state of the nervous system. It is

increased in conditions of hypoxia, malnutrition, and high physical activity, but may be decreased by introduction of some nutrients stimulating energetic resources of brain cells [24, 25].

We have shown that chemically induced hypoxia of vermal cells leads to dramatic increase of the number of hyperchromic Purkinje cells observed in histological slices of the vermis. These cells are characterized by darkened cytoplasm and condensed nuclear chromatin. Hypoxia was induced by sodium azide, an inhibitor of mitochondrial respiratory chain enzymes. Sodium azide solution (1 mM, 5 μl per animal) injected intracranially with direct application to the vermis increased the number of hyperchromic Purkinje cells from 25 to 63% (Fig. 1, Table 1). However, simultaneous application of HLDF-6 or PEDF-6 (10^{-4} M) peptide and sodium azide decreased the number of hyperchromic cells in comparison with animals treated only by sodium azide (Fig. 1, Table 1).

PEDF is a neuroprotective factor whose effects have been shown on several types of neurons *in vivo* and *in vitro*. In particular, PEDF protects rat cerebellar granule cells from glutamate toxicity, and it also maintains survival of these cells in culture [6, 7]. According to Bilak et al. [13], neuroprotective effects of PEDF appear to be associated with the molecular fragment ^{78}V-^{121}T, which is located in the N-terminal region, but we have obtained results confirming the participation of the fragment ^{354}TQVEHR^{359} in the neuroprotective activity of this factor.

We have demonstrated the neuroprotective effects of HLDF-6 and PEDF-6 on the vermis of rat cerebellum on a behavioral level. Chemical hypoxia of the cerebellar vermis leads to failure of some typical behavioral reactions, such as conditional fear formation and long-term habituation of acoustic startle reaction (ASR). Conditional fear intensity was estimated by duration of full animal immobility period observed after the sudden

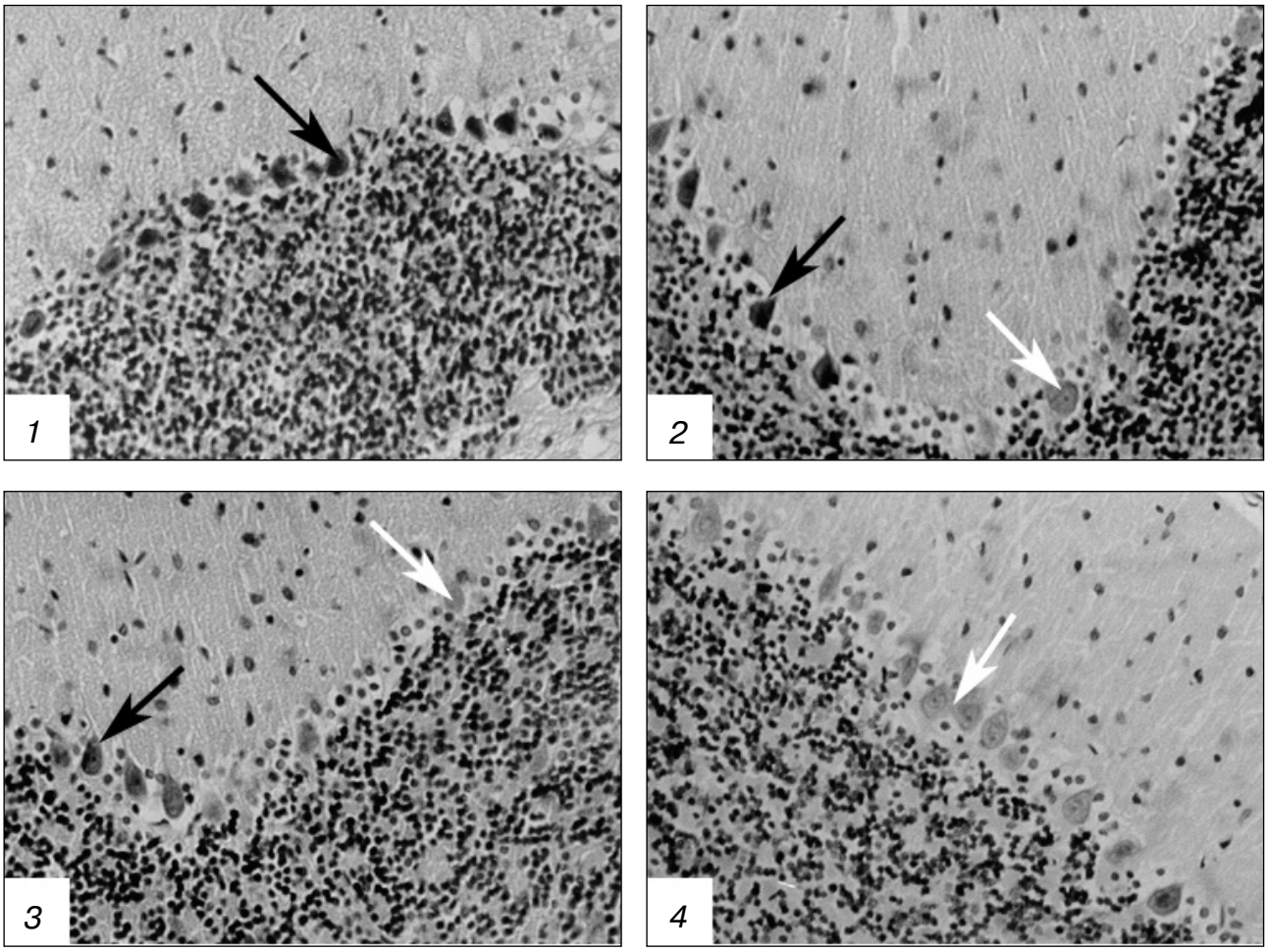

Fig. 1. Rat cerebellar vermis histological slices made 8 days after direct application to the vermis: *1*) sodium azide solution (1 mM) in saline; *2*) sodium azide solution (1 mM) containing HLDF-6 (10^{-4} M); *3*) sodium azide solution (1 mM) containing PEDF-6 (10^{-4} M); *4*) saline (control group). Five microliters of each solution was used per animal. White arrows, normal Purkinje cells. Black arrows, hyperchromic Purkinje cells with darkened vacuolized cytoplasm and round-like form.

Table 1. Hyperchromic Purkinje cells number in the vermis of rat cerebellum (histological slices were made 8 days after the application of agents to the vermis; sodium azide concentration is 1 mM, and peptide concentration is 10^{-4} M; averages of 8 animals ± S.E.M. are shown)

Injected agents	Hyperchromic Purkinje cells, %
Control (saline)	25 ± 6
NaN_3	63 ± 3
NaN_3 + HLDF-6	42 ± 4*
NaN_3 + PEDF-6	47 ± 6*

* $p < 0.05$, significant difference compared to animal group injected with NaN_3.

change in environment. ASR was estimated by pressure on the chamber platform caused by sharp paw extension as a response to the sound impulse. Under normal conditions (without vermal hypoxia) the secondary behavioral testing performed 24 h after the primary one showed some changes in those parameters: full immobility period is increased due to conditional fear formation, ASR amplitude is decreased due to its long-term habituation. As expected, animals whose cerebellar vermis had been treated with sodium azide failed to show any of these reactions—no changes were observed in parameters registered in primary and secondary testing (Table 2). Both HLDF-6 and PEDF-6 peptides recovered conditional fear formation up to the control group levels, while only HLDF-6 peptide recovered ASR long-term habituation, unlike PEDF-6 (Table 2). This suggests that HLDF-6 peptide exhibits protective effects not only on Purkinje cells but also on some other types of vermal neurons that also play a role in ASR long-term habituation.

Effect of HLDF-6 and PEDF-6 on HL-60 cell viability under conditions of sodium azide-induced chemical hypoxia. The protective effect of HLDF-6 and PEDF-6 peptides was tested on HL-60 cell culture (human promyelocytic leukemia cell line) under conditions of chemically induced hypoxia. Sodium azide as well as one of the peptides HLDF-6 or PEDF-6 (10^{-6} M) was added into the culture medium, and cells were cultivated in their presence for 24 h. Viable cells were then quantified by the MTT test. Sodium azide leads to decrease in cell viability in a concentration-dependent manner: its higher concentrations cause higher value of cell death (Fig. 2). HLDF-6 peptide promotes cell viability (the percent of viable cells becomes significantly higher), while PEDF-6 peptide does not exhibit this effect.

Sodium azide can cause either apoptotic or necrotic cell death. According to Bal-Price et al. [26], for cultivated cells the type of their death depends on the presence of glucose in the culture medium. When sodium azide is introduced to the cell culture in glucose-containing medium, the number of necrotic cells is not significantly changed compared to the control level, while the majority of cells show all characteristics of induced apoptotic death, such as cyto-chrome *c* release from mitochondria and caspase activation. Since RPMI-1640 medium used for HL-60 cell cultivation contains glucose, it can be postulated that sodium azide caused apoptotic cell death in our experiments.

Effects HLDF-6 and PEDF-6 on adenylate cyclase in HL-60 cells. We have previously shown that peptides HLDF-6 and PEDF-6 induce differentiation of HL-60

Table 2. Recovering effects of HLDF-6 and PEDF-6 peptides (10^{-4} M) on conditional fear formation and long-term habituation of acoustic startle reaction in rats upon chemical hypoxia induced by sodium azide (1 mM) (primary testing was performed 5 days after application of agents, and secondary testing was performed 24 h later; pressure on chamber platform is recalculated to standard animal mass (250 g); averages of 8 animals ± S.E.M. are shown)

Injected agents	Full immobility duration, sec		Maximal pressure on chamber platform during ASR response, relative units	
	primary testing	secondary testing	primary testing	secondary testing
Control (saline)	68 ± 12	123 ± 21*	463 ± 46	350 ± 31*
NaN_3	77 ± 18	94 ± 20	492 ± 58	509 ± 56
NaN_3 + HLDF-6	87 ± 10	137 ± 13*	445 ± 52	256 ± 29*
NaN_3 + PEDF-6	83 ± 16	129 ± 17*	524 ± 56	580 ± 49

* $p < 0.05$, significant difference compared to primary testing result of the same animal group.

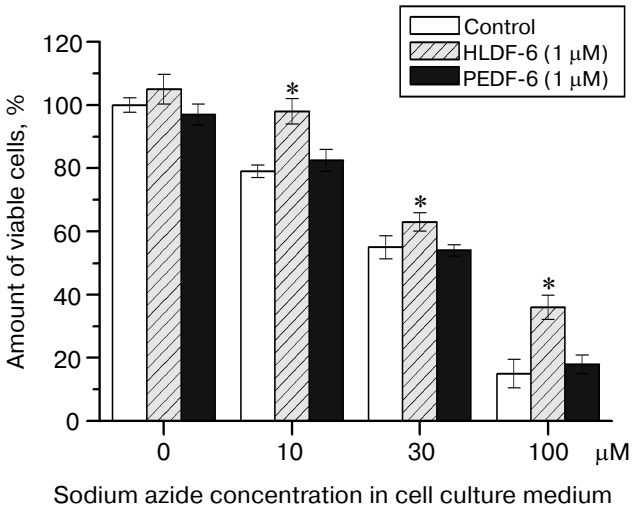

Fig. 2. HL-60 cells viability in hypoxia state induced by different concentrations of sodium azide. The effect of HLDF-6 and PEDF-6 peptides (10^{-6} M) on cell viability. The data are from two independent experiments ± S.E.M; * $p < 0.05$, significant increase of viability compared to control samples.

cells into different phenotypes: granulocyte/neutrophilic and monocyte/macrophagal, respectively [27]. In addition, HLDF-6 peptide increases cell viability upon chemically induced hypoxia, unlike PEDF-6. Based on such observations, we suggested that HLDF-6 and PEDF-6 peptides may mediate their effects on HL-60 cells by different molecular mechanisms. One of the key stages of signal transduction is activation or inhibition of second messenger systems. The effects of the peptides on two major second messenger biosynthesis enzymes, adenylate cyclase and phosphatidylinositol-specific phospholipase C, were studied in the present work.

Sodium azide is known to increase the basal adenylate cyclase activity level in some cell types [28]. Since HLDF-6 peptide protects HL-60 cells from azide-induced apoptosis, the study of its effect on adenylate cyclase in those cells is of interest.

Time-dependent change of cAMP level in HL-60 cells in RPMI-1640 culture medium containing 7% fetal calf serum is shown on Fig. 3. Peptides were used at concentration 10^{-6} M, because their differentiation-inducing effect is maximal at this concentration [14]. HLDF-6 peptide leads to significant decrease in intracellular cAMP level that is visible even after 1 min of incubation, whereas PEDF-6 does not cause any effect. In a serum-free medium, however, the effect of HLDF-6 peptide is less pronounced (Fig. 4). This peptide also inhibits adenylate cyclase activity in isolated HL-60 cell membranes (Fig. 5, *1*). It probably inhibits adenylate cyclase activity by two different pathways: on one hand, by direct enzyme inhibition, and on the other hand, by modulation of serum cytokine binding with cells. We previously showed that HLDF-6 peptide modulates the high-affinity binding of interleukin 1-β to HL-60 cells. Thus, effect of the peptide on adenylate cyclase appears to be mediated by this cytokine, since it has been shown to be an activator of this enzyme [16, 29].

To investigate the mechanism of direct adenylate cyclase inhibition by HLDF-6 peptide, we have analyzed its effect on isolated HL-60 cell membranes in the presence of forskolin, which acts as a specific activator of adenylate cyclase catalytic subunit [30], as well as in the presence of β,γ-imido-GTP, a non-hydrolysable GTP analog. The peptide effect was completely abolished by forskolin (Fig. 5, *2*) but it was preserved in the presence of β,γ-imido-GTP (Fig. 5, *3*).

These observations suggest that the effect of HLDF-6 peptide on adenylate cyclase is mediated by G protein. Most probably, the peptide enhances the interaction between catalytic adenylate cyclase subunit and several G_i proteins (G_i activation leads to adenylate cyclase inhibition). Taking into account our previous observations that HLDF-6 increases lipid mobility in membrane lipid bilayer structure [16], we can suggest that adenylate cyclase inhibition may be caused by increasing the mobility of G_i protein α subunit, which is anchored in the cell membrane by a palmitoyl residue [31]. According to Gudi et al. [32], membrane-bound G proteins can be activated in the absence of G protein-coupled receptor by partial disordering of liquid-crystal membrane structure; such phenomenon has been observed upon "fluid shear stress". Our suggestion about membrane-bound G protein mobility increase by HLDF-6 peptide (through affecting bilayer structure) is also supported by the obser-

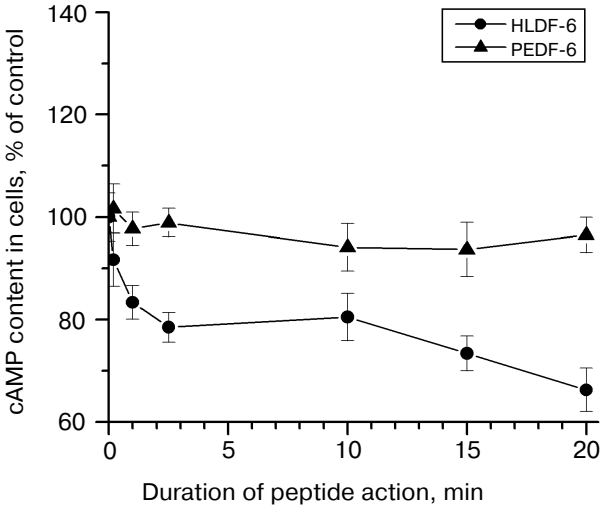

Fig. 3. Time-dependent effect of HLDF-6 and PEDF-6 peptides (10^{-6} M) on cAMP content in HL-60 cells. cAMP content in control samples (10^7 cells) is 6.85 ± 0.45 pmol. The data are from two independent experiments ± S.E.M.

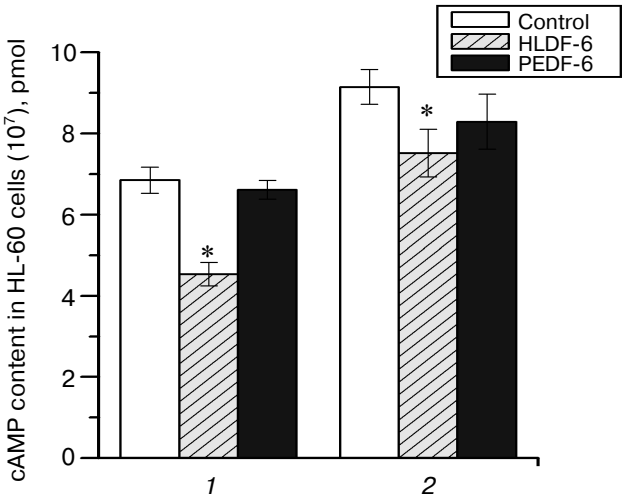

Fig. 4. Effect of HLDF-6 and PEDF-6 peptides (10^{-6} M) on cAMP content in HL-60 cells in RPMI-1640 medium containing 7% fetal calf serum (*1*) or in serum-free medium (*2*). Cells were treated with peptides for 20 min. The data are from three independent experiments ± S.E.M; * $p < 0.05$, significant decrease of cAMP content compared to control samples.

vation that this peptide does not inhibit adenylate cyclase in HL-60 cell membranes solubilized by CHAPS, i.e., in the absence of ordered membrane structure (Fig. 5, *4*).

According to Dessauer et al. [33], adenylate cyclase inhibition by G_i proteins does not occur in the simultaneous presence of forskolin and activated G_s protein because of instability of the quaternary complex G_i–adenylate cyclase–forskolin–G_s [33]. Therefore, the inhibitory effect of HLDF-6 peptide cannot be exhibited in the presence of forskolin since the basal adenylate cyclase activity is formed with the participation of G_s proteins.

G_i proteins affect not all known adenylate cyclase isoforms, but only isoforms I, V, and VI. Isoform I is known to be expressed only in neurons [34, 35]. So, HLDF-6 peptide appears to inhibit only V and VI isoforms in HL-60 cells. We have observed only a slight decrease in total cAMP amount synthesized by membrane-bound adenylate cyclases (10-20% of control), which can be a result of significant inhibition of some adenylate cyclase isoforms on a background of other isoforms whose activity is being unaffected. This may result in a local cAMP decrease in some cellular compartments, which primarily contain isoforms affected by the peptide.

The most important downstream cAMP effector is cAMP-dependent protein kinase A (PKA). Its activation by cAMP occurs through the dissociation of C/R complex, where C is catalytic and R is regulatory PKA subunit. PKA activation is a process dependent on C and R local concentrations in the cell compartment as well as on PKA substrate concentrations [36]. Therefore, at high value of C/R ratio and PKA substrates levels significant PKA activation or inhibition may be caused by even a little change in cAMP concentration.

Effects of HLDF-6 and PEDF-6 on phosphatidylinositol-specific phospholipase C in HL-60 cells. As mentioned above, sodium azide added to HL-60 cell culture induces apoptotic but not necrotic cell death. According to Jarvis et al. [37], the induced apoptosis of this cell line can be prevented by diacylglycerol which is produced by $PtdIns(4,5)P_2$ hydrolysis by phosphatidylinositol-specific phospholipase C (PLC). The direct stimulation of this enzyme also suppresses induced cell apoptosis. Thus, we suggested that the protective effect of HLDF-6 peptide on HL-60 cells as well as the effect of both peptides on Purkinje cells of the vermis of the cerebellum may occur because of PLC activation. It should be also noted that some well-known HL-60 cell differentiation factors such as *trans*-retinoic acid and phorbol ester decrease PLC activity [38, 39].

The effect of the peptides on PLC activity was estimated by change in total intracellular InsP and $InsP_2$ concentration compared to control. As seen from Table 3, neither HLDF-6 nor PEDF-6 significantly affects PLC activity; only a slight tendency to inhibition is observed. To visualize putative peptide effects, PLC was stimulated by aluminum tetrafluoride anions, which are known to activate the enzyme through G_q proteins [40]. In the presence of an activator, HLDF-6 peptide had no effect on

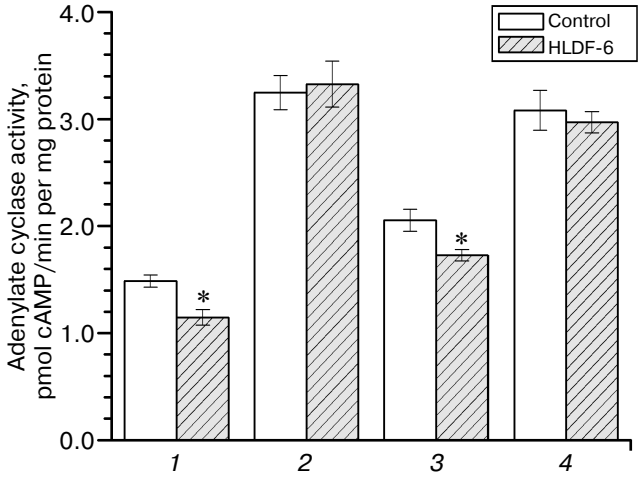

Fig. 5. Effect of HLDF-6 peptide (10^{-6} M) on adenylate cyclase activity in HL-60 cell membranes without any activator of adenylate cyclase (*1*) or in the presence of 1 μM forskolin (*2*) or 10 μM β,γ-imido-GTP (*3*); membranes were solubilized with CHAPS without any activators in the medium (*4*). The experiments were performed in the medium for adenylate cyclase activity assay (see "Materials and Methods"). Duration of peptide action is 20 min. The data are from three independent experiments ± S.E.M; * $p < 0.05$, significant decrease of adenylate cyclase activity compared to control samples.

Table 3. Total inositol mono- and bisphosphate concentration change (% of control without AlF_4^- stimulation) in HL-60 cells caused by HLDF-6 and PEDF-6 peptides in the presence of AlF_4^- (time of incubation is 1 h)

Peptide concentration, M	Non-stimulated PLC		PLC stimulated by AlF_4^-	
	HLDF-6	PEDF-6	HLDF-6	PEDF-6
Control	100.0 ± 2.8%		126.1 ± 6.7%	
10^{-7}	97.2 ± 7.7%	101.1 ± 1.9%	128.3 ± 8.1%	116.6 ± 5.6%
10^{-6}	92.5 ± 2.5%	93.3 ± 6.6%	124.4 ± 5.8%	114.6 ± 2.8%*
10^{-5}	94.8 ± 4.3%	91.0 ± 6.1%	125.6 ± 5.2%	99.5 ± 6.7%*

* $p < 0.05$, significant decrease in concentration of inositol derivatives compared with control.

PLC activity, whereas PEDF-6 caused significant inhibition of this enzyme that was more pronounced at higher peptide concentrations (Table 3).

No tendency to PLC inhibition upon HLDF-6 influence is observed in the presence of AlF_4^- ions. Aluminum tetrafluoride is known to stimulate not only G_q but also G_i proteins, whereas G_s proteins are unaffected [41]. When G_i protein is activated, its α subunit inhibits adenylate cyclase activity while the free complex of β and γ subunits causes PLC activation [42]. Possible inhibitory effect of HLDF-6 peptide on PLC is thus compensated.

PEDF-6 peptide affects PLC activity only in the presence of AlF_4^- ions, which activate this enzyme through G_q proteins. Most probably, the peptide effect is mediated by G proteins of this class. The peptide may either stimulate their GTPase activity or enhance their interaction with some protein of the RGS superfamily (Regulator of G Protein Signaling). The majority of known RGS proteins stimulate GTPase activity by stabilization of the conformation of $G\alpha_q$ subunit, which is required for GTP hydrolysis [43]. RGS4 protein seems to be the most probable candidate for mediating of PEDF-6 effect since its affinity to $G\alpha_q$ subunit has been shown to be dramatically increased by AlF_4^- ions [44]. RGS4 interacts with the $G\alpha_q$ molecule region, which undergoes conformational changes when GDP-bound state is transferred to GTP-bound one. Thus, PLCβ stimulation by aluminum tetrafluoride is prevented by RGS4 protein in the described pathway; the stimulation of this enzyme is completely suppressed in cells overexpressing RGS4 [40]. Maybe PEDF-6 peptide enhances RGS4 interaction with $G\alpha_q$ by some yet unknown mechanism.

When PEDF-6 is applied to the vermis of rat cerebellum simultaneously with sodium azide, the inducer of hypoxia, the number of hyperchromic Purkinje cells is decreased but the functional activity of the vermis is only partially recovered: long-term habituation of ASR is still lacking. According to Ghoumari et al. [45], the pseudosubstrate protein kinase C (PKC) inhibitor Go6976 prevents cell death in organotypical culture of Purkinje cells but does not promote axonal regeneration. Thus, we can speculate that the observed differences in PEDF-6 peptide effect on HL-60 and Purkinje cells may be due to activation of different PKC isoforms in those cells in response to PLC stimulation. At the present time, twelve different PKC isoforms are known. All of them are abundantly expressed in HL-60 cells, while in Purkinje cells PKCγ and δ are prevalent [46-48]. According to Jarvis et al. [37], HL-60 apoptosis is induced by ceramide, a hydrophobic product of sphingomyelin hydrolysis catalyzed by sphingomyelinases. Activation of the classical Ca^{2+}-dependent PKCα isoform leads to stimulation of sphingomyelin hydrolysis and, consequently, ceramide production, while activation of PKCδ isoform causes sphingomyelinase inhibition [49].

Unlike HL-60 cells, ceramide is a survival factor for Purkinje cells [50]. If PEDF-6 leads to PLC inhibition in Purkinje cells such as in HL-60 cells, this leads to PKCδ activity decrease and, in turn, stimulates ceramide production. This is a possible mechanism of PEDF-6 protective effect on Purkinje cells.

In conclusion, it may be postulated that HLDF-6 and PEDF-6 peptides, despite their structural homology, affect different second messenger systems in HL-60 cells, which can be an explanation of differences in their differentiation and protective activities observed on these cells. The results obtained in this study may also explain opposite effects of HLDF-6 and PEDF-6 peptides on the preimplantational development of mice embryos *in vitro* reported by Sakharova et al. [51]: HLDF-6 promotes embryo survival at the stages of 2 and 4 blastomers, whereas PEDF-6 stimulates their death.

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