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The cyclic AMP effects and neuroprotective activities of PACAP and VIP in cultured astrocytes and neurons exposed to oxygen-glucose deprivation

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ABSTRACT

Background: Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are endogenous peptides, widely expressed in the central and peripheral nervous system. The adenylyl cyclase (AC)/cyclic AMP (cAMP) is their main intracellular signal transduction pathway. Numerous data suggest that PACAP and VIP have considerable neuroprotective potential, indicating the possibility for their use as new therapeutic strategies in stroke treatment. The aim of this study was to evaluate the effect of oxygen-glucose deprivation (OGD) – an established *in vitro* model for ischemic cell stress – on PACAP and VIP-evoked receptor-mediated cAMP generation in glial and neuronal cells, and to determine whether PACAP and VIP have neuroprotective activity under these conditions.
Methods: The formation of [^{3}H]cAMP by PACAP, VIP and forskolin (a direct activator of AC) was measured in [^{3}H]adenine prelabeled primary rat glial and neuronal cells under normoxia and OGD conditions. The effects of PACAP and VIP on cell viability were measured using the MTT conversion method, and were compared to tacrolimus (FK506), a well known neuroprotective agent.
Results: The OGD model inhibited the PACAP and VIP-induced cAMP formation in rat astrocytes and neurons. Incubation of neuronal cells with PACAP prevented OGD-induced cell death, more efficiently than VIP and FK506.
Conclusion: The obtained results showed that hypoxia/ischemia may trigger down-regulation of the brain AC-coupled PACAP/VIP receptors, with a consequent decrease of PACAP- and/or VIP-ergic-dependent cAMP-driven signaling. Moreover, our findings indicate that PACAP and VIP can prevent the deleterious effect of OGD on rat neuronal cells.

Introduction

Nowadays, stroke is the most frequent cause of adult disability, as well as death in the elderly [1]. In most cases, the etiology of stroke involves cerebral ischemia, defined as a local disorder that causes a reduction in arterial blood supply to the brain area, which may lead to cerebral atrophy or total necrosis. Ischemia-induced changes in the brain tissue lead not only to its degeneration, but also to the significant activation of cellular mechanisms which protect the affected cells from damage. One of the mechanisms is the expression of such endogenous neuroprotective substances as pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) [2–5].

PACAP and VIP are neuropeptides that belong to structurally-related family of polypeptides, which also includes secretin,

Abbreviations: AC, adenylyl cyclase; cAMP, cyclic AMP; CNS, central nervous system; $CoCl_2$, cobalt chloride; DMEM, Dulbecco's Modified Eagle's Medium; ERK, extracellular signal-regulated kinase; FK506, tacrolimus; GFAP, glial fibrillary acidic protein; GHRH, growth hormone-releasing hormone; GPCRs, receptors coupled with G proteins; HIF-1, hypoxia-inducible factor-1; H_2O_2, hydrogen peroxide; MAP, microtubule-associated protein-2 kinase; MAP-2, microtubule associating protein-2; MTT, 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl-tetrazolium bromide; OGD, oxygen-glucose deprivation; PACAP, pituitary adenylate cyclase-activating polypeptide; PHI, peptide histidine-isoleucine; PKA, protein kinase A; TBI, traumatic brain injury; VIP, vasoactive intestinal peptide.

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glucagon, peptide histidine-isoleucine (PHI), growth hormone-releasing hormone (GHRH) and helodermin [6]. PACAP and VIP are widely distributed throughout the central and peripheral nervous systems, where they are implicated in such phenomena as neurotransmission, neural plasticity and neurotrophy. Both peptides are also endowed with a neuroprotective potential [7,8].

The biological effects of the mentioned peptides are mediated through specific membrane receptors named PAC1, VPAC1 and VPAC2, all belonging to the superfamily of receptors coupled with G proteins (GPCRs). PAC_1 type receptors preferably bind PACAP and recognize VIP poorly, whereas VPAC type receptors recognize both VIP and PACAP to a similar degree [6,9]. The protective mechanism of PACAP and VIP involves many intracellular pathways, which can be generally classified into four categories of action: antiapoptotic, anti-inflammatory, metabolic, and modulation of gene expression. Indeed, literature data indicates that activation of the adenylyl cyclase (AC)/cyclic AMP (cAMP) signaling pathway plays a special role in the neurotrophic activity of PACAP and VIP [10,11].

A large body of evidence provided by many research centers suggests that endo- and exogenous PACAP and VIP, as well as their synthetic derivatives, exert considerable neuroprotective and anti-inflammatory potential. These properties suggest that they might have some value as new therapeutic strategies in stroke treatment [2,8,12,13].

Therefore, the aim of this study was two fold: (1) to evaluate the effect of oxygen-glucose deprivation (OGD) – an established *in vitro* model for ischemic cell stress – on PACAP and VIP-evoked receptor-mediated cAMP generation in glial and neuronal cells, and (2) to determine whether PACAP and VIP have neuroprotective activity under these conditions.

Materials and methods

Chemicals

PACAP-38 (human, ovine, rat), VIP (human, porcine, rat; referred to as mammalian VIP, mVIP) were obtained from Neosystem (Strasbourg, France). Cobalt chloride ($CoCl_2$), glial fibrillary acidic protein (GFAP), microtubule associating protein-2 (MAP-2), DNase I, poly-L-ornithine, trypsin, glutamine, penicillin, streptomycin, amphotericin B, 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), tacrolimus (FK506) and forskolin were obtained from Sigma (St. Louis, MO, USA). Neurobasal culture medium with or without glucose, Dulbecco's Modified Eagle's Medium (DMEM) with or without glucose and B27 were obtained from Gibco (Paisley, Scotland, UK). Multi-well plates and Petri dishes for cell culture were obtained from Nunc (Wiesbaden, Germany). Radiolabeled compounds: [^{3}H]adenine (specific activity 24.2 Ci/mmol) and [^{14}C]cAMP (specific activity 56 mCi/mmol) were purchased from PerkinElmer Life Sciences Inc. (Boston, MA, USA) and Moravek Biochemical Inc. (Brea, CA, USA), respectively.

Animals

Glial and neuronal cell cultures prepared from newborn rats and rat embryos, as described below, were used for the study. All animal procedures were performed in strict accordance with the European Commission guidelines (2010/63/UE) and the Polish governmental regulations concerning experiments on animals (Dz.U.05.33.289). All experimental protocols were approved by the Local Ethical Commission for Experimentation on Animals.

Primary neuronal culture

Primary neuronal cell cultures were prepared from albino Wistar rat embryos at 15–17 days of gestation and were cultivated, as previously described in detail by Jozwiak-Bebenista et al. (2007) and Nowak et al. (2007) [14,15]. Briefly, the rat cerebral cortex was isolated from fetal brain, incubated for 15 min in trypsin/EDTA (0.05%) at 37 °C, triturated in a solution of DNase I (0.05 mg/ml) and fetal bovine serum (20%), and finally centrifuged at 210 × *g* for 5 min at 21 °C. The cells were suspended in Neurobasal medium supplemented with B27, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B, and plated at a density of 2.5×10^5 cells per square centimeter onto multiwell plates coated with 0.01 mg/ml poly-L-ornithine. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and cultivated for 7 days prior to experimentation. The purity of the neuronal cultures was verified by an immunocytochemical staining method using antibodies against MAP-2 for neurons, and against a specific "astrocyte" marker GFAP, for glial cells. The latter analysis revealed the presence of approximately 6–10% of GFAP-positive cells in each new culture, which confirmed that the primary neuronal cultures represented neuron-enriched preparations.

Primary astrocyte culture

Primary astrocyte cultures were prepared from cerebral cortices of 1-day old albino Wistar rat pups, according to the method of Hertz et al. (1985) with some changes [16]. Briefly, the dissected cerebral cortex was cut into small fragments, enzymatically digested and mechanically dissociated as described for the neuronal cultures. The glial cells were grown in 6 cm diameter Petri dishes in DMEM supplemented with 10% newborn calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B, in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For subcultures, glial cells were harvested in trypsin–EDTA (0.25% trypsin, 1 mM EDTA) solution. Cells from the second and third passages were used for experimentation. Immunocytochemical staining for GFAP using anti-GFAP antibodies revealed the homogeneity of the cell population to be approximately 95%. The lack of anti-MAP-2 antibodies confirmed that no neurons were present.

Hypoxic conditions ($CoCl_2$-driven) and OGD

Prior to the experiment, the cells were incubated overnight with fresh medium. To induce a hypoxic ($CoCl_2$-driven) condition, the cultures were placed in medium deprived of serum, and incubated (CO_2 incubator, Heraeus, Germany) for 24 h at 37 °C in the presence of 100 μM $CoCl_2$. To induce OGD, the cells were additionally deprived of glucose and incubated with the same above condition. $CoCl_2$ has been widely used as a hypoxia mimicking agent in both *in vivo* and *in vitro* studies, producing similar biochemical effects as low (1–3%)-oxygen hypoxia [17,18]. In contrast to the hypoxic conditions and OGD, the experiments and results obtained on cell cultures maintained under a humidified atmosphere of 95% air and 5% CO_2 in a culture medium containing all nutrients needed for normal cellular function will be referred to as being performed under normoxic conditions.

Assay of cAMP formation

The glial or neuronal cell cultures were seeded in 12-well plates at a density of 200,000–250,000 cells/well in 500 μl of culture medium and cultured for 2 days. Following 24 h OGD, the culture

medium was removed, fresh serum-free culture medium was added, and the cells were incubated in the presence of [^{3}H]adenine for 90 min at 37 °C. The formation of [^{3}H]cAMP in [^{3}H]adenine-prelabeled cells was assayed according to Shimizu et al. (1969), and the formed [^{3}H]cAMP was isolated by chromatography using a sequential Dowex-alumina column according to Salomon et al. (1974) [19,20]. The results were individually corrected for percentage recovery with the aid of [^{14}C]cAMP added to each column system prior to nucleotide extraction. The accumulation of cAMP during a 15 min stimulation period with peptides or forskolin was assessed as a percentage of the conversion of [^{3}H]adenine to [^{14}C]cAMP.

MTT conversion

Post-hypoxic or post-OGD cellular viability was measured using the MTT conversion method. Cells were seeded (50,000 cells/well) into 96-well plates. Astrocytes and neurons were subjected to 24 h of OGD incubation alone or with PACAP-38, VIP and FK506 or without tested chemicals (control group). All the substances were added simultaneously. After incubation, 50 μl MTT (1 mg/mL, Sigma) was added and the plates were incubated at 37 °C for 4 h. At the end of the experiment, cells were exposed to 100 μl dimethyl sulphoxide, which enabled the release of the blue reaction product: formazan. The absorbance at 570 nm was read on a microplate reader and results were expressed as a percentage of the absorbance measured in control cells.

Data analysis

All results are expressed as mean ± SEM values. Analysis of variance (ANOVA) was used for statistical evaluation, followed by the *post hoc* Students–Newman–Keuls test.

Results

Effect of hypoxia ($CoCl_2$-driven) and OGD on the growth of glial (astrocytes) and neuronal cells

The cultured glial and neuronal cells in normoxic, hypoxic ($CoCl_2$-driven) and OGD conditions were observed. The morphous of glial cells cultured under normoxic conditions was densely covered, exhibited normal cellular morphology with extending neurites. The astrocytes treated with $CoCl_2$ or OGD displayed a thin layer of smaller cells, short protrusion, and a thin and wrinkled shape (Fig. 1). However, the changes were more pronounced in OGD conditions. Similar morphological effects were observed in neuronal cells (data not shown).

Effect of hypoxia ($CoCl_2$-driven) and OGD on PACAP and VIP-evoked receptor-mediated cAMP generation in rat primary glial (astrocyte) and neuronal cell cultures

Glial cells derived from rat cerebral cortex subjected to 24 h OGD showed much weaker reactivity to PACAP-38 (0.1 μM) and VIP (0.1 μM) than those present in normoxic or 24 h hypoxic (100 μM $CoCl_2$-driven) conditions (Fig. 2). In $CoCl_2$-treated cells, the stimulatory actions of PACAP-38 were reduced to 58% compared to normoxia, while under OGD conditions they were reduced to 31%. A similar strong, statistically significant, suppressive effect was achieved for VIP. Under hypoxic ($CoCl_2$-driven) and OGD conditions, the respective effects on cAMP synthesis were only 47% and 18% of that seen under normoxia (Fig. 2).

In contrast to the tested peptides, the effect of the diterpene derivative forskolin, a direct activator of AC, was not affected by hypoxia or OGD. At a concentration of 10 μM, forskolin stimulated cAMP production at a similar efficacy to that seen under normoxic, hypoxic and OGD conditions (Fig. 2).

Another situation was observed in neuronal cells, where OGD induced a strong and statistically significant suppressive effect on cAMP synthesis evoked by both PACAP-38 and VIP, as well as forskolin. Under OGD conditions, the level of cAMP synthesis was reduced to 47% (PACAP-38), 46% (VIP) and 50% (forskolin) compared to normoxia (Fig. 3).

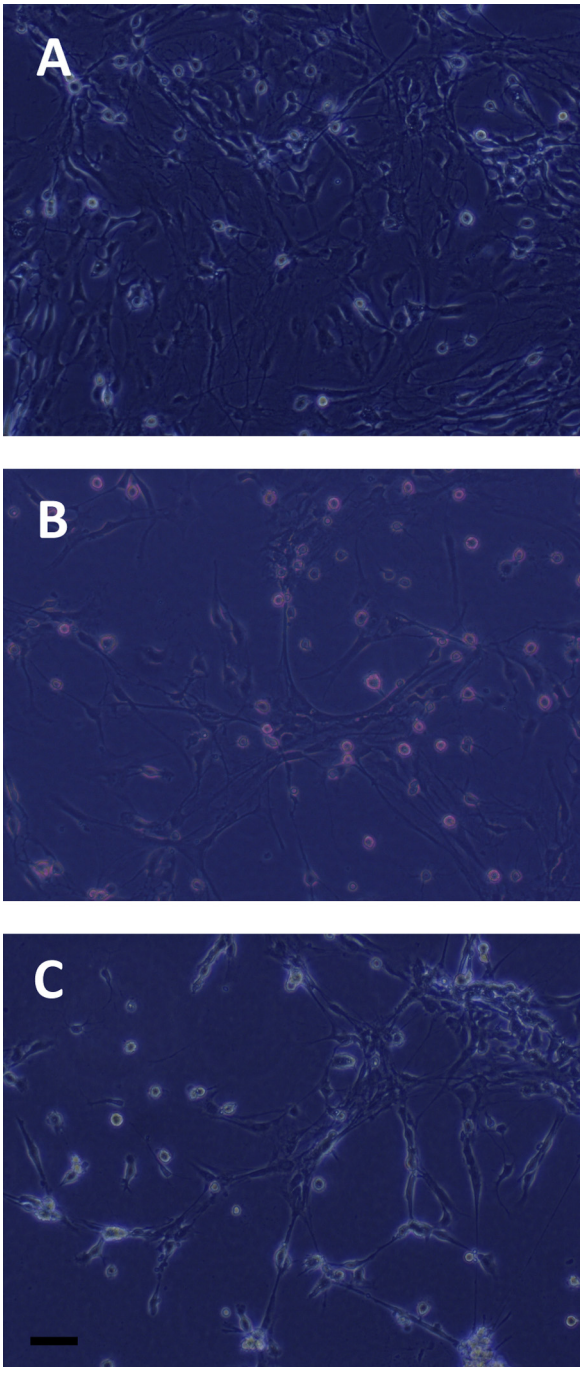

Fig. 1. The growth of rat primary glial (astrocytes) cell cultures in (A) normoxia, (B) hypoxic ($CoCl_2$-driven) and (C) OGD conditions. The glial cells in the control culture were densely covered, exhibited normal cellular morphology with extending neurites. The astrocytes treated with $CoCl_2$ or OGD displayed a thin layer of smaller cells, short protrusion, as well as a thin and wrinkled shape. The pictures from the compact inverted microscope (OLYMPUS CKX41) with reflected fluorescence system (10× objective). Scale bar 40 μm.

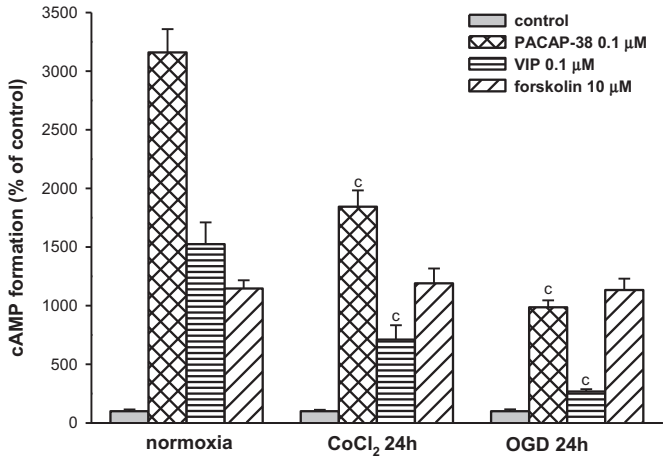

Fig. 2. Effects of PACAP-38, VIP and forskolin on cyclic AMP formation in rat primary glial (astrocyte) cell cultures maintained under normoxic, hypoxic ($CoCl_2$-driven) and OGD conditions. The results are presented as a percentage in relation to the control value (100%). Bars represent the means (±SEM of 9–17 experiments). $^{c}p < 0.001$ *vs.* PACAP-38 and VIP respectively.

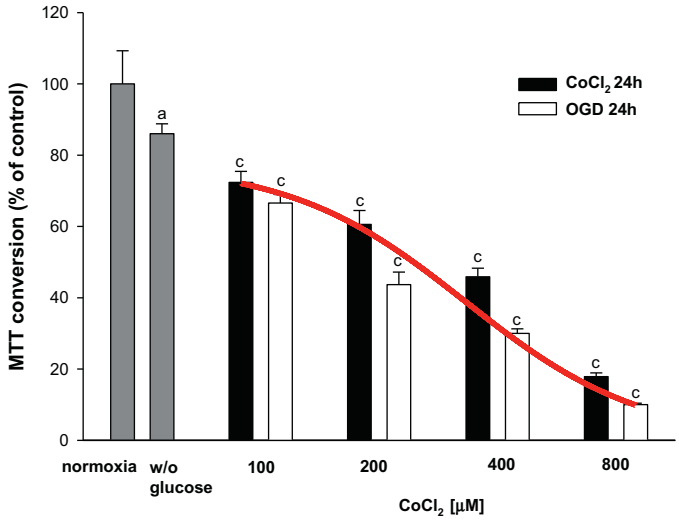

Fig. 4. Effects of 24-h hypoxia ($CoCl_2$, 100, 200, 400 and 800 μM) and OGD on glial cell viability. The results are presented as a percentage in relation to the control value (100%). Bars represent the means (±SEM of 9–16 experiments). $^{a}p < 0.05$ *vs.* normoxia; $^{c}p < 0.001$ *vs.* normoxia.

Assessment of rat primary glial (astrocyte) and neuronal cell viability in response to 24 h of hypoxic ($CoCl_2$-driven) and OGD conditions

In the primary glial cell culture, 24 h chemical hypoxia induced by 100, 200, 400 and 800 μM $CoCl_2$ caused a significant decrease in MTT conversion: the accumulation of formazan was found to be 72%, 60%, 46% and 18% of normoxia value according to the respective increasing concentrations of $CoCl_2$ (Fig. 4). Incubation of glial cells under OGD intensified the observed effect, leading to a 67%, 44%, 30% and 10% decrease in MTT conversion compared to normoxia. All the observed effects reached a high threshold of statistical significance (Fig. 4).

The incubation of neuronal cells with 100, 200, 400 and 800 μM $CoCl_2$ caused a statistically significant decrease of mitochondrial activity – with the observed effect being more pronounced at higher concentrations of $CoCl_2$: 76%, 62%, 36% and 15% compared to normoxia, respectively (Fig. 5). Under OGD, the neuronal cells exhibited even weaker MTT conversion capacity compared to chemical hypoxia. Formazan accumulation was 63%, 54%, 26% and 11% of control values for respective concentrations of 100, 200, 400 and 800 μM $CoCl_2$ (Fig. 5). Although both chemical hypoxia and OGD conditions induced a similar effect in glial and neuronal cells, the OGD model evoked stronger cytotoxicity in both cell cultures.

Effect of PACAP and VIP on the rat primary glial (astrocyte) and neuronal cells viability subjected to OGD condition

Glial cells subjected to 24 h OGD showed a decrease in the conversion of MTT to 72% compared to normoxia (Fig. 6). PACAP-38 used in the concentration range of 0.001–1 μM did not stimulate the accumulation of formazan in the cells subjected to OGD (Fig. 6). Exposure of glial cells to 0.1 and 1 μM VIP resulted in non-significant increases of MTT conversion above the OGD group of 18% and 17%, respectively: these being 85% and 84% of the

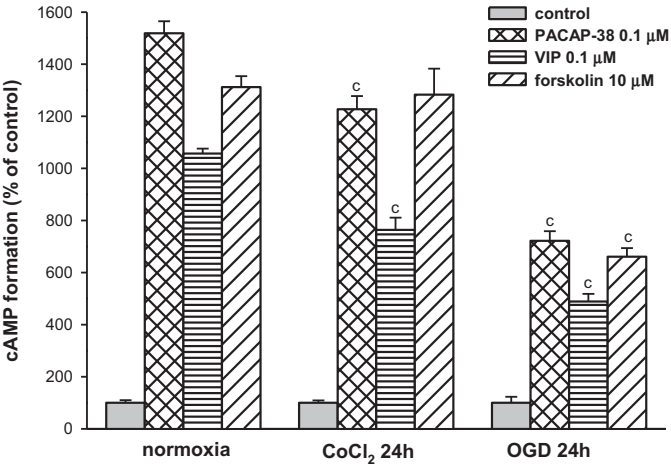

Fig. 3. Effects of PACAP-38, VIP and forskolin on cyclic AMP formation in primary neuronal cell cultures maintained under normoxic, hypoxic ($CoCl_2$-driven) and OGD conditions. The results are presented as a percentage in relation to the control value (100%). Bars represent the means (±SEM of 4–7 experiments). $^{c}p < 0.001$ *vs.* PACAP-38, VIP and forskolin respectively.

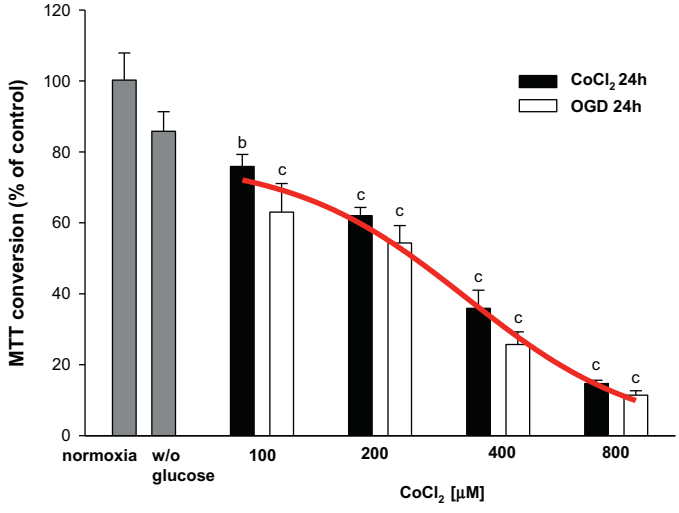

Fig. 5. Effects of 24-h hypoxia ($CoCl_2$, 100, 200, 400 and 800 μM) and OGD on neuronal cell viability. The results are presented as a percentage in relation to the control value (100%). Bars represent the means (±SEM of 5–10 experiments). $^{b}p < 0.01$ *vs.* normoxia; $^{c}p < 0.001$ *vs.* normoxia.

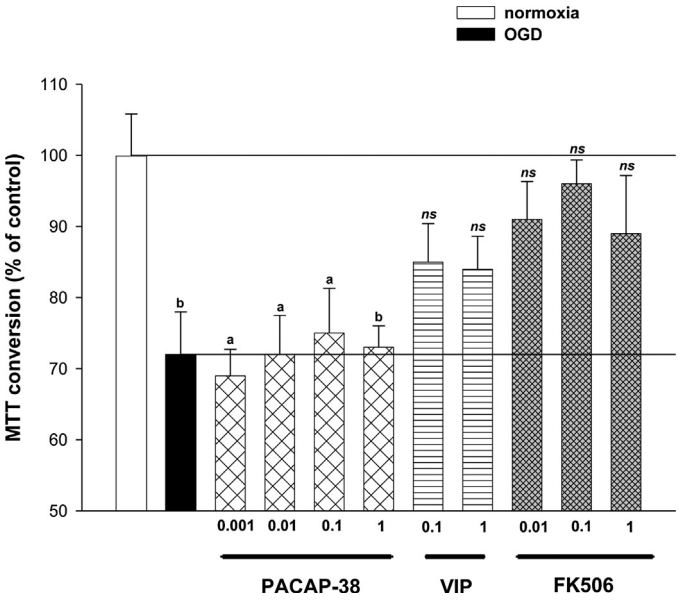

Fig. 6. Effects of PACAP-38, VIP and FK506 on the viability of glial cells subjected to OGD, measured by MTT conversion assay. The results are presented as a percentage in relation to the control value (100%). Bars represent the means (±SEM of 5–12 experiments). $^{a}p < 0.05$ *vs.* normoxia; $^{b}p < 0.01$ *vs.* normoxia; ^{ns}p – statistically insignificant *vs.* normoxia; $^{\#}p$ *vs.* OGD – all obtained results were statistically insignificant *vs.* OGD.

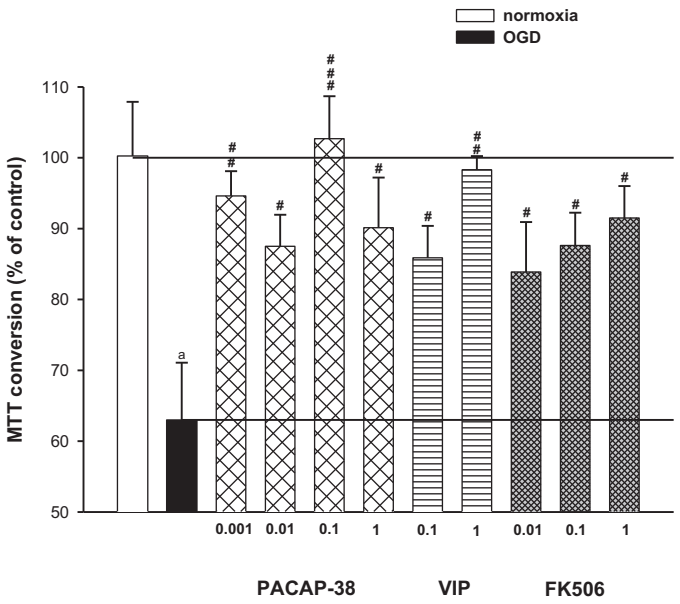

Fig. 7. Effects of PACAP-38, VIP and FK506 on the viability of neuronal cells subjected to OGD, measured by MTT conversion assay. The results are presented as a percentage in relation to the control value (100%). Bars represent the means (±SEM of 6–9 experiments). $^{a}p < 0.05$ *vs.* normoxia; $^{\#}p < 0.05$ *vs.* OGD; $^{\#\#}p < 0.01$ *vs.* OGD; $^{\#\#\#}p < 0.001$ *vs.* OGD.

normoxia values (Fig. 6). The greatest accumulation of formazan in the OGD model was observed after the addition of immunosuppressant agent FK506. FK506, used at concentrations of 0.01, 0.1 and 1 μM, increased the formazan level by, respectively, 26%, 33% and 24% above the OGD group levels, which correspond to 91%, 96% and 89% of normoxia. The resulting differences were not statistically significant (Fig. 6).

Neuronal cells subjected to 24 h OGD exhibited a slightly larger decrease in the conversion of MTT compared to the glial cells: 63% of normoxia (Fig. 7). Incubation of cells treated with 0.001–1 μM PACAP-38 under OGD conditions resulted in a statistically significant increase in the accumulation of formazan by 51%, 38%, 64% and 43% above the OGD group. The peptide was most effective at a concentration of 0.1 μM, raising the level of MTT conversion by 64% above OGD, or 3% above normoxia (Fig. 7). VIP at concentrations of 0.1 and 1 μM added to the OGD treated cells significantly raised the MTT conversion by 36% and 55% above OGD values, *i.e.* 86% and 98% compared to normoxia, respectively (Fig. 7). A similar, statistically significant increase in the accumulation of formazan was observed under the influence of FK506 (0.01–1 μM), which elevated conversion of MTT in a concentration-dependent manner by 33%, 40% and 44% above the OGD group, which corresponds to 84%, 88% and 91% of normoxia, respectively (Fig. 7).

Discussion

Our previous experiments have shown that that PACAP-38 and VIP strongly stimulated cAMP generation in neurons and astrocytes derived from rat cerebral cortex tissue, with PACAP-38 being distinctly more potent than VIP, and glial cell cultures more responsive than neurons [14,15]. However, under hypoxic conditions induced either chemically, with $CoCl_2$, or by using gas mixture with a reduced oxygen level, the effects of PACAP-38 and VIP were less apparent in both biological cell systems. Since the cAMP effects of forskolin were similar under normoxic and hypoxic conditions, it is suggested that hypoxia leads to changes in PACAP- and VIP-driven cAMP-dependent signaling in the rat brain, presumably by influencing molecular processes occurring at the level of receptor protein or receptor-Gs protein coupling [14,15].

The current work uses mature primary cultures of the cerebral cortex under conditions of OGD, an established *in vitro* model for ischemic cell stress, to study signaling and neuroprotection by PACAP-38 and VIP under conditions of hypoxia/ischemia. The choice of such a research model is based on practical reasons, because hypoxic-ischemic brain injury, including stroke and traumatic brain injury (TBI), are the most common acute central nervous system (CNS) neuro-degenerative disorders worldwide, and generally available treatments are still ineffective. Pathologically, these injuries begin with an initial tissue insult and reduction in oxygen and glucose levels in the surrounding extracellular space, developing a core infarct. Experimentally, hypoxia, a condition connected with pathological ischemia, can be induced chemically by adding iron chelators to the culture medium. Many reports have demonstrated that the addition of $CoCl_2$ to the culture medium, or injection of the drug to living animals, creates chemically-induced hypoxia-mimicking conditions in the presence of environmental oxygen. Cobalt substitutes for the iron in the putative hem-containing oxygen sensor, thus preventing or reducing oxygen binding, and stimulating hypoxia. $CoCl_2$ acts also as a pharmacological inducer of the transcription factor hypoxia-inducible factor-1 (HIF-1), which triggers some of the cellular responses to hypoxia [17,21].

OGD is used as an *in vitro* model for ischemic brain damage as cessation of blood flow deprives the brain of essential components, oxygen and glucose.

In neuronal and glial cells subjected to experimental OGD, the stimulatory actions of PACAP-38 and VIP on cAMP synthesis were considerably weaker than those seen under normoxia or hypoxic ($CoCl_2$-driven) conditions (Figs. 2 and 3). However, to compare the effect of OGD on the two cell cultures, PACAP-38 and VIP were found to have a slightly less suppressive effect on cAMP in neuron culture than in glial cell culture. Interestingly, the effect of

forskolin on cyclic nucleotide production was similar under conditions of normoxia and OGD in the glial cells, while the effect of forskolin decreased to 50% in the neuronal cells (Figs. 2 and 3). These results suggest that OGD may trigger down-regulation of the brain AC-coupled PACAP/VIP receptors, with a consequent decrease of PACAP- and/or VIP-ergic-dependent c-AMP-driven signaling. Many reports show that the AC → cAMP pathway is essential for cell fate (survival – death), because it generates a number of both protein kinase A (PKA)-dependent and PKA-independent signals, which modulate the microtubule-associated protein-2 kinase/extracellular signal-regulated kinase (MAP/ERK) cascade, caspases and others, and have a significant impact on the realization of the apoptosis program [11,22]. Based on these results, it can be assumed that OGD induces a change of the PACAP – and VIP – ergic signals in cells or tissues by reducing the activity of the AC → cAMP pathway. These changes might but actually have to influence on further stages of signal transduction in the context of the final neuroprotective effect, which decide cell fate: determine apoptosis and PACAP-and/or/VIP-ergic signal.

The next stage of the work was to assess the effect of chemical hypoxia and OGD on neuronal and glial cell viability by measuring the reduction of MTT to formazan by oxidoreductases present only in living cells. The results revealed that chemical hypoxia induced by a wide range of concentrations of $CoCl_2$ reduced the viability of neurons and astrocytes in a concentration-dependent manner (Figs. 4 and 5). At higher concentrations, *i.e.* 400 and 800 μM, $CoCl_2$ was cytotoxic in both cell cultures. Cultures subjected to OGD were found to have a higher number of dead cells compared to those subjected to $CoCl_2$-induced hypoxia in a $CoCl_2$ concentration-dependent manner (Figs. 4 and 5). The reduced viability of neuronal and glial cells subjected to hypoxia or OGD corresponded with the suppressive effect of cAMP synthesis by PACAP-38 and VIP under the same experimental conditions.

The similar sensitivities of PACAP-38 and VIP receptors in primary neuronal and glial cell cultures observed in the present study, as well as the rates of survival of both cell types under conditions of hypoxia and OGD, suggest that the presence of a small proportion of astrocytes (about 5–10%) in neuronal cultures is not only necessary for the correct growth and development of neurons, but constitutes additional protection against harmful agents. It is known that astrocytes are the principal housekeeping cells of the nervous system. Under physiological conditions their main supportive tasks are to scavenge transmitters released during synaptic activity, control ion and water homeostasis, release neurotrophic factors, shuttle metabolite and waste products, and participate in the formation of the blood–brain barrier. In turn, during ischemia, astrocytes are characterized by additional features such as the ability to store glycogen and synthesize it from lactate, alanine and glutamine: abilities which are absent in neurons [23]. It is clear that neuronal survival in both physiological and pathological conditions, such as brain injury and stroke relies heavily on surrounding astrocytes. A striking example is that of ischemic infarcts, in which neurons do not survive if neighboring astrocytes are lost.

The final series of experiments examined the neuroprotective effect of PACAP-38 and VIP in rat primary glial and neuronal cell cultures in an *in vitro* model of ischemic stroke. The tested peptides have been shown to exert neuroprotective properties in many *in vivo* and *in vitro* studies. In general, PACAP is significantly more effective in protecting neurons or maintaining neuronal survival; however, there are important exceptions to this generalization. In an increasing number of cases, VIP has been demonstrated to have a unique and potent neuroprotective action. Thus, PACAP and VIP protect cells from the neurotoxic effects of ethanol [24], hydrogen peroxide (H_2O_2) [25], β-amyloid [26] and glycoprotein 120 [27]. Moreover, PACAP has been shown to have neuroprotective properties against glutamate [28], human prion protein fragment 106–126 [29] and C2-ceramide [30]. Both peptides reduced brain damage after ischemia, brain injury and ameliorated neurological deficits in a model of Parkinson's disease [13,31,32]. The development of the experimental OGD model used in this study was based on previous findings from other centers regarding the effects of hypoxia on CNS cells, as well as those from our own previous studies [33–36]. As 100 μM $CoCl_2$ was observed to have a cytotoxic effect in both glial and neuron cell cultures subjected to OGD, with 33% and 37% respective reductions in cell number, this concentration was chosen for further experimental work (Figs. 4 and 5).

FK506 was used as reference. It is a known immunosuppressant compound which has been demonstrated to have neuroprotecive potential by many *in vitro* and *in vivo* studies [33,37,38]. Its protective effect has been confirmed in various models of ischemia, such as transient focal cerebral ischemia, where FK506 was found to have neuroprotective activity even 2 h after ischemia was induced [39]. Although the precise mechanism of action of FK506 is not completely understood, it is known that it may protect cells by inhibiting calcineurin activity and pro-inflammatory cytokines synthesis [38,40].

The results of the MTT assay demonstrate that PACAP-38 (0.001–1 μM) bestows no protective effect in glial cells subjected to OGD. In turn, VIP exerted neuroprotective potency at both 0.1 and 1 μM, but as the results were not statistically significant, interpretation is difficult (Fig. 6). The FK506 used at concentrations ranging from 0.01 to 1 μM exhibited neuroprotective properties much stronger than VIP (Fig. 6). In contrast both tested peptides and the immunosuppressive agent were found to exhibit clear neuroprotective properties in the primary neuronal cell cultures (Fig. 7). PACAP-38 (0.1 μM) was found to exert the strongest protective effect on neurons: it increased hypoxic cell viability to levels above those of the OGD cultures, even reaching normoxia levels. The effects of VIP and FK506 on MTT conversion were similar with respective maximum levels of 55% and 44% above the OGD group at concentration of 1 μM (Fig. 7). The obtained results correlate with those of Gabryel et al. (2006), who observed that FK506 had a strong neuroprotective effect on the viability of rat cortical astrocytes subjected to 8 h OGD [33]. On the other hand, little data exists regarding the effects of PACAP and VIP on CNS cells in a model of hypoxia/ischemia. The first *in vitro* experimental work investigating the neuroprotective properties of PACAP in rat cortical neurons subjected to hypoxic gas appeared in 2007 [41]. It indicated that PACAP-27 (0.001 μM and 0.005 μM) bestowed a protective influence only during moderate hypoxia (90 min). At a higher concentration, 0.1 μM, and for longer period of ischemia (120 min), this peptide caused a significant increase in neuronal death. The observed effect was completely blocked by the PAC1 receptor antagonist, PACAP6-38, which is a shortened derivative of the native peptide [41].

Moreover, it is worth noting that the greater influence of PACAP-38 than VIP observed in the prevention of OGD-induced neuronal damage, may suggest that PAC1 receptors are involved in the neuroprotective activity of PACAP-38. These findings correspond to those of a previous study provided by Vaudry et al., that PACAP exerts a potent protective effect against neuronal degeneration induced by hydrogen peroxide by acting through the PAC1 receptor [25].

In conclusion, the present work demonstrates that rat neuronal and glial cells subjected to $CoCl_2$-driven hypoxia or OGD exhibited a reduced viability and ability to stimulate cAMP synthesis under the influence of PACAP and VIP. Indeed, the OGD condition was characterized by greater cytotoxicity and resulted in greater changes in both cell cultures compared to chemical hypoxia. These changes, manifested as deficiencies in PACAP-/VIP-ergic signaling, might contribute to the overall ischemia-driven pathology. If so, a

normalization of disturbed PACAP-/VIP-ergic mechanisms in the ischemic brain could represent part of a therapeutic strategy by which post-ischemia pathological symptoms may be counteracted. Furthermore, in the experimental *in vitro* model of ischemia, PACAP and VIP had positive effects on cell viability (especially in neurons), which confirms that these peptides are potential neuroprotective agents.

Conflicts of interest

All the authors declare no conflicts of interest.

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