Pharmacological Reports
2008, 60, 1019–1024
ISSN 1734-1140

Short communication

Effects of PEDF and VEGF on PACAP-/VIP-induced cAMP formation in rat brain derived astrocyte cultures

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Abstract:
Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are two structurally related peptides acting on their specific receptors. Both were shown to concentration-dependently (0.001 nM – 1 μM) stimulate cyclic 3',5' adenosinomonophosphate (cAMP) formation in rat primary glial cell (astrocyte) cultures, with PACAP being distinctly more potent than VIP. The acute effects of the peptides were significantly suppressed (25% and 36% for PACAP and VIP, respectively) when tested in cell cultures preincubated for 24 h (but not 2 h) in the presence of pigment epithelium-derived factor (PEDF, 50 ng/ml). Both 24 h and 2 h preincubation of cells with vascular endothelial growth factor (VEGF, 50 ng/ml) had no influence on PACAP or VIP actions. The addition of PEDF and VEGF together for 24 h preincubation, produced suppression of the PACAP- or VIP-evoked cAMP responses similar to that seen with PEDF alone. Neither PEDF nor VEGF significantly affected cAMP generation in an assay with a 15-min incubation, which is a standard incubation period for PACAP and VIP. The findings show that PEDF, displaying by itself no effect on cAMP generating system in rat astrocytes, significantly affected biological activity of both PACAP and VIP. The reported observation may be of significance, considering the neurotrophic and/or neuroprotective activity of the tested polypeptides.

Key words:
PACAP, VIP, PEDF, VEGF, cAMP, astrocytes, rat brain

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are members of the same superfamily of peptides that includes, among others, secretin, glucagon, helodermin, peptide histidine-isoleucine (PHI) and its human analog peptide histidine-methionine (PHM). PACAP occurs in tissues in two biologically active forms, PACAP-38 and the C-terminally truncated PACAP-27, whereas VIP occurs in only one form which shares an amino acid sequence identity of 68% with PACAP-27. All three molecules exert their functions *via* three types of G protein-coupled receptors class B, named PAC_1, $VPAC_1$ and $VPAC_2$. PAC_1 receptors specifically rec-

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ognize PACAP (PACAP-specific receptors), whereas VPAC type receptors are equally sensitive to PACAP and VIP. Their signaling mechanisms mainly involve the activation of adenylyl cyclase, yet, depending on receptor subtype and splice variant, phospholipase C (PLC)- and phospholipase D (PLD)-directed signaling cascades may also operate in different cells/tissues. Consequently, the binding of PACAP/VIP to PAC_1 or VPAC type receptors produces multidirectional cell activation, giving rise to cAMP production, calcium mobilization, protein kinase C activation and phosphorylation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK). Having a potential to activate an array of signaling pathways, PACAP and VIP exert pleiotropic functions, acting in the central nervous system as a hormone, a neuromodulator, a neurotransmitter and a trophic factor. They also play a role in the control of diverse phenomena, including neurogenesis, gliogenesis, or neuroprotection [1, 7, 10, 11, 19, 28, 33, 35, 41].

In such complex processes as those mentioned above, the published data concerning the role of PACAP and VIP are sometimes contradictory, sometimes unpredictable (e.g. in terms of concentration-specific windows of activity) or show conflicting roles of the peptides, as observed in proliferation, differentiation or neuroprotection. Thus, an emerging idea is that the actual role of either PACAP or VIP, at a given moment of organism development or in a given cell/tissue model system and experimental condition, is highly influenced by the presence of other trophic or protective factors or signal transduction molecules. Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) are among factors exhibiting both neurotrophic and neuroprotective activity; additionally, they display an antiangiogenic and proangiogenic potential, respectively [4, 18, 23, 29, 36–38, 40, 42]. Due to their unique biological profiles these two factors have been selected for the present study carried out on rat astrocytes. Interestingly, VEGF is capable of stimulating astrocyte growth [20] and astrocytes exposed to hypoxia are able to express VEGF [34]. PEDF, a 50-kDa secreted glycoprotein and a non-inhibitory member of the serpin (serine protease inhibitor) superfamily of proteins, evokes many effects including those linked to astrocyte biochemistry [4, 5, 12, 21, 40]. The receptoric mode of action of VEGF is rather well recognized [13, 23, 42], whereas the mechanism(s) by which PEDF initiates its diverse biological activities is/are still largely unclear due to difficulties in identifying its receptor [9, 24, 40]. Earlier data have suggested the existence of a putative 85-kDa receptor for PEDF occurring exclusively on retinal cells [3]. Other suggestions concerning the nature of the endothelial cell-localized PEDF receptor, which is responsible for the inhibition of VEGF-driven angiogenesis, include PACAP interaction with γ-secretase and translocation of the intracellular domain of type 1 VEGF receptor (known as VEGFR-1), as well as phosphorylation of VEGFR-1 [9]. A recently identified lipase-linked cell membrane receptor for PEDF (PEDF-R; which, upon binding with PEDF, stimulates the enzymatic phospholipase A2 activity), will surely help to understand the molecular processes underlying the multifunctional nature of this molecule [24].

This report presents our first results showing that PEDF, but not VEGF, significantly suppressed PACAP and VIP ability to stimulate cAMP formation, a phenomenon likely to have functional consequences for glia-dependent brain processes.

Materials and Methods

Chemicals

PACAP-38 (human, ovine, rat) and VIP (human, porcine, rat; referred to as mammalian VIP, mVIP) were from Neosystem (Strasbourg, France). Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) were from Chemicon (Temecula, CA, USA). 3-Isobutyl-1-methylxanthine (IBMX), poly-L-ornithine, trypsin, glutamine, penicillin, streptomycin, amphotericin B, culture media, and most of other non-specified compounds, were from Sigma (St. Louis, MO, USA). Multi-well plates and Petri dishes for cell culture were from Nunc (Wiesbaden, Germany).

Radiolabeled compounds: [^{3}H]adenine (specific activity 24.2 Ci/mmol) and [^{14}C]cyclic AMP (specific activity 56 mCi/mmol) were purchased from PerkinElmer Life Sciences Inc. (Boston, MA, USA) and Moravek Biochemicals Inc. (Brea, CA, USA), respectively.

Cell culture

Experiments were performed on glial cell cultures from newborn rats. All animal procedures were in

strict accordance with the Polish governmental regulations concerning experiments on animals (Dz.U. 97.111.724), and all experimental protocols were approved by the Local Ethical Commission for Experimentation on Animals.

Primary astrocyte cell culture was prepared from cerebral cortices of 1-day old albino Wistar rat pups using the method previously described [17, 26]. Briefly, a dissected cerebral cortex was cut into small fragments, enzymatically digested and mechanically dissociated. The glial cells were grown in 6-cm diameter Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 2 mM glutamine, penicillin (100 U/ml)-streptomycin (100 μg/ml) solution, 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. The cells from the second or third passage were used for experiments. The homogeneity of cell population was approximately 95%, as verified by staining immunocytochemically for glial fibrillary acidic protein (GFAP), using the anti-GFAP antibodies; no neurons, as confirmed by an anti-MAP-2 antibodies, were detected [17, 26].

Assay of cyclic AMP formation

Details of this procedure were reported by us in previous publications [17, 26]. The glial cells used in the experiments 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. One day prior to the experiment, the culture medium was removed, fresh serum-free culture medium was added and cells were preincubated with PEDF (50 ng/ml), VEGF (50 ng/ml), or with PEDF plus VEGF for either 2 h or 24 h. On the day of the experiment, the cells were incubated with [^{3}H]adenine for 30 min at 37°C. The medium was then removed, the cells were rinsed two times with pre-warmed phosphate-buffered saline (PBS) and serum-free culture medium was added. Cells were preincubated for 10 min at 37°C with 3-isobutyl-1-methylxanthine (IBMX; 100 μM), followed by a further 15 min incubation with PACAP-38 (0.001 μM) or mammalian VIP (mVIP, 1 μM). Some experiments also included concurrent incubation with PEDF or VEGF (each at 50 ng/ml concentration). The reaction was stopped by adding 500 μl of cold 10% trichloroacetic acid (TCA). The cell mixture was then transferred into test tubes, centrifuged and the formed cAMP was quantified in a supernatant fraction. The formation of [^{3}H]cAMP in [^{3}H]adenine-prelabeled cells was assayed according to Shimizu et al. [32]. The formed [^{3}H]cAMP was isolated by sequential Dowex-alumina column chromatography according to Salomon et al. [30]. The results were individually corrected for percentage recovery with the aid of [^{14}C]cAMP added to each column system prior to the nucleotide extraction. The accumulation of cAMP during a 15-min stimulation period was assessed as a percentage of the conversion of [^{3}H]adenine to [^{14}C]cAMP.

Data analysis

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

Results and Discussion

In agreement with our previous results [10, 17, 26], and those of others [15], PACAP and VIP potently stimulated cAMP formation in cultured rat astrocytes, with PACAP being distinctly more potent than VIP. PACAP at concentrations as low as 10^{-12} M produced measurable increases of cAMP production (285% of control). At 10^{-9} M cAMP production was increased to the level of 2812% of the control value (taken as 100%); the obtained results expressed in % conversion were: control – 1.08 ± 0.14(9); PACAP: 10^{-12} M – 3.08 ± 0.27(6), 10^{-9} M – 30.37 ± 1.56(13), 10^{-6} M – 39.49 ± 4.45(5).

In parallel experiments, VIP at 10^{-9} M and 10^{-6} M produced values of 2.31 ± 0.27(6) and 12.78 ± 0.55(7) % conversion, which expressed in percent of control value (0.88 ± 0.09(10)% conversion) gave stimulations of 262.5 and 1452.3. The cAMP generating system occurring in primary cultures of rat astrocytes clearly appeared to be more responsive to the studied peptides, especially to PACAP, than those operating in primary neuronal cultures or cerebral cortical slices [17, 26]. The observed difference in potency between PACAP and VIP as stimulators of cAMP synthesis in cultured astrocytes indicates that both peptides exerted their effects by acting *via* different receptors, i.e.

PAC_1 receptor for PACAP and VPAC type receptor(s) for VIP. Since the latter receptor type is also a target for PACAP, the global effects of this peptide likely resulted from stimulation of adenylyl cyclase-linked PAC_1 and VPAC receptors. The presence of both PAC_1 and VPAC type receptors on rat astrocytes has been reported earlier by other authors [2, 15, 16, 22, 27].

A high potency and effectiveness of PACAP and VIP in stimulating adenylyl cyclase-driven signaling pathway agrees with subnanomolar-nanomolar range of concentrations that are required for these peptides to strongly manifest their biological potential, especially in the field of neurotrophism and/or neuroprotection [7, 31, 33]. Based on the present results, one concentration of both PACAP (10^{-9} M) and VIP (10^{-6} M) was selected for further study.

As stated in the Introduction, the idea of the present work was to determine whether biological activity of PACAP and VIP in astrocytes can be influenced by endogenous molecules known to possess established neurotrophic and/or neuroprotective potential. This series of experiments were initiated with pigment epithelium-derived factor (PEDF), a multifaceted neurotrophic factor possessing powerful neuroprotective, anti-angiogenic and anti-tumorigenic activity [4, 5, 12, 21, 40].

Despite the unknown receptor mechanism, in our experiments, PEDF (50 ng/ml) by itself – after 15 min incubation, or 2 and 24 h preincubation – did not modify the activity of the cAMP generating system in cultured astrocytes. Futher, only in the 24 h treatment did PEDF lead to significant suppression of the effects evoked by both PACAP (by 25%) and VIP (by 36%) (Tab. 1; Fig. 1, 2).

In further experiments we turned to another growth factor, namely vascular endothelial growth factor (VEGF), a well recognized pro-angiogenic molecule, and a key regulator of angiogenesis in physiological and in most pathological conditions [8, 13]. VEGF is endowed with marked neurotrophic and neuroprotective activity [23, 29, 37, 38, 42], and shows a functional antagonism with PEDF in some phenomena, especially those linked to neovascularization [6, 9, 25, 39]. Similar to PEDF, VEGF (50 ng/ml) by itself did not modify the activity of the astrocyte cAMP generating system (Tab. 1; Fig. 1, 2). Moreover, VEGF did not significantly influence the PACAP- and VIP-driven cAMP responses, although there was a small (up to 22%) but consistent tendency to enhance the PACAP effect in the 24 h treatment (Fig. 2). The co-preincubation of VEGF with PEDF also failed to significantly modify the suppressive action of the latter factor (Fig. 2).

Tab. 1. The effects of acute treatment with VEGF and PEDF on PACAP- and VIP-evoked cAMP formation in primary cultures of rat astrocytes

Group	cAMP, % conversion	% of respective control
Control	0.89 ± 0.08(6)	100
VEGF 50 ng/ml, 2 h	0.94 ± 0.14(5)	106
PEDF 50 ng/ml, 2 h	1.01 ± 0.1(6)	113
PACAP 1 nM, 15 min	27.7 ± 2.12(6)	100
+ VEGF 50 ng/ml, 2 h	28.38 ± 1.62(6)	102
+ PEDF 50 ng/ml, 2 h	27.2 ± 2.1(6)	98
VIP 1 μM, 15 min	15.54 ± 1.78(6)	100
+ VEGF 50 ng/ml, 2 h	15.05 ± 2.41(6)	97
+ PEDF 50 ng/ml, 2 h	16.04 ± 2.85(6)	103

Results are expressed in percent conversion and represent the means ± SEM from the number of experiments given in parentheses. All experiments were done in the presence of 100 μM IBMX applied to 30 min preincubation period (i.e. 15 min prior to PACAP or VIP). The differences between the results are not statistically significant

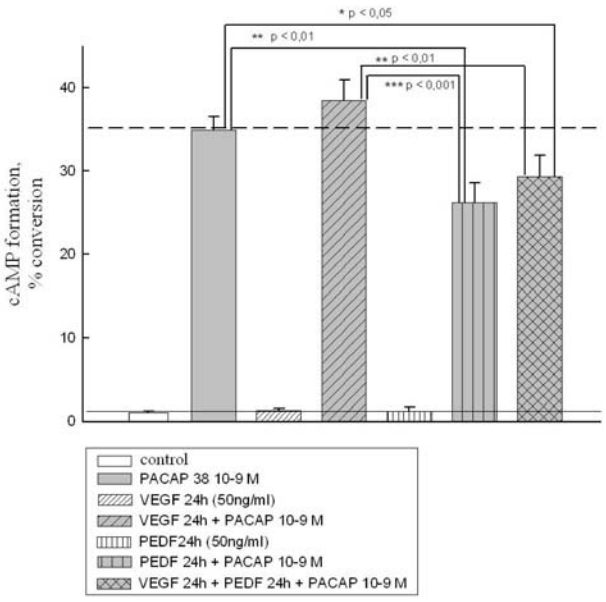

Fig. 1. Effects of vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) on pituitary adenylate cyclase-activating polypeptide (PACAP)-induced cyclic adenosinomonophosphate (cAMP) generation in primary cultures of rat astrocytes. Results are shown as the mean ± SEM from the number of 6–22 experiments. For methodological details see Materials and Methods

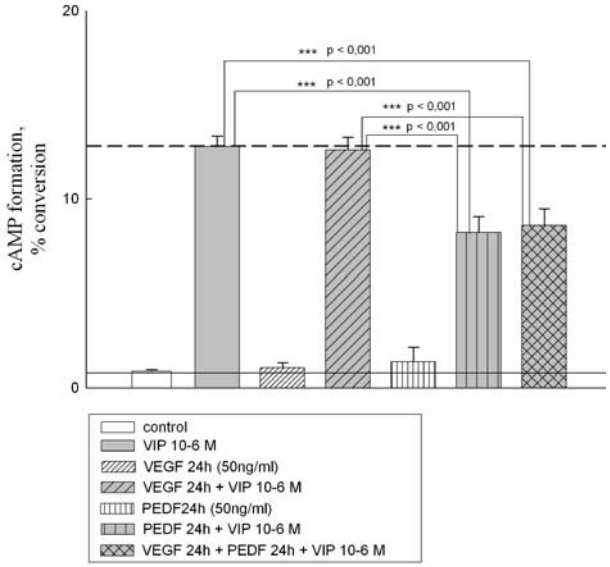

Fig. 2. Effects of vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) on vasoactive intestinal peptide (VIP)-induced cyclic adenosinomonophosphate (cAMP) generation in primary cultures of rat astrocytes. Results are shown as the mean ± SEM from the number of 5–10 experiments. For methodological details see Materials and Methods

In the present study we focused on cAMP generation, as PACAP and VIP are powerful activators of this signaling system. The present unexpected observation that PEDF suppresses the PACAP- and VIP-evoked cAMP effects in rat astrocytes opens new directions for the study of a possible interplay between endogenous molecules possessing neurotrophic and neuroprotective potential. The mechanism, as well as the meaning of the reported suppressive effect of PEDF on PACAP/VIP-induced receptor-related actions, remains to be established. It would be highly interesting to analyze, under similar experimental conditions, other signaling pathways that are known to be commonly affected by both PACAP/VIP and PEDF, and such experiments are in progress in our lab.

In conclusion, we have observed in rat primary astrocyte cultures that PEDF, having by itself no action on the cAMP-generating system, led to a marked suppression of PACAP- and VIP-induced nucleotide synthesis. Further, the effects of these peptides were not modified by another growth factor, VEGF, showing that there is no functional antagonism between these two trophic factors, at least in rat cerebral cortex-derived glial cells and PACAP/VIP-sensitive cAMP related system. PACAP/VIP and PEDF, as well as VEGF, are endogenously occurring molecules endowed with neurotrophic and neuroprotective potential in addition to other biological activities. All are considered therapeutically useful molecules that are currently undergoing preclinical and/or clinical testing [12–14]. Therefore, any details characterizing their functioning on different levels of molecular/cellular organization of an organism will broaden our knowledge on the complex nature of the studied peptides.

Acknowledgments:
This study was supported by funds from Medical University of Łódź (grants No. 503-1023-1 and No. 502-11-462).

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Received:
August 1, 2008; in revised form: December 9, 2008.