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Effects of PACAP, VIP and related peptides on cyclic AMP formation in rat neuronal and astrocyte cultures and cerebral cortical slices

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Abstract:
The effects of pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), peptide histidine-isoleucine (PHI) and peptide histidine-methionine (PHM) on cyclic AMP formation were studied in parallel on rat cerebral cortical slices, primary neuronal cultures and primary glial (astrocyte) cultures. PACAP appeared to be the most potent agent in all biological systems. The rank order of the peptides' potency was as follows: PACAP > VIP > PHI = PHM for cortical slices and neuronal cell cultures, and PACAP >> PHM ≈ VIP > PHI for glial cell cultures. The cyclic AMP responses to the tested peptides, especially to PACAP, were distinctly larger in glial cell cultures than in neuronal cell cultures or brain slices. In an additional study, the cyclic AMP response to helodermin and secretin, as well as isoprenaline, histamine and forskolin, were tested in parallel on glial and neuronal cell cultures, and directly compared with the actions of PACAP. Helodermin and isoprenaline showed clearly stronger activity in glial cell cultures, yet their activity was much weaker than that of PACAP, whereas the effect of forskolin was only 2 times larger in glial cells than in neuronal cultures; histamine had no effect in any cell culture, while secretin produced a small but significant effect only in glial cells. The obtained results suggest that the astrocyte compartment of the rat brain may be the main target for such peptides as PACAP, VIP, or stucturally related PHI/PHM or helodermin.

Key words:
PACAP, pituitary adenylate cyclase activating polypeptide, VIP, vasoactive intestinal peptide, helodermin, secretin, neuronal cell culture, glial cell culture, brain slices, cyclic AMP, rat

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are members of the structurally related family of polypeptide hormones, which also includes peptide histidine-isoleucine (PHI), peptide histidine-methionine (PHM), secretin, glucagon-like peptide (GLP), glucose-dependent insulinotropic polypeptide (GIP), growth hormone releasing hormone (GHRH) and helodermin [1, 6, 27, 31]. PACAP and VIP are widely distributed both in the central nervous system and peripheral organs of various vertebrates, and exert pleiotropic physiological functions. They have been described to act as regulators of the pituitary gland, pancreas and adrenal glands, relaxants of smooth muscles in blood vessels, respiratory system, gastrointestinal tract, reproductive system, and factors affecting immune system elements. In the central nervous system, PACAP

and VIP act as neuroregulators, neurotransmitters, as well as neurotrophic or neuroprotective factors [1, 3, 5, 6, 22, 31]. Both neuropeptides exert their actions *via* common receptors: VPAC1 and VPAC2, which bind VIP and PACAP with similar affinity; PHI/PHM may also act through these receptors. Besides, PACAP stimulates PAC1-type receptors, which bind PACAP with much higher affinity than VIP or PHI/PHM [10, 31]. The adenylyl cyclase (AC)/cyclic AMP cascade is the main intracellular signal transduction pathway coupled with all mentioned types of receptors [1, 10, 31]. The current work is a continuation of our previous studies on the effects of various neuropeptides, including PACAP and VIP, on the AC → cyclic AMP-linked signaling pathway, carried out on slices of rat cerebral cortex [4, 16–21, 32–34]. The aim of the present work was to study the effects of PACAP, VIP, PHI and PHM, as well as several other peptide and non-peptide agents, on cyclic AMP production in neuronal and glial cell cultures, and to directly compare the peptides' effects with those obtained in a functionally integral tissue represented by cerebral cortical slices.

Materials and Methods

Chemicals

The following neuropeptides were used: PACAP-38 (human, ovine, rat), VIP (human, porcine, rat; referred to as mammalian VIP or mVIP), peptide histidine-isoleucine (porcine form – pPHI, and human form – PHM), helodermin and secretin, were from Neosystem (Strasbourg, France). Forskolin, isoprenaline, histamine, poly-L-ornithine, DNase I, trypsin, glutamine, penicillin, streptomycin, amphotericin B, antibodies against glial fibrillary acidic protein (GFAP) and microtubule associating protein-2 (MAP-2), were from Sigma (St. Louis, MO, USA). Neurobasal culture medium and B27 were from Gibco (Paisley, Scotland, UK). 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 Biochemical Inc. (Brea, CA, USA), respectively. Other chemicals were of analytical purity and were obtained mainly from Sigma (St. Louis, MO, USA).

Animals

Experiments were performed on glial and neuronal cell cultures prepared from newborn rats and rat embryos, and on cerebral cortical slices prepared from adult albino Wistar rats, as described below. 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 neuronal culture

Primary neuronal cell cultures were obtained from Wistar rat embryos on days 15–17 of gestation and were maintained according to Brewer [3] and Halonen et al. [9], described in detail by Nowak et al. [15]. Briefly, pregnant females were anesthetized with ether vapor, killed by cervical dislocation and subjected to cesarean section in order to remove fetuses and their brains. Cerebral cortex was dissected, incubated for 15 min in trypsin/EDTA (0.025%) 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 μg/ml streptomycin and 0.25 mg/ml amphotericin B, and plated at a density of 2.5×10^5 cells per square centimeter onto poly-L-ornithine (0.01 mg/ml) coated multi-well plates. The cultures were maintained at 37°C in a humidified atmosphere containing a 5% CO_2 and cultivated for 7 days prior to experimentation. The purity of neuronal cultures was verified by means of the immunocytochemical staining method using antibodies against microtubule associating protein-2 (MAP-2) for neurons, and against a specific "astrocyte" marker, i.e. glial fibrillary acidic protein (GFAP), for glial cells. The latter analysis carried out in each new culture revealed the presence of approximately 6–10% of GFAP-positive cells, which indicates that the primary neuronal cultures represented in fact neuron-enriched preparations.

Primary astrocyte culture

Primary astrocyte cultures were prepared from cerebral cortices of 1-day old Wistar rat pups essentially according to the method of Hertz et al. [11], as described by us in detail earlier [15]. Briefly, 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 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 mg/ml amphotericin B, in 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 and third passage were used for experiments. The homogeneity of cell population was approximately 95%, as verified by staining immunocytochemically for GFAP, using the anti-GFAP antibodies. No neurons, as confirmed by an anti-MAP-2 antibodies, were detected.

Cerebral cortical slices

Male albino Wistar rats (180–200 g) kept under 12-h light (150 lux)/dark cycle with standard food and tap water *ad libitum* were used. On the day of experiment, the animals were killed by decapitation between 9.00–9.30, their brains were removed, and cerebral cortex (without white matter) was isolated and processed for the measurement of cyclic AMP generation. In brief, the tissue pieces (consisting of a part of parietal cerebral cortex) were rapidly cross-sliced (0.25 mm) with the aid of a McIlwain tissue chopper and suspended in cold, O_2/CO_2 (95:5)-gassed, glucose containing modified Krebs-Henseleit medium (KMH; mmol/l): 118, NaCl; 5, KCl; 1.3, $CaCl_2$; 1.2, $MgSO_4$; 25, $NaHCO_3$; 11.7, D-glucose; pH = 7.4).

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. On the day of experiments, the culture medium was removed, fresh serum-free culture medium was added and cells were incubated in the presence of [^{3}H]adenine for 1.5 h at 37°C. Cerebral cortical slices were prepared just before the experiment (as stated above) and then incubated with [^{3}H]adenine. The formation of [^{3}H]cyclic AMP in [^{3}H]adenine prelabeled preparations was assayed according to Shimizu et al. [28], and the formed [^{3}H]cyclic AMP was isolated by a sequential Dowex-alumina chromatography according to Salomon et al. [26]. The results were individually corrected for percentage recovery with the aid of [^{14}C]cyclic AMP added to each column system prior to the nucleotide extraction. The accumulation of cyclic AMP during a 15-min stimulation period with peptides was assessed as a percentage of the conversion of [^{3}H]adenine to [^{14}C]cyclic AMP.

Statistical 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* Student-Newman-Keuls test.

Results

PACAP, VIP, PHI/PHM – effects on cyclic AMP formation in cerebral cortical slices

In our previous experiments carried out on brain slices from different species (birds – chick, duck, goose, turkey; mammals – rat, guinea pig), the cyclic AMP responses to PACAP were usually larger than those to VIP and PHI, and their maximal values ranged between ≈ 300–2000% of respective control value; there were also marked differences between the results of similar experiments performed on different occasions [4, 16–21, 32–34].

In the current experiments performed on rat cerebral cortical slices (Fig. 1 *top*) and carried out in parallel on primary cell cultures, the maximal effects produced by PACAP-38 and VIP were similar (i.e. 550% of respective control value), although this level of stimulation was reached by both peptides at 0.1 and 3 μM, respectively; the calculated EC_{50} values for PACAP and VIP were, respectively, 30 nM and 180 nM, which is in line with our earlier data [16]. PHI and PHM appeared to be the least potent peptides, producing stimulation of 233 and 329% of respective control value at 3 μM concentration (Fig. 1 *top*).

PACAP, VIP, PHI/PHM – effects on cyclic AMP formation in glial cell cultures

As shown in Figure 1 (*middle panel*), the cyclic AMP responses to PACAP-38, VIP and PHI/PHM were distinctly larger than those observed in brain slices. In fact, PACAP-38 appeared to be a powerful stimulator of the cyclic AMP generation, being more potent than VIP by approximately 4 orders of magnitude. At 0.001 nM PACAP-38 already significantly stimulated the nucleotide production, reaching maximal effect at 10 nM (449 ± 26 and 4639 ± 309% of control, respectively). The calculated EC_{50} value for PACAP-38 was 0.25 nM. In contrast to PACAP, the ability of VIP and PHI/PHM to stimulate cyclic AMP synthesis in this cell preparation was comparatively poor, as these peptides evoked a measurable response at 10–100 nM, producing, however, a relatively (compared to tissue slices or neuronal cultures) marked effects at the highest tested concentrations, i.e. 3–5 μM, with the following rank order of potency: PHM > VIP > PHI.

PACAP, VIP, PHI/PHM – effects on neuronal cell cultures

The results are shown in Figure 1 (*bottom panel*). Although PACAP was also a potent stimulator of cyclic AMP synthesis in neuronal cell cultures (EC_{50} = 0.25 nM), its effectiveness appeared to be rather moderate (compared to glial cells) as its maximal effect reached at 10–1000 nM was about 650% of respective control value. In this cell system, VIP started to enhance the cyclic AMP generation at 1 nM, nearly approaching the PACAP-evoked maximal level at 1 μM concentration. Assuming that this level was the maximal response (plateau level) for VIP, the calculated EC_{50} for the peptide would be 20 nM. PHI and PHM run in parallel were at least one order of magnitude less potent than VIP.

Cyclic AMP response to various agents in glial and neuronal cell cultures

Since the cyclic AMP responses to PACAP, VIP and PHI/PHM appeared to be less pronounced in neuronal cell cultures than in glial cell cultures, we decided to test in parallel how the neuronal and glial cyclic AMP generating systems will respond to several other agents (peptides and non-peptides) capable of activating the nucleotide formation in different cell/tissue preparations. We selected two peptides belonging to the VIP-PACAP family of neuropeptides, i.e. secretin and helodermin (both at 1 μM concentration), as well as a selective β-adrenoceptor agonist isoprenaline

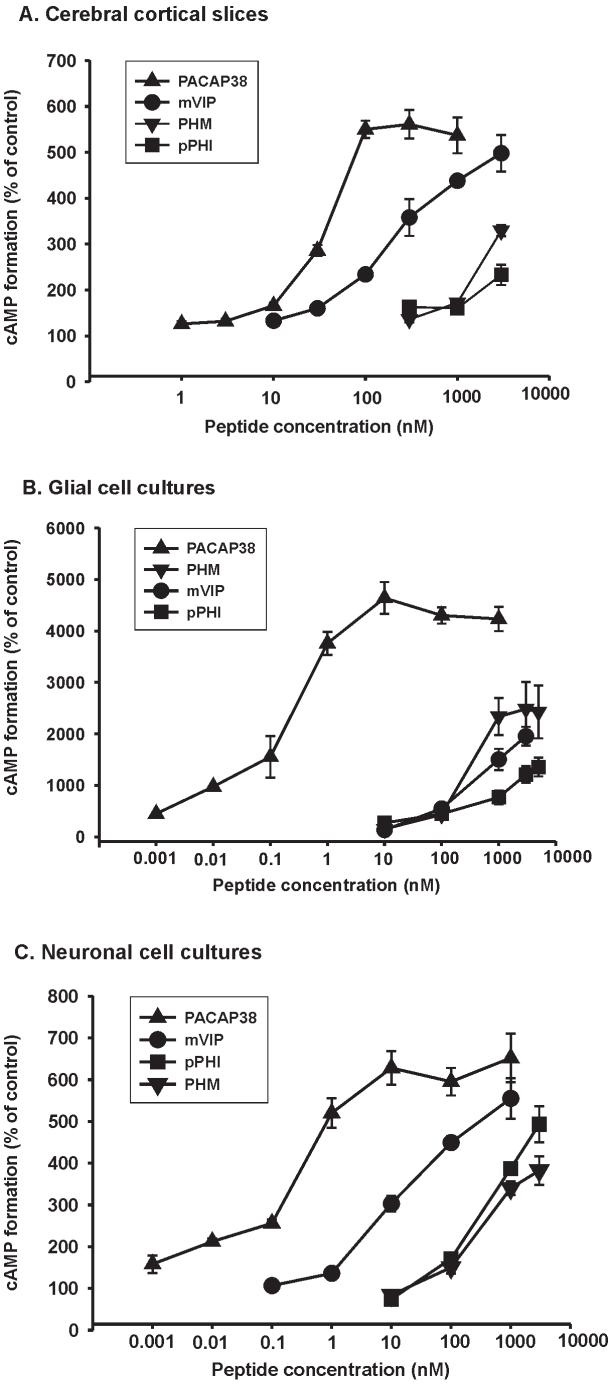

Fig. 1. Effects of PACAP, VIP, PHI and PHM on cyclic AMP formation in rat cerebral cortical slices (*top*), primary glial-astrocyte cell cultures (*middle*) and primary neuronal cell cultures (*bottom*). Results are expressed as the mean ± SEM of 6–24 experiments, and show percent of respective control value

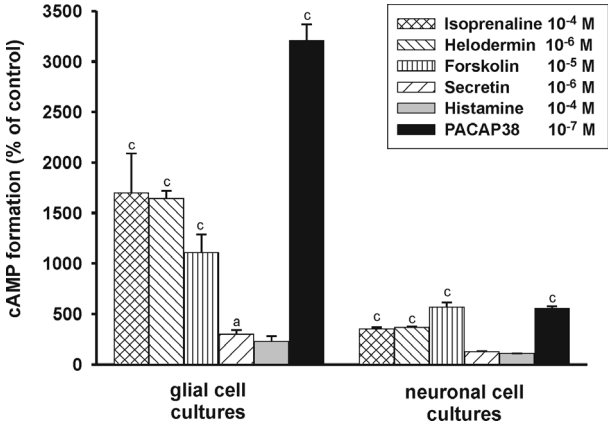

Fig. 2. Effects of peptide (PACAP-38, helodermin, secretin) and non-peptide (forskolin, isoprenaline, histamine) drugs on cyclic AMP formation in rat primary glial-astrocyte and neuronal cell cultures. Results are expressed as the mean ± SEM of 5–12 experiments, and show percent of respective control value; p value: a < 0.05; c < 0.001

(100 μM), histamine (100 μM), and forskolin (10 μM), a diterpene derivative acting directly on catalytic domain of AC.

Of the tested agents, histamine and secretin had very poor activity in both cell preparations (although the secretin effect in glial cells reached the level of statistical significance; Fig. 2 left), whereas other drugs evoked the cyclic AMP responses generally larger in glial cell cultures than in neuronal cell cultures, with the forskolin effect showing only two-fold difference (Fig. 2). PACAP-38 (0.1 μM), taken as a reference drug, again displayed a distinct biological activity in glial cell cultures, with the cyclic AMP responses being decisively lager than that of any drug tested in this set of experiments, and distinctly larger than the responses observed in neuronal cell cultures (Fig. 2). Interestingly, while the effect of 0.1 μM PACAP was clearly greater than that of 10 μM forskolin in astrocytes, these two agents used at the same concentrations produced comparable stimulations of the cyclic AMP formation in neurons (Fig. 2 bottom).

Discussion

PACAP and VIP are well recognized agents capable of stimulating cyclic AMP synthesis/accumulation in various tissues, including the central nervous system of many mammalian and non-mammalian species [1, 6, 21, 31]. In contrast to these two peptides, much less is known about biological effects of PHI and its human analog PHM. According to published data, at least some effects of PHI/PHM may result from their interaction with VPAC type receptors positively coupled with AC [31]. In this work, we have directly compared the cyclic AMP effects of PACAP and VIP with those of PHI and PHM, and additionally with the effects of several other peptide (helodermin, secretin) and non-peptide (forskolin, isoprenaline, histamine) drugs in three biological systems originating from rat brain (cerebral cortical slices, glial cell cultures, and neuronal cell cultures).

The present study clearly showed the glial cyclic AMP generating system to be the most responsive to both PACAP and VIP, suggesting that glial cell compartment may constitute the primary target for both peptides. Although such a suggestion may be valid also under *in vivo* – physiological conditions, nevertheless there is an unanswered issue concerning the role (if any) of "developmental" factor underlying the differences described here, as the material for neuronal cultures derived from brains of rat embryos from 15–17 day of gestation, while the material for glial cell cultures – from brains of newborn animals. Indeed, some functional differences occurring at the level of receptor-effector coupling between embryo- and newborn-derived cells/tissues might contribute to the picture observed in the present study as the effects of forskolin (a post-receptor AC activator) and PACAP (an agent requiring specific receptors for its action) produced in the two cell cultures markedly varied, i.e. < 2 for forskolin and ≈ 6 for PACAP when expressed as the "glia/neuron" ratio (Fig. 2). On the other hand, a small difference in the effects of forskolin between neuronal and glial cultures may suggest that the activity of the nucleotide generating system operating in neuronal and astrocyte preparations exploited in the present study is similar.

Alternatively, the smaller cyclic AMP responses to the tested peptides in neurons than in astrocytes may be explained by the different expression of PACAP/VIP receptors in these cells. The presence of PACAP- and VIP-sensitive receptors and receptor-driven signaling mechanisms in cultured glial cells (astrocytes) has earlier been shown [2, 8, 12, 14, 30]. Furthermore, the observation made by Gozes et al. [7] revealed that neuronal cells exhibited only the lower affinity VIP receptor, whereas glial cells contained both high and low affinity receptors.

PACAP, being distinctly more potent than VIP (a difference of two orders of magnitude in neuronal cultures and four orders of magnitude in glial cultures) likely acted through its specific adenylyl cyclase-linked PACAP type I receptor, PAC1 [10, 24]. Yet, the question about a role of PACAP type II receptors: VPAC1 and VPAC2 which have approximately equal affinity for VIP and PACAP [10, 29], still remains open. Under physiological conditions, VIP stimulates cyclic AMP production through VPAC type receptors, as its activity at "classic" PAC1 receptors is comparatively poor.

It is interesting to mention that very recently a VIP-sensitive hop2 splice variant of the PAC1 receptor (which differs from the commonly known PAC1 only by the addition of the 27 amino acids containing hop2 cassette) has been identified. This receptor, linked to cyclic AMP production, mediated VIP-driven protective effects in astrocytes and neuronal cells [23]. In addition, novel splice variants of the VIP- and PACAP-sensitive AC-coupled PAC1 receptor were shown to exist in human neuroblastoma cells. The cyclic AMP responses evoked by VIP or PACAP markedly varied depending on the receptor variant, with some variants showing enhanced sensitivity to VIP [13]. Furthermore, VPAC2 receptors showing atypical pharmacological properties (PHI-sensitive and PACAP-insensitive) have been proposed to mediate cyclic AMP-independent protein kinase C-mediated VIP neuroprotective effects in newborn mice [25]. Summarizing the cited data, it can be inferred that, despite the commonly approved view accepting the existence of three types of receptors for PACAP and VIP [10], the picture emerging from recent work on PACAP/VIP receptors' subtypes creates some space for more detailed analysis, and perhaps re-investigation of numerous biological effects these peptides produce in different biological systems.

In conclusion, the results of the present work demonstrate the existence of differences between the members of the PACAP-VIP family of polypeptides in their ability to stimulate receptor-driven cyclic AMP production in three biological systems, i.e. tissue slices, primary neuronal cultures and primary glial (astrocyte) cell cultures, with PACAP being the most potent drug in all tested conditions. The results also suggested that, at least in the rat brain, the astrocyte compartment may be the main target for the studied peptides in evoking diverse cyclic AMP-dependent physiological responses occurring in the central nervous system.

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