Chapter 2
Occurrence, Distribution, and Physiological Function of Pituitary Adenylyl Cyclase-Activating Polypeptide in Invertebrate Species

Zsolt Pirger, Nora Krajcs, and Tibor Kiss

Abstract The occurrence and distribution of pituitary adenylate cyclase-activating polypeptide (PACAP) are summarized in invertebrate species with special attention to annelids, mollusks, and arthropods in this review. Furthermore, the role of PACAP is highlighted in physiological and behavioral processes of oligochaete (*Lumbricus*), gastropods (*Helix*, *Lymnaea*), insect (*Drosophila*), as well as malacostraca (*Litopenaeus*). Since its discovery PACAP has become increasingly recognized for its important and diversified roles in the central and peripheral nervous system and in several peripheral organs of a variety of vertebrate and invertebrate species. Twenty-six years after its discovery, PACAP is now one of the most extensively studied neuropeptides both in invertebrate and vertebrate species. This review surveys the importance of PACAP or PACAP-like peptide(s) in invertebrates. The relevance of studies on lower vertebrates and invertebrates, which do not have a pituitary gland like higher vertebrate, is to contribute to the unraveling of fundamental effects of PACAP or PACAP-like peptide(s) and to provide a comparative view.

Keywords Invertebrate PACAP-like molecules • Morphology and physiology • Oligochaetes • Gastropods • Insects

Z. Pirger (✉) • N. Krajcs
MTA-ÖK BLI NAP_B Adaptive Neuroethological Research Group, Department of Experimental Zoology, Balaton Limnological Institute, MTA Centre for Ecological Research, Tihany 8237, Hungary
e-mail: pirger.zsolt@okologia.mta.hu

T. Kiss
Chemical Ecology and Neurobiology, Department of Experimental Zoology, Balaton Limnological Institute, MTA Centre for Ecological Research, Klebelsberg K. u. 3., Tihany 8237, Hungary

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Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was isolated first 26 years ago from ovine hypothalamic extract on the basis of stimulating cAMP formation in anterior pituitary cells [1, 2]. Thereafter, it was also discovered that PACAP has wide distribution not only in the hypothalamic nuclei, but also in the whole central (CNS) and peripheral nervous system (PNS) as well as in several peripheral organs. These include endocrine glands, gonads, respiratory and urogenital tracts, digestive system, skin, and muscles suggesting a broader function of PACAP than only the stimulation of pituitary gland [3–9]. PACAP has two biologically active isoforms, PACAP-38 [1] and PACAP-27 [2], which share the same N-terminal amino acids (AA) and are post-translational modifications of a single precursor, the preproPACAP [2, 10, 11]. In vertebrates the PACAP-38 form is the predominant isoform both in brain and peripheral tissues [4, 12, 13]. In contrast, in several invertebrate species the concentration of PACAP-27 is significantly higher, suggesting its pronounced role [4, 12, 14–18]. The discovery of PACAP was soon followed by identification of its receptors. Two types of receptors were characterized according to their relative affinities for PACAP: PAC1-R as well as VPAC1 and VPAC2 [4, 19]. PAC1-R is specific for PACAP and VPAC1, and VPAC2 receptors are activated by both PACAP and VIP molecules. PACAP receptors are members of G-protein-coupled receptor family and are unique in the sense that their complex genes are able to generate receptor splice variants, which have been reported for all three receptor types [20]. The possibility of genes being processed differently and thereby generating receptor splice variants could lead to alterations in pharmacology and signal transduction mechanisms [21]. Expression and distribution of PACAP and its receptors in the CNS and PNS of mammals have been described in detail [4]. Broad distribution of PACAP and its receptors is also observed in nonmammalian vertebrate species such as avians [22–24], reptiles [25, 26], amphibians [27–30], and fish [31–34]. Widespread occurrence and distribution of PACAP and its receptors suggest that the polypeptide exerts pleiotropic physiological functions [3, 4]. PACAP and its receptors are involved in numerous physiological functions, for example, as regulators of metabolism in the nervous, endocrine, cardiovascular, muscular, and the immune system. The physiological effects of PACAP in vertebrates are discussed by several excellent reviews [4, 35, 36]. Soon after the discovery of PACAP in vertebrates, the presence of PACAP-like peptide(s) is described in the fruit fly (*Drosophila melanogaster*) body wall neuromuscular junction and its possible role in neuronal plasticity as well as in the memory storage and retrieval [37, 38]. Thereafter, a number of studies have been published showing the presence and physiological role of PACAP or PACAP-like peptide(s) in several invertebrate species. In this review we summarize data obtained so far on the occurrence, distribution, and physiology of PACAP-like peptide(s) and its receptors in different invertebrates, such as oligochaetes, mollusks, insects, as well as malacostraca.

Expression and Localization of PACAP

Oligochaetes

The distribution of PACAP-like immunoreactivity (PACAP-IR) was studied in the CNS and PNS of three oligochaete (Annelida) worms with immunocytochemistry (IHC) [14]. Using PACAP-27 (n° 88121-5 antibody; [39, 40]) or PACAP-38 antibodies (anti-PACAP-38, Peninsula, CA, USA, [14, 39]) immunopositive cells and fibers were observed in cerebral, medial, and lateral parts of the subesophageal and ventral cord ganglia. In the peripheral nervous system, PACAP-IR was found in the enteric nervous system, epidermal sensory cells, and clitellum (reproductive organ). The distribution pattern of PACAP-IR was similar in all three species (*Lumbricus terrestris*, *Eisenia fetida*, *Lumbricus polyphemus*), suggesting a common distributional pattern of PACAP-like peptide(s) in oligochaete.

The levels of PACAP-27 (n° 88123-3 antibody) and PACAP-38 (n° 88111-3 antibody, [12]) isoforms were also measured in the nervous, intestinal, excretory, and reproductive systems of adult *Lumbricus* by RIA [15]. PACAP-27 and -38 isoforms were detected at significant amount in all of the examined tissues; however, their distribution was very heterogeneous. For example, the amount of PACAP-27 and PACAP-38 ranged between 0.31–17.12 and 0.02–1.51 ng/mg total protein, respectively. The level of PACAP-27-like immunoreactivity was approximately ten times higher than that of PACAP-38. In comparison, the highest PACAP-38 level in human is 4.7 ng/mg total protein in the bed nucleus of the stria terminalis [41]. These results suggest that, in contrast to vertebrates, the dominant isoform of the peptide is PACAP-27 in Oligochaeta.

PACAP immunopositive compounds were also observed in early stages (E1) of the embryonic development of the earthworm *Eisenia* using RIA (n° 88111-3 antibody), dot blot, and IHC (n° 9211-4 antibody, [42]) methods [17]. During embryonic development, the level of PACAP-like compounds decreased in cocoon fluids, while PACAP-IR cell bodies appeared in the developing body wall, prostomium, pharyngeal wall, and CNS. Furthermore, it was found that the clitellum of sexually mature worms contained significantly higher levels of PACAP-IR than other regions of the same animals or the clitellar region of a non-reproducing animal [17].

The presence and distribution of PAC1-R were described in the ventral nerve cord (VNC) of the adult *Eisenia* using IHC and commercial anti-PAC1-R [43]. Based on light and electron microscopic observations, the exact anatomical positions of labeled structures were established suggesting that PACAP mediates the activity of some interneurons, a few small motoneurons, and certain sensory fibers. High number of PAC1 receptors was found in both pre- and postsynaptic membranes in addition to extrasynaptic sites suggesting that PACAP acts as a neurotransmitter and neuromodulator in the earthworm nervous system. In early embryonic stages the first appearance of PAC1-R-like immunoreactivity was revealed by WB and Far WB methods as early as the E2 developmental stage. Immunolabeled CNS

neurons were seen in the supraesophageal ganglion and distally in the subesophageal and ventral nerve cord ganglia. Ultrastructurally, PAC1-Rs were located mainly on plasma membranes and intracellular membranes, especially on cisternae of the endoplasmic reticulum [43, 44]. The authors conclude that PACAP-like compounds may affect the differentiation of germinal layers (at least that of the ectoderm) and of some neurons and they act as signaling molecules during earthworm embryonic development. Further details on the occurrence and functions of PACAP in Annelida can be found in another chapter of this book (Chap. 3).

Gastropods

IHC, RIA, WB, and mass spectrometric (MS) analysis revealed the presence of both the 27 and 38 AA isoforms of PACAP-like IR elements in CNS, PNS, and peripheral organs of simple garden snail, *Helix pomatia* [9, 16] and pond snail, *Lymnaea stagnalis* [45]. PACAP-containing neurons were present in each ganglion of the CNS but their distribution pattern was not homogenous. The majority of PACAP-IR neurons were observed in the areas where different peptide-containing neurons are located [46]. PACAP-like IR was observed in non-neuronal cells of the salivary gland, perineurium of the cerebral ganglion located around the blood vessel lacunae, and the wall of anterior aorta suggesting that non-neuronal PACAPs enter the circulatory system and thus may have humoral functions [8, 16]. Using IHC, it was shown that PACAP was absent in the muscle itself, but present in nerve fibers innervating the columellar, foot, heart, and tentacle flexor muscles [9, 16].

The concentration of PACAP-27-like polypeptide was significantly higher than that of PACAP-38, in contrast mammals, where PACAP-38 is the dominant isoform. However, the data obtained in gastropods correspond well those obtained in an oligochaete species (*Lumbricus*, *Eisenia*). Interestingly, both IHC and RIA studies revealed different expression levels of PACAP in active and non-active (hibernated or aestivated states) suggesting a dependence on behavioral state of the animal.

In a WB study a 14 kDa protein band was detected by PACAP-27 (n° 92112-4 antibody) and PACAP-38 antibodies (n° 88111-3). In addition, the anti-PACAP-38 reacted with a protein at 4.5 kDa. It is speculated that data obtained by both antibodies at 14 kDa represents an extended PACAP-like molluscan peptide or the precursor form of PACAP. In human prostate and prostate cancer cells a 14.6 kDa product was described, which is likely a product of the prePACAP protein (19.9 kDa), partially processed by convertases [47]. The assumption that extended PACAP-like molecules may exist is not unique. For example, in lower vertebrates, such as the stingray and catfish, 44- and 64-AA-long PACAP molecules were observed [42, 48]. Using the MS/MS fragment ion calculator the molecular weight based on sequence can be predicted accordingly, and the average mass of protonated quasimolecular ion ($[M^+H]^+$) of stingray and catfish PACAP would be *m*/*z* 5338.25 (5.3 kDa) and *m*/*z* 7856.25 (7.8 kDa), respectively. Based on the MALDI TOF/TOF measurement similar sequences of PACAP-27 and PACAP-38 can be identified from hemolymph and CNS

samples of the snail with a molecular weight of 3147.1 and 4535.2, respectively. In addition, fragments of a PACAP-like molecule were found in *Helix* CNS homogenate with an identical AA sequence to mammalian PACAP-27 and -38 at positions 1–10 and 20–27 [16]. The AA sequence at 27–38 differs by only one AA (an iso-leucine to valin substitution) according to the mass calculation. Mass spectra of tryptic digest obtained by MALDI-TOF MS from *Lymnaea* CNS homogenate revealed complete sequence similarity of fragments between 1 and 32 AAs compared to mammalian PACAP-38 [45]. The average mass of $[M^+H]^+$ of synthetic mammalian PACAP-38 is *m*/*z* 4535.47 while in the pond snail, squid, planarian, and hydra the hypothetical average $[M^+H]^+$ of the PACAP-38-like molecule is *m*/*z* 4656.37. The reason for this difference could be the discrepancy in three AAs between synthetic mammalian PACAP-38 and isolated invertebrate PACAP-38-like molecule [49].

The PAC1-like receptor was also identified in the snail by IHC and biochemical methods [16, 45]. Similarly to vertebrate, PACAP activates a G-protein-coupled receptor and acts through the AC-cAMP pathway [45, 50]. In *Lymnaea* cerebral ganglia, the synthetic PACAP-38 incubation increased cAMP level by 82 %. In addition, both maxadilan, a specific PAC1-R agonist [51], and VIP, an agonist of VPAC1 and VPAC2 receptors also binding PACAP [19], increased cAMP synthesis by 47 % and 79 %, respectively. About 50 % of the cAMP-stimulating effect of PACAP-38 could be blocked by co-application of PACAP6-38 or maxadilan antagonist (M65). The biochemical results confirmed that PACAP-like peptides could increase cAMP levels through PACAP receptors in CNS or PNS. PAC1-like receptor expressing neuronal elements were observed in the CNS and a number of peripheral organs such as columellar muscle, heart, tentacles, and epithelial glandular cells. Far-WB experiments revealed three binding sites in snail brain homogenate. Two of these corresponded well to the VPAC1 (~45 kDa) and PAC1 (~60 kDa) receptors of vertebrates [16].

Insects

Using a vertebrate PACAP-38 antiserum (RIN-8920, Peninsula, CA, USA) PACAP-38-like IR was found in a subset of larval CNS neurons of *Drosophila* [37]. Furthermore, PACAP38-like IR was also found in nerve terminals innervating almost all muscle fibers in wild-type and NF1 mutant larvae [37, 52]. The staining appeared to be concentrated in varicosities where synaptic vesicles are localized. A comparison of the patterns of IR with previous anti-horseradish peroxidase staining, which reveals all nerve terminals arborized on muscle fibers, suggests that PACAP38-like IR is restricted to large-sized-type varicosities typical for neuropeptides.

In contrast to oligochaetes and gastropods, only the PACAP-38 isoform was present in the CNS of Insects [37]. In WB experiments an IR band was observed at 5.4 kDa that compared well with calculated mass of 4.5 kDa of mammalian PACAP-38. In addition a 19 kDa band was detected which may represent a possible PACAP precursor polypeptide in *Drosophila*. It is concluded that antibodies raised against mammalian PACAP38 identify an insect polypeptide with similar size [37].

In *Drosophila* a neuropeptide gene was identified that has some identity to PACAP [38]. This gene, named amnesiac, encodes a signal peptide followed by several possible peptides depending on the cleavage sites. One of the peptides deduced from the gene had 10 % identity with human PACAP-38 or 18 % with PACAP-27. This identity is too low to claim that amnesiac is homologous to PACAP in tunicates or vertebrates. However, the authors showed that an inserted space in PACAP after both amino acids 23 and 27 would increase the identity to 21 % for PACAP-38 and 30 % for PACAP-27. If amino acid similarity is used for the calculation, the relationship is higher [35].

Malacostraca

In some protostomes, such as the planarian (*Dugesia japonica*), the american cockroach (*Periplaneta americana*), and the bigfin reef squid (*Sepioteuthis lessoniana*) partial mRNAs corresponding to the highly conserved PACAP coding exon have been deposited in public databases [53]. Based on this public sequence information, Lugo and his coworkers [54] proposed a degenerative primer pair (F-LvPACAP and R-LvPACAP) and isolated for the first time the cDNA encoding the mature PACAP molecule from neural eyestalk tissue of a crustacean species, the white shrimp (*Litopenaeus vannamei*) by RT-PCR. Its high degree of sequence conservation is corroborated, when compared with sequences reported from tunicates (*Chelyosoma productum*) to mammalian vertebrates.

Functions of PACAP

The eukaryote *Tetrahymena thermophila* is a free-living ciliate protozoon widely used as an animal model in biological and biomedical research and exhibits a behavioral avoidance to PACAP-38. For example, the antagonists PACAP6-27 and 6-38, which inhibit PACAP receptors, serve as agonists for *Tetrahymena* [55]. The possibility cannot be excluded that PACAP is able to exert its action by directly activating the AC-cAMP pathways penetrating the cell membrane.

The high structural conservation and interphyletic distribution of a PACAP-like peptide and its receptor molecules suggest that this peptide is involved in the regulation of several basic physiological functions in invertebrates similar to those observed in vertebrates.

Effect in Regeneration

PACAP is involved in an array of physiological functions; thus, the role of the peptide is thought to be essential for cell survival. This is supported by the observation that the mortality of PACAP or PACAP receptor knockout mice is much higher than their

wild-type mates [4, 19, 35, 56, 57]. Studies in PACAP knockout animals provide further evidence for the involvement of endogenous PACAP in regeneration processes. Upregulation of PACAP following nervous injuries has been shown in vertebrates by numerous previous studies [58]. It has been shown by RIA and IHC methods that the concentration of PACAP-like compounds increases in regenerating CNS and peripheral tissues of the earthworm *Eisenia* following injury indicating the possible role of PACAP in the regeneration [40]. Significant increase in the concentration of PACAP-like compounds was also observed in coelomocytes of regenerating earthworm [59]. Electron microscopic immunocytochemistry showed that PAC1 receptors are located on coelomocytes (mainly on amebocytes and on some granulocytes). Authors hypothesize a link between PACAP and coelomocytes, suggesting that PACAP modulates the function of amebocytes and certain granulocytes that play a role in regenerating earthworms. The data show that PACAP-like peptide(s) accumulate in the regenerating tissues of the earthworm suggesting trophic functions of these compounds in invertebrate tissues similarly to those observed on vertebrates.

Anti-apoptotic Effect

The anti-apoptotic effect of PACAP on vertebrate neuronal and non-neuronal cells is well documented [6, 7, 60, 61]. The anti-apoptotic effect of PACAP is mainly mediated by PAC1 receptor. The results imply that the anti-apoptotic effect of PACAP may be one of the basic functions of the peptide through evolution; both the peptide structure and this function have been conserved. PACAP has anti-apoptotic effect in the salivary gland cells of the snail [8]. In several gastropod species saliva or mucus release is performed by the holocrine release mechanism leading to cell destruction [62]. It has been suggested that cell death is indeed the physiological method of saliva release which takes place through a form of programmed cell death that is regulated by transmitters. It has been observed that stimulation of the salivary nerve or external application of dopamine elicits a change of mitochondrial membrane potential, and translocation of cytochrome-c from mitochondria to the cytoplasm is typical for the intrinsic mitochondrial pathway of programmed cell death. It has been observed that PACAP significantly attenuates the dopamine- and colchicine-induced apoptosis [8, 63].

Effect on Ion Channels

In snail (*Helix*) neurons expressing PAC1-like receptors synthetic PACAP-27 and -38 elicited membrane potential changes (both hyper- and depolarization) leading to significant changes in action potential frequency. PACAP6-38, as a receptor antagonist, powerfully antagonized the membrane effect of PACAP [16]. These results may suggest that PACAP is able to modulate the ion channels responsible for membrane and action potential generation. PACAP-like peptide has been found to

modulate ionic conductance at the neuromuscular junction [37, 64]: in *Drosophila* larval muscles synthetic PACAP-38 enhanced L-type Ca^{2+}-current via AC-cAMP-PKA pathway [64]. Focal application of vertebrate PACAP-38 to the neuromuscular junction of *Drosophila* larval muscle triggered two temporally distinct responses: an immediate depolarization and a large enhancement of K-current. The enhancement occurred 12–14 min after the early depolarization. The effect of external PACAP-38 could be mimicked by high-frequency stimulation of motor nerve suggesting that PACAP or PACAP-like peptide is co-released and is functionally present in nerve terminals [37]. In the tentacle flexor muscles of the land snail, *Helix*, a potentiating effect of synthetic PACAP-27 was observed on cholinergic neuromuscular transmission. PACAP-27 presynaptically enhanced the release of acetylcholine by activating the AC-cAMP-PKA pathway. Postsynaptically, PACAP-27 enhanced muscle contractility by PKC-mediated signaling pathway resulting in an increased Ca^{2+} release from intracellular stores. These findings suggest that regulation of Ca^{2+} release may contribute to the stimulatory effect of PACAP [9].

Role of PACAP in Learning and Memory

PACAP activates molecular cascades leading to the execution of many physiological processes, including learning and memory [50, 65]. Feany and Quinn [38], cloning the memory gene in *Drosophila* responsible for the amnesiac mutation, observed that one of the "amnesiac" potential neuropeptides (AMN) had homology to the gene that encodes mammalian PACAP. The amn gene encodes a homolog of vertebrate PACAP, the AMN, strongly expressed in dorsal paired medial (DPM) neurons which is required for stable memory [66]. Furthermore, DMP activity is needed for middle-term memory, so suggesting that the PACAP-like AMN peptide release from the DPM neurons contributes to memory persistence [11, 66, 67].

The *Lymnaea* homologue of PACAP was found necessary for the acquisition and consolidation of long-term memory in the snail. We showed that systemic application of synthetic PACAP-27 or -38 accelerated the formation of transcription-dependent memory during single-trial reward chemical or multiple aversive tactile conditioning in *Lymnaea*. Using the antagonist PACAP6-38 it was also shown that the memory-accelerating effect of PACAP depended on G-protein-coupled PAC1-like receptors [50].

The age-related decline in memory performance could be reversed by administration of PACAP. Exogenous PACAP-38 boosted memory formation in aged *Lymnaea*, where endogenous PACAP-38 levels were significantly lower than in young snail based on WB experiments. In aged *Lymnaea*, there was a significant deficit of both intermediate- and long-term memory formation after one-trial reward conditioning. The deficit in both of these types of memory, however, was rescued by the application of the synthetic PACAP-38 peptide before training. Due to the evolutionarily conserved nature of these polypeptides and their established role in memory and synaptic plasticity, there was a very high probability that they could also act as "memory-rejuvenating" agents [68].

The role of PACAP and/or PACAP-like peptide(s) in acquisition and memory consolidation and recall in invertebrate model animals is discussed in detail in another chapter (Chap. 4).

Summary

The primary structure of PACAP has proved to be remarkably conserved during evolution not only in higher and lower vertebrates but also in invertebrates. In Table 2.1 different sequences of invertebrate PACAP molecules are aligned with human PACAP using ClustalW2—multiple sequence alignment. Detailed analysis revealed a high homology (>89 %) of inferred amino acid sequences: 35 AAs are conserved at the N-terminus and 3 AAs are variable at the C-terminus. Unfortunately, there is currently no definite sequence information about invertebrate PACAP or PACAP-like molecule(s) [3, 4, 69, 70]. Therefore, in IHC experiments different types of vertebrate antibodies are used which raises questions about the authenticity of the reported data even though antibodies with different epitopes produce the same effect. In WB experiments applying the same vertebrate antibodies the masses of immunopositive WB bands differ from those of expected. On the contrary, in most of the physiological experiments powerful and clear effect of the externally applied PACAP is observed suggesting the presence of specific receptor able to recognize synthetic PACAP. Although partial cDNA encoding PACAP-like peptide(s) in protostomes has been reported, no cDNA or gene encoding a PACAP-like peptide has been identified so far in species with fully/partially sequenced genomes [19, 53]. However, a highly conserved partial sequence corresponding to the exon encoding the mature PACAP peptide has been isolated in *Hydra magnipapillata*, in the tunicate, *Halocynthia roretzi* and in several protostomes such as

Table 2.1 Sequence comparison between in silico AA sequence of invertebrate PACAP peptides and human PACAP

		Identity (%)
Q8IU39_DUGJA	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNK	92
Q75W94_HALRO	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNE	89
Q8IU38_HYDMA	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNK	92
Q75W88_EUCA	HSDGIFTDSYSRYREQMAVKKYLAAVLGKRYRQRYRNK	89
Q8IU37_SEPLE	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNK	92
Q8IU36_PERAM	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRSK	89
PACAP_HUMAN	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK	100
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DUGJA—*Dugesia japonica* (AB083649), HALRO—*Halocynthia roretzi* (AB121759), HYDMA—*Hydra magnipapillata* (AB083650), EUCA—*Eriocheir japonica* (AB121765), SEPLE—*Sepioteuthis lessoniana* (AB083651), PERAM—*Periplaneta americana* (AB083652)

*—amino acid identity; :—replaceable amino acid

planarian (*Dugesia japonica*), crab (*Eriocheir japonica*), squid (*Sepioteuthis lessoniana*), and cockroach (*Periplaneta americana*).

In conclusion, PACAP or PACAP-like peptide(s) are present in invertebrates but the existence of a PACAP gene or peptide homologue remains to be convincingly demonstrated. However, the reported physiological effects of PACAP further confirm the presence of PACAP(-like) signaling pathways in invertebrates.

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