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MULTIFUNCTIONAL ROLE OF PACAP-LIKE PEPTIDES IN MOLLUSCS

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Abstract. The purpose of this review is to highlight the role of pituitary adenylate cyclase-activating polypeptide (PACAP) in a range of physiological and behavioural processes of gastropod molluscs, *Helix* and *Lymnaea*. Since its discovery in 1989 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-two years after its discovery, PACAP is now one of the most extensively studied of the neuropeptides. This review surveys the importance of PACAP and PACAP-like peptides in invertebrates, focusing mainly on the gastropod molluscs. The relevance of studies on lower vertebrates and invertebrates, which do not have a pituitary gland, is to contribute to the unraveling of fundamental effects of PACAP or PACAP-like peptides and to provide a comparative view.

Pituitary adenylate cyclase-activating polypeptide

Pituitary adenylate cyclase-activating polypeptide (PACAP) is the member of the growth hormone releasing factor (GRF) superfamily [1]. PACAP was first isolated 22 years ago from ovine hypothalamic extract, which had been found to stimulate cAMP formation in anterior pituitary cells [2, 3]. PACAP shows a remarkable amino acid (AA) sequence similarity at the N-terminal domain across higher and lower vertebrate species (Table **1**). Particularly, the first 1-27 AAs of the peptides N-terminus have been completely conserved in all vertebrate species investigated until now, except chicken, stargazer and sturgeon, which have one AA substitution at different positions. The sequence similarity is not limited to the peptide sequence as the nucleotide sequence similarity is 96% between the human and tunicate PACAP-27 cDNAs (Table **2**). Such a high degree of sequence homology represents conservation over 700 million years of evolution (the estimated time when the stem line

leading to vertebrates separated from Urochordata) [1, 4] and indicates that PACAP fulfills important biological functions.

Table **1**. Sequence comparison of vertebrate PACAP peptides

	* . ******. ** *** : * * ** ** * ** * ** * : * * : . *
human	**HSDGIFTD**S**YSRYR**K**QMA**VK**KYLA**A**VLG**K**RY**KQ**RV**K**NK**
chicken	- I - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
lizard	- - - -- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . . .
frog	- - - - - - - - - - - - - - - - - - - - - - - -- - - - - - - - - - I - - -
zebrafish	- - - - - - - - I - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
salmon	- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - R - - YRS -
catfish	- - - - - - - - - - - - - - - - - - - - - - - - - - - - R -- R - -FR - - GRRLVVPSVWGIRDWVIWPEKRGKRY
stingray	- - - - - - - - - - - - - - - - - - - -Q - - - - - - - - R – R - - - R - -
sturgeon	- G
tunicate I	- - - - - - -- - - - - - - N - - - - - - - - - - - - - - - - -
tunicateII	HSDGIFTDSYSRYRNQMAVKKYINALLGKRYQ

*, amino acid identity, :, replaceable amino acids

The discovery of PACAP was soon followed by identification of its receptors in different vertebrate species. It is believed that peptide-receptor pairs would have had a better chance of survival through evolution if duplication of the ligand and its receptor occurred simultaneously. Generally, neuropeptides and their cognate receptors are encoded by different genes but their co-evolution is strongly implied in order to maintain the system functioning [5]. The absence of PACAP or PACAP-like receptors in tunicates however raises an intriguing question about agonist-receptor co-evolution and function in Deuterostomia. The PACAP and vasoactive intestinal peptide (VIP, another member of the GRF/secretin/glucagon superfamily) receptors are suggested to have arisen only in the vertebrate lineage, probably via genome duplication [4]. Two types of receptors have been characterized according to their relative affinities for PACAP and VIP: PAC1-R and VPAC1 and VPAC2, respectively [4, 6]. These receptors are members of the 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 [7]. The PAC1-R is specific for PACAP and the VPAC1 and VPAC2 receptors are activated by both PACAP and VIP. In vertebrates PACAP and its receptors are widely distributed not only in the hypothalamic nuclei, but in the whole central (CNS) and peripheral nervous system (PNS) and several organs (eye, different glands, gonads, placenta, uterus, respiratory and urogenital tracts, digestive system, skin, and muscles) suggesting a broader function to PACAP than the stimulation of the pituitary gland [6, 8, 9, 10, 11]. The physiological effect assigned to the peptide is its ability to stimulate the activity of adenylate cyclase (AC). Recently however, owing to the involvement of PACAP in an array of physiological functions, the role of the peptide is thought to be essential for cell survival. This statement is supported by the observation that the majority of PACAP or PACAP receptor knockout animals die [1, 4, 11, 12]. 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 [13]. Recently, it has been shown that the concentration of PACAP-like compounds increase in regenerating tissues of the earthworm following injury indicating the possible role of PACAP in the regeneration [14].

Table **2**. mRNA comparison of invertebrate PACAP

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AB083650|Hydra              CATTCGGATGGGATCTTCACAGATAGCTACAGCCGCTACCGAAAGCAAAT 50
AB121759|Halocynthia        CATTCGGATGGGATCTTCACAGATAGCTACAGCCGCTACCGAAAGCAAAT 50
AB083651|Sepioteuthis       CATTCGGATGGGATCTTCACAGATAGCTACAGCCGCTACCGAAAGCAAAT 50
AB121765|Eriocheir          CATTCGGATGGGATCTTCACAGATAGCTACAGCCGCTACCGAGAGCAAAT 50
AB083649|Dugesia            CATTCGGATGGGATCTTCACAGATAGCTACAGCCGCTACCGAAAGCAAAT 50
AB083652|Periplaneta        CACTCGGATGGGATCTTCACAGACAGCTACAGCCGCTACCGAAAGCAAAT 50
                            ** ******************* ****************** *******

            GGCAGTCAAGAAATACCTGGCGGCAGTCCTTGGGAAAAGGTATAGACAGAGATATAGAAACAAA 114
            GGCAGTCAAGAAATACCTGGCGGCAGTCCTTGGGAAAAGGTATAGACAGAGATATAGAAACGAA 114
            GGCAGTCAAGAAATACCTGGCGGCAGTCCTTGGGAAAAGGTATAGACAGAGATATAGAAACAAA 114
            GGCAGTCAAGAAATACCTGGCGGCAGTCCTTGGGAAAAGGTATAGACAGAGATATAGAAACAAA 114
            GGCAGTCAAGAAATACCTGGCGGCAGTCCTTGGGAAAAGGTATAGACAGAGATATAGAAACAAA 114
            GGCAGTCAAGAAATACCTGGCGGCAGTCCTTGGGAAAAGGTATAGACAGAGATATAGAAGCAAA 114
            ************************************************************ * **
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***** – identity

GRF-superfamily: VIP, PACAP and related peptides

Upon closer examination, peptides with similar structures are frequently observed and can be derived from common or different precursors. Sets of peptides with similar AA

layout are identified and classified into families based on conserved sequence homologies, where several members may occur in one species or may extend both inter- or intraphyletically [15, 16]. Identification of peptide families and their members offers an insight into phylogenetic relationships and the evolution of neuropeptides and their precursor molecules [12]. The phylogenetic tree shows (Fig.1.) that the variability between biologically active PACAP peptides is less that between PACAP and VIP peptide families.

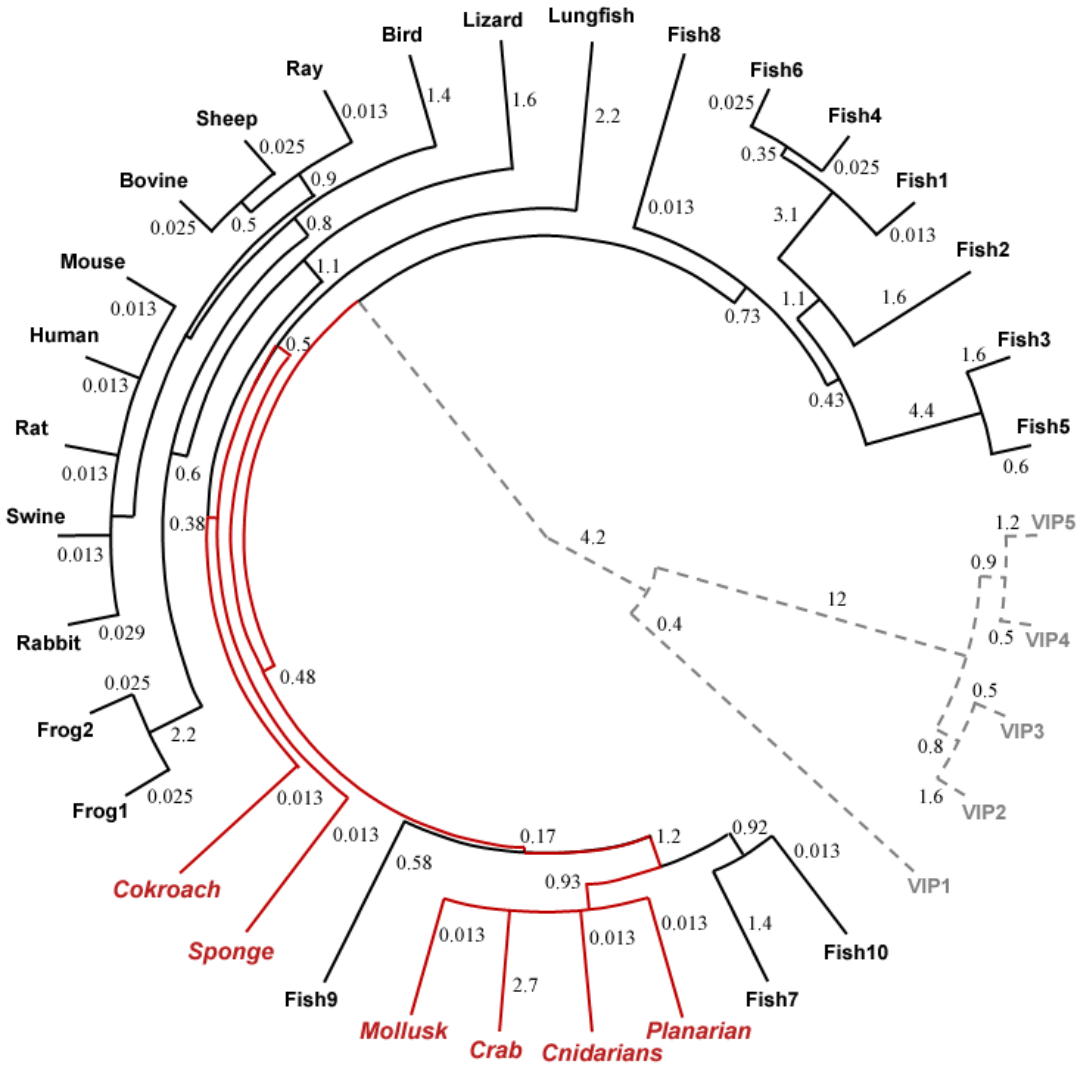

Fig.1. – Distance phylogenetic tree from protein sequences of PACAP. For analyzes the specific "phyligenetical tree option" of program of Computational Biochemistry Research Group (ETH, Zurich, http://www.cbrg.ethz.ch/services/index) was used. Evolutionary distances (numbers) were calculated based on 38, 44 or 65 AA sequences

of PACAP from different vertebrate and invertebrate animals. Sequences were obtained from NCBI and ExPASy database: cnidarians – *Hydra vulgaris* [Q8IU38]; sponge – *Halocynthia roretzi* [Q75W94]; planarian - *Dugesia japonica* [Q8IU39]; mollusk - *Sepioteuthis lessoniana* [Q8IU37]; cockroach - *Periplaneta americana* [Q8IU36]; crab - *Eriocheir japonica* [Q75W88]; lizard - *Podarcis siculus* [ABD77494]; frog1 - *Xenopus laevis* [AAD56956]; frog2 - *Rana ridibunda* [AAB20402]; ray - *Torpedo marmorata* [ADP00547]; lungfish - *Protopterus dolloi* [ACI25366]; fish1 - *Uranoscopus japonicus* [P81039]; fish2 - *Clarias macrochephalus* [CAA55684]; fish3 - *Ctenopharyngodon idella* [ABQ81649]; fish4 - *Haplochromis burtoni* [ACB29679]; fish5 - *Danio rerio* [AAG59830]; fish6 - *Cynoglossus semilaevis* [ACM43290]; fish7 - *Oncorhynchus mykiss* [AAK28558]; fish8 - *Mola mola* [AAV85450]; fish9 - Acipenser schrenckii [BAC21154]; fish10 - *Trachurus japonicus* [BAC21153]; bird - *Gallus gallus* [AAX56089]; sheep - *Ovis aries* [AAB21469]; bovine - *Bos taurus* [AAY16443]; rabbit - *Oryctolagus cuniculus* [XP_002713481]; swine - *Sus scrofa* [NP_001001544]; rat - Rattus norvegicus [AAA41791]; mouse – *Mus musculus* [NP_033755]; human – *Homo sapiens* [AAB21470]; VIP1 - *Oncorhynchus mykiss* [AAB34607]; VIP2 – *Homo sapiens* [CAI21764]; VIP3 – *Bos taurus* [DAA26008]; VIP4 – *Rattus norvegicus* [EDL92841]; VIP5 – *Mus musculus* [AAH89511]. Asterisks indicate the invertebrate animals (bold italic). Dash grey lines indicate the member of VIP peptide family.

The high degree of homology in some examples strongly suggests that the precursors were derived from a common ancestral gene. The GRF superfamily provides a good example of how gene and exon duplication with tandem insertion led to the evolution of a family of related peptides [17]. The phylogenetic distribution of each peptide is thought to be generally restricted within the Protostomia and Deuterostomia groups, because neuropeptides have changed with phylogenetic evolution [18, 19, 20]. However, many peptides are widely distributed among several phyla or even between Protostomia and Deuterostomia. Examples of this kind of interphyletic distribution are members of the oxytocin/vasopressin, tachykinin/substance P, opioid peptides and VIP/PACAP families. Members of the GRF superfamily such as VIP, peptide histidine methionine (PHM), halospectin (HS), halodermin (HD), and glucagons have also been described in the CNS and periphery of *Helix* and *Lymnaea* species.

The cell specific distribution and seasonal variations of these peptides imply that they may also act as transmitters, modulators and hormones in the nervous and sensory

system of gastropods. The presence of PAC1, VPAC1 and VPAC2 receptors has not been demonstrated earlier in gastropods due to the high specificity of monoclonal antibodies used in these experiments [21, 22, 23].

Expression and localization of PACAP and its receptors in molluscs

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 **3** different sequences of invertebrate PACAP molecules are aligned with human PACAP using ClustalW2 - Multiple Sequence Alignment (http://www.ebi.ac.uk/tool/msa/clustalw2). 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. It has been found that the N-terminus plays a crucial role in the biological activity of the peptide. Site-directed mutagenesis has revealed that the N-terminus is essential for receptor activation but is not involved in the recognition of the receptor-binding site, which instead seems to involve

Table **3**. - Sequence comparison of invertebrate PACAP peptides with human PACAP

Identity (%)

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Q8IU39_DUGJA      HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNK
Q75W94_HALRO      HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNE
Q8IU38_HYDMA      HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNK
Q75W88_EUCA       HSDGIFTDSYSRYREQMAVKKYLAAVLGKRYRQRYRNK
Q8IU37_SEPLE      HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNK
Q8IU36_PERAM      HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRSK
PACAP_HUMAN       HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK
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***-** amino acid identity; **:** - replaceable amino acid

AAs in the C-terminal domain [6, 24, 25]. The occurrence, localization and distribution of PACAP-like peptide and its receptor was recently described in the CNS and peripheral organs of gastropods, *Helix* and *Lymnaea*. Some nerve fibers in the neuropil and peripheral nerves but not the soma of neurons in the CNS were immunostained with PACAP AB [21]. Mass spectrometric analysis, radio-immune assay (RIA), western-blot (WB) and immunohistochemistry revealed the presence of both the 27 and 38 forms of PACAP [26, 27]. The concentration of PACAP27 is significantly higher than PACAP38 in the snail, which contrasts to data obtained on mammals, where PACAP38 is the dominant form. However, the

data obtained in gastropods corresponds well with those obtained in an oligocheta species (*Lumbricus polyphemus, Eisenia fetida)*, where the nervous system contains about ten-fold higher concentration of PACAP27 than PACAP38 [11, 28, 29, 30, 31]. PACAP-like immunoreactivity is observed at very early stages of the embryonic development. It has been also found that the clitellum of sexually mature worms contains significantly higher levels of PACAP-like immunoreactivity than other regions of the same animals or the clitellum of a non-reproducing animal. The observations suggest a role of PACAP or PACAP-like peptides in the reproduction and development of invertebrates. Conversely, only the PACAP38 form is present in the CNS of insects [32]. Unfortunately there is currently no sequence information about molluscan PACAP or PACAP-like molecules. It is speculated, that a 14 kDa protein band, detected by PACAP27 and PACAP38 antibodies using WB in molluscs represents an extended PACAP-like molluscan peptide of larger molecular weight than the vertebrate 4-6 kDa PACAP molecules. In human prostate and prostate cancer cells however, a 14.6 kDa product is described, which likely to be a product of the prePACAP protein (19.9 kDa) that has been partially processed by convertases [33]. 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 are observed [34, 35]. Using the MS/MS Fragment Ion Calculator the molecular weight based on sequence could be predicted. 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 to PACAP27 and PACAP38 can clearly be identified from hemolymph and brain samples of the snail with a molecular weight signal of 3147.1 and 4535.2, respectively. Fragments of a PACAP-like molecule are found in *Helix* brain homogenate with an identical AA sequence to mammalian PACAP27 and 38 at positions 1-10 and 20-27. 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 from *Lymnaea* brain homogenate revealed complete sequence similarity of fragments between 1-32 AAs [26]. These data confirm the conclusion that PACAP is the most conserved member of the GRF peptide superfamily [6]. Interestingly, the identity of the *Drosophila* amnesiac gene product with human PACAP38 and PACAP27 is only 10% and 18%, which is too low to accept as homologue of PACAP in vertebrates. The existence of the PACAP and PACAP receptor gene in invertebrates remains to be demonstrated. The average mass of protonated quasimolecular ion ($[M+H]^+$) of synthetic mammalian PACAP38 is m/z 4535.47 while in the pond snail, squid, planarian and hydra the hypothetical

average $[M+H]^+$ of the PACAP38-like molecule is m/z 4656.37 (MS/MS Fragment Ion Calculator). The reason for this difference could be the three AA differences between synthetic mammalian PACAP38 and invertebrate PACAP38-like molecule.
The presence of PACAP receptor in invertebrates has only been demonstrated by immunological methods so far, and no direct evidence is available for the occurrence of this extremely conserved molecule. In snails, similar to vertebrate and other invertebrate species, the PACAP acts through a G protein-protein coupled receptor and activate the AC-cAMP pathway[26] . The PAC1-like receptor has been identified in the snail by immunohistochemistry and biochemical methods[26, 27]. PAC1-like receptor expressing neuronal elements have been observed in the CNS and a number of peripheral organs such as columellar muscle, heart, tentacles and epithelial glandular cells. Far western blot 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. The observation supports the notion that only one type of VIP receptor existed earlier in evolution [1]. In addition the findings favor the presence of specific PACAP receptors, the PAC1-like and VPAC1-like receptors in the snail, which however should be isolated and sequenced.

Functions of PACAP in molluscs

Neuropeptides have different activities that are dependent on the target tissue, developmental stage or interactions with other modulators. In vertebrates PACAP and its receptors are widely distributed in different tissues and cells. They are also involved in numerous physiological functions and regulator of metabolism in the nervous, endocrine, cardiovascular, and muscular and the immune system [1, 4]. The wide distribution of PACAP and its receptors suggests that the peptide may exert pleiotropic physiological functions [6]. Several good reviews discuss the physiologic effects of PACAP in vertebrates [1, 11, 36, 37]. In contrast in this review we would like to focus on the data obtained so far on molluscs. The high structural conservatism and interphyletic distribution of PACAP and its receptor molecules suggest that this peptide could also be involved in the regulation of several basic physiological functions in snails similar to those observed in vertebrates.

Antiapoptotic effect

Recently it was shown for the first time, that PACAP has an anti-apoptotic effect in the salivary gland cells of the snail [38]. In several gastropod species saliva or mucus release

is performed by the holocrine release mechanism leading to cell destruction [39, 40]. 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. Indeed, 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 typical for the intrinsic mitochondrial pathway of programmed cell death [41, 42]. It has been observed that PACAP significantly attenuates the dopamine- and colchicine–induced apoptosis. The anti-apoptotic effect of PACAP on vertebrate neuronal and non-neuronal cells is well documented [8, 9, 43, 44]. The protective effects of PACAP are based on the capacity of the peptide to prevent apoptosis by acting directly on caspases and Bax or indirectly through the release of cell-protective factors by astrocytes. The anti-apoptotic effect of PACAP is mediated by PAC1 receptor [44]. These 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.

Possible role in hibernation

In active snails the level of PACAP is fourfold higher compared to the brain of hibernating snails, as revealed by immunohistochemistry and radioimmunoassay analysis [27]. Terrestrial snails possess a variety of behavioural, physiological and biochemical adaptation strategies to overcome unfavourable environmental conditions [45]. The overall depression of metabolic rate is a general strategy for animals during hibernation. Evidence showing PACAP is a metabolic regulator makes it an ideal candidate for a role in hibernation. Since PACAP is considered to be a metabolic regulator its participation in hibernation is suggested. Whether the observed decrease in PACAP is a consequence of the overall hypometabolism typical for hibernating animals, or is indicative of a decisive role in regulating the hibernated state is still an unanswered question [46]. Seasonal changes in the other members of the GRF superfamily have also been observed. For example hibernating snails (*Helix*) contain higher numbers of VIP immunoreactive neurons than active animals [21]. It is speculated that peptides present at higher expression levels or maintained exclusively in a particular state might contribute of a physiological fate which may be to either attenuate [46, 47]. However, expression level alone is not necessarily an indicator of any given peptide's immediate

participation in establishing the physiology. It could be simply be a reduced metabolic rate and increased intensity as a consequence of a decreased release.

Effect on ion channel function

In snail neurons expressing PAC1-like receptors PACAP27 and PACAP38 elicit membrane potential changes (both hyperpolarisation and depolarisation) leading to changes in action potential frequency. PACAP6-38, a specific PACAP-R antagonist, powerfully antagonizes the membrane effect of PACAP [27]. These results may suggest that PACAP is directly able to modulate the ion channels responsible for membrane and action potential generation. Indeed, in insect larval muscles PACAP enhances K^+-current and modulates L-type Ca^{2+}-current via cAMP-PKA pathway [32, 48]. PACAP-like peptide has been identified in the insect *Drosophila melanogaster* [49], and this peptide has been found to modulate ionic conductance at the neuromuscular junction[32] . In human pituitary adenoma cells PACAP can activate voltage dependent tetrodotoxin sensitive Na^+-channels via the adenylate cyclase protein kinase A pathway [50]. The effect is antagonized by PACAP6-38 showing that the effect of the peptide is mediated by its specific receptor. In mouse olfactory epithelia PACAP reduces the expression of Kv1.4 and Kv4.1 channel subunits underlying A-type current. PACAP induced reduction of A-type K^+-channels is completely blocked by a phospholipase C pathway antagonist, however the channel is still dowregulated by PACAP when the cAMP pathway is inhibited [51]. The results available to date suggest that PACAP changes ion channel activity directly or via a down regulation of mRNA expression.

Effect on secretion

In vertebrates, the salivary gland is innervated by PACAP containing neurons of the parasympathetic ganglia [1]. In order to study the stimulus-secretion mechanism, the salivary gland in snails is highly amenable [52] but surprisingly, PACAP immunoreactive nerve fibres are not detected in the snail; PACAP immunopositivity is localized exclusively to certain types of gland cells. In addition, PACAP or PACAP-like receptors have not been found in snail salivary gland with the antibody used in experiments to date [38]. Despite it is observed that PACAP increases the cAMP concentration in the homogenate of snail salivary glands and it protects cells from apoptosis: however its exact role in secretion remains largely unknown.

The role of PACAP in learning and memory

The molluscan homologue of PACAP is found to be necessary for the aquisition and consolidation of long-term memory in the snail. It has been demonstrated, that systemic application of exogenous PACAP accelerates the formation of transcription-dependent memory during single trial reward chemical or multiple aversive tactile conditioning in *Lymnaea*. Using the PACAP6-38 antagonist it has also been shown that the memory accelerating effect of PACAP is dependent on G-protein coupled PAC1-like receptors [53].These observations are not altogether surprising because previous behavioral studies on vertebrates have found that PACAP is necessary but not sufficient for memory formation and consolidation [54, 55, 56].

Conclusion:

The presence of PACAP-like sequences in of molluscs establishes the origin of the PACAP/glucagon superfamily in invertebrates. The protochordates are the major group from which the vertebrates are thought to have arisen. In tunicates a cDNA has been identified that encodes the PACAP-27 but not the extended PACAP-38 [1]. Based on these observations it is suggested that PACAP27 could have been the first molecular form to evolve [1]. Although the existence of PACAP receptors have not been identified and sequenced in invertebrate animals their existence is highly suggested. Considering the data obtained in invertebrates an earlier appearance or parallel evolution of the PACAP38 molecule could be implicated. The Eukaryota *Tetrahymena* is a free-living ciliate protozoa widely used as an animal model in biological and biomedical research and exhibits a behavioural avoidance to PACAP-38. PACAP has been observed to act through a common AC activating pertussis sensitive G-protein receptor. However, the pharmacological profile of the receptor is different from known PACAP-R in other systems. For example, the antagonists PACAP 6-27 and 6-38, which competitively inhibit many PACAP receptors actually serve as agonists for *Tetrahymena* [57]. PACAP-like peptides are also reported in cnidarians and other Protostomia, their function has been demonstrated but identification of the receptor is missing [58]. The possibility cannot be excluded that PACAP is able to exert its action by directly activating the AC penetrating the cell membrane. Certainly, more studies on invertebrates, regarding both the molecular structure and function of peptides and receptors will largely provide an important contribution to establishing the evolutionary origin of PACAP.

References

[1] Sherwood, N. M.; Krueckl, S. L.; McRory, J. E., The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. Endocr Rev 2000**,** 21 (6), 619-670.
[2] Miyata, A.; Arimura, A.; Dahl, R. R.; Minamino, N.; Uehara, A.; Jiang, L.; Culler, M. D.; Coy, D. H., Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun 1989**,** 164 (1), 567-574.
[3] Miyata, A.; Jiang, L.; Dahl, R. D.; Kitada, C.; Kubo, K.; Fujino, M.; Minamino, N.; Arimura, A., Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). Biochem Biophys Res Commun 1990**,** 170 (2), 643-648.
[4] Cardoso, J. C.; Vieira, F. A.; Gomes, A. S.; Power, D. M., PACAP, VIP and their receptors in the metazoa: Insights about the origin and evolution of the ligand-receptor pair. Peptides 2007**,** 28 (9), 1902-1919.
[5] Stefano, G. B., Stereospecificity as a determining force stabilizing families of signal molecules within the context of evolution. in Comparative aspects of neuropeptide function, Eds. E. Florey, G.B. Stefano, Manchester University Press 1991; Vol. 11, 14-28.
[6] Vaudry, D.; Gonzalez, B. J.; Basille, M.; Yon, L.; Fournier, A.; Vaudry, H., Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacol Rev 2000**,** 52 (2), 269-324.
[7] Dickson, L.; Fynlayson, K., VPAC and PAC receptors: From ligands to function. Pharmac Therap 2009**,** 121, 294-316.
[8] Shioda, S.; Ohtaki, H.; Nakamachi, T.; Dohi, K.; Watanabe, J.; Nakajo, S.; Arata, S.; Kitamura, S.; Okuda, H.; Takenoya, F.; Kitamura, Y., Pleiotropic functions of PACAP in the CNS: neuroprotection and neurodevelopment. Ann N Y Acad Sci 2006**,** 1070, 550-560.
[9] Somogyvari-Vigh, A.; Reglodi, D., Pituitary adenylate cyclase activating polypeptide: a potential neuroprotective peptide. Curr Pharm Res 2004**,** 10 (23), 2861-2889.
[10] Zhou, C. J.; Shioda, S.; Yada, T.; Inagaki, N.; Pleasure, S. J.; Kikuyama, S., PACAP and its receptors exert pleiotropic effects in the nervous system by activating multiple signaling pathways. Curr Protein Pept Sci 2002**,** 3 (4), 423-439.
[11] Vaudry, D.; Falluel-Morel, A.; Bourgault, S.; Basille, M.; Burel, D.; Wurtz, O.; Fournier, A.; Chow, B. K.; Hashimoto, H.; Galas, L.; Vaudry, H., Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. Pharmacol Rev 2009**,** 61 (3), 283-357.
[12] Hoyle, C. H. V., Neuropeptide families: evolutionary perspectives. Regul Pept 1998**,** 73 (1), 1-33.
[13] Waschek, J. A., Multiple actions of pituitary adenylyl cyclase activating peptide in nervous system development and regeneration. Dev Neurosci 2002**,** 24, 14-23.
[14] Varhalmi, E.; Somogyi, I.; Kiszler, G.; Nemeth, J.; Reglodi, D.; Lubics, A.; Kiss, P.; Tamas, A.; Pollak, E.; Molnar, L., Expression of PACAP-like compounds during the caudal regeneration of the earthworm Eisenia fetida. J Mol Neurosci 2008**,** 36 (1-3), 166-174.
[15] Kiss, T.; Pirger, Z., Neuropeptides as modulators and hormones in terrestrial snails: Their occurence, distribution and physiological significance. in: Invertebrate neuropeptides and hormones:Basic knowledge and recent advances. Ed. H. Satake, Transworld Research Network: Kerala, India, 2006; p 75-110.
[16] Holmgren, S.; Jensen, J., Evolution of vertebrate neuropeptides. Brain Res Bull 2001**,** 55 (6), 723-735.
[17] Hoyle, C. H. V., Evolution of neuronal signalling: Transmitters and receptors. Autonomic Neuroscience:Basic and Clinical 2010, 165, 28-53.

[18] Muneoka, Y.; Morishita, F.; Furukawa, Y.; Matsushima, O.; Kobayashi, M.; Ohtani, M.; Takahashi, T.; Iwakoshi, E.; Fujisawa, Y.; Minakata, H., Comparative aspects of invertebrate neuropeptides. Acta Biol Hung 2000**,** 51 (2-4), 111-132.
[19] Muneoka, Y.; Takahashi, T.; Kobayashi, M.; Ikeda, T.; Minakata, H.; Nomoto, K., Phylogenetic aspects of structure and action of molluscan neuropeptides. National Research Council of Canada: Toronto, 1994; p 109-118.
[20] Price, D. A.; Davies, N. W.; Doble, K. E.; Greenberg, M. J., The Variety and Distribution of the Fmrfamide-Related Peptides in Mollusks. Zool Sci 1987**,** 4 (3), 395-410.
[21] Kaufmann, W.; Kerschbaum, H. H.; Hauser-Kronberger, C.; Hacker, G. W.; Hermann, A., Distribution and seasonal variation of vasoactive intestinal (VIP)-like peptides in the nervous system of Helix pomatia. Brain Res 1995**,** 695, 125-136.
[22] Schot, L. P. C.; Boer, H. H.; Swaab, D. F.; Van Noorden, S., Immunocytochemical demonstration of peptidergic neuros in the central nervous system of the pond snail Lymnaea stagnalis with antisera raised to biologically active peptides of vertebrates. Cell Tissue Res 1981**,** 216, 273-291.
[23] Roberts, M. H.; Speh, J. C.; Moore, R. Y., The central nervous system of Bulla gouldiana: Peptide localization Peptides 1988**,** 9 (6), 1323-1334.
[24] Gourlet, P.; Woussen-Colle, M. C.; Robberecht, P.; de Neef, P.; Cauvin, A.; Vandermeers-Piret, M. C.; Vandermeers, A.; Christophe, J., Structural requirements for the binding of the pituitary adenylate-cyclase-activating peptide to receptors and adenylate-cyclase activation in pancreatic and neuronal membranes. Eur J Biochem 1991**,** 195 (2), 535-541.
[25] Vandermeers, A.; Vandeborre, S.; Hou, X.; de Neef, P.; Robberecht, P.; Vandermeers-Piret, M.; al., e., Antagonistic properties are shifted back to agonistic properties by further N-terminal shortening of pituitary adenylate-cyclase-activating peptides in human neuroblastoma NB-OK-1 cell membranes. Eur J Biochem 1992**,** 208 (3), 815-880-819.
[26] Pirger, Z.; Laszlo, Z.; Hiripi, L.; Hernadi, L.; Toth, G.; Lubics, A.; Reglodi, D.; Kemenes, G.; Mark, L., Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors are present and biochemically active in the central nervous system of the pond snail Lymnaea stagnalis. J Mol Neurosci 2010**,** 42, 464-471.
[27] Hernadi, L.; Pirger, Z.; Kiss, T.; Nemeth, J.; Mark, L.; Kiss, P.; Tamas, A.; Lubics, A.; Toth, G.; Shioda, S.; Reglodi, D., The presence and distribution of pituitary adenylate cyclase activating polypeptide and its receptor in the snail Helix pomatia. Neuroscience 2008**,** 155 (2), 387-402.
[28] Reglodi, D.; Lengvari, I.; Szelier, M.; Vigh, S.; Arimura, A., Distribution of PACAP-like immunoreactivity in the nervous system of oligochaeta. Peptides 2000**,** 21 (2), 183-188.
[29] Somogyvari-Vigh, A.; Reglodi, D.; Li, M.; Lengvari, I.; Vigh, S.; Arimura, A., Tissue distribution of PACAP27 and -38 in oligochaeta: PACAP27 is the predominant form in the nervous system of Lumbricus polyphemus. Peptides 2000**,** 21 (8), 1185-1191.
[30] Arimura, A.; Somogyvari-Vigh, A.; Miyata, A.; Mizuno, K.; Coy, D. H.; Kitada, C., Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes. Endocrinology 1991**,** 129 (5), 2787-2789.
[31] Boros, A.; Reglodi, D.; Herbert, Z.; Kiszler, G.; Nemeth, J.; Lubics, A.; Kiss, P.; Tamas, A.; Shioda, S.; Matsuda, K.; Pollak, E.; Molnar, L., Changes in the expression of PACAP-like compounds during the embryonic development of the earthworm Eisenia fetida. J Mol Neurosci 2008**,** 36, 157-165.
[32] Zhong, Y.; Pena, L. A., A novel synaptic transmission mediated by a PACAP-like neuropeptide in Drosophila. Neuron 1995**,** 14 (3), 527-536.
[33] Garcia-Fernandez, M. O.; Bodega, G.; Solano, R. M.; Ruiz-Villaespesa, A.; Sanchez-Chapado, M.; Carmena, M. J.; Prieto, J. C., Expression and distribution of pituitary adenylate

cyclase-activating peptide in human prostate and prostate cancer tissues. Regul Pept 2002**,** 110 (1), 9-15.
[34] McRory, J. E.; Parker, D. B.; Ngamvongchon, S.; Sherwood, N. M., Sequence and expression of cDNA for pituitary adenylate cyclase activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH)-like peptide in catfish. Mol Cell Endocrinol 1995**,** 108, 169-177.
[35] Matsuda, K.; Yoshida, T.; Nagano, Y.; Kashimoto, K.; Yatohgo, T.; Shimomura, H.; Shioda, S.; Arimura, A.; Uchiyama, M., Purification and primary structure of pituitary adenylate cyclase activating polypeptide (PACAP) from the brain of an elasmobranch, stingray, Dasyatis akajei. Peptides 1998**,** 19 (9), 1489-1495.
[36] Arimura, A., Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. Jpn J Physiol 1998**,** 48 (5), 301-331.
[37] Shioda, S.; Shimoda, Y.; Hori, T.; Mizushima, H.; Ajiri, T.; Funahashi, H.; Ohtaki, K.; Ryushi, T., Localization of the pituitary adenylate cyclase-activating polypeptide receptor and its mRNA in the rat adrenal medulla. Neurosci Lett 2000**,** 295 (3), 81-84.
[38] Pirger, Z.; Nemeth, J.; Hiripi, L.; Toth, G.; Kiss, P.; Lubics, A.; Tamas, A.; Hernadi, L.; Kiss, T.; Reglodi, D., PACAP has anti-apoptotic effect in the salivary gland of an invertebrate species, Helix pomatia. J Mol Neurosci 2008**,** 36 (1-3), 105-114.
[39] Pirger, Z.; Elekes, K.; Kiss, T., Functional morphology of the salivary gland of the snail, Helix pomatia: a histochemical and immunocytochemical study. Acta Biol Hung 2004**,** 55 (1-4), 221-232.
[40] Moura, K. R. S.; Terra, W. R.; Ribeiro, A. F., The functional organization of the salivary gland of Biomphalaria straminea (Gastropoda: Planorbiade): secretory mechanisms and enzymatic determinations. J Moll Stud 2004**,** 70, 21-29.
[41] Kiss, T., Apoptosis and its functional significance in molluscs. Apoptosis 2010**,** 15, 313-321.
[42] Pirger, Z.; Racz, B.; Kiss, T., Dopamine-induced programmed cell death is associated with cytochrome c release and caspase-3 activation in snail salivary gland cells. Biol Cell 2009**,** 101 (2), 105-116.
[43] Rácz, B.; Gasz, B.; NBorsiczky, B.; Gallyas, F.; Tamás, A.; R., J.; Lubics, A.; Kiss, P.; Rőth, E.; Ferencz, A.; Tóth, G.; Hegyi, O.; Wittmann, I.; Lengvári, I.; Somogyvari-Vigh, A.; Reglődi, D., Protective effects of pituitary adenylate cyclase activating polypeptide in endothelial cells against oxidative stress-induced apoptosis. Gen Comp Endocrinol 2007**,** 153 (1-3), 115-123.
[44] Bourgault, S.; Vaudry, D.; Dejda, A.; Doan, N. D.; Vaudry, H.; Fournier, A., Pituitary adenylate cyclase-activating polypeptide: focus on structure-activity relationships of a neuroprotective peptide. Curr Med Chem 2009**,** 16 (33), 4462-4480.
[45] Storey, K. B.; Storey, J. M., Metabolic rate depression in animals: transcriptional and translational controls. Biol Rev Camb Phil Soc 2004**,** 79 (1), 207-233.
[46] Pirger, Z.; Lubics, A.; Reglodi, D.; Laszlo, Z.; Mark, L.; Kiss, T., Mass spectrometric analysis of activity-dependent changes of neuropeptide profile in the snail, Helix pomatia. Neuropeptides 2010**,** 44, 475-483.
[47] Kiss, T., Diversity and abundance: The basic properties of neuropeptide action in molluscs. Gen Comp Endocrinol 2011**,** 172, 10-14.
[48] Bhattacharya, A.; Lakhman, S. S.; Singh, S., Modulation of L-type calcium channels in Drosophila via a pituitary adenylyl cyclase-activating polypeptide (PACAP)-mediated pathway. J Biol Chem 2004**,** 279 (36), 37291-37297.
[49] Feany, M. B.; Quinn, W. G., A neuropeptide gene defined by the Drosophila memory mutant amnesiac. Science 1995**,** 268, 869-873.

[50] Koshimura, K.; Murakami, Y.; Mitsushima, M.; Hori, T.; Kato, Y., Activation of Na+ channels in GH3 cells and human pituitary adenoma cells by PACAP. Peptides 1997**,** 18 (6), 877-883.
[51] Han, P.; Lucero, M. T., Pituitary adenylate cyclase activating polypeptide reduces expression of Kv1.4 and Kv4.2 subunits underlying A-type K(+) current in adult mouse olfactory neuroepithelia. Neuroscience 2006**,** 138 (2), 411-419.
[52] Pirger, Z.; Elekes, K.; Kiss, T., Electrical properties and cell-to-cell communication of the salivary gland cells of the snail, Helix pomatia. Comp Biochem Physiol A-Molecular & Integrative Physiology 2006**,** 145 (1), 7-19.
[53] Pirger, Z.; László, Z.; Kemenes, I.; Toth, G.; Reglodi, D.; Kemenes, G., A homolog of the vertebrate pituitary adenylate cyclase-activating polypeptide is both necessary and instructive for the rapid formation os associative memmory in an invertebrate. J Neurosci 2010**,** 30 (41), 13766-13773.
[54] Sacchetti, B.; Lorenzini, C. A.; Baldi, E.; Bucherelli, C.; Roberto, M.; Tassoni, G.; Brunelli, M., Pituitary adenylate cyclase-activating polypeptide hormone (PACAP) at very low dosages improves memory in the rat. Neurobiol Learn Mem 2001**,** 76 (1), 1-6.
[55] Jozsa, R.; Hollosy, T.; Tamas, A.; Toth, G.; Lengvari, I.; Reglodi, D., Pituitary adenylate cyclase activating polypeptide plays a role in olfactory memory formation in chicken. Peptides 2005**,** 26 (11), 2344-2350.
[56] Telegdy, G.; Kokavszky, K., The action of pituitary adenylate cyclase activating polypeptide (PACAP) on passive avoidance learning. The role of transmitters. Brain Res 2000**,** 874 (2), 194-149.
[57] Lucas, J.; Riddle, M.; Barttholomew, J.; Thomas, B.; Forni, J.; Nickerson, L. E.; Van Heukelum, B.; Paulick, J.; Kuruvilla, H., PACAP-38 signaling in Tetrahymena thermophila involves NO and cGMP. Acta Protozool. 2004**,** 43, 15 - 20.
[58] Grimmelikhuijzen, C. J. P.; Leviev, I.; Carstensen, K., Peptides in the nervous system of Cnidarians: structure, function, and biosynthesis. International Review of Cytology, 1996**,** 167, 37-89.