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PAC1 receptor localization in a model nervous system: Light and electron microscopic immunocytochemistry on the earthworm ventral nerve cord ganglia

L. Molnár [a,*], E. Pollák [a], A. Boros [a], S. Shioda [b], S. Nakajo [c], A. Tamás [d], I. Lengvári [d], D. Reglődi [d,1], A. Lubics [d,1]

[a] Department of General Zoology (Adaptation Biology Research Group of the Hungarian Academy of Sciences), Pécs University, Hungary
[b] Department of Anatomy, Showa University, Japan
[c] Biological Chemistry, Showa University, Japan
[d] Department of Anatomy, Pécs University, Hungary

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Abstract

The presence and pattern of pituitary adenylate cyclase activating polypeptide (PACAP) type I (PAC1) receptors were identified by means of pre- and post-embedding immunocytochemical methods in the ventral nerve cord ganglia (VNC) of the earthworm *Eisenia fetida*. Light and electron microscopic observations revealed the exact anatomical positions of labeled structures suggesting that PACAP mediates the activity of some interneurons, a few small motoneurons and certain sensory fibers that are located in ventrolateral, ventromedial and intermediomedial sensory longitudinal axon bundles of the VNC ganglia. No labeling was located on large interneuronal systems such as dorsal medial and lateral giant axon systems and ventral giant axons. At the ultrastructural level labeling was mainly restricted to endo- and plasma membranes showing characteristic unequal distribution in various neuron parts. An increasing abundance of PAC1 receptors located on both rough endoplasmic reticulum and plasma membranes was seen from perikarya to neural processes, indicating that intracellular membrane traffic might play a crucial role in the transportation of PAC1 receptors. High number of PAC1 receptors was found in both pre- and postsynaptic membranes in addition to extrasynaptic sites suggesting that PACAP acts as neurotransmitter and neuromodulator in the earthworm nervous system.
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1. Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) was isolated from ovine hypothalami on the basis of its ability to stimulate adenylate cyclase in anterior pituitary cells [1,2]. Since its discovery, widespread distribution and diverse effects of PACAP have been described in the central and peripheral nervous systems (CNS and PNS) and also in various other organs [3–5]. PACAP exists in two forms, with 38 and 27 amino acid residues [1,2]. PACAP38 is the predominant form in the vertebrate nervous system.

PACAP shows a remarkable sequence similarity among species implying that the peptide is involved in basic physiological processes [3–7]. The presence and distribution of PACAP have been described in detail in the central nervous system of mammalians, including humans [3,4]. PACAP shows a widespread distribution also in various other vertebrate species: in avians [8,9], reptiles [10,11], amphibians [12,13], and in different fishes [14–17].

PACAP-like immunoreactivity is present in Annelids, and by means of radioimmunoassay, PACAP27 has been shown to represent the major form of the peptide in earthworms [6,18,19]. In a molluscan species, PACAP immunoreactive fibers have been found in the neuropil and in peripheral nerves [20]. PACAP38-like immunoreactivity is present in Drosophila

* Corresponding author. Department of General Zoology, University of Pécs, H-7624 Pécs, Ifjúság u. 6., Hungary. Tel.: +36 72 503600x4596; fax: +36 72 501517.
E-mail address: molnar@ttk.pte.hu (L. Molnár).
[1] Lubics A and Reglődi D made equal contribution to the present work.

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nervous system, where PACAP modulates synaptic activity [21–23]. The peptide encoded by the Drosophila amnesiac gene shows homology to PACAP which also implies a function of PACAP in memory processes, including odor memory [24–28]. In protozoans, the peptide has been demonstrated to function as a chemorepellent [29,30]. Furthermore, maxadilan, a peptide in the salivary gland of the sand fly (which obtains blood from vertebrates) has been shown to be a specific agonist of PAC1 receptors, the activation of which results in vasodilation [31].

PACAP exerts its effects through two types of G-protein coupled receptors. VPAC1 and VPAC2 bind vasoactive intestinal peptide (VIP) and PACAP with similar affinities while PAC1 binds PACAP with a much higher affinity than VIP [3–5,32,33]. The distribution of PACAP receptors has been thoroughly studied in the nervous system of different vertebrate species [34–36] but not in invertebrate species. Invertebrates, like annelids, crustaceans, insects and mollusks are widely used model animals in neurobiological experiments due to the relatively simple organization of their nervous system. The investigation of the occurrence and pattern of PAC1 receptors in the nervous system of a model animal could serve not only to increase our knowledge of the neurochemical organization of an ancient nervous system but also to understand the physiological significance of PACAP during evolution.

The ventral nerve cord (VNC) ganglia of the earthworm *Eisenia fetida* was the focus of the present study due to their well-known anatomical organization (Fig. 1) [37]. Each VNC ganglion connects to peripheral organs and tissues with three pairs of segmental nerves and has some landmark structures such as dorsal and ventral giant axons, giant interneurons, giant and small motoneurons and a few central sensory cells [37]. Central processes of primary sensory cells, located in body wall epithelium, form sensory longitudinal axon bundles in the neuropil where distinct pathways of motoneurons and small interneurons have also been identified [38]. Our recent results have revealed the exact anatomical positions of PACAP38 immunoreactive structures in the VNC ganglia [18]. The present study gives a detailed description of the distribution of PAC1 receptor-like immunoreactivity in earthworm VNC ganglia and is the first report on its ultrastructural localization in labeled neurons applying both pre- and post-embedding immunocytochemistry.

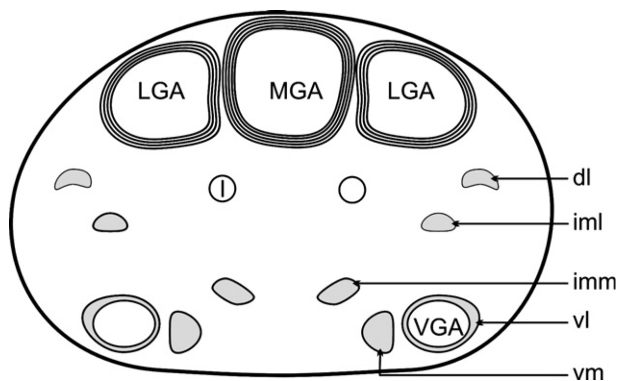

Fig. 1. Schematic drawing of the ventral nerve cord ganglion cross section of earthworms showing landmark structures. LGA: lateral dorsal giant axons; MGA: medial dorsal giant axon; I: large segmental interneuron; VGA: ventral giant axon; dl: dorsolateral, iml: intermediolateral, imm: intermediomedial, vl: ventrolateral and vm: ventromedial sensory longitudinal axon bundles.

2. Materials and methods

2.1. Animals and tissue processing

Sexually matured (clitellated) specimens of *E. fetida* Sav. (Annelida, Oligochaeta), kept at standard condition in our breeding stock [39] were hand-sorted and placed to plastic boxes containing wet filter paper. All experiments were carried out on intact depurated animals selected under a stereo-binocular microscope.

After anesthesia with carbon dioxide and chilling, 10–12 post-clitellar segments were cut from each experimental animal. VNC ganglia were dissected and fixed in freshly prepared 4% paraformaldehyde (PFA) for whole mount immunocytochemistry, or in a mixture of 0.1% glutaraldehyde (GA) and 4% PFA for both light and electron microscopic post-embedding immunocytochemistry. All fixatives were dissolved in 0.1 M (pH 7.4) phosphate buffered saline (PBS), and fixation was carried out for 3 h at room temperature. A part of GA-PFA fixed samples was post-fixed with 1% osmium tetroxide for one hour at 4 °C. For post-embedding immunohistochemistry and cytochemistry, VNC ganglia were dehydrated and embedded into epoxy resin (Durcupan ACM, Sigma Co., Budapest, Hungary). Serial semithin (1 µm) and ultrathin (60–70 nm) sections were cut with a Reichert ultramicrotome.

2.2. Whole mount immunocytochemistry

The fixed VNC ganglia were thoroughly washed in PBS and the perineural connective tissue was removed with fine forceps and scissors. After permeabilization with 0.5% Triton X-100, a three-step immunoreaction was carried out. Briefly, sections were incubated with PAC1 receptor antiserum raised in rabbit [40] (diluted in PBS, 1:500), followed by staining with a rabbit ExtrAvidin kit (Sigma Co, Hungary, Budapest). Immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB) and hydrogen peroxide solution. Stained preparations were thoroughly washed in PBS, cleared and coverslipped in 87% glycerol.

2.3. Post-embedding immunocytochemistry

Serial semithin sections of two ganglia heated on chrome–aluminium–gelatine coated slides were etched in sodium-ethanolate and thoroughly washed in distilled water and Tris–HCl, then in 0.5% Triton-X 100 diluted in Tris–HCl. After a pre-incubation step in 2.5% normal goat serum, sections were layered with anti-PAC1 receptor antiserum (diluted in Tris–HCl 1:500) and incubated in a wet chamber overnight. The immunoreaction was finalized with the above mentioned rabbit ExtrAvidin staining kit and visualized in 0.03% DAB solution containing 0.01% hydrogen peroxide under a continuous light microscopic control. Sections were washed, dehydrated, cleared in xylol and coverslipped in DePex (Fluka Co., Budapest, Hungary).

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