Immunocytochemical Demonstration of a Novel System of Neuroendocrine Peptidergic Neurons in the Pond Snail Lymnaea stagnalis, with Antisera to the Teleostean Hormone Hypocalcin and Mammalian Parathyroid Hormone

Sjoerd E. Wandelaar Bonga,* Floris P. J. G. Lafeber,* Gert Flik,* Toyoji Kaneko,† and Peter K. T. Pang†

*Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands, and †Department of Physiology, Faculty of Medicine, University of Alberta, Edmonton, Canada T6G 2H7

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Immunocytochemical staining with antisera raised against trout hypocalcin, the hypocalcemic hormone of the Stannius corpuscles and against bovine parathyroid hormone (bPTH$_{1-84}$), revealed a new system of neuroendocrine neurons in the pond snail Lymnaea stagnalis. The neurons are located in small groups or single cells in the visceral, parietal, and pedal ganglia of the central nervous system. The axons of these cells are running to the periphery of the pleuroparietal, visceroparietal, and pleuropedal connections, the dorsoparallel commissure, and to several nerves originating in the visceral, parietal, and pedal ganglia. The axons are ending with characteristic axonal distensions in the periphery of these connectives, commissure, and nerves. These regions probably act as neurohaemal areas. The affinity of this neuroendocrine system for both the anti-hypocalcin and anti-PTH sera is another indication for a special relationship between hypocalcin and PTH, which possess some immunological resemblance and similar biological activities, although no similarity in primary structure. © 1989 Academic Press, Inc.

Many biological active peptides are synthesized and released by neuroendocrine cells and conventional neurons. They have been shown to act as neurohormones, neuromodulators, or neurotransmitters. In the last decade the presence has been indicated of peptides structurally related to vertebrate hormones and neurohormones, such as hypophyseal and pancreatic peptides, in the nervous systems of invertebrates (Duve and Thorpe, 1980a,b; Boer et al., 1980; Hansen et al., 1982; Smit et al., 1988). Conversely, immunoreactivity to peptides first identified in invertebrates, e.g., FRMF amide, has subsequently been demonstrated in the vertebrate nervous system (Boer et al., 1980; Boer and Van Minnen, 1985). In the nervous system of the pond snail Lymnaea stagnalis, one of the most extensively studied invertebrate species, many peptidergic cells have been characterized with antisera raised against vertebrate and invertebrate biologically active peptides (Schot et al., 1981, 1984).

The aim of the present study was to investigate the distribution of immunoreactivity to antisera raised against two calcitropic vertebrate hormones, hypocalcin and parathyroid hormone (PTH). We have recently isolated hypocalcin from trout, where it is produced in the corpuscles of Stannius (Lafeber et al., 1988). These corpuscles are endocrine glands typical for teleostean and holostean fishes. Hypocalcin (or teleocalcin, as the same hormone isolated from salmon has been called, Wagner et al., 1986) most likely is the main hypocalcemic hormone in teleost fish (Wagner et
al., 1986; Wendelaar Bonga et al., 1986; Lafeber et al., 1988). Lopez et al. (1984) and Tisserand-Jochem et al. (1987) have postulated that the corpuscles of Stannius are homologous with the parathyroid glands, which are restricted to the tetrapods. The structure of the hormone of the corpuscles of Stannius in eels has recently been predicted on the basis of DNA sequence analysis (Butkus et al., 1987). Whereas the eel hormone showed a high degree of homology with the N-terminal part of trout hypocalcin (Lafeber et al., 1988) and of the hormone of the Stannius corpuscles isolated from salmon (Wagner et al., 1986), it had no sequential homology with other known vertebrate or invertebrate peptides, including PTH (Butkus et al., 1987). Nevertheless, hypocalcin shows a marked similarity in bioactivity with mammalian PTH in both mammalian and teleostean assay systems (Milet et al., 1979, 1980; Wendelaar Bonga et al., 1986; Lafeber et al., 1988). In this paper we describe a novel system of neuroendocrine cells in the central nervous system of _L. stagnalis_, which shows affinity to hypocalcin as well as PTH antisera.

**MATERIALS AND METHODS**

*Preparation of tissues.* Specimens of _L. stagnalis_ (shell height, 30-40 mm) were collected in ponds near Edmonton, Canada and near Nijmegen, The Netherlands. The central nervous system, including buccal, cerebral, pedal, pleural, parietal, and visceral ganglia, and the proximal parts of the nerves originating from these ganglia, were dissected immediately after collection. They were fixed overnight in either Bouin's fixative without acetic acid or in a mixture of 1% glutaraldehyde and paraformaldehyde in phosphate buffer (pH 7.3), and embedded in paraplast. Serial sections (5 μm) were mounted consecutively on four different slides. This was done for control purposes and for staining of the same cells with different antisera.

*Immunocytochemical staining.* Immunocytochemical staining was carried out according to the ABC method (Hsu et al., 1981), using commercial reagents (Vector Laboratories). After removal of the paraplast the sections were treated, at room temperature, with 0.6% hydrogen peroxide in distilled water for 15 min to inactivate endogenous peroxidase activity, and incubated for 30 min with normal goat serum (1:25) to reduce nonspecific staining. The sections were incubated with specific antisera for 18 hr at 4°C, then with biotinylated antibody (1:220) for 30 min, and ABC reagent for 60 min at room temperature. Staining followed for 2-5 min with freshly prepared 0.075% 3,3'-diaminobenzidine tetrahydrochloride in TRIS-HCl buffer containing 0.003% hydrogen peroxide, and was subsequently dehydrated and mounted in Canada balsam. Specificity of the staining was tested as: 1, omission of the primary antibody; 2, substitution of the primary antisera with preimmune serum; and 3, incubation with the primary antibody after absorption with the appropriate peptide. Experimental and control sections were processed simultaneously.

*Antisera production and specificity.* The following peptides were used for raising antisera: bovine parathyroid hormone 1-84 (bPTH_1-84); antisera recognizes the sequence 48-64), human parathyroid hormone 1-34 (hPTH_1-34), salmon calcitonin (the antisera was purchased from Bachem), and trout hypocalcin. The antisera were raised in guinea pig with exception of the hypocalcin antisemur, which was raised in rabbit. Working dilution of the antisera varied from 1:1500 to 1:2500. Preabsorption of the antisera was effected by incubation (12-16 hr; 4°C) of the appropriate peptides at a concentration of 10 μg/ml with antisemur at the working dilution. The bPTH_1-84 antisemur was preabsorbed with purified as well as synthetic bPTH_1-84. The specificity of the antisemur has been tested extensively. The hypocalcin antisemur did not cross-react with various vertebrate glycoproteins and peptides, including PTH (Kaneko et al., 1988). The specificity of the bPTH antisemur was demonstrated in the same way by Harvey and Pang (1988). Preabsorption of hypocalcin antisemur with PTH did not prevent the immunostaining of Stannius corpuscles of trout. Similarly, the staining of rat parathyroid glands with PTH antisemur was not affected by preabsorption with hypocalcin.

*Electron microscopy.* For electron microscopy, the tissues were fixed for 2 hr in a freshly prepared mixture of 0.8% glutaraldehyde and 1% O_3O_4 in veronal buffer (pH 7.4), and postfixed for 30 min in a solution of 1% uranylnitrate. Ultrathin sections were stained with lead citrate.

**RESULTS**

**General Anatomy**

The central nervous system consists of 11 ganglia, which are interconnected by connectives and commissures, and are located around the esophagus: the paired cerebral, pleural, parietal, pedal, and bucca ganglia, and the unpaired visceral ganglion
On top of each cerebral ganglion two small endocrine glands are located, the mediadorsal and laterodorsal bodies. Many nerves originate from the ganglia and innervate all parts of the body. The general histology and ultrastructure of the central nervous system have been described earlier (Wendelaar Bonga, 1970). A diagram (without buccal ganglia) is presented in Fig. 1.

**Immunocytochemical Staining**

**Anti-hypocalcin.** With an antiserum raised against the hormone isolated from trout Stannius bodies, many nerve cells are stained in the parietal, visceral, and pedal ganglia (Fig. 1).

In the right parietal ganglion, three groups of about two to six cells each are found, with cell bodies varying in diameter. Most cells are small or medium sized ($\phi$: 150–200 $\mu$m; Fig. 2). The axons of the immunoreactive cell bodies are oriented toward the central neuropile of the ganglion, where they intermingle and can be followed toward the parietopleural and parietovisceral connectives, and to both right pallial nerves (Fig. 1). The periphery of these connectives and the proximal parts of these nerves contain many distended axon endings with immunopositive material, that is located close to or is in contact with the perineurial connective tissue, that surrounds the central nervous system. The left parietal ganglion contains only a few anti-hypocalcin positive cell bodies. Some positive fibers are running to both parietal connectives and to the left pallial nerve. They end in the periphery of the connectives and nerve, similar as in the right parietal ganglion (Fig. 1).

The visceral ganglion contains three groups of three to six anti-hypocalcin positive cells, with cell bodies showing the same size distribution as in the right parietal ganglion (Fig. 4), and with axons that can be traced into the central neuropile and from there to both visceroparietal connectives and the four visceral nerves (Fig. 1). As in the parietal region, the axons end in the periphery of the connectives and nerve (Fig. 6).

In each of the pedal ganglia, groups of 5 to 10 cells each ($\phi$: 10–50 $\mu$m) are found. One group is located in the region between the inferior pedal nerve and the pedal commissures, and another in the region between this nerve and the pleuropedal connective (Figs. 1 and 5). Smaller cell groups (2–5 cells) are present more dorsally, in the region between the pedal commissure and the cerebropedal connective. The axons of all these cell groups are directed toward the

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**Fig. 1.** Diagram of the central nervous system of *L. stagnalis* showing the location of the anti-hypocalcin and anti-bPTH positive cell bodies (black dots) in parietal, visceral, and pedal ganglia; their axon tracts running to and ending in the periphery of the dorsopedal commissure; and of several connectives and nerves (neurohemal areas, IITTTT); l. cer, left cerebral ganglion; r. cer, right cerebral ganglion; l. pl, left pleural ganglion; r. pl, right pleural ganglion; l. par, left parietal ganglion; r. par, right parietal ganglion; visc, visceral ganglion; l. pe, left pedal ganglion; r. pe, right pedal ganglion; db, dorsal bodies; II, lateral lobes; 1, cerebral commissure; 2, subcerebral commissure, 3, dorsopedal commissure; 4, ventropedal commissure; 5, cerebropleural connectives; 6, pleuropedal connectives; 7, pleuroparietal connectives; 8, parietovisceral connectives. Nerves: 9, left pallial; 10, cutaneous pallial; 11, anal; 12, intestinal; 13, genital; 14, right pallial; 15, right external pallial; 16, superior pedal; 17, superior cervical; 18, median pedal; 19, inferior cervical; 20, columellar; 21, inferior pedal.
central neuropile of the pedal ganglia (Fig. 8), and from there they are running to the dorsopedal commissure (Fig. 9), to the pleuropedal connectives, and to some of the pedal nerves. Most are entering the inferior pedal nerve (Fig. 10), some others the columellar nerve, the median pedal (Fig. 11), and inferior cervical nerves. In the periphery of the pedal commissure and of all the connectives and nerves mentioned, anti-hypocalcin positive axon endings are present.

Positive nerve fibers are further present in small numbers in the connective tissue surrounding the parietal, visceral, and pedal ganglia, their connectives, and the proximal parts of their nerves.

Anti-bPTH\textsubscript{1-84}. The central nervous system contains many neurons that stain with anti-bPTH serum. The distribution of the cell bodies and axonal tracts is similar to that of the cells staining positively with the anti-hypocalcin serum. Using adjacent sections that contain profiles of the same cell bodies and axons, it can be demonstrated that the immunoreactivity to anti-bPTH and anti-hypocalcin sera is located in the same cells (Figs. 3 and 7).

Anti-hPTH\textsubscript{1-34}. With an antiserum raised against the N-terminal of hPTH, no reactivity is observed.

Control procedures. All control procedures, including the use of antisera preabsorbed with the respective antigens, result in extinction of the immunoreactivity. On the other hand, preabsorption of the hypocalcin antiserum with bPTH\textsubscript{1-84}, or of PTH antiserum with hypocalcin, does not prevent the immunostaining.

**Electron Microscopy**

Examination of the periphery of the pedal commissure and of some pedal nerves shows distended axons and axon terminals that are close to or in contact with the perineurial connective tissue. Many axons contain electron-dense secretory granules with a diameter varying from 100 to 220 nm (Fig. 12). With respect to the structure and size distribution of the dense granules, only one type of axon could be distinguished. Occasionally axons containing the same type of granules are found in the perineurium. Since only one type of granule-containing axon terminals is found in the periphery of most pedal nerves, these axons are equated with the anti-hypocalcin and anti-PTH positive material observed in these regions in the light microscope. Axons with the same type of granules are present in the connectives and nerves of the pleural (Fig. 13), parietal, and visceral ganglia, where they are found between fibers and terminals of axons originating from other types of neuroendocrine cells present in these ganglia (dark green cells, light green cells, and yellow cells). The granules of the light green and dark green cells are larger than those of the presumptive hypocalcin and PTH positive cells, but only slightly smaller than those of the yellow cells (Wendelaar Bonga, 1970). They differ

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**Figs. 2 and 3.** Neuronal cell bodies in adjacent sections of the right parietal ganglion; immunoreactivity to anti-hypocalcin (Fig. 2) and anti-bPTH (Fig. 3) antisera is colocated in the cytoplasm of a large cell body. 200×.

**Figs. 4 and 5.** Immunoreactivity to anti-hypocalcin antiserum in small and medium-sized neuronal cell bodies in visceral ganglion (Fig. 4; note positive fibers in neuropile, arrows; 300×) and in right pedal ganglion (Fig. 5; 450×).

**Figs. 6 and 7.** Adjacent (oblique) sections of the intestinal nerve; immunoreactivity to anti-hypocalcin (Fig. 6) and anti-bPTH (Fig. 7) antisera is colocated in distended axons and axon endings at the periphery of the nerve, close to the perineurial sheath of connective tissue (p). 320×.

**Fig. 8.** Section of the right pedal ganglion showing immunoreactivity to anti-bPTH antiserum; the staining is located in axonal fibers in the central neuropile of the ganglion and in an axon tract running to the inferior pedal nerve (arrow). 320×.
Fig. 9. Section of the left and right pedal ganglia and the dorsopedal commissure (c); anti-hypocalcin positive fibers are present in the neuropile (n) and in the commissure; a positive axonal swelling is present in the periphery of the commissure (arrow). 210×.

Fig. 10. Cross-section of the inferior pedal nerve showing anti-hypocalcin immunoreactivity in axonal swellings and axon endings in the periphery of the nerve and in two small branches of this nerve (arrows); p, perineurial sheath of connective tissue. 475×.

Fig. 11. Longitudinal section of the columellar nerve showing anti-bPTH immunoreactivity in axonal swellings and axon endings in the periphery of the nerve; p, perineurial sheath of connective tissue. 450×.

from the latter by the presence, within a single axon, of granules of high and low electron density (Figs. 12 and 13).

DISCUSSION

Anti-hypocalcin and Anti-bPTH Positive Cells

The parietal, visceral, and pedal ganglia contain a surprisingly large number of cells that react positively with both anti-hypocalcin and anti-bPTH (HP-cells). These cells apparently form an important type of hitherto unknown peptidergic neuroendocrine cells. None of the various types of neuroendocrine cells reported so far by light or electron microscopy in one or more of these ganglia shows a similar type of distribution (Wendelaar Bonga, 1970; Schot et al., 1981). In the parietal and vis-
ginal ganglia, two types of neuroendocrine cells have been described by the Alcian blue/Alcian yellow staining procedure: the yellow green cells (YGC) and the yellow cells (YC). The axons of these cells are ending in the periphery of the parietopleural and parietovisceral connectives in the periphery of the proximal parts of all the nerves originating from the parietal and visceral ganglia and in the adjacent connective tissue (Wendelaar Bonga, 1970). The periphery of connectives, commissures, and the proximal parts of many nerves, as well as the adjacent connective tissue, represent the typical neurohemal areas of pulmonate snails (Boer et al., 1968; Wendelaar Bonga, 1970). In these regions, axon swellings and terminals are in contact with the connective tissue and blood spaces. Here the secretory granules accumulate and finally the granular contents are released by exocytosis (Wendelaar Bonga, 1970; Roubos, 1976; Roubos and Buma, 1982). The HP-cells, although also present in the parietal and visceral ganglia, differ in size and location from the YGC and the YC. However, their axons are ending in the same neurohemal areas. With the electron microscope, the periphery of the pallial and visceral nerves contains axon endings with at least three

Fig. 12. Part of the periphery of the dorsopedal commissure showing distended axons containing electron-dense secretory granules, most likely of the anti-hypocalcin and anti-bPTH positive cells. 12,500x.

Fig. 13. Part of the periphery of the right pleuropedal connective showing distended axons containing electron-dense granules characteristic of the dark green cells (dgc) and of the anti-hypocalcin and anti-bPTH positive cells (hp). 17,900x.
types of secretory granules. Two types have been identified before as belonging to the YGC and YC (Wendelaar Bonga, 1970). The third type probably contains the anti-hypocalcin and anti-bPTH positive material. This identity is supported by our electron microscopic observations of the pedal nerves, which revealed the same type of granulated axon terminals (see below).

A large number of HP-cells are further located in the pedal ganglia. Typical neuroendocrine cells have not been reported before in these ganglia, although they contain many neurons that react positively to antiserum raised against vertebrate peptides such as insulin, pancreatic polypeptide, gastrin, α-MSH, met-enkephalin, vasopressin, vasotocin, oxytocin, and calcitonin. None of these neurons shows the structural characteristics of neuroendocrine cells (Schot et al., 1981). They may have a neurotransmitter or neuromodulator function. Our results show that neuroendocrine cells are also present in the pedal ganglia, and that the periphery of the proximal parts of several pedal nerves as well as the periphery of the pedal connectives, the dorsopedal commissure, and the adjacent connective tissue function as important neurohemal areas for these cells. In the periphery of the pedal nerves, only one type of granulated axon terminal is found, and therefore we conclude that these axons belong to the HP-cells. The presence of the cell bodies of these neurons in five ganglia and the distribution of their axons and axon terminals in a commissure and many connectives and nerves make the HP-cells the most extensive neuroendocrine system described so far in L. stagnalis.

**The Relationship between Hypocalcin and PTH**

In recent years, several vertebrate hormones have been identified in the neurones of invertebrates. Recently the expression of a gene coding for an insulin-related peptide has been demonstrated in the light green cells of the cerebral ganglia of L. stagnalis. The DNA-derived structure revealed the presence of A and B chains, a C-peptide equivalent and a signal sequence, indicating the early evolutionary origin of an insulin superfamily (Smit et al., 1988). It is common knowledge, however, that it is impossible to identify peptides only on the basis of immunoreactivity with one or two antisera. Therefore, the identity of the immunoreactive substance(s) localized in the present study remains to be clarified. The distribution of the staining of cell bodies, axons, and axon terminals indicates that the immunoreactivity to hypocalcin and PTH is present in the secretory granules. It is possible that different molecules with antigenic sites in common with either hypocalcin or PTH—in particular the 48–64 sequence of bPTH—are located in the secretory granules of the HP-cells. Another explanation is that the antigenic sites are located on the same molecule. This possibility needs attention because there is a striking resemblance in bioactivity between hypocalcin and PTH when tested in the same assay systems. This is rather surprising since hypocalcin most likely is the main hypocalcemic hormone in fish, whereas PTH is the hypercalcemic hormone of the terrestrial vertebrates. Nevertheless, both hormones stimulate osteoclastic resorption of rat and mouse bone (Milet et al., 1979; Lafeber et al., 1986), induce hypocalcemia in several fish species (Wendelaar Bonga et al., 1986), and inhibit branchial calcium influx in eels (Milet et al., 1979). Since the hypocalcin-producing cells also showed cross-reactivity to some antisera raised against mammalian PTH, it has been suggested that the corpuscles of Stannius of fish are homologous with the parathyroid glands of terrestrial vertebrates (Milet et al., 1980; Lopez et al., 1984; Tisserand-Jochem et al., 1987). However, hypocalcin and PTH have no aminoacid sequences in common (Butkus et al., 1987). The similar-
ity in bioactivity and the limited immunological similarity between both hormones may originate from some steric resemblance. Our results, showing anti-hypocalcin and anti-PTH positive material in the same molluscan neurons, represent further evidence for a specific relationship between hypocalcin and PTH, although the nature of this relationship is unknown.

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