Calcitonin gene-related peptide-like nervous system in the optic lobe and peduncle complex of the octopus, *Octopus vulgaris*

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Summary

Calcitonin gene-related peptide (CGRP)-like immunoreactivity was examined in the octopus optic lobe and peduncle complex by immunohistochemistry and biochemistry. In the optic lobe cortex, CGRP-immunoreactive somata were scattered in the inner granular cell layer but not in the outer granular cell layer. In the optic lobe medulla, scattered CGRP-immunoreactive somata were seen in the cell island and many immunoreactive varicose fibers were observed in the surrounding neuropil. In the peduncle lobe, immunoreactive somata were seen in the cell layer of the basal zone, but not in that layer of the spine. Based on the size of the immunoreactive somata, these CGRP-immunoreactive cells may correspond to the projection neurons from the basal zone to the motor areas of the central brain. Immunoreactive varicose fibers were observed in the basal zone neuropil, but only a few in the spine neuropil. In the olfactory lobe, many immunoreactive somata were seen in the posterior olfactory lobule, but only a few in the median olfactory lobule. We found no immunoreactive somata in the anterior olfactory lobule. However, immunoreactive varicose fibers and puncta were abundant in the neuropils of these three lobules. Western blot analysis indicated that the used anti-rat CGRP antiserum detected an approximate 31.6 kDa band from optic lobe extracts, which may be a precursor form or the active form of a CGRP-like peptide in the octopus.

These results suggest that a CGRP-like substance is present in the octopus optic lobe and peduncle complex and that the substance functions as a neuromodulator in the octopus nervous system similar in the vertebrate brain.

Introduction

Calcitonin, one of the Ca-regulating hormones, is secreted from C-cells of the thyroid gland in mammals or from parenchymal cells of the ultimobranchial gland in nonmammalian vertebrates. The calcitonin gene encodes another peptide known as calcitonin gene-related peptide (CGRP) (Amara et al., 1982). CGRP is a 37-amino acid neuropeptide produced by alternative splicing from the primary transcript of the calcitonin gene, which acts as a neuromodulator in the central and peripheral nervous systems (Zaidi et al., 1987). CGRP-like substances are widely distributed in the central nervous systems of invertebrates as well as vertebrates (Zaidi et al., 1987; Sasayama et al., 1991).

Cephalopods have well developed image-forming eyes that are remarkably similar to vertebrate eyes. The nervous systems of cephalopods are highly complex and centralized for invertebrates. The optic lobe is a center of analysis for visual input (Young 1962). The peduncle complex, composed
of the peduncle lobe (a visuo-motor center), olfactory lobe (innervated by olfactory nerves) and optic gland (an endocrine gland), is situated on the dorsal surface of the optic tract and lies at the hilus of the optic lobe (Fig. 1). The neuroanatomy of the optic lobe and peduncle complex has been extensively investigated and the presence of classical neurotransmitters has been reported (Florey and Winesdorfer, 1968; Boadle, 1969; Cory and Rose, 1969; Juorio, 1971; Kleinschuster and Morris, 1972; Matus, 1973; Tansey, 1980; Kito-Yamashita et al., 1990; Cornwell et al., 1993). In addition, we and others have described the differential distribution of some neuropeptides in these organs and suggested the associations of these neuropeptides with the organs’ functions (Feldman, 1986; Osborne et al., 1986; Le Gall et al., 1988; Di Cosmo and Di Cristo, 1998; Suzuki et al., 2000, 2002, 2003; Iwakoshi et al., 2002).

In the present study, we examined CGRP-like immunoreactivity in the optic lobe and peduncle complex of the octopus (Octopus vulgaris) by immunohistochemistry and biochemistry. A preliminary report on CGRP-immunoreactive neurons of the optic lobe cortex was published elsewhere (Suzuki and Yamamoto, 2002).

**Materials and Methods**

*Octopus vulgaris* specimens (about 1 kg) of both sexes, collected between April and November 2003 in the sea of Genkai in Japan, were used in the present study. They were anesthetized on ice and the optic lobe with the peduncle complex was dissected out. The tissue was fixed for 1 day in 0.1 M sodium phosphate buffer (PB; pH 6.9) containing 4% paraformaldehyde and 0.2% picric acid. After washing with PB, it was cut into sections 20 μm thick with a sliding microtome equipped with a freezing stage. Free-floating sections were collected in 0.1 M PB (pH 7.4) containing 0.9% sodium chloride (PBS) and washed overnight in the same buffer. According to the avidin-biotin-horseradish peroxidase complex (ABC) method, sections were incubated with rabbit anti-rat CGRP (Cambridge Research Biochemicals, Cambridge, England) diluted 1:1,000 in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (PBS-BSAT) for 48 hours at 4 °C. After washing in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:100 in PBS-BSAT for 1 hour at room temperature. The sections were then washed again.
in PBS, and incubated with ABC (Vector Laboratories) diluted 1:200 in PBS-BSAT for 30 minutes at room temperature. After a final wash in PBS, the sections were reacted with a 0.005 M Tris-HCl buffer solution (pH 7.4) containing 0.002% 3,3′-diaminobenzidine hydrochloride (DAB) and 0.005% hydrogen peroxide. Thereafter, sections were mounted on gelatin-coated glass slides, dried and counterstained with thionine. Controls were prepared by omitting the antiserum in the first incubation or by using antiserum preabsorbed with synthetic rat CGRP (10 μg/ml; Peptide Institute, Osaka, Japan).

For Western blotting, one optic lobe (178 mg, wet weight) was homogenized in 200 μl of 1.0 M acetic acid at 4 °C and heated in boiling water for 5 minutes. The homogenates were rapidly cooled in ice and centrifuged at 10,000xg for 1 hour at 4 °C. The supernatant was lyophilized and 200 μl of Laemmli’s sample buffer (Laemmli, 1970) were added. This sample and synthetic rat CGRP were analyzed by sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis (SDS-PAGE). After SDS-PAGE, separated proteins were transferred to Clear Blot Membrane-p (Atto Corporation, Tokyo, Japan) and probed with anti-rat CGRP antiserum after blocking non-specific protein binding with 3% BSA. The probed membranes were then incubated with biotinylated anti-rabbit IgG (1:1,000) and ABC (1:1,000) diluted in PBS-BSAT. After washing with PBS, the membranes were finally reacted with DAB solution containing 0.005% hydrogen peroxide and 0.6% nickel sulfate for 5 minutes. Rainbow marker (Amersham Pharmacia Biotech, Bucks., England) was simultaneously electrophoresed as a molecular marker.

Results

In retinal bundles consisting of nerve fibers connecting the retina and the optic lobe, scattered CGRP-immunoreactive fibers were observed (Fig. 2A). The optic lobe cortex (the so-called deep retina) consisted of two cell layers (the outer and inner granular cell layers) and one plexiform layer which was intercalated between these two cell layers (Fig. 2B). Ovoid CGRP-immunoreactive somata (about 10 μm in diameter) were scattered in the inner granular cell layer but not in the outer granular cell layer (Fig. 2B). In the plexiform layer, CGRP-immunoreactive puncta were condensed in a zone of the inner one quarter of this layer (Fig. 2B).

The optic lobe medulla is composed of many cell islands and neuropils surrounding the cell islands. Many CGRP-immunoreactive somata (14–23 μm in diameter) were observed in these cell islands (Fig. 2C). The size of CGRP-immunoreactive somata located in the deeper medulla tended to be larger than those in the superficial medulla. In the surrounding neuropils, many CGRP-immunoreactive varicose fibers were running back and forth (Fig. 2C). In contrast, those fibers in the superficial medulla were running radially (Fig. 2B). In neuropils that were continuous from the neuropil of the deep optic lobe medulla to the basal zone neuropil of the peduncle lobe, several CGRP-immunoreactive varicose fibers were observed and some of these fibers were continuous between the medulla and the basal zone (Fig. 2D).

The peduncle lobe is composed of the spine and basal zone and each structure has a peripheral cell layer and a central neuropil. CGRP-immunoreactive somata (about 19 μm in diameter and pyramidal in shape) were scattered in the cell layer of the basal zone but not in the cell layer of the spine
Figure 2. **A.** Retinal bundles. Arrows indicate CGRP-immunoreactive varicose fibers in the bundles. **B.** Optic lobe cortex and superficial medulla. Small arrows indicate CGRP-immunoreactive puncta in an inner one quarter of the plexiform layer (pfl) of the optic lobe cortex. Large arrows indicate radially-arranged CGRP-immunoreactive varicose fibers in the superficial medulla (m) under beneath the inner granular cell layer (igc) of the optic lobe cortex. Arrowheads indicate CGRP-immunoreactive somata in the inner granular cell layer. Abbreviation: ogc, outer granular cell layer. **C.** Optic lobe medulla. Arrows indicate CGRP-immunoreactive varicose fibers and puncta in the neuropil and arrowheads indicate immunoreactive somata in a cell island. **D.** Transitional area between the medulla (m) and the basal zone. Arrows indicate continuous immunoreactive varicose fibers between these structures. Arrowheads indicate immunoreactive somata in the cell layer of the basal zone (bzc). Abbreviation: bzn, neuropil of the basal zone. Scale bars indicate 50 μm (A) and 100 μm (B–D).

(Fig. 3 and 4A). Each immunoreactive soma protruded an apical process to the centrally located neuropil (Fig. 4B). Many CGRP-immunoreactive varicose fibers and puncta were seen in the basal zone neuropil (Fig. 3, 4A and 4B), but only a few in the spine neuropil (Fig. 3).

The olfactory lobe consisted of three lobules, namely anterior, median and posterior lobules, and each lobule had a peripheral cell layer and a central neuropil (Fig. 4A). In the anterior olfactory lobule, CGRP-immunoreactive fibers were seen in the neuropil but immunoreactive somata were not observed in the cell layer (Fig. 4C). CGRP-immunoreactive somata (about 13 μm in diameter and ovoid in shape) were scattered in the cell layer of the median olfactory lobule (Fig. 4D). In the neuropil of the median olfactory lobule, abundant CGRP-immunoreactive varicose fibers and puncta were seen (Fig. 4D). In contrast to the median olfactory lobule, abundant, large CGRP-immunoreactive somata (about 28 μm in diameter and pyramidal in shape) were observed in the cell layer of the posterior olfactory lobule (Fig. 3 and 4E).
These cells protruded apical processes to the adjacent neuropil (Fig. 4E). In the neuropil of the posterior olfactory lobule, many immunoreactive varicose fibers and puncta were also observed (Fig. 3 and 4E). Neuropil of three olfactory lobules and the peduncle lobe were continuous in deep areas of these structures (Fig. 3 and 4A). Some CGRP-immunoreactive fibers in the neuropil of the posterior olfactory lobule seemed to be continuous to those in the neuropil of the anterior and median olfactory lobules (Fig. 4A). In addition, some immunoreactive fibers in the posterior olfactory lobule also seemed to be continuous to those in the neuropil of the basal zone (Fig. 3). We found no CGRP-like immunoreactivity in the optic gland (Fig. 4A).

The preabsorption of anti-rat CGRP antiserum with synthetic rat CGRP inhibited these staining profiles. Sections processed from the incubation step with biotinylated anti-rabbit IgG showed no immuno-staining (results not shown).

Western blot analysis indicated that the anti-rat CGRP antiserum recognized synthetic rat CGRP (MW 3,806) that had migrated to an approximate 4.0 kDa-position (Fig. 5). From optic lobe extracts, this antiserum labeled an approximate 31.6 kDa band, although the antiserum did not label a 4.0 kDa band (Fig. 5). No immunopositive bands were seen when the probed membranes were processed from the incubation step of the second antibody (Fig. 5).
Figure 4. A. Low magnification showing the continuity of CGRP-immunoreactive fiber tracts among three olfactory lobules and the basal zone in a horizontal section. Abbreviations: aolc, cell layer of the anterior olfactory lobule; aoln, neuropil of the anterior olfactory lobule; bzc, cell layer of the basal zone; bzn, neuropil of the basal zone; molc, cell layer of the median olfactory lobule; moln, neuropil of the median olfactory lobule; opg, optic gland; polc, cell layer of the posterior olfactory lobule; poln, neuropil of the posterior olfactory lobule. B. Basal zone of the peduncle lobe. Arrows indicate immunoreactive puncta in the neuropil. Large arrowheads indicate immunoreactive somata in the cell layer and small arrowheads indicate immunoreactive protrusions from the immunoreactive somata to the neuropil. C. Anterior olfactory lobule. Arrows indicate immunoreactive varicose fibers and puncta. Note no immunoreactive somata in the cell layer of the anterior olfactory lobule (aolc). D. Median olfactory lobule. Arrows indicate immunoreactive varicose fibers and puncta in the neuropil, and arrowheads indicate immunoreactive somata in the cell layer. E. Posterior olfactory lobule. Arrows indicate immunoreactive varicose fibers and puncta in the neuropil. Large arrowheads indicate immunoreactive somata in the cell layer and small arrowheads indicate immunoreactive apical processes to the neuropil. Scale bars indicate 500 μm (A) and 50 μm (B–E).
Discussion

Antibody specificity was supported by the results of preabsorption of the antiserum with synthetic CGRP. In addition, Western blot analysis indicated that the presence of an immunoreactive band in the optic lobe extract, although the band was a far larger form than that of 37-amino acid CGRP. Further study is required to elucidate biochemical characteristics of octopus CGRP-like peptides.

CGRP-like immunoreactivity has been detected in some invertebrate nervous systems such as the planarian, slug, leech and pill bug, but not in sea anemone nor hydra (Sasayama et al., 1991). The present report has added a new species of invertebrates exhibiting a CGRP-like peptide in the nervous system, and we suggest that CGRP-like peptides are ubiquitous neuropeptides, not only in vertebrates, but also in invertebrates. Although the physiological data of CGRP functions in the invertebrate nervous systems are not available, the peptide increase intracellular cyclic adenosine-3', 5'-monophosphate (cAMP) levels in the rat spinal cord (Parsons and Seybold, 1997). Cyclic AMP-dependent protein kinase phosphorylates inositol 1, 4, 5-trisphosphate (IP3) receptor-binding protein and subsequently inhibits Ca²⁺ mobilization (Berridge and Irvine, 1984; Supattapone et al., 1988). Cyclic AMP and IP3 are candidates as intracellular messengers in invertebrate photoreceptor cells (Szuts et al., 1986; Tsuda, 1987; Yarfitz and Hurley, 1994). Alternatively, CGRP significantly inhibits substance-P (SP)-induced Ca²⁺ mobilization from intracellular Ca²⁺ storage sites without affecting IP3 production (Tanabe et al., 1996). SP-immunoreactive fibers were observed in the octopus (unpublished data) and squid optic lobes (Osborne et al., 1986). Therefore, the CGRP-like peptide in the octopus may function as a neuromodulator through interference in the Ca²⁺ mobilization similar in the vertebrate nervous systems. We previously demonstrated that CGRP-immunoreactive cells, of which the somata were located in the inner granular cell layer of the optic lobe cortex, were one type of centrifugal cell projecting to the retina (Suzuki and Yamamoto, 2002). Some of the CGRP-immunoreactive fibers in the retinal bundles may be axons of these CGRP-immunoreactive centrifugal neurons. These findings suggest that the possibility of the modulation of a CGRP-like substance to photoreceptor cell’s functions.

The optic lobe provides a system for coding, storing and decoding visual input to produce relevant motor responses (Young, 1962). Most photoreceptor
axons terminate at the outer border of the plexiform layer (Young, 1962; Saidel, 1979), and excitation of the optic nerve is passed on from the plexiform layer to the medulla by centripetal cells (Young, 1962). In the optic lobe medulla of the octopus, not only CGRP-, but also other neuropeptides, such as galanin-, neuropeptide Y (NPY)-, FMRFamide-, corticotropin-releasing factor (CRF)- and gonadotropin releasing hormone-immunoreactive neuron systems were located (Di Cosmo and Di Cristo, 1998; Suzuki et al., 2000, 2002, 2003). These neuropeptide-immunoreactive neuron systems in the optic lobe may modulate visual information by making nerve connections with the fibers forming the visual input pathways.

The basal zone of the peduncle lobe receives input from the optic lobe medulla (Woodhams, 1977), and there are two types of projecting neurons. One type of neuron has a relatively large somata (20 μm in diameter) and projects to the motor areas of the central brain. The other one has a relatively small somata (4–10 μm in diameter) and projects to the spine of the peduncle lobe (Messenger, 1967; Woodhams, 1977). The size of the CGRP-immunoreactive somata in the basal zone was that of the former type. Therefore, CGRP-immunoreactive neurons in the basal zone may directly project to the central brain.

The olfactory lobe receives inputs from chemoreceptor cells of the olfactory pit, however, its concrete function is still unknown. The three lobules of this lobe interconnect with each other via their neuropils, and medially these neuropils become confluent with that of the basal zone of the peduncle lobe (Messenger, 1967). Furthermore, fibers projecting to the optic lobe are most abundantly from the posterior lobule (Saidel, 1982). The continuity of CGRP-immunoreactive fibers in the deep neuropil suggests that some of the CGRP-immunoreactive somata in the posterior olfactory lobule project to the optic lobe. Based on these nerve connections, the olfactory lobe may participate in the interaction between chemoreceptive and visuo-motor functions. The CGPR-immunoreactive nervous system in the olfactory lobe, therefore, may possibly modulate this interaction. CGRP-immunoreactive somata, like NPY-immunoreactive somata (Suzuki et al., 2002), were seen in the median and posterior olfactory lobules, but not in the anterior lobule. However, galanin-, CRF-, and FMRFamide-immunoreactive somata were present in the anterior lobule in addition to the median and posterior lobules (Di Cosmo and Di Cristo, 1998; Suzuki et al., 2000, 2003). The differences in the distribution patterns of these neuropeptides within the olfactory lobe suggest the functional differentiation of three lobules.

References
Boadle, M. C. (1969) Observation on a histaminase of invertebrate origin: a contribution to the


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