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Kase, Yoichi
Noto Marine Laboratory, Institute of Nature and Environmental Technology, Division of Marine Environmental Studies, Kanazawa University | Laboratory of Marine Environmental Science, Faculty of Agriculture, Kyushu University

Ogiso, Shouzo
Noto Marine Laboratory, Institute of Nature and Environmental Technology, Division of Marine Environmental Studies, Kanazawa University | Laboratory of Marine Environmental Science, Faculty of Agriculture, Kyushu University

Ikari, Takahiro
Noto Marine Laboratory, Institute of Nature and Environmental Technology, Division of Marine Environmental Studies, Kanazawa University | Laboratory of Marine Environmental Science, Faculty of Agriculture, Kyushu University

Sekiguchi, Toshio
Noto Marine Laboratory, Institute of Nature and Environmental Technology, Division of Marine Environmental Studies, Kanazawa University | Laboratory of Marine Environmental Science, Faculty of Agriculture, Kyushu University

他

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Immunoreactive Calcitonin Cells in the Nervous System of Polychaete *Perinereis aibuhitensis*

Yoichi KASE[1], Shouzo OGISO[1], Takahiro IKARI[1], Toshio SEKIGUCHI[1], Yuichi SASAYAMA[1], Yoichiro KITANI[1], Yohei SHIMASAKI, Yuji OSHIMA, Akira KAMBEGAWA[2], Yoshiaki TABUCHI[3], Atsuhiko HATTORI[4] and Nobuo SUZUKI[1]*

Laboratory of Marine Environmental Science, Faculty of Agriculture, Kyushu University, Hakozaki 6–10–1, Higashi–ku, Fukuoka, 812–8581, Japan
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We examined immunoreactive calcitonin (iCT) cells in the nervous system of the polychaete *Perinereis aibuhitensis* using immunohistochemical methods. We found the most iCT cells (53–70 cells) in the cerebral ganglion. We also detected iCT cells (4–6 cells) in the subpharyngeal ganglion. Furthermore, it was noted that iCT cells were present bilaterally in each segment of the ventral nerve cord. These results suggest that iCT cells play some functional roles in the nervous system. Next, the molecular weight (MW) of the iCT substance was examined using the western blotting method. Cerebral ganglia were collected from 200 individuals. These ganglia were homogenized and centrifuged. The separated supernatants were treated with 66% acetone and then dialyzed to remove low MW substances (less than 2,000) at 4°C. After lyophilization, the sample was reconstituted with 1 M acetic acid and then fractionated with an ultrafiltration membrane system into MWs of 3,000 to 10,000. Thereafter, the specimen was separated by SDS–polyacrylamide gel electrophoresis and analyzed by western blotting with anti–salmon calcitonin (CT) antiserum. Our results indicated that the MW of the iCT substance was close to that of teleost fish CT (3.5 kDa). The annelid *Capitella teleta* has two genes encoding CT–like peptides. This suggests that the iCT substance in *P. aibuhitensis* includes amino acid residues similar to fish CT and belongs to the CT family.

Key words: immunoreactive calcitonin cells, polychaete, central nervous system, western blotting

INTRODUCTION

Calcitonin (CT) is a peptide hormone composed of 32 amino acids with two N–terminal cysteine residues. This hormone is secreted from C–cells of thyroid glands in mammals or from parenchymal cells of ultimobranchial glands in non–mammalian vertebrates (see a review, Sasayama, 1999). CT has a hypocalcemic function resulting from the suppression of osteoclasts in mammals (see a review, Wimalawansa, 1997). Also, CT suppressed osteoclastic activity in goldfish (freshwater teleosts) and nibbler fish (marine teleosts) (Suzuki *et al*., 2000a). In addition, CT plays a role in the excretion of extra calcium after calcium is injected in eels (Suzuki *et al*., 1999) and stonefish (Kaida and Sasayama, 2003).

On the other hand, CT is present in various tissues such as the gut, gonads, lungs, and brain in vertebrates (Azria, 1989). Immunoreactive calcitonin (iCT) was also detected in the central nervous systems of several vertebrate classes (humans: Fischer *et al*., 1983; rats: Flynn *et al*., 1981; pigeons: Galan Galan *et al*., 1981a; lizards: Galan Galan *et al*., 1981b; bullfrogs: Yui, 1983; lampreys and hagfish: Sasayama *et al*., 1991). Furthermore, CT receptors have been detected in the brain of flounders (Suzuki *et al*., 2000b), stingrays (Suzuki *et al*., 2012), rats (Becskei *et al*., 2004), and humans (Bower *et al*., 2016), indicating that CT has some neurophysiological functions in addition to calcium regulation. In mammals, CT actually has an analgesic effect, probably caused by increases in plasma β–endorphin levels and interaction with the endogenous opiate system (see a review, Hamdy and Daley, 2012).

In a species of protochordate (*Ciona intestinalis*), CT has been determined (Sekiguchi *et al*., 2009). This protochordate CT was composed of 30 amino acids with two N–terminal cysteine residues and acted to suppress osteoclasts in goldfish (Sekiguchi *et al*., 2009). Furthermore, this molecule is expressed in the neural complex of juveniles (Sekiguchi *et al*., 2009). Taking these facts into consideration, CT also plays some roles in the nervous systems of invertebrates.

We focused on one species of polychaeata *Perinereis aibuhitensis*. This worm is easy to collect because this species is imported from Korea for fishing bait. Therefore, we examined CT–producing cells by immunohistochemical methods. Furthermore, to analyze the biochemical characterization of the iCT substance, its molecular weight (MW) was investigated by western blotting with anti–salmon CT antiserum.

[1] Noto Marine Laboratory, Institute of Nature and Environmental Technology, Division of Marine Environmental Studies, Kanazawa University, Housu–gun, Ishikawa 927–0553, Japan
[2] Kambegawa Laboratory, Tokyo 201–0013, Japan
[3] Division of Molecular Genetics Research, Life Science Research Center, University of Toyama, Sugitani, Toyama 930–0194, Japan
[4] Department of Biology, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Ichikawa, Chiba 272–0827, Japan
* Corresponding author (E–mail: nobuos@staff.kanazawa–u.ac.jp)

MATERIALS AND METHODS

Animals

Polychaetes were purchased from a fishing shop. For acclimation, the worms were kept in an aquarium for three days with aeration.

Detection of iCT cells

The bodies of worms (n = 10) were separated into 12 portions. One portion consisted of nine segments. These portions were fixed with Bouin solution without acetic acid (Okuda *et al*., 1999) and then were preserved in 70% ethyl alcohol. Tissues embedded in paraffin were serially sectioned at 10 μm by a routine paraffin method. The paraffin sections were incubated with an anti–salmon CT polyclonal antibody (1/100,000 dilution). This antiserum was prepared in the rabbit by the injection of synthetic salmon CT (Teikokuzouki Co. Ltd., Tokyo, Japan) which had been combined with bovine serum albumin. The specificity of this antiserum has been analyzed by enzyme–linked immunosorbent assay (Suzuki, 2001). Then, the paraffin sections were incubated with biotinylated anti–rabbit immunoglobulins goat antibody (1/2,000 dilution) (E 432, Dako Japan Co. Ltd., Kyoto, Japan). Thereafter, iCT cells were detected using a labeled streptavidin–biotin kit (Dako Japan Co. Ltd.). All steps were incubated on a horizontal shaker.

Determination of iCT substance

The cerebral ganglia were dissected out under a binocular microscope. The tissues were immediately frozen and kept at −80°C until use. The procedures for preparing the crude extracts and fractionating them are indicated in Figure 1. To prepare the crude extract, the ganglia were boiled for 10 min with 5 ml of distilled water to deactivate the endogenous proteases. The resultant suspension was immediately cooled and acidified with glacial acetic acid to make a final concentration of 1 M. Then, the acid–treated samples were homogenized at 4°C with a glass homogenizer and centrifuged at 25,000 × g for 10 min at 4°C.

The crude extract was treated with 66% acetone to remove high molecular proteins. After centrifugation, the supernatant was dialyzed (Spectra/Por, MWCO 2000, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) to remove low MW substances (less than 2,000) at 4°C. After lyophilization, the sample was reconstituted with 1 M acetic acid and then fractionated with ultrafiltration membrane systems into MWs of 3,000 to 10,000 (Centricon, Merck Millipore Corporation, Darmstadt, Germany). Namely, the sample was filtered with a MW of 10,000 (Centricon, Merck Millipore Corporation), and the filtrated sample was then subjected to a MW of 3,000 (Centricon, Merck Millipore Corporation).

The fractionated samples (MWs of 3,000 to 10,000) were lyophilized and then solubilized in lysis buffer containing 4% sodium dodecyl sulfate, 4% 2–mercaptoethanol, 8 M urea, and 10 mM Tris–HCl (pH 6.8) and subjected to electrophoresis. Eel CT (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a positive control. The separation gel was prepared with a linear gradient from 10% to 20% polyacrylamide. Samples separated by electrophoresis were transferred to a polyvinylidene difluoride membrane (Clear Blot Membrane–P; Atto Co. Ltd., Tokyo, Japan) at room temperature for 2.5 h at 16 V/cm (Suzuki *et al*., 1995). Thereafter, the membrane was washed three times in a 10 mM phosphate buffer solution containing 0.05% Tween 20 (PBST) adjusted with HCl to pH 7.2. Then, PBST containing 1% normal swine serum was used to block the non–specific binding of the antibody onto the membrane for 30 min at room temperature. After washing with PBST, the membrane was then treated with salmon CT antiserum for 12–15 hours at room temperature. The unbound antiserum was removed by washing with PBST. Then, the membrane was incubated with biotinylated anti–rabbit immunoglobulin goat antibody (E 432, Dako Japan Co. Ltd.). After washing with PBST, the target protein was detected using a labeled streptavidin–biotin kit (Dako Japan Co. Ltd.) to immunostain the membrane.

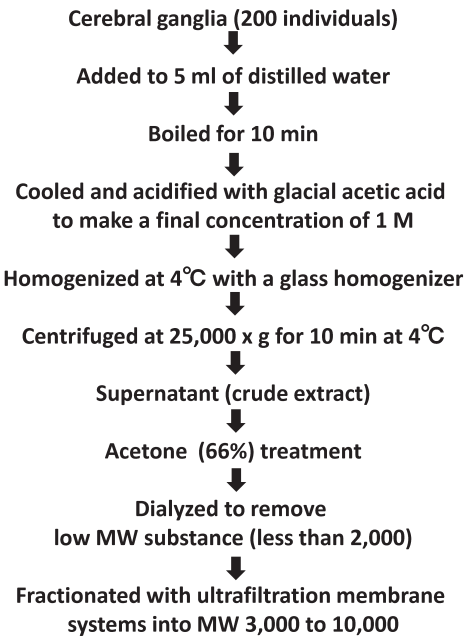

Fig. 1. The procedure of preparing and fractionating of crude extracts from cerebral ganglia in a polychaete *P. aibuhitensis*.
The cerebral ganglia were collected from 200 individuals. These ganglia were homogenized and centrifuged. The separated supernatants were treated with 66% acetone and then dialysis to remove low molecular weight (MW) substances (less than 2,000). Thereafter, the sample was fractionated with an ultrafiltration membrane system into MWs of 3,000 to 10,000.

RESULTS

Detection of iCT cells by immunohistochemical methods

The distribution of iCT cells is summarized in Figure 2. The most iCT cells (53–70 cells) were in the cerebral ganglion (Figs. 3A and 3B), the portion including head.

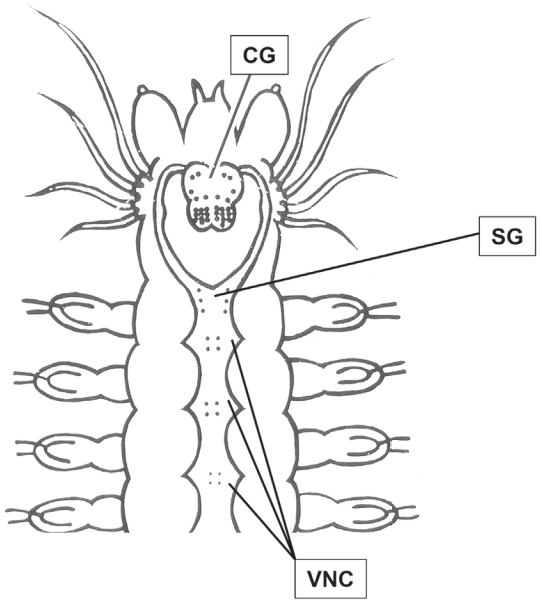

Fig. 2. Schematic drawings showing the distribution and number of immunoreactive calcitonin cells (black spots) in a polychaete *P. aibuhitensis*.
The size of the black spots is shown according to the strength of the positive reaction. CG: cerebral ganglion; SG: subpharyngeal ganglion; VNC: ventral nerve cord.

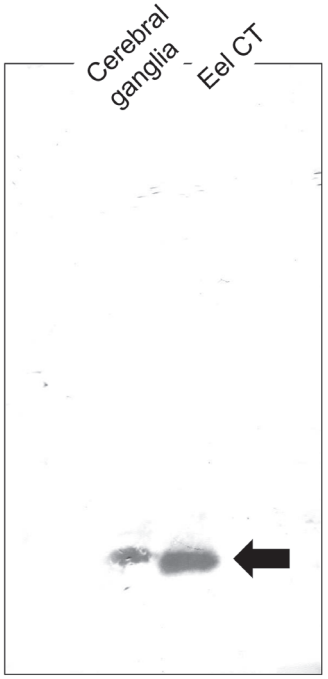

Fig. 4. Analysis of immunoreactive calcitonin (iCT) substance by western blotting.
Molecular weight of the iCT substance in the polychaete *P. aibuhitensis* was close to that of eel calcitonin (CT) (3.5 kDa) (Arrow).

In other parts, iCTs were detected in the subpharyngeal ganglion (4–6 cells). Furthermore, 4 iCT cells were found in each segment of the ventral nerve cord (Fig. 3C). These iCT cells were detected in two pairs—right and left sides—at each segment (Fig. 2).

Analysis of the iCT substance in polychaetes

The results of western blotting of the cerebral ganglia (200 individuals) are indicated in Figure 4. We found that the MW of the iCT substance in this polychaete was close to that of teleost fish CT (3.5 kDa) (Fig. 4).

DISCUSSION

iCT cells were detected not only in cerebral ganglia, but also in subpharyngeal ganglia and ventral nerve cord of the polychaete *P. aibuhitensis* (Figs. 2 and 3). It is known that in a gastropod *Physella heterostropha*, synthetic somatostatin and salmon CT promoted neurite formation in a dose–dependent manner, although other hormones, such as thyrotropin–releasing hormones, arginine vasotocin, and eledoisin, failed to promote neurite outgrowth (Grimm–Jørgensen, 1987). In another species of gastropod *Aplysia kurodai*, the effect of salmon CT on the neurons (R9 and R10) of abdominal ganglia has also been reported (Sawada *et al*., 1993). This examination using *A. kurodai* indicated that micropressure–ejected salmon CT induced a slow outward current associated with a decrease in Na^+ conductance, causing membrane hyperpolarization (Sawada *et al*., 1993). In *A. kurodai*, therefore, CT may act as an inhibitory neurotransmitter that regulates the firing pattern of major neurons in abdominal ganglia. In the present study, it is remarkable that iCT cells were also present in the subpharyngeal nerve and ventral nerve cord in addition to

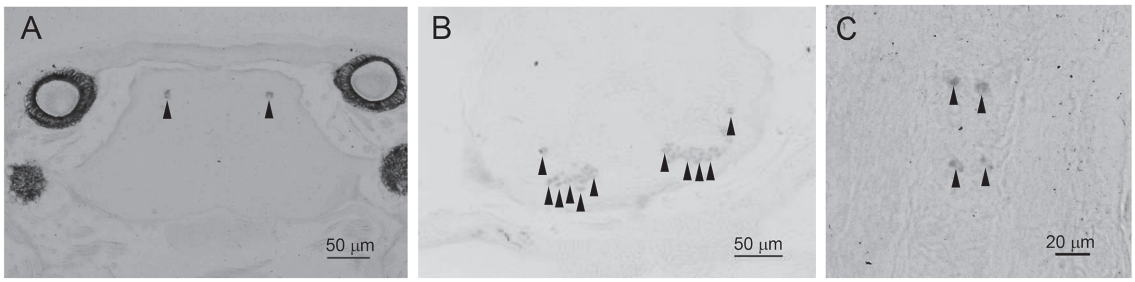

Fig. 3. Immunoreactive calcitonin (iCT) cells in a polychaete *P. aibuhitensis*.
A: cerebral ganglion (anterior region); B: cerebral ganglion (posterior region); C: ventral nerve cord. Arrowheads indicate iCT cells.

the central ganglion. Moreover, we found out that those cells looked to be in a row systematically in each body segment (Fig. 2). This arrangement of iCT cells has some physiological meaning in this worm.

In *A. kurodai*, Sawada *et al*. (1993) reported that extra–cellular micropressure ejection of forskolin (an activator of cyclic adenosine 3',5'–monophosphate) induced hyperpolarization in the R9 neuron of abdominal ganglia. This phenomenon seems to be mediated by a receptor that regulates increase of intracellular cyclic adenosine 3',5'–monophosphate. In the polychaete *P. aibuhitensis*, therefore, the iCT substance may function as a neurohormone *via* CT receptor because CT increases intracellular cyclic adenosine 3',5'–monophosphate after binding CT receptor (see a review, Goldring *et al*., 1993). The CT family receptor has been sequenced in invertebrates such as the bivalve mollusc (*Crassostrea gigas*: Dubos *et al*., 2003) and chordates (*C. intestinalis*: Sekiguchi *et al*., 2009; *Branchiostoma floridae*: Sekiguchi *et al*., 2016). The CTs of both *C. intestinalis* and *B. floridae* are bioactive in fish osteoclasts (Sekiguchi *et al*., 2009; Sekiguchi *et al*., 2017). In the annelid *Capitella teleta*, two genes encode CT–like peptides, one encodes a CT–type peptide (31 amino acid residues) with two N–terminal cysteine residues, and one encodes a diuretic hormone 31–type peptide without two N–terminal cysteine residues (Rowe *et al*., 2014). We found that the MW of the iCT substance in this polychaete was close to that of teleost fish CT (3.5 kDa) (Fig. 4). Thus, the iCT substance in *P. aibuhitensis* may include amino acid residues similar to fish CT and may belong to the CT family.

AUTHOR CONTRIBUTIONS

Y. Kase, Y. Sasayama, A. Kambegawa and N. Suzuki designed the study. Y. Kase and N. Suzuki performed the histological experiments and western blotting. S. Ogiso, Y. Kitani, T. Ikari, T. Sekiguchi, Y. Tabuchi, and A. Hattori analyzed the data and wrote the paper. Y. Oshima and Y. Shimasaki supervised the work. All authors assisted in editing of the manuscript and approved the final version.

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