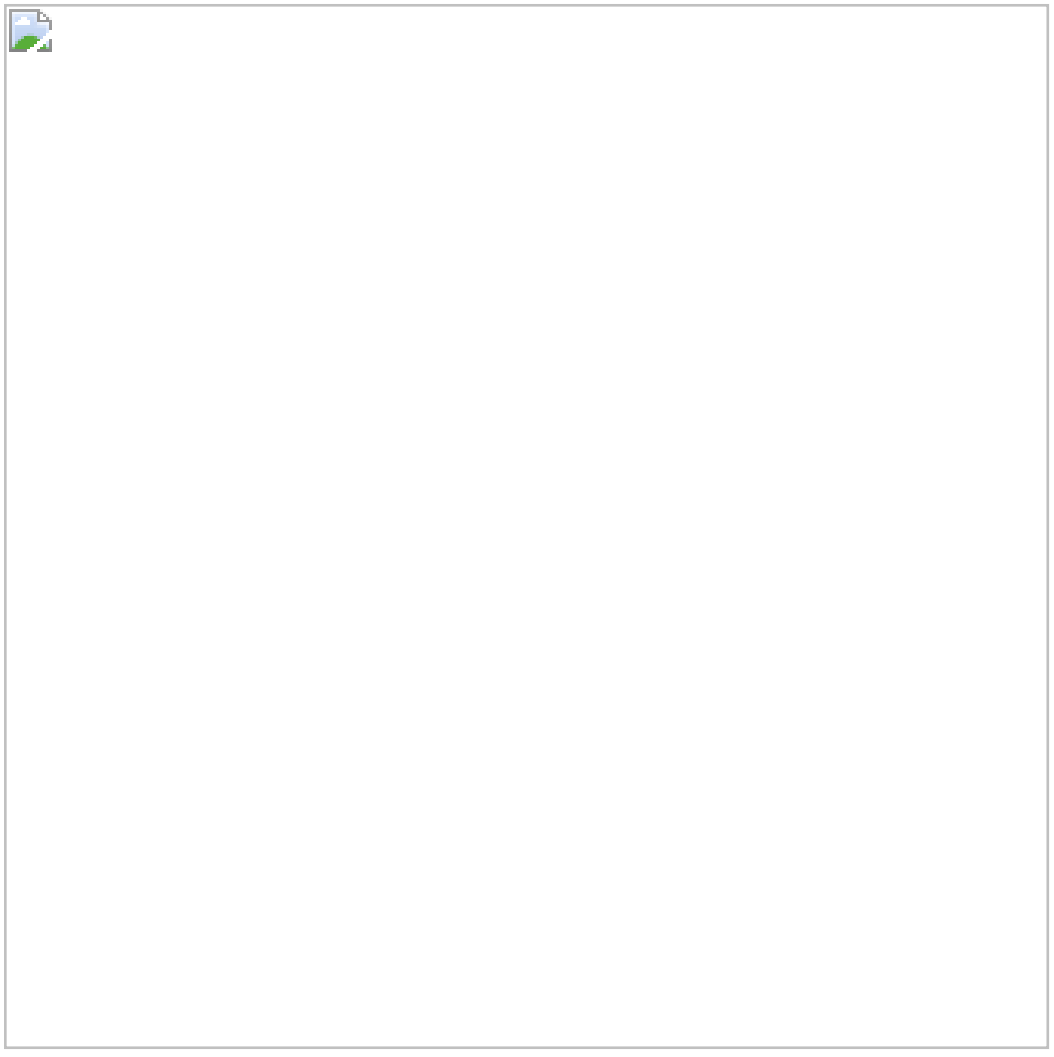

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Adrenocorticotropin-Like Immunoreactivity in the Granules of Neural Complex Cells of the Ascidian *Halocynthia roretzi*

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ABSTRACT—Immunohistochemical studies on the neural complex (neural gland, dorsal strand, and cerebral ganglion) of an ascidian, *Halocynthia roretzi*, were performed by using an antiserum against porcine ACTH. The antiserum recognized a considerable number of the cells scattered along the tubular structure of the dorsal strand and a few cells in the cerebral ganglion. Immunoelectron microscopic studies revealed that the ACTH-like substance resided within secretory granules with diameter of 300–500 nm. Furthermore, those ACTH-immunoreactive cells were demonstrated to be different from PRL-immunoreactive cells, the presence of which had previously been reported.

Key words: adenohypophysis, adrenocorticotropic hormone, ascidian, neural complex

INTRODUCTION

Several series of experimental studies have demonstrated that the hypophysis in vertebrates from amphibians to mammals is of neuroectodermal origin (Couly and Le Douarin 1985, 1987; Eagleson *et al*., 1986; Kawamura and Kikuyama 1992; Kouki *et al*., 2001). This suggests that cells equivalent to the vertebrate adenohypophyseal cells in protochordates, if any, might be found in neuroectodermal derivatives. In ascidians, the neural complex (neural gland, cerebral ganglion, and dorsal strand) is known to be derived from the embryonic neural tube during metamorphosis (Dodd and Dodd 1966).

There are a few earlier reports describing the presence of substances immunoreactive with antisera against pituitary hormones such as prolactin (PRL; Fritsch *et al*., 1982; Pestarino 1983) and adrenocorticotropic hormone (ACTH; Georges and Dubois 1979, 1985; Pestarino 1988) in the cerebral ganglion of ascidians. However, it has not been examined whether these immunoreactive substances exist within secretory granules. Recently we demonstrated the presence of PRL-immunoreactive substance within the secretory granules in the cells of the dorsal strand as well as in the neuronal cells in the cerebral ganglion of *Halocynthia roretzi* (Terakado *et al*., 1997).

As one step to search for the prototype of the vertebrate pituitary, the present experiment was conducted to see whether ACTH- immunoreactive substance exists, as in the case of PRL-like substance, in the cells of the cerebral ganglion and/or the dorsal strand of *Halocynthia roretzi*, and if so, whether the substance is contained in cells different from the PRL-immunoreactive cells and whether it is localized in the secretory granules.

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MATERIALS AND METHODS

Animals

Adult specimens of the ascidian *Halocynthia roretzi* (weighing 250–450 g) that had been cultured in Mutsu Bay in Aomori City, Japan were purchased from a commercial dealer.

Immunohistochemistry

The neural complexes were excised, fixed with Bouin's fluid without acetic acid for 15 hr, dehydrated through an ethanol series, and embedded in Paraplast. Sections (6 μm in thickness) were mounted on gelatin-coated slides and stained immunohistochemically by the ABC (streptavidin-biotin-peroxidase) method using a polyclonal rabbit antibody against porcine ACTH (Tanaka and Kurosumi, 1986) or bullfrog PRL (Yamamoto and Kikuyama, 1982). Dewaxed and rehydrated sections were treated with 0.5% H_2O_2 in 70% ethanol for 30 min to block endogenous peroxidase activity, washed, and treated with normal swine serum (1: 50; Dako, Copenhagen, Denmark) for 30 min to reduce non-specific staining. The primary antisera against ACTH and PRL, diluted 1: 2000 and 1: 1000, respectively, in 0.01 M phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), pH 7.5, were reacted with the sections overnight at 4°C. Biotinylated swine antibody against rabbit immunoglobulins (1: 300; Dako) and peroxidase-con-

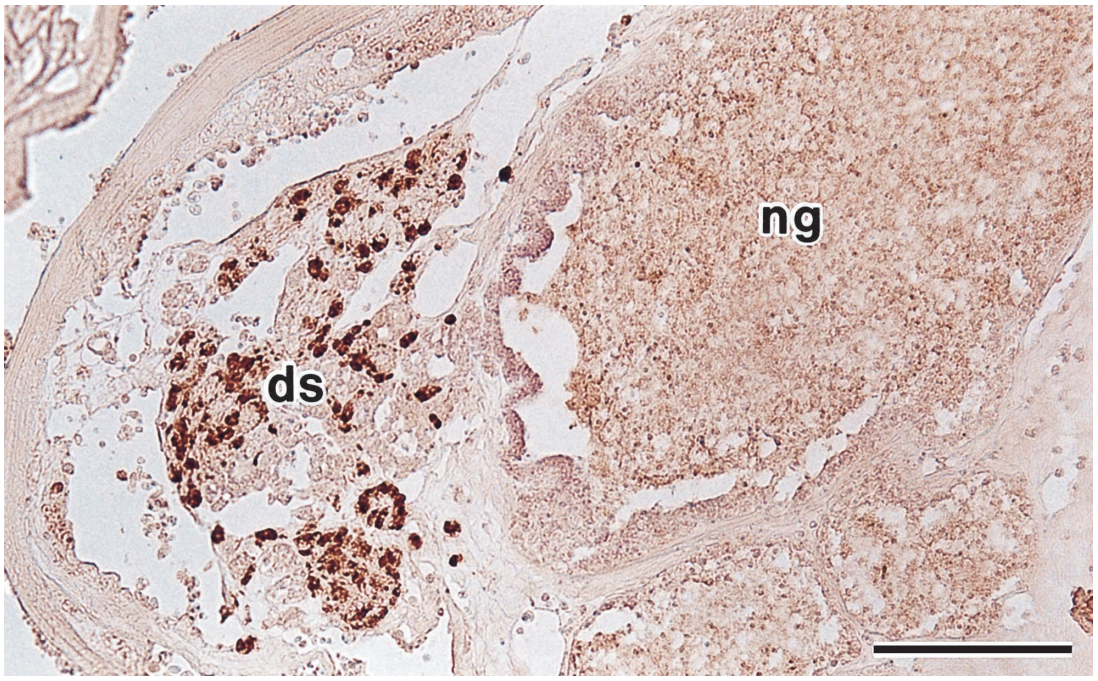

Fig. 1. Immunocytochemical staining of the dorsal strand with anti-porcine ACTH serum. The ACTH-imunoreactive cells are distributed among the tubular structures of the dorsal strand. Empty spaces within and around the tissues of the dorsal strand constitute the dorsal blood sinus. ds, dorsal strand, ng, neural gland. Bar: 100 μm

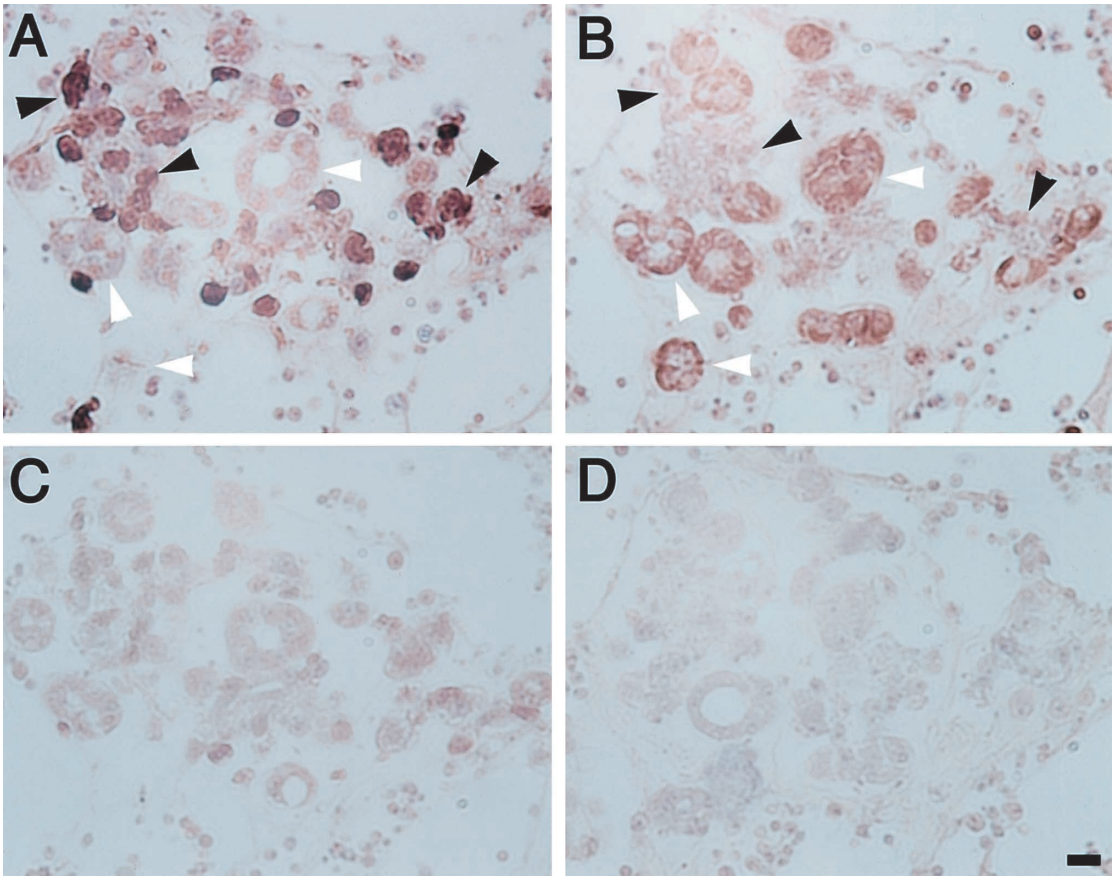

Fig. 2. Four adjacent sections stained with anti-ACTH (A), anti-PRL (B), anti-ACTH preabsorbed with antigen (C) and anti-PRL preabsorbed with antigen (D). Equivalent cells in “A” and “B” are indicated by black (ACTH- immunoreactive cells) and white (PRL- immunoreactive cells) arrowheads. Bar: 10 μm

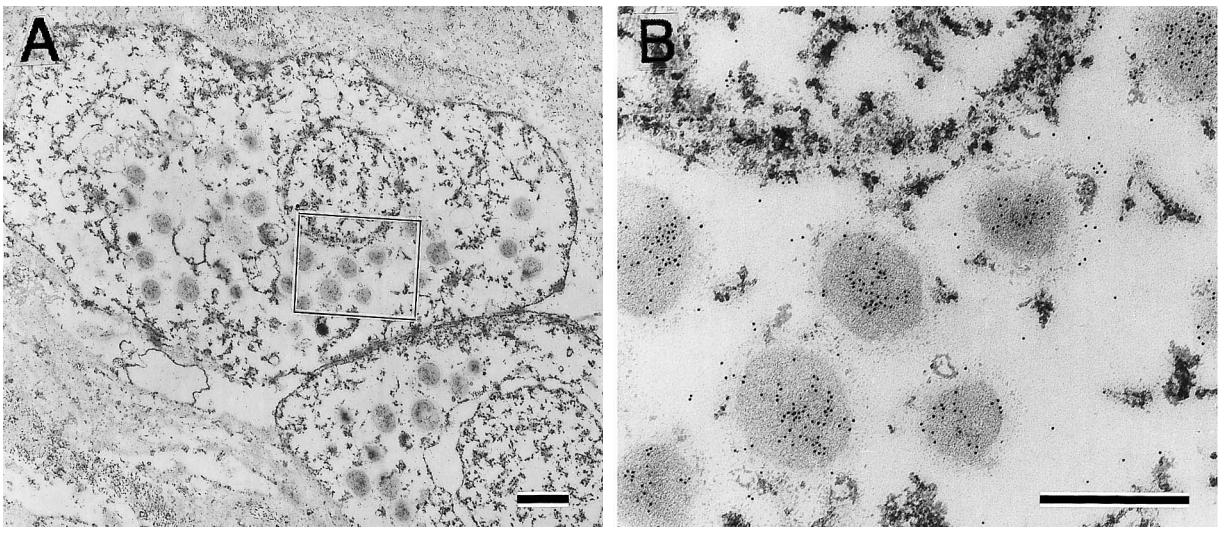

Fig. 3. Immunoelectron microscopy of dorsal strand. (A) Immunoelectron micrograph of ACTH-immunoreactive cells distributed in the dorsal strand. Bar: 1 μm. (B) A higher magnification of the region indicated by the rectangle in "A". Bar: 0.5 μm. Gold particles are localized over secretory granules that measure about 300–500 nm in diameter.

jugated streptavidin (1: 300; Dako) were applied successively, each for 1 hr at room temperature. Immunoreactivity was visualized by exposing the sections to 0.02% 3,3′-diaminobenzidine containing 0.006% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.5) at room temperature for 3-10 min, after which the sections were stained lightly with Mayer's hematoxylin. Control staining was carried out by incubating sections overnight at 4°C in antiserum against ACTH (1: 2000, 1 ml) or antiserum against PRL (1: 1000, 1 ml) that had been preabsorbed with the corresponding antigen (10 μg).

Immunoelectron microscopy

Dissected neural complexes were cut into small pieces and fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 4 hr at 4°C. These tissue pieces were washed overnight at 4°C in 0.1 M cacodylate buffer containing 7% sucrose, postfixed with 1% osmium tetraoxide in 0.15 M posphate buffer for 1 hr at 4°C, dehydrated in a graded ethanol series, and embedded in LR white resin (London Kes in Company Ltd, Berkshire, UK), which was then incubated at 55°C for 48 hr. Ultrathin sections were placed on Formvar-coated nickel grids and treated with 1% meta- periodic acid for 10 min, followed by 1% BSA in PBS for 1 hr. The primary antibody used was the polyclonal antiserum against porcine ACTH (1: 200). Sections were incubated overnight with this antigen at 4°C, washed with PBS, treated with gold-labeled (10 nm) secondary antibody (1: 20; British BioCell International, Cardiff, UK) for 1 hr, washed, and observed with a Hitachi H700H electron microscope operated at 100 kV. Control staining was performed by treating sections with the primary antiserum (1: 200, 1 ml) preabsorbed with antigen (100 μg) as described above.

RESULTS

A considerable number of the cells scattered around the tubular structure of the dorsal strand were immunoreactive with the antiserum against ACTH (Figs. 1 and 2A). A few cells in the cerebral ganglion were also immunopositive with the antiserum (data not shown). However, no cells in the neural gland were ACTH-immunopositive (Fig. 1). Staining of adjacent sections with the antiserum against PRL revealed that the ACTH-immunopositive cells are different from the PRL-immunopositive ones (Fig. 2B). The immunoreaction was prevented by preabsorption of the anti-ACTH (Fig. 2C) or the anti-PRL (Fig. 2D) with the appropriate antigen.

Immunoelectron microscopic study revealed that the gold particles were mostly localized in the secretory granules with diameters of about 300–500 nm (Fig. 3A, B). When the antiserum preabsorbed with ACTH was applied, the gold particles on the secretory granules were reduced in number to the back ground level (data not shown).

DISCUSSION

In a previous communication, we reported that some of the cells in the dorsal strand and the cerebral ganglion contained a PRL-like immunoreactive material within their secretory granules (Terakado *et al*., 1997). In the present experiment, ACTH-like immunoreactivity was also demonstrated in a considerable number of cells in the dorsal strand and in a few cells in the cerebral ganglion. The preservation of membranous structures and cell organella of our preparations for immunoelectron microscopic study was rather poor. This was due to the use of LR white resin for embedding of the tissue, which is known to preserve immunoreactivity. However, it was obvious that the immunoreactivity resided within the secretory granules. Moreover, it was apparent that ACTH-like substance and PRL-like substance were localized in different cells. Of note, the ACTH-like substance was contained within the granules with a diameter of approximately 300–500 nm, which are the largest among the granules found in the cerebral ganglion and the dorsal strand. On the other hand, the PRL-like substance was detected in the granules of about 100–250 nm in diameter (Terakado *et al*., 1997).

Earlier studies by Pestarino (1984, 1985a, b) showed

the presence of ACTH-like immunoreactive substance and PRL-like immunoreactive substance in the neural gland of another ascidian, *Styela plicata*. Although the existence of these substances in the cerebral ganglion of the same species was also reported by the same investigator (Pestarino, 1983, 1988), the occurrence of pituitary hormone-like immunoreactive substances in the dorsal strand had never been pointed out. On the contrary, we have observed the presence of neither secretory granules nor of ACTH-like and PRL-like immunoreactivities in the neural gland of *H. roretzi*, whereas a considerable number of the cells in the dorsal strand of this species did contain these PRL-like and ACTH-like substances. At moment it is not clear whether the discrepancy between these two species of ascidians in terms of localization of ACTH-like and PRL-like immunoreactive cells in the neural complex is due to the species difference, difference in some physiological conditions of the specimens when examined, difference in properties of antisera used in each experiment or some other reason.

More recently, Pestarino and his group provided an evidence that some of the cerebral ganglion cells of *S. plicata* expressed the mRNA of a POMC-like molecule (Masini *et al*., 1998). In vertebrates, the pituitary POMC molecule is cleaved by prohormone convertases to generate bioactive peptides such as ACTH, α-melanophore-stimulating hormone (α-MSH) and β-endorphin. In fact, the occurrence of immunoreactivities indicating the presence of POMC-related peptides such as ACTH (Fritsch *et al*., 1982, Georges and Dubois 1979, Pestarino 1988) α-MSH (Pestarino, 1988) and β-endorphin (Pestarino, 1985c) in the cerebral ganglion of two species of ascidians, *Ciona intestinalis* and *S. plicata* has been reported. The prohormone convertases that generate the POMC-related peptides are known to be PC1 and PC2 (Benjannet *et al*., 1991; Thomas *et al*., 1991). Very recently we recognized that some cells in the cerebral ganglion as well as in the dorsal strand possessed PC1 and PC2-like substances (Kawahara *et al*., 2001).

On the other hand, immunoreactive GnRH has been localized in the dorsal strand and the cerebral ganglion of *H. roretzi* (Terakado and Ogawa, 1995). Subsequently, two forms of GnRH have been isolated from the neural complex of an ascidian, *Chelyosoma productum* and one of them has been localized in the dorsal strand (Powell *et al*., 1996).

In view of these results, it is highly probable that the cerebral ganglion and dorsal strand of ascidians contain a cell population equivalent to the hypothalamic and/or pituitary cells in vertebrates.

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