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Complement Receptor 3-Like Immunoreactivity in the Superior and Inferior Tentacles of Terrestrial Slug, *Limax marginatus*

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ABSTRACT—Complement receptor 3 (CR3), one of cell adhesion molecules, plays a crucial role in secretion of mammalian neutrophils. To study whether CR3 is also involved in neurosecretion of gastropod molluscs, we examined the CR3-like immunoreactivity in the central nervous system (CNS) and the superior and inferior tentacles of the terrestrial slug, *Limax marginatus*. In the CNS of *L. marginatus*, we did not detect the specific immunoreactivity at all. In contrast, the CR3-like immunoreactivity was observed in the cell bodies and processes of collar cells of the superior and inferior tentacles. In particular, granules contained in the cell bodies of collar cells exhibited the CR3-like immunoreactivity. Retrograde labeling of horseradish peroxidase applied on the sensory epithelia (SE) of superior and inferior tentacles showed that the collar cells of these tentacles project their processes to the SE. In the previous study, homogenate of the superior tentacles injected into the body cavity of slugs stimulated spermatogenesis and simultaneously inhibited egg-laying behavior, suggesting that the tentacular hormone acts as gonadotropic hormone. Taken together, CR3 expressed in the collar cells is considered to be involved in the secretion of tentacular hormone in *L. marginatus*.

INTRODUCTION

Complement receptor 3 (CR3) is a C3bi-specific receptor, which was identified in rosette formation of C3bi-coated erythrocytes with neutrophils and other blood cells in human (Ross and Lambris, 1982). Intracellular CR3 is localized in specific granules in human neutrophil (O'shea *et al*., 1985; Brown *et al*., 1991). Treatment of human neutrophils with phorbol myristate acetate leads to the highest rates of specific granule exocytosis and doubling of average density of CR3 in the membrane (Pryzwansky *et al*., 1991). These dense concentrations of CR3 are co-localized with secreted lactoferrin, a specific granule marker. Therefore, incipient CR3 appears on the cell surface in high concentration at the sites of specific granule exocytosis. In invertebrates, the CR3-like immunoreactivity was observed in the neurosecretory cells, the light green cells (LGCs) and the canopy cells (CCs), in the central nervous system (CNS) of the pond snail, *Lymnaea stagnalis* (gastropod mollusc) (Hatakeyama *et al*., 2000). The CR3-like immunoreactivity expressed in the LGCs and CCs exhibited meshwork-like structure. The immunoreactivity was also observed along the edges of median lip nerves. The immunoreactive regions in the median lip nerves appeared to form the axonal plates, from which the LGCs and the CCs release molluscan insulin-related peptides (MIPs) into the blood. However, the presence of CR3 has not yet been confirmed in other invertebrates.

In the terrestrial slug, *Limax*, neuropeptides in the neurosecretory cells of the CNS and the superior and inferior tentacles regulate the egg-laying behavior (Lane, 1962, 1964; Van Minnen and Sokolove, 1981; Van Minnen *et al*., 1992). An immunohistochemical study has shown that a putative egg-laying hormone is contained in neurons of the CNS of *L. maximus* (Van Minnen *et al*., 1992). In the superior and inferior tentacles of slugs, so-called "collar cells", which surround the tentacular ganglia and disperse in their digits and whose dendritic processes extend to the sensory epithelia (SE), i.e. the tip of tentacles, contain many granules and release their secretory materials at the SE (nonsynaptic sites) (Lane, 1962, 1964; Bierbauer and Török, 1968; Bierbauer and Teichmann, 1970; Kataoka, 1976; Takeda *et al*., 1987). The granules in collar cells were defined as type II granules (Bierbauer and Török, 1968). These type II granules seem to include phospholipid and lipoprotein (Bierbauer and Teichmann, 1970).

The aim of our present work was to examine whether CR3 is involved in the secretion of secretory materials from the collar cells of *L. marginatus*. We thus studied CR3-like immunoreactivity in the entire CNS and the superior and infe-

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rior tentacles of *L. marginatus* by an immunohistochemical procedure.

MATERIALS AND METHODS

Slugs

Adult specimens of the terrestrial slug, *Limax marginatus*, were used. Slugs were obtained from the laboratory colony or collected in the open field, maintained in the laboratory with 12:12 light-dark cycle at 20°C and fed on paste of rat chow. Anesthesia of slugs weighing about 1.2 g was due to injection of 0.2–0.3 ml of an anesthetizing solution (see Ito *et al*., 1999 for composition).

Immunohistochemistry

Staining method was modified from Hatakeyama and Ito (1999, 2000). The specimens of CNSs and tentacles isolated from anesthetized slugs were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) at 4°C and washed in PBS containing 30% sucrose. After embedding the CNS and tentacles in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA), serial 20 μm frozen longitudinal sections were cut horizontally using a cryostat (Leica, Nussloch, Germany) and collected on aminopropyltriethoxysilane-coated slides. The preparations were left at 4°C overnight, washed in PBS at 4°C for 15 min, and incubated with the primary anti-CR3 antibody at 4°C overnight. The primary mouse anti-CR3 antibody (OX-42; BMA Biomedicals, Augst, Switzerland) was diluted at 1:1000 in PBS. As a control, the sections of CNSs and tentacles were incubated only in the buffer without the primary antibody. The preparations and the control sections were next washed in PBS at 4°C for 15 min, and subsequently incubated in a 1:50 dilution of fluorescein-labeled secondary antibody (sheep anti-mouse antibody; ICN Pharmaceuticals, Aurora, OH) at 4°C overnight in PBS. Finally, they were rinsed with PBS and mounted in a 9:1 dilution of glycerol and 50 mg/ml *p*-phenylenediamine for observation under a fluorescence microscope (Zeiss, Oberkochen, Germany). In Figs. 2D and 3D, a graphic editing software “Corel PHOTO-PAINT” (Ver. 7, Corel Corporation, Ottawa, Canada) was used to eliminate autofluorescence.

Horseradish-peroxidase staining

The 20% solution of horseradish peroxidase (HRP; Roche, Basel, Switzerland) dissolved in distilled water was repeatedly brought into contact with the SE of both the superior and inferior tentacles of anesthetized slugs during a 10-min period. The superior and inferior tentacles were dissected away 2 d later. The tentacles were fixed with 0.1 M phosphate buffer (pH 7.6) containing 1% paraformaldehyde and 1% glutaraldehyde for 4 hr. Serial 20 μm longitudinal sections were cut horizontally on a cryostat. Sections were washed in 0.05 M Tris-HCl buffer at pH 7.6 for 2 min and incubated in 0.1% diaminobenzidine chloride in 0.05 M Tris-HCl buffer at pH 7.6. The histochemical reaction was started by gently adding 30% H_2O_2 (70 μl) into the incubation medium (100 ml). Sections were incubated with this solution for 10 min at room temperature, rinsed in distilled water, dehydrated in ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific, Fair Lawn, NJ). They were observed by a light microscope (Nikon, Tokyo, Japan).

RESULTS AND DISCUSSION

The CNS of *L. marginatus* consists of 11 ganglia (Fig. 1A, B). The tentacular ganglia are located in the two pairs of tentacles, the superior and inferior tentacles (Fig. 1B). Both the tentacles contain the collar cells surrounding the tentacular ganglia (Bierbauer and Török, 1968; Bierbauer, 1970; Bierbauer and Teichmann, 1970), but the eyes and the optic nerves are included only in the superior tentacles (Fig. 1C, D). The collar cells also disperse in the digits of both tentacles (Fig. 1C, D; Bierbauer and Török, 1968; Bierbauer, 1970; Bierbauer and Teichmann, 1970).

We examined the CR3-like immunoreactivity in the CNS and tentacles of *L. marginatus*. In the CNS of *L. marginatus*, no specific immunoreactivity was detected (data not shown). The CR3-like immunoreactivity was observed in the collar cells, which disperse in the digits and surround the tentacular ganglia of the superior and inferior tentacles (Figs. 2A and 3A). Yellow and orange autofluorescence was exhibited mainly in the nuclei (Figs. 2 and 3). In the digits of the superior and inferior tentacles, the immunoreactivity was detected in the cell bodies and processes of collar cells (Figs. 2B and 3B). We could also observe the immunoreactivity in the granules in the cell bodies of collar cells surrounding the tentacular ganglia (Figs. 2C and 3C). By eliminating the autofluorescence by the help of a graphic editing software, the true CR3-like immunoreactivity could be observed (Figs. 2D and 3D).

Silver impregnation has shown that the collar cells of another pulmonate mollusc, *Helix aspersa*, extend their processes to the SE, suggesting that they release the gonadotropic hormone at the SE (Lane, 1964). To examine this morphology in the superior and inferior tentacles of *L. marginatus*, the cell bodies of collar cells of both tentacles were labeled by application of HRP to the SE of tentacles (Figs. 2E and 3E). The collar cells in both tentacles were confirmed to expand their processes to the SE.

In general, the entire periphery of the nervous systems of gastropods is used for hormone release. For instance, the SE of *L. marginatus* is used for the release of secretory materials produced in the collar cells (Takeda *et al*., 1987). In the present study, the granules in the cell bodies of collar cells exhibited the CR3-like immunoreactivity (Figs. 2C,D and 3C,D); the CR3-like immunoreactive processes of collar cells reached the SE of superior and inferior tentacles (Figs. 2B,E and 3B,E). Therefore, the release of secretory materials produced in the collar cells, such as gonadotropic hormone, are probably regulated by CR3. HRP staining showed that all the collar cells were not necessarily stained with HRP, suggesting that collar cells also release their secretory materials at other sites. However, because no CR3-like immunoreactivity was observed in other tissues except for the cell bodies and processes to the SE of collar cells, different mechanisms may be employed in the release at other sites.

In the previous study, we observed the CR3-like immunoreactivity in the CNS of the pond snail, *Lymnaea stagnalis* (Hatakeyama *et al*., 2000). In the CNS of *L. stagnalis*, the immunoreactivity was detected in some neurosecretory cells (LGCs and CCs), which release the MIPs into the blood, as described in Introduction. The immunoreactivity was also observed along the edges of median lip nerves (MLNs), which are the release sites of LGCs and CCs. Although the CCs use the surfaces of MLNs and cerebral commissure for the release of their MIPs, the cerebral commissure was not immunopositive to anti-CR3 antibody. In the present study,

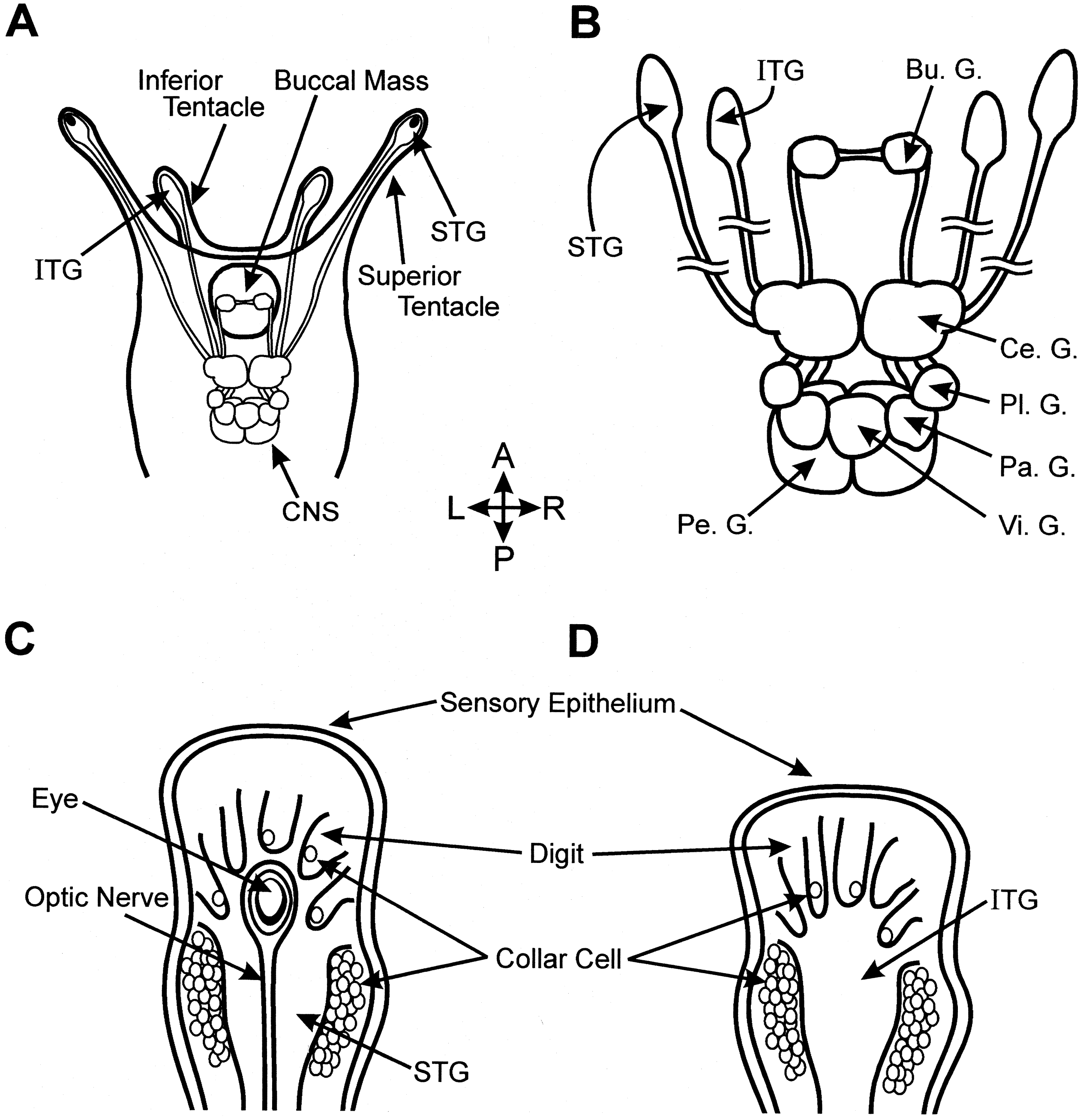

Fig. 1. Schematic drawings of CNS and superior and inferior tentacles in *L. marginatus*. (A) Dorsal view of the *Limax* head. (B) Dorsal view of the CNS of *L. marginatus*. The CNS of *L. marginatus* consists of 11 ganglia, and the superior and inferior tentacles contain the tentacular ganglia, STG and ITG, respectively. (C) Dorsal view of the superior tentacle of *L. marginatus*. Superior tentacle contains the eye and the optic nerve. (D) Dorsal view of the inferior tentacle of *L. marginatus*. The collar cells not only surround the both tentacular ganglia but also disperse in the digits of tentacles. Bu. G.; Buccal ganglion, Pe. G.; pedal ganglion, Ce. G.; cerebral ganglion, Pl. G.; pleural ganglion, Pa. G.; parietal ganglion, and Vi. G.; visceral ganglion. A; anterior, P; posterior, L; left, R; right.

the immunoreactivity was not observed in the CNS of *L. marginatus*, either. Taking into account that Van Minnen *et al.* (1981) suggested the presence of the cells producing a growth hormone in the CNS of *L. marginatus*, the CR3 may not be involved in the hormone release in the CNSs of gastropod molluscs.

This issue can be supported by the following data. The neurosecretory cells were also identified in the CNS of *L. maximus* by a histological procedure (Van Minnen and Sokolove, 1981; Van Minnen *et al.*, 1992). Takeda (1977) demonstrated that the injection of the brain hormone (homogenate of CNS) of *L. flavus* into the body cavity of slug stimulated egg laying. In contrast, the collar-cell homogenate produced from the superior tentacles was shown to have a stimulating effect on spermatogenesis, which is controlled directly by gonadotropic hormone secreted from the hermaphrodite gland (Takeda, 1977, 1982). In the present study, the CR3-like immunoreactivity was detected in the collar cells of

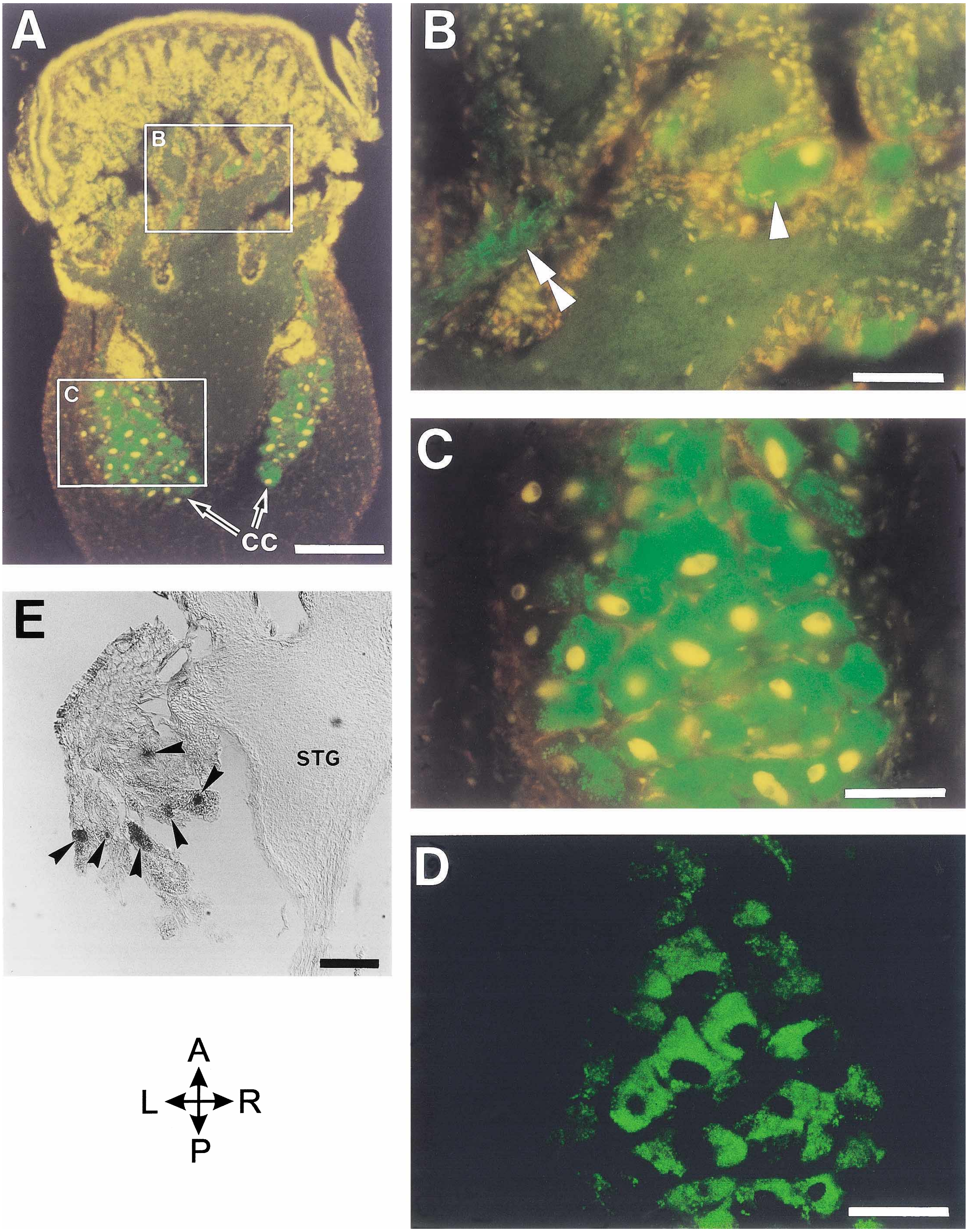

Fig. 2. CR3-like immunoreactivity in superior tentacle of *L. marginatus*. (A) CR3-like immunoreactivity (green) in the right superior tentacle. The autofluorescence was yellow and orange. CC; collar cell. (B) Magnified view of the white-squared area indicated "B" in (A). The immunoreactivity was detected in the cell body (arrowhead) and the processes (double arrowhead) of collar cells in the digit. (C) Magnified view of the white-squared area indicated "C" in (A). The granules in collar cells surrounding the tentacular ganglion exhibited CR3-like immunoreactivity. (D) Elimination of autofluorescence in (C) by the graphic editing software. The true CR3-like immunofluorescence remained. (E) Cell bodies (arrowheads) of collar cells labeled by application of HRP to the SE of superior tentacles of *L. marginatus*. STG; tentacular ganglion of the superior tentacle. Bars = 200 μm (A), 50 μm (B–D), 100 μm (E).

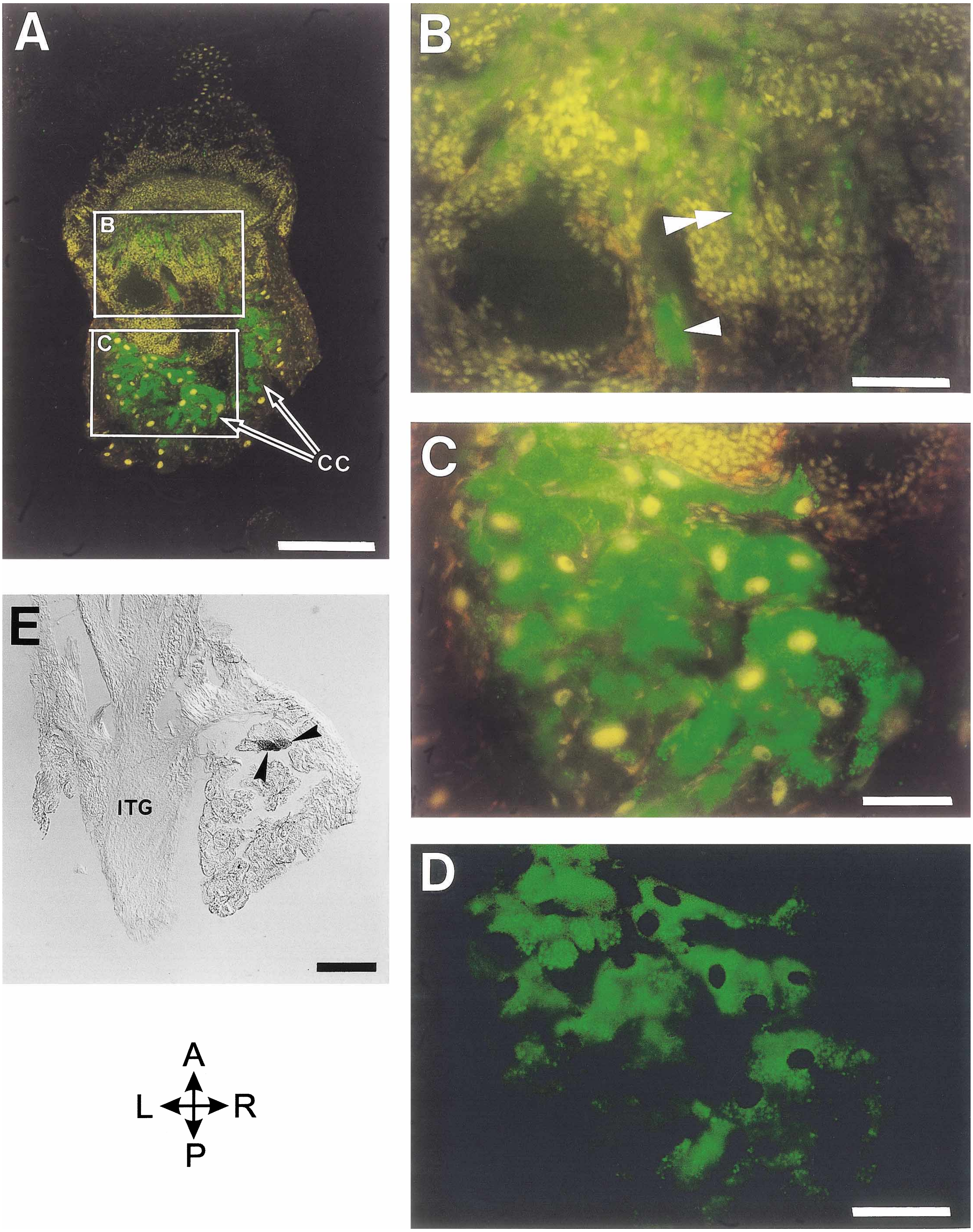

Fig. 3. CR3-like immunoreactivity in inferior tentacle of *L. marginatus*. (A) CR3-like immunoreactivity (green) in the right inferior tentacle. The autofluorescence was yellow and orange. CC; collar cell. (B) Magnified view of the white-squared area indicated “B” in (A). The immunoreactivity was detected in the cell body (arrowhead) and the processes (double arrowhead) of collar cells in the digit. (C) Magnified view of the white-squared area indicated “C” in (A). (D) Elimination of autofluorescence in (C) by the graphic editing software. The true CR3-like immunofluorescence remained. The granules in collar cells surrounding the tentacular ganglion exhibited CR3-like immunoreactivity. (E) Cell bodies (arrowheads) of collar cells labeled by application of HRP to the SE of inferior tentacles of *L. marginatus*. ITG; tentacular ganglion of the inferior tentacle. Bars = 200 µm (A), 50 µm (B–D), 100 µm (E).

the superior tentacles of *L. marginatus*, but not in the CNS. Therefore, CR3 may be involved in the release of gonadotropic hormone in the superior tentacles. The CR3-like immunoreactivity was also detected in the collar cells in the inferior tentacles.

Ito *et al*. (2000) stained the neurons in the superior and inferior tentacles in *L. marginatus* by backfilling of the tentacular nerves with Lucifer yellow. They showed no differences in the morphological features of stained neurons between the superior and inferior tentacles in *L. marginatus*. Although the function of inferior tentacles of slugs is not yet clear (Ito *et al*., 2000), the CR3 of the collar cells in inferior tentacles is suggested to play some roles in the release of their secretory materials as well as in the superior tentacles. The same physiological analyses for the inferior tentacles as those for the superior tentacles may answer the roles of the collar cells in the inferior tentacles, such as ultrastructural observation (Kataoka, 1976; Takeda *et al*., 1987), injection of homogenate of collar cells (Takeda, 1982) and analyses of physiological and ethological changes by removal of tentacles (Takeda, 1979, 1982). Finally, we should insist that the studies for neurosecretion mechanism, as well as those for olfactory-processing mechanism, are needed in the tentacles and CNS of slugs (Suzuki *et al*., 1997; Kimura *et al*., 1999; Sekiguchi *et al*., 1999, Watanabe *et al*., 1999).

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