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Substance P-, Neurotensin- and Bombesin-like Immunoreactivities in the Gill Epithelium of *Ciona intestinalis* L.

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Summary. Substance P-, neurotensin- and bombesin-like immunoreactivities were localised in some gill epithelial cells in the pharynx of *Ciona intestinalis* L. No immunoreactivity was obtained with antisera to gastrin, glucagon, insulin, pancreatic polypeptide or calcitonin. Some of the epithelial cells of the gills were shown to be argyrophilic with the Grimelius technique.

Key words: Immunocytochemical staining – Endocrine cells – Gill epithelium – *Ciona intestinalis* L.

The occurrence of polypeptide hormone-producing cells in the gastrointestinal tract and nervous system of lower vertebrates and invertebrates is well established (Wilson and Falkmer 1965; Mehrotra and Falkmer 1969; Falkmer 1972; Davidson et al. 1972; Falkmer et al. 1973; Van Noorden and Pearse 1974; Fritsch et al. 1976, 1978, 1979; Fritsch and Sprang 1977; Falkmer et al. 1977; Sundler et al. 1977; Van Noorden et al. 1977, 1979; Van Noorden and Falkmer 1980; Van Noorden and Patent 1978; Thorndyke and Bevis 1978; Bevis and Thorndyke 1978, 1979; Thorndyke and Probert 1979). Of special interest are the many peptides which have been found both in endocrine cells of the gastroenteropancreatic system and in neurones of the central and peripheral nervous systems (Pearse 1976, 1977, 1978). In vertebrates neurotensin, substance P and bombesin all belong to this category, and for substance P and neurotensin the dual localisation seems to occur also in invertebrates (Fritsch et al. 1979, 1980b). Since in some protochordates the endostyle region of the pharynx has already been shown to resemble a vertebrate endocrine gland, the thyroid, by virtue of its iodine-concentrating ability (*Branchiostoma lanceolatum*; Barrington 1958) and the groups of granulated

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calcitonin-like cells (*Styela clava*; Thorndyke and Probert 1979), it seemed reasonable to investigate the possibility of the production of peptide hormone-like substances in the prominent pharynx or gill basket of *Ciona intestinalis*.

An immunocytochemical study was therefore undertaken using a variety of antisera to vertebrate peptides in the expectation that positive results would provide insight into the regulatory mechanisms of the gill region of *Ciona* and the evolution of peptide hormonal function in higher vertebrates.

Materials and Methods

Animal Materials. Ciona intestinalis were collected by a diver in the western Baltic sea. The animals (30–50 mm long) were aggregated in colonies which were either attached to brown algae or to secondary sediment. They were kept in aerated sea water (16‰) in glass aquaria at 5°C.

Histological Methods. The gill basket was dissected out and fixed for a short period (3 h) in either Bouin's fluid, methanol-free formaldehyde (Polak et al. 1971) or 6% glutaraldehyde, followed by standard dehydration through graded alcohols, embedding in Paraplast and serial sectioning.

Antibodies. Antibodies to gastrin, glucagon, pancreatic polypeptide, substance P, bombesin, calcitonin and neurotensin were produced in rabbits and to insulin in the guinea pig. They were tested by radioimmunoassay and by immunostaining in mammalian tissues (Polak et al. 1976).

Immunostaining. The indirect immunofluorescence technique (Coons et al. 1955) and the peroxidase-anti-peroxidase (PAP) technique (Sternberger 1974) were used. The primary antibodies were applied for 48 h at 4°C in a range of dilutions from 1/200 to 1/2,000, depending on the titre of the antibody. Fluorescein-conjugated goat anti-rabbit globulin or rabbit anti-guinea pig globulin was used as the second layer for immunofluorescence or, for the PAP method, unlabelled goat anti-rabbit globulin followed by rabbit PAP complex. The attached peroxidase was revealed by the diaminobenzidine reaction. Sections were mounted in buffered glycerine for immunofluorescence or in DEPEX for the PAP stain.

Controls. Specific immunostaining was quenched by preabsorption of the diluted primary antibody with 10 nmol/ml of its specific antigen.

Argyrophilia. Argyrophilic cells were revealed by the silver impregnation technique of Grimelius (Grimelius 1968).

Results

The gill basket of *Ciona intestinalis*, which must be regarded as the pharynx, occupies the main volume of the animal. The terminal buds of the gills, which project into the lumen of the pharynx, are covered by simple, ciliated, columnar epithelium.

With antisera to substance P, bombesin and neurotensin, immunoreactivity was found in these terminal buds in presumptive endocrine cells of the epithelial layer (Figs. 1, 2a, b). No immunostaining was revealed by the other antisera used, and no immunoreactive cells were present in the epithelium of the filament connecting the terminal bud with the pharyngeal wall.

The immunoreactive cells were of wide triangular shape, resting on the basal lamina and with an apical projection that was occasionally seen to reach the ciliated surface of the epithelium (Fig. 1c, d).

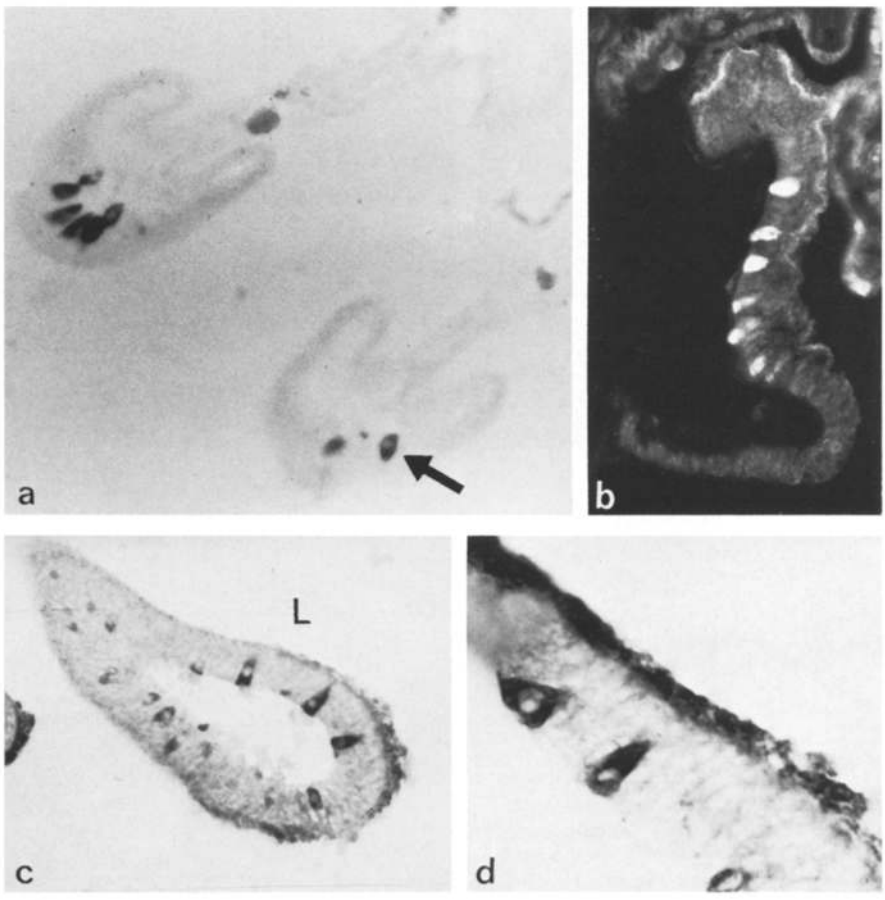

Fig. 1a–d. Immunostaining of the gill epithelium of *Ciona intestinalis*. (**a, b**) Substance P-like immunoreactivity in the terminal bud of the gills. The cells form either clusters (**a**) or appear as single elements (**a**, *arrow*). (**a**) PAP-method, ×800; (**b**) immunofluorescence, ×800. (**c, d**) Neurotensin-like immunoreactive cells distributed evenly within the epithelium. *L* lumen of the pharynx. PAP-method: (c) ×800; (d) ×1,900

The cells immunoreactive with antiserum to substance P occurred either singly or in clusters, but without a recognisable distribution pattern. Sometimes only one or two substance P-positive cells were present in a single cross section of the terminal bud; nevertheless the total number of substance P-immunoreactive cells in each terminal bud seemed to be rather constant.

By comparison, a larger number of cells reacted positively with antisera to neurotensin and bombesin. They showed a regular distribution pattern with approximately equal distances between reactive cells. No cluster formation was observed. The total number of cells reacting positively with either bombesin or neurotensin antiserum was decidedly larger than the number of cells immunoreactive for substance P.

No immunostaining was observed after preabsorption of the antibodies with their specific antigens; preabsorption with albumin did not affect the staining.

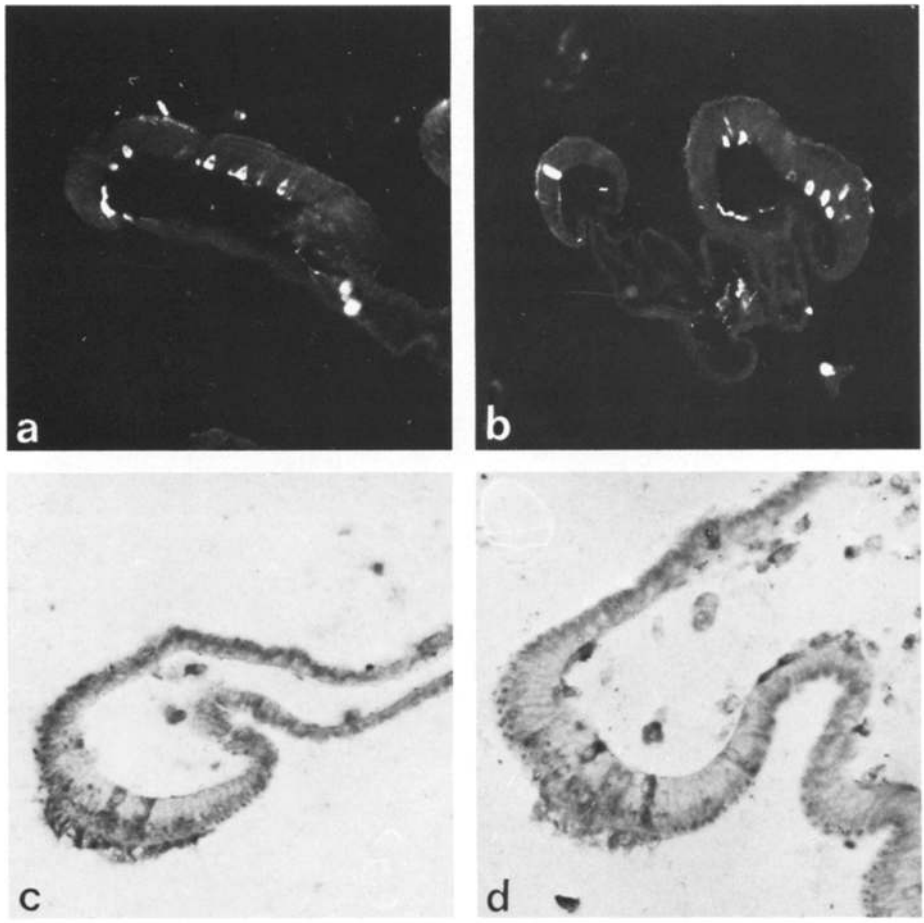

Fig. 2a–d. Immunostaining and silver impregnation of the gill epithelium of *Ciona intestinalis*. (**a, b**) Photomicrographs of the terminal buds of the gills. Immunofluorescence of cells after incubation with anti-bombesin serum. × 390. (**c, d**) Staining for argyrophilia according to Grimelius. Fine granular cells can be observed. × 750

Some cells were argyrophilic with the Grimelius method (Fig. 2c, d). They were triangular single elements in the epithelium of the terminal bud, and thus resembled the immunoreactive cells described above. No cluster formations of argyrophilic cells were found. No particular distribution pattern could be discerned; however, the cells were present in all sections where immunostaining was observed.

Discussion

Unlike the gills of hemichordates, which are primarily respiratory in function, those of *Ciona intestinalis* seem to be involved in mechanisms other than respiration (Millar 1953). It is therefore not surprising to find presumptive endocrine cells in the

pharynx of this animal. Surprising, however, is the exposed localisation of these cells in the terminal buds of the gills and the precise identity of their peptide hormone-like products.

Substance P-like immunoreactivity has been found in the cerebral ganglion of *Ciona intestinalis* (Fritsch et al. 1979), as well as in ganglion cells and nerve fibres of this animal and of the mollusc *Achatina fulica* (Van Noorden et al. 1980). Furthermore, substance P has been identified in extracts of *Ciona intestinalis* (Dahlstedt et al. 1959), as well as in the brain and intestine of bony and cartilaginous fishes (von Euler and Östlund, 1956) and the hagfish (Dahlstedt et al. 1959). Creagh et al. (1979) have recently shown that the immunoreactive substance P-like material of some lower vertebrates and invertebrates is similar to but not identical with synthetic mammalian substance P in radioimmunoassay of extracts. Nevertheless the substance P-like peptide of these animals, and possibly also that of *Ciona intestinalis*, may well resemble substance P in its actions. Substance P has been shown to stimulate intestinal motility in teleosts (von Euler and Östlund 1956) and can be extracted not only from the brain and gut of vertebrates but also from a number of other tissues, including salivary glands, thyroid gland, trachea, pancreas, urinary bladder, prostate gland, smooth muscle and skin (Polak and Bloom 1978a). It is thus possible that the role of substance P as a contractile agent in a wide variety of vertebrate organs is anticipated in the pharynx of *Ciona intestinalis*; this animal probably evolved from an ancestral stock that also gave rise to the vertebrates.

Although neurotensin was originally extracted from the mammalian hypothalamus, larger quantities of the peptide were later found to be present in gastrointestinal tract (Carraway and Leeman 1976). In mammals neurotensin is present in intestinal endocrine cells. Its dual localisation probably reflects two different roles: (i) in the nervous system, that of a peptidergic neurotransmitter or releasing factor, and (ii) in epithelial endocrine cells, that of a circulating or locally acting (paracrine) hormone. The specific localisation of neurotensin-like immunoreactivity in the pharynx of *Ciona* may indicate a physiological role of this peptide in digestion, as suggested for the other hormone-like peptides found in the alimentary tract (Fritsch et al. 1978).

Bombesin was originally extracted from the skin (cutaneous glands) of the anuran amphibian, *Bombina bombina* (Anastasi et al. 1971). Bombesin-like immunoreactivity also occurs in the myenteric plexus of some teleost fishes, particularly in the stomach (Langer et al. 1979; Van Noorden and Falkmer 1980) and in both cells and nerve fibres in the gut of other vertebrates (Polak et al. 1976; Walsh and Holmquist 1976). It is found in very numerous cells in the chicken proventriculus (Timson et al. 1979) and in sparsely distributed endocrine cells of the mammalian lung (Wharton et al. 1978). Its role in mammals is not known but one of its actions is to promote contraction of the smooth musculature of the gastrointestinal tract, the bronchi and the urinary tract (Polak and Bloom 1978b). Further, Erspamer and Melchiorri (1973) have shown that the effect of bombesin on intestinal contractility may be associated with changes in the myoelectrical activity.

The secretion of the bombesin-like cells in the pharynx of *Ciona intestinalis* may exert a similar effect. As ciliary feeders, ascidians possess a highly contractile

pharynx. The presence of immunoreactivity to three peptides in its epithelial cells together with the previously demonstrated localisation of one of them, substance P, in the cerebral ganglion (Fritsch et al. 1979), supports the assumption (i) that there are dual-role hormones in *Ciona*, and (ii) that substances resembling vertebrate hormones must have been present at much earlier stages in evolution.

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