Localization of calbindin D28K-like immunoreactivity in fish gill: a light microscopic and immunoelectron histochemical study

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(Received 10 April 1992; revised version received and accepted 7 July 1992)

Key words: Fish gill; Neuroendocrine cell; Calbindin D28K; Immunohistochemistry

Summary

The presence of calbindin D28K in fish (\textit{Heteropneustes fossilis}) gill was studied by use of specific antibodies raised against chick duodenal 28 kDa calbindin in immunoperoxidase and electron-microscopic labelling experiments. Immunoreactivity for calbindin D28K, which has been observed in the intestine of a number of avian and mammalian species, is reported for the first time in the gill. It was primarily located in neuroendocrine (NE) cells. Some immunoreactivity was also located in the glyco­calyx of the non-endocrine cells, i.e., the pavement cells, which have ultrastructural characteristics quite different from those of endocrine cells.

The calbindin-immunopositive NE cells were ascertained in both gill filamental and lamellar epithelium. All the NE cells contained secretory granules as the most distinctive feature of these cells. Ultrastructurally, two types of NE cells were distinguished according to the morphology of their secretory granules.

The calbindin immunoreactivity in the NE cells was stimulated when the calcium concentration of the ambient water was reduced. The present findings suggest that NE
cells exert some as yet unidentified function related to calcium-mediated processes involving the expression of calbindin.

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**Introduction**

Calbindin D28K is a member of the family of calcium-binding proteins which further includes calmodulin, parvalbumin and calretinin. Calbindin D28K is a cytosolic calcium-binding protein occurring in all the vertebrate species, as well as in some invertebrates. It was first described by Wasserman and Taylor [1] in the chick intestinal mucosa. Certain neurons of the central nervous system, and enterocytes of mammalian and non-mammalian vertebrate species contain high concentrations of this protein [2–5]. Calbindin D28K synthesis in intestine, kidney and pancreas is regulated by 1,25-dihydroxyvitamin D-3. In other tissues, including brain, the expression of the protein seems to be independent of this steroid.

Recently, calbindin D28K was detected by immunohistochemistry in the absorptive cells and the neuroendocrine (NE) cells of gut epithelium of higher and lower vertebrates [3–5]. The protein may play a key role in the calcium transport in these tissues. It was also found in some endocrine glands, and in the diffuse endocrine cells of the gut and the pancreas by Buffa et al. [6], thus suggesting a correlation of calbindin and hormone action.

Calbindin, calretinin and parvalbumin are largely found in neurones [7–10]. A phylogenetic survey of calbindin distribution shows that this protein has an old history and is highly conserved in the vertebrates including fish. In the latter calbindin has been described in the brain and not in kidney or gut [11].

Information on the distribution of calcium-binding proteins in fish epithelia is limited to the epidermal distribution of the protein calmodulin in skin and its mucous layer [12–14]. We have studied the distribution of calbindin in the gills of a teleost fish.

Fish gills are multifunctional organs. While serving primarily as gas exchangers, they are also deeply involved in the acid-base regulation, osmoregulation and calcium uptake [15]. Recent investigations from our laboratory have demonstrated for the first time that there are neuroendocrine cells in the gill epithelium of certain fish species. This was first reported by Zaccone et al. [16] using peptide and serotonin immunohistochemistry.

We studied the localization of calbindin DK28 immunoreactivity in the gill epithelium of the Indian catfish, *Heteropneustes fossilis*. Because of the relation reported in the higher vertebrates between calbindin and the transepithelial calcium transport, we investigated the possible relationship between the presence of calbindin and the calcium concentration of the ambient water. To this end fish were exposed to reduced concentrations of calcium in the water. This procedure has been shown to increase both the calcium uptake across the branchial epithelium as well as the calmodulin content of the mucus in teleost fish [12,15,17].
Materials and Methods

Animals and tissue preparation

Indian catfish, *Heteropneustes fossilis* (Bloch) were provided by a commercial supplier (Euraquarium, Bologna) and acclimated to laboratory conditions before experimentation. The fish were kept in 100-l aquaria at 25°C and fed throughout the experiments with Tetramin tropical fish food and minced beef liver. A first group of fish was maintained in tap water (composition in mmol/l: Na⁺ 3.02, K⁺ 0.22, Ca²⁺ 1.085, Mg²⁺ 1.03, SO₄⁻ 0.50) and served as control. Two other groups of fish were exposed to low ambient calcium levels (freshwater composition in mmol/l: Na⁺ 0.72, K⁺ 0.069, Ca²⁺ 0.2, Mg²⁺ 0.098, SO₄⁻ 0.0208) for 1 month. Some catfish were adapted for 1 month to freshwater containing 0.8 mM Ca²⁺, instead of 0.2 mM Ca²⁺.

At the end of the experimental period the fish were anaesthetized in MS222 and the

![Fig. 1. Section of the gill of *Heteropneustes fossilis* kept in normal tap water. gf = gill filament; gl = gill lamellae. Original magnification × 470.](image-url)
Fig. 2. (a) Calbindin immunostaining of neuroendocrine (NE) cells scattered through the gill filamental and lamellar epithelium. Original magnification $\times 470$. Inset: same structures outlined in a at higher magnification showing presence of two calbindin-immunoreactive NE cells at the basis of the gill lamellae and one within the epithelium lining the terminal branching of the gill. Original magnification $\times 1170$. (b) The proliferative pattern of NE cells in the gill epithelium of fish adapted to low calcium environment (0.2 mM) in comparison with the control fish kept in normal tap water (c). Original magnification $\times 400$. 
gill tissues collected and rinsed in phosphate-buffered saline (PBS) before fixation for both light microscopic and ultrastructural immunohistochemistry.

Antibodies and immunoperoxidase staining

Antisera against chick intestinal calbindin D28K were generously donated by Dr. P. Emson (Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK). The antisera have been successfully used for immunohistochemistry and Western blot experiments in epithelia of various vertebrate species [4,5].

Light microscopic immunohistochemistry

Gill tissues were fixed in a 4% paraformaldehyde solution in 0.1 M PBS, pH 7.4 for 24 h, embedded in Paraplast and sectioned serially at 4 µm thickness. Immunohistochemical staining was carried out using the peroxidase-antiperoxidase (PAP) technique [18]. All the incubations were performed in a humid atmosphere. Hydrated

Fig. 3. Calbindin staining after pre-absorption with calbindin. The immunostaining is completely abolished. No immunoreactive NE cells are visible in this picture. Original magnification x 470.
sections were equilibrated in PBS, pH 7.4 and immersed in 0.1 M hydrogen peroxide solution in PBS for 30 min to block endogenous peroxidase activity, followed by treatment with normal, non-immune 20% goat serum (NGS) for 60 min to reduce non-specific background staining. Then, the sections were covered with the primary antiserum (diluted 1:500–1:1000) for 24 h at +4°C. After washing extensively in PBS for 30 min, the sections were incubated in goat anti-rabbit IgG peroxidase conjugate (Sigma) diluted 1:100 in PBS containing 1% NGS.

After washing the peroxidase sites were revealed by incubating the slides in a freshly prepared solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) (25 mg in 100 ml Tris-HCl (pH 7.6) with 0.1 ml 30% H₂O₂) for 3–8 min.

Controls included replacement of the specific sera by the non-immune goat serum or by the diluted calbindin D28K antiserum preincubated overnight with 6 mg/ml of purified brain calbindin.

**Immunochemistry**

Gill tissues were immersed in a fixative containing freshly prepared 4% paraformaldehyde and 0.1% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 1 h at +4°C. Sections of fixed tissues (40–75 μm) were prepared with a microtome (Sorvall, Inc., Newton, CT). The sections were collected in PBS and the best were selected under a dissecting microscope for cytochemical study.

The tissues sections were treated with reagents according to the following labelling sequence:

1. Blocking of the non-reacted aldehyde groups by glycine 0.02 M in PBS, 30 min.
2. Washing in PBS (three changes).
3. Blocking of the non-specific sites by 20% NGS for 60 min.
4. Labelling by the primary antibody at the dilution 1:1000 for 24 h at +4°C.
5. Washing (three changes) in 20% NGS in PBS.
6. Incubation with peroxidase-labelled goat anti-rabbit IgG (Sigma) diluted 1:100 in PBS containing 1% NGS.
7. Peroxidase labelling with DAB (Sigma) substrate.
8. Washing in PBS (three times).
9. Postfixation in 1% OsO₄ and embedding in Epon 812 and ultrastructural examination without counterstaining with uranyl acetate and lead citrate.

The immunological specificity was demonstrated using the two types of controls adopted for light microscopic immunohistochemistry.

Sections were examined in a Zeiss 902 EM operating at 100 kV.

**Results**

**Structural and ultrastructural morphology**

The gill filaments possess two rows of gill lamellae. The epithelium consists of three major cell types, namely the squamous pavement cells, the chloride cells and the neuroendocrine cells which have been discovered in the species under study very recently by immunohistochemistry [16]. Mucous cells are not seen in these locations.

With the electron microscope the external boundary is lined by squamous pavement...
cells displaying a system of microridges. They are polygonal or columnar in shape and have a conspicuous Golgi apparatus, cytoplasmic vesicles rising toward the apical membrane and deeply infolded parietal membranes. The so-called chloride cells is one of the main features of the gill filament epithelium. The reader is referred for histology to the excellent description provided by Laurent [19].

Neuroendocrine cells are mainly featured by their capacity to be stained by serotonin antibodies and neuropeptide markers [16]. These cells are seen to occur in the basal part of the gill epithelium or resting on the epithelial basal lamina, but some solitary NE cells are also observed near the vicinity of the gill surface. Their main characteristic is the presence of dense-cored vesicles packed in different parts of the cell. Another most significant feature of the NE cells is in their innervation. The electron microscope reveals the occurrence of intraepithelial nerve endings in contact with these cells.

*Light microscopic immunohistochemistry*

The morphology of the catfish gill is shown in Fig. 1. Calbindin D28K immunoreactivity was mainly found in the neuroendocrine (NE) cells (Fig. 2). However, patches of squamous pavement cells in both filamental and lamellar gill epithelium appeared positive, but showed a less intense immunolabelling that could only be observed at higher magnification. The NE cells were scattered along the gill filamental and lamellar epithelium. They were ovoid or triangular and filled with immunoreactive cytoplasmic granules. Most of the NE cells were seen isolated or clustered and facing apparently the mucosal surface. Their distribution in the two epithelia showed that these cells have no preferential localization or clustering. The positive staining with calbindin antiserum was totally inhibited by pre-absorption with calbindin in control preparations (Fig. 3). The distributional patterns of calbindin-immunopositive NE cells are very similar to the serotonin and neuropeptide immunoreactive NE cells found in the gill of species studied [16].

![Fig. 4a, for legend see page 204.](image-url)
Immunoelectron microscopy

In the electron microscope immunolabelling was dominant in the NE cells. It was associated with the secretory granules in their cytoplasm. However, some immunoreactivity for calbindin D28K was also detected in the glycocalyx of some pavement cells of the external layer in the filamental and lamellar epithelium.

Based on the ultrastructure of the secretory granules, the calbindin D28K immunoreactive NE cells in the gill were classified into two types (Fig. 4a–d). Type A cells contained round or ovoid granules with a moderate electron density, often with an
Fig. 4. Electron micrographs of the two types of NE cells immunoreactive for calbindin D28K in the gill epithelium. (a) Type A NE cell within the lamellar epithelium with round and ovoid immunopositive endocrine granules. Labelling is also visible in the glycocalyx (G) of a pavement surface cell (PC). Original
irregularly shaped core. In these cells the granules sometimes showed several morphological forms, probably representing different stages of maturation (Fig. 4a). The contents of the granules were homogeneous and separated from the limiting membrane by a distinct electronlucent zone. The diameter of the granules ranged from 140 to 300 nm. Type B cells contained a mixture of small and large rounded granules. Some large granules were irregularly shaped. Most granules exhibited a granular, highly electron-dense material closely bound by a membrane. A narrow electron-lucent rim separated the core and the limiting membrane of some granules. They were 35–270 nm in diameter.

Some calbindin immunoreactive NE cells show a narrow process near to the gill surface epithelium, but no images are available to show their opening, and so confirm their open morphology. In some specimens these cells were covered by a thin layer of superficial pavement cells and thus were located in the second cell tier from the surface.

Calbindin D28K immunoreactive NE cells increased in number in the gill epithelium of fish adapted to low calcium water (0.2 mM and 0.8 mM Ca$^{2+}$). Fig. 2b depicts the proliferative pattern for NE cells in the gills of experimental fish as compared to controls (Fig. 2c).

**Discussion**

In mammals and birds calbindins occur in several tissues. In lower vertebrates and specially in fish their distribution has been reported to be restricted to neural circuits [11,20]. However, the present investigation demonstrates for the first time the presence of calbindin D28K in the gill epithelium of fish.

We show the occurrence of calbindin immunoreactivity in the glycocalyx of the surface epithelial lining and the NE cells of fish gill. The highest intensity of labelling is associated with the NE cells that are easily recognizable by their shape, the occurrence of dense-cored vesicles and the pattern of their innervation. They are found both in lamellar and filamental epithelium and sometimes are seen to be in contact with the mucosal surface epithelium. These cells were seen in these locations in the species studied using serotonin and neuropeptide markers [16]. In order to clarify whether serotonin and calbindin coexist in the same cells double labeling experiments are being investigated.

The NE cells possess membrane bound vesicles as the most distinctive cytoplasmic feature, and vary in morphology and size. Two types of NE cells are identified at the ultrastructural level.

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magnification × 13,500. (b) Typical NE cell (type A) from the gill filament epithelium with round granules. M, mitochondria; CC, chloride cell. Original magnification × 28,600. (c) A cluster of two type A NE cells within the gill filament epithelium with immunopositive secretory granules. CC, chloride cell. Original magnification × 16,500. (d) Type B NE cell from gill lamellar epithelium. One notices a mixture of the round large and small granules containing an immunoreactive granular material. PC, pavement surface cell. Original magnification × 28,600. The immunohistochemical pictures (Figs. 2–4) are taken from the gill of adult specimens kept in low calcium ambient.
Many, although not all, endocrine cells of higher vertebrates that contain calbindin are known or suspected to be involved in calcium metabolism. Previous studies conducted on the kidneys and intestines of mammals and lower vertebrates point to a role of calbindin in calcium translocation, including the immunopositive calbindin NE cells of the gut epithelium [21–24]. In this respect the occurrence of calbindin in the NE cells of both lamellar and filamental epithelium is of interest. In fact, it has been proposed that the lamellar epithelium, as well as the filamental epithelium participate in the uptake of ions through the chloride cells [25]. Thus, it is possible that both epithelia where calbindin is expressed represent a site of calcium uptake. This implies that a direct or indirect role of the NE cells of the gills in the regulation of calcium homeostasis in fish is possible.

That the NE are functionally related to ion-transport in the gills is indicated by our observation of an increase of the number of cells in catfish exposed to a reduction of the water calcium concentration. Such a reduction leads to an increase of the branchial calcium uptake in the cichlid Oreochromis mossambicus [26]. However, a reduction of the water calcium concentration also increases the permeability of the gills to water and ions [17]. The resulting passive losses of monovalent ions will elicit compensatory responses such as an increased active uptake of monovalent ions via the gills. Thus, although there are indications for an involvement of the NE in calcium transport, a more general action of these cells in the regulation of branchial ionoregulation can certainly not be excluded.

All the immunohistochemical features of the NE cells described here leave open the question of how the presumed local endocrine function of these cells is affected. Calbindin immunopositive cells of the gills qualify as paraneurons based on the basis of their immunohistochemical and ultrastructural characteristics, or as receptosecretory cells – a term proposed very recently by Bailly et al. [27].

Calbindin D28K distribution in the gill agrees with the immunohistochemical findings reported by Parmentier [4] and Pochet et al. [5] in the gut mucosa, i.e., immunolabelling was mainly restricted to the NE cells.

Apart from the NE cells we found calbindin immunoreactivity at the glycocalyx of the epithelial lining of the gill filaments and lamellae. The active uptake of calcium across the gills is probably concentrated in the chloride cells. Biochemical analysis of the gills of fish resulted in the demonstration of an ATP-dependent Ca\(^{2+}\) -transporting enzyme and a sodium gradient driven Ca\(^{2+}\) -exchanger in the basolateral cell membranes of these cells [28]. It is possible that the calbindin present at the surface of the epithelial lining is functionally related to the transport of calcium across the gill epithelium. The amount of calbindin was increased after reduction of the water calcium concentration. However, the location of calbindin does not coincide with the chloride cells but with only the neuroendocrine cells which do proliferate in this medium in comparison with the control fish showing a few number of these cells in the gill. Thus, the question whether calbindin is functionally related to the calcium transport in the gills as well as whether differences in the calbindin contents of NE cells and in the proliferation status correspond to a real distinction of two classes of cells, need further investigation. Nevertheless, the presence of substantial amounts of calbindin in the gills, and the circumstance that their amounts can be changed by manipulating the
calcium concentration of the water, make these gills a useful model to study the function of calbindin in both NE cells and in calcium transporting epithelia.

Acknowledgements

We thank Prof. G. Cimino for his help with the production of analytical data of the freshwater during the course of the experiments, Mr. A. Raffa for the excellent EM technical assistance and Mr. V. Sidoti for the photographic work. This investigation was supported by MURST Grants (40% and 60%), Rome, and an operating grant of the Dutch Organization for Scientific Research (NWO) to Prof. S.E. Wendelaar Bonga.

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