Peanut lectin binds to a subpopulation of mitochondria-rich cells in the rainbow trout gill epithelium

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Received 9 January 2001; accepted in final form 11 June 2001

Goss, Greg G., Sophia Adamia, and Fernando Galvez. Peanut lectin binds to a subpopulation of mitochondria-rich cells in the rainbow trout gill epithelium. Am J Physiol Regulatory Integrative Comp Physiol 281: R1718–R1725, 2001.—Fluorescently labeled peanut lectin agglutinin (PNA-FITC) was used to identify a subtype of mitochondria-rich (MR) cells in the gills of freshwater rainbow trout. In situ binding of PNA-FITC was visualized by inverted fluorescence microscopy and found to bind to cells on the trailing edge of the filament epithelium as demonstrated by differential interference contrast optics. The amount of PNA-FITC binding on the filament epithelium increased with cortisol pretreatment concomitant with an increased chloride cell fractional area as demonstrated by scanning electron microscopy. Dispersed gill cells were isolated by trypsinization and separated using a discontinuous Percoll density gradient. Cells migrating to the 1.06–1.09 g/ml interface were found to be MR as demonstrated by staining with the vital mitochondrial dye 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide and transmission electron microscopy (TEM). However, only ~40% of the MR cells were found to bind PNA-FITC. Cortisol pretreatment increased the relative numbers of MR cells isolated from the dispersed gill cell population, but the relative proportions of PNA binding cells remained unchanged. Ultrastructural analysis of isolated cells in the TEM demonstrated that the MR cell fraction was comprised of a mixed population of chloride cells and pavement cells.

chloride cells; pavement cells; peanut lectin; peanut lectin agglutinin; scanning electron microscopy; transmission electron microscopy; fish

IN FRESHWATER FISH, THE GILLS are the primary sites for Na⁺ and Cl⁻ uptake, with both the cellular localization and identity of transporters being the subject of much debate. The freshwater fish gill epithelium (FFGE) is a heterogeneous epithelium primarily composed of pavement cells (PVC) and chloride cells (CCs; Ref. 19). The heterogeneous nature of the gill epithelium and the lack of identified transporters make it difficult to definitively assign function to each particular cell type or determine if there are subtypes of these cells. It is generally accepted that the location of Cl⁻ uptake/base excretion is the CC, based on correlations between CC fractional area and unidirectional Cl⁻ uptake (3, 5, 6). However, considerable debate exists on both the mechanism and the cellular localization of Na⁺ uptake/H⁺ excretion. Traditionally, the CC was viewed as the sole site of ion transport (10, 11, 14). However, recent studies using immunocytochemical localization suggest that the PVC is the site of H⁺ excretion and Na⁺ uptake (21, 23).

On the basis of staining patterns in the transmission electron microscope (TEM) and distinct cellular localizations, Pisam and co-workers (19, 20) proposed existence of two CC subtypes. According to the nomenclature proposed by these authors, the α-CCs are localized at the base of lamellae and can be characterized by light staining and a smooth apical membrane, whereas β-CCs are located in the interlamellar portion of the filaments, are smaller in size, have an invaginated or wavy apical membrane, are stained darker, and have the nucleus and mitochondria located toward the base of the cell. To try to assess a functional difference between these cell subtypes, Wong and Chan (24, 25) used a density Percoll separation to isolate relatively pure populations of mitochondria-rich (MR) cells from the gill epithelium of Japanese eel. On the basis of flow cytometry, these authors also suggest that there are two subtypes of CCs in freshwater eels, with corresponding changes in cell size, cell internal complexity, and autofluorescence during seawater adaptation, resulting in a single population. The potential existence of PVC subtypes has not been thoroughly investigated. PVCs, comprising roughly 90–95% of all gill cells, can be generally characterized in the TEM having dense peripheral chromatin (9) and few mitochondria. However, a very small proportion of PVCs are characterized by having larger numbers of mitochondria, a key feature found in ion transporting cells (3). The identity or function of these MR PVCs remain unknown. As a result of this observation, it was suggested that there may a small subpopulation of PVCs that are MR and may be involved in ion and acid/base regulation (3). However, recent studies using immunocytochemical localization suggest that the PVC is the site of H⁺ excretion and Na⁺ uptake (21, 23).

The FFGE shares a number of physiological and morphological features with other well-characterized epithelia, including the cortical collecting duct of the mammalian kidney (19). The cortical collecting duct
epithelium of the mammalian kidney consists primarily of principal cells, similar in ultrastructure to the PVC, and two subtypes of intercalated cells (α-IC and β-IC) that are ultrastructurally nearly identical to the CC of freshwater fish. Peanut lectin agglutinin (PNA), a 120-kDa protein that specifically binds to terminal β-galactosyl residues of glycosylated membrane proteins, has been successfully used as a diagnostic tool to differentiate between the β-IC subtype and the α-IC subtype of the kidney epithelium (12). PNA will bind only to the β-subtype of IC in mammalian kidney collecting duct and not to the α-subtype. Further research identified the PNA binding β-subtype as the base excreting cell (possessing an apical Cl−/base exchange), whereas the α-subtype was shown to possess an apical H+−ATPase (1). This arrangement is similar to the structural separation of Na+/acidic equivalent and Cl−/basic equivalent transport proposed for the fish gill.

The goal of this study was to determine if there is apical binding of PNA to the fish gill in situ and to identify if there are subpopulations of MR cells in FFGE similar to that found in the mammalian cortical collecting duct. We report that PNA binds specifically to the apical membrane of the fish gill, with binding primarily localized to the base of the lamellae and the interlamellae spaces. Of the MR cells isolated using Percoll density gradients (24, 25), PNA binding was found on ~40% of the cells. TEM analysis of this MR fraction demonstrated that it was comprised mainly of two cell types, an MR CC and an MR PVC. We were unable to definitively identify which cell type is PNA positive, but this remains the subject of future investigations. This finding presents an important step for the future isolation and functional identification of the transporters on the different subtypes of MR cells.

MATERIALS AND METHODS

Experimental animals and holding conditions. Freshwater rainbow trout (Oncorynchus mykiss) of either sex were obtained from Alberta Trout Growers and were held indoors in fiberglass tanks with flowing dechlorinated water. The water temperature was maintained at 15°C throughout. The fish were fed daily with commercial fish pellets. Photoperiod was maintained similar to the natural photoperiod in Edmonton, Alberta, Canada.

Cortisol pretreatment. To stimulate a higher number of CCs cells in the gill epithelium, a cortisol injection protocol similar to that previously published was followed (9, 11). Briefly, adult rainbow trout (250–300 g) were lightly anesthetized (MS-222, 1:20,000) and intramuscularly injected with cortisol (hydrocortisone 21-hemisuccinate; 10 mg/kg body wt) daily for up to 6 days. After treatment, fish were killed by ephelial blow, and the gills were removed for further study.

In situ binding of PNA to gill filaments. A portion of the second gill arch was excised from the fish (control or cortisol treated, n = 6 for each treatment) and placed in ice-cold Ca2+/Mg2+-free PBS. All further experimental manipulation was then carried out at 4°C unless otherwise noted. The gill rakers were removed, and individual filaments were separated in such a manner that the pair of filaments was attached to the septum. The filaments could then be incubated in solution containing PNA bound covalently to the fluorescent molecule FITC (PNA-FITC, 20 min; 40 μg/ml in PBS). The filaments were then washed three times to remove any nonspecific staining. As a control for nonspecific staining, filaments were incubated in 0.1 M galactose-PBS solution to saturate the galactose-binding sites of the PNA. These filaments showed no binding of PNA-FITC.

PNA binding was then visualized using a Nikon TE300 inverted fluorescent microscope equipped with ×100 differential interference contrast (DIC) optics, a digital camera (Sensicam, Cooke), and digital capture software (Intelligent Imaging Slidebook). A fluorescent image of PNA-FITC binding was first obtained (excitation 495 ± 10 nm, emission 540 ± 25 nm, dichroic LP 515 nm), and then a DIC image was captured for the same field. This procedure allows for determination of PNA binding sites in isolated filaments.

Gill preparation for scanning electron microscopy. To prepare gill filaments for scanning electron microscopy (SEM), a few filaments from the second gill arch of each of the fish isolated above were immediately fixed with 5% glutaraldehyde paraformaldehyde buffered with sodium cacodylate (0.15 mM, pH 7.4, 290 mOsM) for 1 h at 4°C. After fixation, filaments were washed three times with a sodium cacodylate buffer (0.15 M) followed by dehydration in an ethanol series. The filaments were dried by critical point drying, mounted on an SEM stub, coated with gold particles, and observed in the SEM (Joel model JSM-6301F).

Isolation and fractionation of gill epithelial cells. Trout were removed from the aquarium and killed by ephelial blow. The pericardial cavity was opened, and the gills were perfused through the bulbous arteriosus with 50 ml heparinized (25 IU/ml) Ca2+/Mg2+-free Cortland’s saline (in mM: 139 NaCl, 5 KCl, 5 NaHCO3, 3 Na2PO4, 5 glucose, pH 7.8, 290 mOsM). The gill arches were excised from the fish and immediately placed into ice-cold PBS and washed three times for 5 min each. The gill filaments were then carefully cut from the gill rakers and incubated three times (each 30 min) at 18°C in trypsin solution (trypsin 0.05% in PBS + 0.02% EDTA) with vigorous agitation (~200 rpm). The resulting cell suspension was passed through a series of nylon meshes (335, 94, 60, and finally 25 μm mesh) to isolate single cells. The mixed single cell population was then centrifuged (250 g for 5 min, 18°C), and the cell pellet was washed three times and resuspended in PBS (Ca2+/Mg2+-free). The cells were layered over the step gradient consisting of 1.03, 1.06, 1.09 mg/ml (2 ml each) Percoll (Sigma) in PBS in a similar protocol to that recently published (24, 25). The cells layered on the Percoll gradient were then centrifuged (45 min, 2000 g, 18°C), and the bands from each interface were collected, washed two times, and used for analysis as appropriate.

Inverted fluorescent and confocal laser scanning microscopy. Dispersed cells obtained from each fraction of the discontinuous Percoll gradient (1.03, 1.06, 1.09 g/ml) were incubated in PNA-FITC in PBS (20 min, 40 μg/ml) to determine PNA binding. To determine if the cells were MR cells, a common marker of MR cells, 4-(4-(dimethylamino)styryl)-1-methylpyridinium iodide (DASPMI, 20 min, 25 μM), was also used either parallel or in combination with PNA-FITC. After being washed to remove the dye(s) and any nonspecific binding, the cells were placed on glass slides, and fluorescent images were obtained using either a Nikon inverted fluorescent microscope or a confocal laser scanning microscope (PNA-FITC: excitation 494 ± 15; emission 520 ± 20, DASPMI: excitation 475 ± 15 nm, emission 605 ± 15 nm).

Quantification of PNA binding cells. For quantification of PNA binding in each of the fractions, dispersed cells of each fraction and of the initial total dispersed cell population were
obtained from four untreated and three cortisol-treated fish and stained with PNA as above. Total cell counts and the fraction of cells displaying PNA binding were determined by counting >20 fields of view per fish (>500 cells/fish/fraction). Total cell number and distribution to the various fractions during Percoll gradient separation were determined by counting cells using a hemocytometer and bright-field optics.

Preparation of isolated cells for TEM. Dispersed cells obtained from the interfaces of the Percoll gradient were washed, pelleted in a microcentrifuge tube, and then fixed by bathing the pelleted cells in a 2.5% glutaraldehyde-3% paraformaldehyde solution (0.15 mM sodium cacodylate, pH 7.4, 290 mOsM; 2 h, 4°C). The cell pellet was then superficially washed twice in PBS and postfixed in 2% osmium tetroxide in distilled water for 1 h at room temperature. The fixed cell pellet was dehydrated through a graded ethanol series (20, 40, 70, 90, and 100% and propylene oxide (PO) (3 times, 10 min each)). Samples were incubated with Epon-PO (1:3 2 h, 1:1 overnight, 100% Epon 1 h) and embedded into Epon at 60°C for 2 days. Ultrathin sections (~90 nm) of the embedded cells were cut using an automatic ultramicrotome (Reichert Ultracut E) using a diamond knife. Sections were collected on nickel grids (300 mesh) and poststained with 1% uranyl acetate for 1 h at room temperature followed by 0.02% lead citrate staining for 1 min. Grids were then examined in the TEM (Philips model 201).

RESULTS

Figure 1 is a series of SEM images of the filament epithelium of trout showing the effect of experimental elevations of circulating cortisol. Under control conditions, there were substantial numbers of CCs displayed on the filament epithelia (Fig. 1A), with additional differences within those CC populations in the degree of apical ornamentation. After only 4 days of cortisol treatment (Fig. 1B), there were significant increases in the apical exposure of CCs, with large increases in the exposed average area. These results are similar to that found previously by our group and demonstrate that we can use cortisol pretreatment to manipulate CC fractional area on the fish gill. In addition, substantial differences in the degree of apical ornamentation of the CCs were evident in both control and cortisol-treated epithelia (Fig. 1C).

Incubation of whole filaments with fluorescently labeled PNA demonstrated specific binding to cells at the base of the lamellae and in the interlamellar epithelium of the filamental epithelium (Fig. 2). Figure 2A shows a DIC image of the filament epithelium of a control trout, with the corresponding fluorescent image of gill PNA-FITC binding shown in Fig. 2B. DIC optics can be used to focus through multiple optical planes and determine when we are directly imaging at the apical surface of the gill in situ. We are able to clearly differentiate when we are imaging directly at the base of the lamellae and can visualize both the lamellar and filamental epithelia readily using this technique. This allows us to determine the parallel location of any fluorescence. Figure 2A shows a typical DIC image of the trout gill filament epithelium, with the base of a lamellae indicated by a bracket. A fluorescent image of the same field showed distinct patterns of binding of PNA-FITC to cells at the base of the lamellae and in the interlamellar region only (Fig. 2B). Little PNA binding was found on the trailing edge of the filament despite the known presence of CCs in this area (Fig. 1). PNA binding to the leading edge of the filament was also negligible (data not shown). To further evaluate
whether PNA was directly binding to some CCs, we used cortisol pretreatment as a tool to induce CC proliferation. Significantly increased PNA staining was observed on the filamental epithelium from cortisol-treated fish (compare Fig. 3A with Fig. 3, B-D) corresponding with the increase in CC fractional area noted in Fig. 1. The intensity and area of the staining increased with increasing time of cortisol pretreatment, with the staining principally found at the base of the filaments and in the interlamellar regions.

To determine if the cells that bind PNA in situ are indeed MR CCs, we used a technique recently published (25) to isolate MR cells by density fractionation and to test PNA binding on each of these fractions. A discontinuous gradient (1.03, 1.06, 1.09 g/ml) was used to separate cells into three different fractions and each fraction could then be examined for PNA binding, whether the cells are MR, and for positive identification of the cell types in each fraction by TEM. Figure 4, A, C, and E, are phase contrast images from each fraction, whereas Fig. 4, B, D, and F, demonstrate the corresponding fluorescent image found for each of those phase images. Cells obtained from the 1.00–1.03 g/ml interface of the Percoll gradient were a heterogeneous mixture of cells, including contaminating red blood cells, mucous cells, some PVCs, and other mixed cell types and cellular debris (Fig. 4A). Approximately 45% of all the dispersed cells migrated to this interface in both untreated and cortisol-treated fish (Table 1), yet there were almost no PNA-positive cells found in this fraction in both control and cortisol-treated fish (Fig. 4B; Table 1). Some nonspecific PNA staining of mucous and smaller particles in this fraction was observed, but there was no cellular staining. Cells obtained from the 1.03–1.06 g/ml interface of the Percoll gradient were more homogeneous in nature, with a
large proportion of these cells having similar morphological characteristics when viewed under phase contrast microscopy (Fig. 4C). Approximately 36% of all cells migrated to this fraction in untreated fish, with very few of the cells demonstrating PNA-FITC binding (Table 1; Fig. 4D). After cortisol pretreatment, the percentage of cells migrating to this layer was reduced slightly from 36 to 28%. However, <3.5% of the 1.03–1.06 g/ml interface demonstrated significant PNA binding in either untreated or cortisol-pretreated fish (Table 1). The cells obtained from the interface of the 1.06–1.09 g/ml layers of the Percoll gradient were larger than those in the 1.03–1.06 g/ml interface (Fig. 4E). Cells that migrate to this interface have the highest density, which would correspond to the distinct ultrastructural characteristics of having a larger number of mitochondria. In the recently published paper by Wong and Chan (25), this fraction was found to contain primarily MR cells. In this fraction, we noted significant binding of PNA-FITC to only some of the cells, giving us at least two MR cell subtypes, one that is PNA positive and one that is PNA negative. Direct quantification of the cells showed that ~20% of all cells migrated to this fraction, yet only ~40% of those cells are PNA positive. Cortisol pretreatment resulted in an increase in the percentage of cells migrating to this fraction (to 30%) corresponding to an overall increase in MR cells. However, the percentage of cells in the 1.06–1.09 g/ml fraction that bound PNA remained the same after cortisol pretreatment, suggesting that both cell types increased in number concomitantly. To determine if indeed all cells in the 1.06–1.09 g/ml interface were MR, we double stained cells with both DASPMI and PNA-FITC and examined them with a confocal laser-scanning microscope (Fig. 5). DASPMI is a vital mitochondria dye that can cross a membrane and enter into the mitochondria in live cells. DASPMI has been used numerous times to identify MR CCs in both freshwater and seawater fishes (8, 18, 22). All cells isolated from the 1.06–1.09 g/ml interface stained positively for DASPMI (Fig. 5A) with approximately equal intensity in all cells. This is noted as the orange/red color in Fig. 5A due to capture of a wide band of fluorescence emission wavelengths. However, a fraction of the cells in this interface also stained positively for both DASPMI and PNA-FITC. Closer examination of the two cells cell located at the top right corner of Fig. 5A revealed both PNA-positive, DASPMI-positive and PNA-negative, DASPMI-positive cells. Separation of the emission wavelengths specific to detect DASPMI and FITC independently using cut-off filters allowed us to see that, whereas both cells in Fig. 5B are DASPMI positive, only one is PNA-FITC positive (Fig. 5C), with the PNA-FITC staining clearly outside of the cell. Cells incubated with PNA-FITC in the presence of 100 mM galactose, a competitive inhibitor of PNA binding, did not show binding.

Table 1. Distribution of total cells and %PNA positive in fractions isolated by Percoll density centrifugation

<table>
<thead>
<tr>
<th>Fraction, g/ml</th>
<th>% of Cells Migrating to Fraction</th>
<th>%PNA-Positive Cells in Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated / Cortisol treated</td>
<td>Untreated / Cortisol treated</td>
</tr>
<tr>
<td>1.00–1.03</td>
<td>46 ± 2 / 42 ± 2</td>
<td>0.16 ± 0.08 / 0.25 ± 0.1</td>
</tr>
<tr>
<td>1.03–1.06</td>
<td>36 ± 3 / 28 ± 3</td>
<td>2.95 ± 1.7 / 3.5 ± 1.1</td>
</tr>
<tr>
<td>1.06–1.09</td>
<td>20 ± 3 / 30 ± 3*</td>
<td>43.62 ± 1.7 / 42 ± 1.9</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 3). *Significantly different from untreated value (P < 0.05).
not show any staining demonstrating specificity of PNA binding.

To definitively examine the cell types present in the 1.06–1.09 g/ml fraction, we isolated cells and fixed them for examination of their ultrastructure in the TEM. There were two cell types predominantly found in the 1.06–1.09 g/ml interface. MR CCs, with a relatively homogeneously stained ovoid nucleus formed a significant fraction of the cells (Fig. 6A). These MR CCs have a dense network of intracellular tubules and vesicles clearly visible in the cytoplasm (Fig. 6B). However, there was also a substantial population of MR cells that had different features from the MR CC. While these cells also had numerous mitochondria present in their cytoplasm, they possessed large irregularly shaped nuclei and had dense chromatin located both in the center and at the periphery of the nucleus (Fig. 6C), yet they did not possess the typical vesiculotubular network normally associated with CCs (Fig. 6D). These features, other than the presence of large numbers of mitochondria, are more typical of PVC morphology. Therefore, we have termed these cells MR PVCs.

**DISCUSSION**

This paper demonstrates that PNA binds specifically to a subtype of MR cell in the gill epithelium of rainbow trout. Binding occurs in both isolated dispersed cells

$$\text{Fig. 5. Dispersed cells harvested from the 1.06–1.09 g/ml Percoll interface were double stained with both the mitochondrial stain 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (DASPMI, 25 \mu M, 20 min) and PNA-FITC (40 \mu g/ml) for 20 min, washed 3 \times \text{in ice-cold PBS, and examined using a confocal laser scanning microscope. A: all cells from this fraction stain positively for DASPMI while others are double stained with both FITC and DASPMI. B: DASPMI staining of the cell at top right of A; C: PNA-FITC only staining pattern of the double-stained cell at the top right of A. Scale bar = 5 \mu M.}$$

$$\text{Fig. 6. Dispersed cells harvested from the 1.06–1.09 g/ml Percoll interface were fixed (2.5\% glutaraldehyde, 3\% paraformaldehyde), poststained in osmium (2\%), imbedded in Epon, and sectioned for examination in the transmission electron microscope. There were 2 principal cellular ultrastructures found in the mitochondria rich (MR) 1.06–1.09 g/ml Percoll interface. A: an MR CC with an inset of high magnification demonstrating the vesicular tubule network present in these cells. C: an MR pavement cell with a high-magnification inset of the cytoplasm demonstrating an absence of a vesiculotubule network. Magnification approximately } \times10,200 (A), \times41,500 (B), \times6,500 (C), \times39,250 (D). \text{Scale bar = 2 \mu M (A and C); 250 nm (B).}$$
and in situ on isolated filaments. Using Percoll density separation to isolate total MR cells, we demonstrated that there are at least two distinct subpopulations of MR cells in the fish gill based on differential staining with PNA. Electron microscopy of the MR cell fraction also showed that this fraction was primarily composed of two distinct cell types. According to a recently published report on the eel gill, this fraction was supposed to be primarily composed of MR CCs (25). However, the assays used by these authors would only differentiate MR cells from non-MR cells and could not be used to identify subtypes of MR cells. In a subsequent paper (24), these same authors suggested subtypes of CCs exist based on characteristics using flow cytometry. However, no TEM analysis of the MR fraction was carried out. It is possible that the two types of cells noted in eels correspond to the two types of MR cells noted in our study. TEM analysis used in the present study definitively demonstrates MR CCs and MR PVCs migrating to this fraction in rainbow trout. It is still possible that there is more than one subtype of CC present in rainbow trout and that we are unable to distinguish it using the current methods. However, the presence of an MR PVC is a novel and interesting finding in our study. Furthermore, differential staining with PNA can now be used as a cellular marker to distinguish functional difference(s) between MR cell subtypes.

While unable to definitively assign which cell type binds PNA from this data, we suggest PNA binds to the MR CC for the following reasons. The pattern of in situ PNA binding to the base of the lamellae closely parallels the location of CCs in the fish gill. Furthermore, cortisol pretreatment, which is known to result in significant CC proliferation (3, 5, 11), resulted in a substantial increase in fluorescently labeled PNA binding in the interlamellar area only. Finally, the kidney collecting duct, a tissue involved in acid-base regulation with similar morphological characteristics to the fish gill (15), showed a similar pattern of differential PNA binding to IC cells. In the kidney, only the base secreting (apical Cl-/HCO3- containing; β-type IC) binds PNA (12). Correlations between Cl- uptake and CC fractional area (3, 4) suggest that the CC in freshwater fish is also the base-secreting (apical Cl-/HCO3- containing) cell type. The functional similarity between the MR CC and the mammalian kidney β-type IC and the fact that only β-type IC binds PNA suggest that the filamental PNA binding cell type in the fish gill is the MR CC.

We propose that there exists a small but significant population of MR PVCs in the fish gill. This classification is based on the classical ultrastructural characteristics noted for PVCs, an irregularly shaped nucleus, dense peripheral chromatin, and a lack of significant vesiculotubular network. However, the cells migrating to the 1.06–1.09 g/ml interface of the Percoll density gradient all have substantial numbers of mitochondria, a feature not classically associated with PVCs. Presumably, MR PVCs have an ionoregulatory function similar to the ionoregulatory function of the MR CC.

There exists some disagreement regarding the localization of the H+/ATPase in fish gill. While most evidence suggests that H+/ATPase is localized on some lamellar PVCs (21), some authors have suggested that the transporter is also localized to CCs (14, 23). It is possible that the disagreement arises because Na+/K+-ATPase immunoreactivity and/or DASPMI staining was used as an indicator of CCs in these studies. Because MR PVCs have elevated numbers of mitochondria (and presumably Na+/K+-ATPase), this would lead to an erroneous conclusion that apical H+/ATPases are located on CCs.

Sullivan and colleagues (21) showed that immunoreactivity to the 31-kDa subunit of the kidney ATPase was primarily on the lamellar epithelium, and this staining increased during exposure to hypercapnia. This staining was found in a region where MR CCs do not appear to be present and was localized to a very small proportion of PVCs in the lamellar epithelium. Furthermore, it has been previously shown that lamellar MR PVCs could be found in the gill epithelium of hypercapnic bullheads (3). It is possible that the MR PVC is the location of the apical H+/ATPase activity in freshwater fish gill. Future experiments will focus on trying to assess this hypothesis.

Cortisol treatment has been shown numerous times to increase the unidirectional rate of both Na+ and Cl- uptake respectively (11), as well as being associated with a large increase in the fractional area of CCs expressed on the apical surface of the gill (7, 16). This is usually represented by an increase of CCs on both the filamental and lamellar epithelium as seen in the SEM. Our data clearly demonstrate an increase in apical PNA binding during cortisol treatment and give credence to our hypothesis that the PNA binding cell is the MR CC. However, we were unable to see an increase in the lamellar PNA binding during cortisol pretreatment. This is despite an expected increase in CCs on the lamellar epithelium during cortisol pretreatment as has been seen previously. It is possible that the MR PVC may be the cell type increasing on the lamellar epithelium. A balance between the relative rate of Na+ and Cl- uptake (i.e., MR PVC and MR CCs) must be maintained during normal acid-base status to prevent relative acidification or alkalinization of the fish due to discrepancies between Cl-/HCO3- exchange and H+/ATPase/Na+ channel activity. In fact, cortisol pretreatment under normal acid-base conditions increases the rates of both Na+ and Cl- uptake (17). We found an increase in the relative number of cells in the 1.06–1.09 g/ml fraction during cortisol pretreatment, yet there was no change in the relative proportions of PNA binding and nonbinding cells. This indicates that the MR CC and MR PVC populations increase concomitantly during cortisol pretreatment.

Lectins have been used previously to localize MR cells in freshwater fish and have demonstrated binding to CCs by Concanavalin A (ConA) and PNA (2, 13, 22). We used differential PNA binding to define subpopulations of MR cells in the freshwater fish gill. The specificity of other lectins (e.g., ConA) has not been
investigated in our preparation. Future experiments will determine if the ConA-positive CCs noted in other studies are the same or a different cell type from the PNA-positive CC. It is possible that other lectins may be used as indicators of different cellular subtypes in freshwater fish.

This study demonstrates the existence of at least two types of MR cells in freshwater fish, one that is PNA positive and one that is PNA negative. Ultrastructural analysis using the TEM demonstrates that these two MR cells types likely represent MR CCs and MR PVCs, respectively. Future research will focus on purification of independent populations and the functional analysis of these cell types. Determination of the types of transport proteins present in the cell membrane should allow us to definitely assign function and identity the various MR cell subtypes found in the fish gill.

We thank Dr. S. D. Reid for critical reading of the manuscript. This work is supported by an National Sciences and Engineering Research Council (NSERC) operating grant and an Alberta Heritage Foundation for Medical Research Independent Establishment grant to G. Goss and an NSERC postdoctoral fellowship to F. Galvez.

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