Wong, Chris K. C., and D. K. O. Chan. Isolation of viable cell types from the gill epithelium of Japanese eel Anguilla japonica. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R363–R372, 1999.—High-purity viable cells with low mitochondria (pavement cells) and mitochondria-rich content (chloride cells) were successfully isolated from the gill epithelium of Japanese eels, using three-step Percoll gradient low-speed centrifugation. Cytochemistry (silver staining for chloride, rhodamine-123, and Mitotracker for mitochondria and actin/spectrin immunofluorescence) and scanning electron microscope images were used to identify the cell types in the gill epithelium of the eel. Pavement cells were isolated at 97 and 98% purity for freshwater- and seawater-adapted eels, respectively, and chloride cells were obtained at 89 and 92% purity. The enzymatic activities of the isolated cells were determined. Na\(^{\text{+}}\)-K\(^{\text{+}}\)-ATPase, Mg\(^{\text{2+}}\)-ATPase, and succinate dehydrogenase were found mainly in the chloride cell. Alkaline Ca\(^{2+}\)-ATPase and low- and high-affinity Ca\(^{2+}\)-ATPase were about twice as high in the chloride cell compared with the pavement cell. Transfer of eels to seawater resulted in enlargement of chloride cell sizes and significant increases in Na\(^{\text{+}}\)-K\(^{\text{+}}\)-ATPase, Mg\(^{\text{2+}}\)-ATPase, and succinate dehydrogenase activities, while all Ca\(^{2+}\)-ATPases declined by ~60–80%. This is the first report demonstrating the successful isolation of freshwater chloride cells and also an exclusive method of getting high-purity seawater chloride cells. The isolated cells are viable and suitable for further cytological and molecular studies to elucidate the mechanisms of ionic transport.

chloride cell; pavement cell; Percoll; mitochondria; adenosinetriphosphatase

The freshwater eel actively takes up Cl\(^{-}\), Na\(^{+}\), and Ca\(^{2+}\) from the water to balance out losses of electrolytes in the urine (2). In seawater, the eel achieves electrolyte homeostasis by actively secreting Cl\(^{-}\) and Na\(^{+}\) (21) into the water, but the role of the gill in Ca\(^{2+}\) homeostasis is not known. The seawater-adapted kidney excretes high levels of Ca\(^{2+}\) (3), which can account for the elimination of the Ca\(^{2+}\)-absorbed along with drinking.

A series of experiments has been used to study ionic transport across the gill epithelia. Using the perfused head and opercular membrane preparations, freshwater chloride cells have been implicated as the possible site of Ca\(^{2+}\) uptake (23, 25, 29), while seawater chloride cells were responsible for Cl\(^{-}\) extrusion (34). The cellular location of Na\(^{+}\) and Cl\(^{-}\) uptake in freshwater environment is still controversial (27). Nevertheless, it is believed that chloride cells should play a principal role in it.

The “chloride-secreting cell” in the gill epithelium was first shown by Keys and Willmer (19) to undergo marked morphological changes following transfer of the eel from fresh to sea water. Since then, the chloride cell has been described in other teleost fishes and shown to respond to a rise in salinity with an increase in number and/or size (15, 31, 35) and to undergo marked ultrastructural changes, including increased mitochondria content, proliferative extension of the tubular system by invagination the basolateral plasma membrane (31, 35), and enlargement of the apical pit into which Cl\(^{-}\) was excreted (6). Na\(^{+}\)-K\(^{+}\)-ATPase/ouabain binding sites of the gill tissue also increased (8, 17, 18, 33). Although a great deal of information has been gathered, the cellular mechanisms involved in ion transport in freshwater and seawater are still not fully understood. One inherent difficulty is the complexity of the gill epithelium, making it impossible to assign a particular function to a specific cell type. The gill is certainly involved in gaseous exchange, acid-base balance, and nitrogen excretion. Some of these actions no doubt take place across the “pavement” epithelial cells, but suggestions have been made that the chloride cell may also be involved.

A relatively enriched chloride cell fraction had been isolated from the gill epithelium of Anguilla japonica (16), A. anguilla (24, 33), Lagodon rhomboides (15), and Opsanus beta (28). However, only limited biochemical and physiological studies had been made on these cells. The present study was conducted to develop methods to isolate and characterize viable chloride cells of high purity from the gills of Japanese eels that had been adapted to different salinities for varying periods of time.

**MATERIALS AND METHODS**

**Animals**

Japanese eels (Anguilla japonica), weighing between 500 and 600 g, were reared unfed in fiberglass tanks supplied with charcoal-filtered aerated tap water for at least 3 wk. The fish were then transferred to 50% seawater and full seawater and killed at predetermined intervals from 1 day to 3 wk.

**Scanning Electron Microscopy**

The excised gill arch (n = 12) was fixed overnight in 2.5% glutaraldehyde in Hanks’ balanced salt solution (HBSS) at room temperature. After dehydration in ethanol and critical point drying with CO\(_2\), the tissue was mounted onto aluminum stubs and coated with gold palladium. The preparation was then examined under a Leica Stereoscan-440 scanning electron microscope.

**Cytochemical Characterization**

Chloride. Gill arches (n = 5) were fixed briefly in buffered Formalin and then immersed in a solution of 1% AgNO\(_3\) and 2% HNO\(_3\) overnight. The tissue was rinsed in distilled water, blotted dry on filter paper, and refixed in Bouin’s fluid. Five-micrometer wax sections were prepared and examined (6).
Mitochondria. Mitochondria in fresh gill tissues (n = 6) were stained by incubation with 1 µM rhodamine-123 (Calbiochem) or Mitotracker (MTMTRos-H2) (Molecular Probes) for ~45 min at room temperature. After washing in 10 mM PBS, the preparation was examined in a laser confocal microscope using an argon light source (Bio-Rad, MRC-600). Tissues stained with Mitotracker were postfixed in 4% (wt/vol) paraformaldehyde in 10 mM PBS (pH 7.2) at 4°C overnight, and the whole tissue was examined under the laser confocal microscope.

Actin and spectrin. Bouin-fixed wax sections of the gill tissue (n = 8) were dewaxed, rehydrated in graded ethanol, and rinsed in PBS. The staining procedure involved pretreatment of tissue sections with 10% normal goat serum in PBS to reduce nonspecific staining, followed by a 1-h incubation at room temperature with antiserum [rabbit anti-actin (1:100) or mouse anti-spectrin (1:500)] and a 1-h incubation with goat anti-rabbit or mouse IgG coupled to FITC (1:40) and then incubation with antiserum [rabbit anti-actin (1:100)] and a 1-h incubation with goat anti-rabbit or mouse IgG coupled to FITC (1:40) and then mounted in a 9:1 mixture of glycerol and PBS and examined by the confocal microscope. The slides were washed 3 × 15 min in the PBS after each antiserum application. Control procedures included the application of preimmune rabbit serum.

Acid mucoid. Acid mucoid material was stained with 1% toluidine blue to detect metachromasia.

Cell Isolation

The eels (n = 46) were anesthetized by tricaine methansulphonate (1–2%). Gill was perfused with a buffered saline (in mM: 130 NaCl, 2.5 KCl, 5 NaHCO3, 2.5 glucose, 2 EDTA, 10 HEPES, pH 7.0), to remove blood cells. Gill arches were excised and washed. Epithelia were scraped off with an underlying cartilage with a glass slide into Ca2+-, Mg2+-free HBSS (Sigma). The scrapings were dispersed by passing through two stainless steel filters with mesh size of 104 and 73.7 µm. They were then digested by 1.25 mg/ml collagenase (Sigma) and 2 mg/ml hyaluronidase (Sigma type I-S) at room temperature for 10–15 min. The suspension was washed by Ca2+-, Mg2+-free HBSS and finally resuspended in 1.06 g/ml Percoll solution. Initially, a step gradient of 1.09, 1.08, 1.07, 1.06, 1.05, 1.04, and 1.03 g/ml was prepared and was centrifuged at 2,000 g, 15°C, for 15–20 min. For subsequent studies, a three-step gradient of 1.09, 1.06, and 1.03 g/ml was used instead. Isolated cell fractions were concentrated by centrifugation, and the pellet was processed for histochemical and scanning electron microscopic examination as in fixed tissues.

Cell Sizing and Counting

Isolated cells were counted and sized using a Coulter Multisizer II, with an orifice tube 70 µm in diameter and with isoton II as electrolyte. The cell count signal was the change of conductance of the electrolyte induced by particle resistance. Isoton II was used as blank, and calibration was carried out with monodiameter particles (PDVB latex 5.06 µm, Coulter). An aperture coincidence correction was below 2%.

Enzymatic Assays

Isolated cells were homogenated in a buffer (250 mM sucrose, 50 mM imidazole-HCl, 2 mM sodium EDTA, and 2 mM β-mercaptoethanol, pH 7.2). The homogenate was centrifuged at 300 g for 10–15 min. The supernatant was further centrifuged at 14,000 g for 20 min. The final supernatant was stored at ~76°C and assayed for Na+-K+-ATPase, Mg2+-ATPase, and Ca2+-ATPase. Protein content was measured by Bio-Rad protein assay kit.
Fig. 1. Scanning electron micrographs showing distribution of chloride cells (arrowheads) on filamental and lamellar epithelia of freshwater (A)- and seawater (B)-adapted eels. Pavement cells and chloride cells are characterized by microridge (single arrows) and microvilli (double arrows) appearance, respectively. C illustrates that apical area (single arrow) was shared by 2 seawater chloride cells (*). Scale bars represent 3 µm.
were located mainly on the trailing (caudal) edge or afferent filamental surface, at the base of the secondary lamellae, and in the interlamellar space. In freshwater gills (Fig. 1A), chloride cells tend to occur singly, whereas in seawater gills (Fig. 1, B and C), two or three chloride cells shared a common depression between pavement cells. The crypt openings measured $\sim 4.34 \pm 0.92 \mu m \times 1.98 \pm 0.41 \mu m$ in freshwater eels. In seawater-adapted eels, the crypt openings were reduced and the longer diameter measured only $3.43 \pm 0.6 \mu m$ ($n = 41$, $P < 0.05$).

In the cross section, the chloride cell could be easily identified by the dense precipitation of silver in the apical pit (Fig. 2A). Inside the cell, there was also a graded concentration of silver precipitates, with densities increasing toward the apical pit ($n = 72$, $r = 0.847$, $P < 0.05$). The silver-impregnated cell was also positive for anti-actin-FITC immunofluorescence. The FITC-green fluorescence was located in the whole cell (total pixel $= 0.27 \pm 0.03 \times 10^6$, $n = 48$) except the nuclear region (Fig. 2B). Chloride cells also showed intense mitochondria staining (Fig. 2C). Red blood cells showed intense staining for spectrin (Fig. 2D). The mucous cell stained with toluidine blue to yield a pink color (metachromasia), but did not stain with mitochondria stains, silver, actin, or spectrin. The pavement cell lining of the gill surface did not stain with silver, mitochondria stains, actin (total pixel $= 0.07 \pm 0.002 \times 10^6$, $n = 38$), or spectrin.

Step Percoll Gradient and Cell Identification

Seven-step Percoll gradient centrifugation yielded cells in eight layers. According to the distribution of cell size, there were four main populations of cells. The top layer (density < 1.03 g/ml) consisted of mucous (showing toluidine blue metachromasia) and dead cells (trypan blue positive). Mucous cells had large and numerous storage vesicles in the scanning electron microscope image (Fig. 3G). Layers 2–4 (1.03 g/ml <
resulted in a 16-fold increase of Na⁺-K⁺-ATPase activities in both cases) (Table 1). Thus adaptation to seawater surface (Fig. 3, A and B). Layers 5–7 (1.06 g/ml < ρ < 1.09 g/ml) were composed of cells with diameter of 10–16 µm. In gills of freshwater eels, these cells had a mean size of 11.5 µm and accounted for 85–98% of harvested cells in these fractions (Fig. 3, C and D). In gills of seawater eels, the mean size was 15.5 µm and accounted for 85–98% of harvested cells. The predominant cell had a granular appearance and stained for mitochondria (freshwater cell: total pixel density 0.18 ± 0.13 × 10⁶; n = 41; seawater cell: total pixel density = 4.21 ± 0.13 × 10⁶; n = 86, P < 0.001) (Fig. 3, E and F), silver, and actin. They were “chloride cells.” The apical pit was well preserved in isolated cells. On the basis of the mitochondria density, two types of chloride cells could be discerned.

Layer 8 (density > 1.09 g/ml) was composed of cells that were ~5 µm in diameter and were identified as red blood cells by their spectrin staining and their ultrastructure (Fig. 3H).

For higher efficiency and yield, a three-step gradient (1.03, 1.06, and 1.09 g/ml) was designed. Again, the top cell layer consisted of mucous cells and dead cells. Layer 2, at the interface between 1.03 and 1.06 g/ml, consisted predominantly of pavement cells (8–10 µm diameter), with the cell purity measuring 97% for freshwater eels and 98% for seawater eels. Layer 3, at the interface between 1.06 and 1.09 g/ml, consisted mainly of chloride cells (10–16 µm), with cell purity of ~89% for freshwater eels and 92% for seawater eels.

Multisizer counting demonstrated that layer 3 cells increased in mean size from 11.5 to 15.5 µm within 4 days after seawater transfer (Fig. 4). Furthermore, for the same weight of starting materials, the yield of layer 3 cells from the seawater-adapted eel (2.95 ± 0.12 × 10⁶) was ~10-fold higher than that of freshwater eel (0.31 ± 0.02 × 10⁶). In contrast, no change in size was recorded for cells in layer 2 following transfer to seawater.

Enzymatic Assays

Enzymatic analyses were based on the cells separated by three-step Percoll gradient centrifugation. In both freshwater and seawater gills, layer 3 cells had significantly higher Na⁺-K⁺-ATPase activities than the corresponding layer 2 cells (6-fold difference in freshwater eel and 30-fold difference in seawater eel, P < 0.001 in both cases) (Table 1). Thus adaptation to seawater resulted in a 16-fold increase of Na⁺-K⁺-ATPase activity in the layer 2 cells (P < 0.001) and an 84-fold increase in the layer 3 cells (P < 0.001). For Mg²⁺-ATPase activity, there was no significant difference between freshwater layers 2 and 3 cells. Following adaptation to seawater, the enzyme activity increased significantly in layer 3 cells (40-fold increase).

The enzyme alkaline Ca²⁺-ATPase was highest in freshwater layer 3 cells, at a level more than twice that in layer 2 cells. Following full adaptation to seawater, the enzyme activity was reduced to ~25% in both groups. For low-and high-affinity Ca²⁺-ATPase, the dependence of Ca²⁺-ATPase activity on Ca²⁺ concentration revealed a relationship representing the sum of two saturable components (Table 2). A high-affinity component was evident below 0.7 µM. Between 0.7 and 1.462 µM, there was minimal change in reaction rates. Above 5 µM, the ATPase activity again rose sharply.

The affinity for Ca²⁺ was determined by presenting the results on an Eadie-Hofstee plot. In freshwater, the Kₘ and Vₘₐₓ of the low-affinity Ca²⁺-ATPase of low-mitochondria cells and mitochondria-rich cells were 13.14 µM Ca²⁺ and 11.93 nmol Pᵢ·mg⁻¹·min⁻¹ and 9.87 µM Ca²⁺ and 48.43 nmol Pᵢ·mg⁻¹·min⁻¹, respectively. For the high-affinity Ca²⁺-ATPase, the Kₘ and Vₘₐₓ of the low-mitochondria cells and mitochondria-rich cells were 0.043 µM Ca²⁺ and 2.57 nmol Pᵢ·mg⁻¹·min⁻¹ and 0.087 µM Ca²⁺ and 12.1 nmol Pᵢ·mg⁻¹·min⁻¹, respectively. Following adaptation to seawater, there were significant declines in the activities of both low- and high-affinity Ca²⁺-ATPase. The Kₘ values were unaltered in most cases, except for the low-affinity enzyme in the layer 3 cells (chloride cells), where seawater adaptation increased the Kₘ from 9.87 to 31.3 µM.

Activities of succinate dehydrogenase (Table 3) were higher in layer 3 than the corresponding layer 2 cells (1.2-fold in freshwater and 3.8-fold in seawater, P < 0.001). The enzymatic activities increased in both layers following adaptation to seawater. Layer 3 cells had eightfold higher succinic dehydrogenase activity than those in freshwater.

Trypan blue exclusion test showed that cell viability was ~90% for the isolated layer 2 cells and 92% for the layer 3 cells. Transfer to seawater had no effect on the proportion of viable cells harvested, although the total number of layer 3 cells harvested increased dramatically.

**DISCUSSION**

Several isolation methods have been used to harvest chloride cells from the fish gill in the past 20 years, such as velocity sedimentation (24), step dextran gradient centrifugation (16), step Ficoll gradient centrifugation (15, 33), and continuous Percoll gradient centrifugation (28). Some methods were applied to seawater fish only and some were applicable to both freshwater and seawater fish. These results strengthen the view that the chloride cells in branchial epithelia are important in mediating the processes of ionic transport. However, the methods produced the isolated cells with either low purity (45–76%) and/or low cellular integrity (16, 33),
which severely limit future cytological and physiological studies. In the present study, we make a great improvement on the methodology, which minimizes the cellular damage and maximizes the purity of the isolated cells. The purity of seawater chloride cells was 92%, which is better than that obtained by Naon and Mayer-Gostan (24) (76%), Hootman and Philpott (15) (60%), Sargent et al. (33) (45%), Kamiya (16) (enriched fraction), and Perry and Walsh (28) (enriched fraction). In addition, this is the first report demonstrating the successful isolation of freshwater chloride cell with purity over 89%. Furthermore, the isolated cells could be used in other functional studies (5), indicating a breakthrough in this area of study.

Among all the gradient forms, Percoll has been selected in this study because of its low viscosity and adjustable osmolarity, making it superior in terms of the maintenance of the cellular integrity. In combination with the step-gradient method, the present results showed that separation could be carried out under a relatively mild gravitational force. To get rid of blood cells, perfusing the gill with heparinized saline was essential. The scrapped epithelium was sieved to remove large clumps and filamental fragments. The high reaggregation tendency of the gill cells produced a problem in cell isolation. Trypsinization tends to produce a viscous complex of isolated cells. To minimize this effect, our preliminary trials showed distinct advantages to omitting trypsin as the tissue dissociation enzyme. A combination of collagenase and hyaluronidase was found to yield a suspension of free single cells. In early studies, a seven-gradient centrifugation was designed. Eight separated layers of cells were obtained. Comparing the freshwater and seawater sample, we noticed that there was an increase in the yield of large-size and high-density cells in the latter. However, the seven-step gradient had low cell recovery, and a longer centrifugation time was required to reach equilibrium. Some of the cells would span among the gradient solution rather than at the interface of the gradient. The three-step gradient centrifugation was found to be more satisfactory. The cells isolated in layer 2 and layer 3 reached in excess of 95% homogeneity, and a high percentage in excess of 90% were shown to be viable by the trypan blue exclusion test.

Colocalization of silver and mitochondria staining provided unequivocal identification of the mitochondria-rich cell as the chloride cell. The high content of actin fibers in the chloride cell probably played an essential role in maintaining cell shape and apical pit architecture for this ion-transporting cell. All three staining procedures confirmed that the chloride cell could be isolated in high purity in the layer 3 fraction of the three-gradient Percoll gradient centrifugation. Biochemical data on Na\(^{+}\)-K\(^{+}\)-ATPase and succinic dehydrogenase also provided support for the identity of this cell. Among the layer 3 cells, some had a very dense concentration of mitochondria whereas others had distinctly fewer mitochondria. Thus two types of chloride cells coexisted in both freshwater- and seawater-adapted eels (unpublished results). These could correspond with the two-cell type previously described in many teleost species on the basis of transmission electron microscopy studies (32, 35). Because pavement cells were not positively stained with any test in this study, surface ridges provided the only reliable criterion for the identification of these cells after isolation. The presence of concentric ridges on the surface of the pavement cell implied an essential function such as increasing surface area for respiration.

Apart from mitochondria-specific fluorescence dyes (rhodamine-123 and Mitotracker). Mg\(^{2+}\)-ATPase (7) and succinic dehydrogenase (33) were also located exclusively in the mitochondria and hence provide a marker for the mitochondria-rich chloride cell. In freshwater eels, chloride cells had 16 and 22% more succinic dehydrogenase activity and Mg\(^{2+}\)-ATPase activity per milligram cellular protein, respectively, compared with the pavement cells. Following adaptation to seawater, both enzymes increased about twofold in the pavement
cells. However, in the chloride cells, succinic dehydrogenase activity increased 8-fold while Mg\(^{2+}\)-ATPase activity increased 40-fold. Thus the seawater chloride cell appeared to be more efficient in generating ATP than succinic acid oxidation. This shift suggested that seawater adaptation actually involved not only an increase in the cell size for the chloride cell but also significant reorganization of the structural units making up the mitochondria inner membranes, boosting its efficiency in ATP production.

The Na\(^+-\)K\(^+-\)ATPase enzyme has been shown to be associated exclusively with the basolateral plasma membrane and its invaginated system of tubules. The enzyme increased by 84-fold in the chloride cell following adaptation to seawater; this must have arisen from the proliferative extension of the intracellular tubule system, which must form an essential component in the Na\(^+-\)extrusion mechanism that operates in the seawater-adapted chloride cell. The results of multisizer counting clearly demonstrated that entry to seawater caused significant increases in the size and number of chloride cells that took place over several days. The increases in size and the yield of chloride cells during seawater transfer provide unequivocal proof of the importance of this cell type in ionic transport in seawater. Cortisol has been shown to be essential in triggering seawater adaptation and Na\(^+\) extrusion in the teleost fish (22). Indeed, cortisol injection into eels maintained in freshwater stimulated production of the seawater-type chloride cell (high mitochondria autofluorescence, large cell size) within 4–6 days (4). On a proportional basis, using the freshwater system as the baseline, the increase in Na\(^+-\)K\(^+-\)ATPase activity following seawater adaptation was much more significant compared with the increase in mitochondria enzymes, e.g., succinic dehydrogenase and Mg\(^{2+}\)-ATPase.

Chloride cells had higher levels of all three Ca\(^{2+}\)-ATPase enzymes compared with the pavement cells. All three enzymes decreased to about the same extent in both chloride cells and pavement cells following transfer of the eel from freshwater to seawater. Considering that there was ~10-fold increase in the yield of chloride cells after adaptation to seawater, the total Ca\(^{2+}\)-ATPase in the gill epithelium could actually increase. Ma et al. (20) first reported there was no distinct difference in gill total alkaline Ca\(^{2+}\)-ATPase activity when freshwater rainbow trout were adapted to seawater. Burdick et al. (1), on the other hand, demonstrated a higher gill alkaline Ca\(^{2+}\)-ATPase activity in seawater-adapted killifish. Ho and Chan (14), working on the Japanese eel, reported a significant rise in serum Ca\(^{2+}\) concentration during the initial 2–4 days following transfer to seawater, but this declined and remained regulated from day 6 onward when branchial alkaline Ca\(^{2+}\)-ATPase also increased and remained high as the eel fully acclimated to the seawater. However, Fenwick (10) reported that alkaline Ca\(^{2+}\)-ATPase activity was higher in gills of freshwater-acclimated American eel than in the gills of seawater-acclimated eel. These contradictory results cast serious doubts on the direct role alkaline Ca\(^{2+}\)-ATPase plays in the active transepithelial transport of calcium through the gills. A better understanding on the role of this enzyme must depend on direct measurements of Ca\(^{2+}\) transport itself.

Table 1. Transport ATPase in gill cells isolated by a three-step Percoll gradient (layer 2 and layer 3): enzymatic activity

<table>
<thead>
<tr>
<th></th>
<th>Na(^+)K(^+)-ATPase</th>
<th>Mg(^{2+})-ATPase</th>
<th>Ca(^{2+})-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkaline</td>
<td>Low affinity</td>
<td>High affinity</td>
</tr>
<tr>
<td>Freshwater eel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer 2</td>
<td>0.55 ± 0.05</td>
<td>1.52 ± 0.06</td>
<td>43.9 ± 1.3</td>
</tr>
<tr>
<td>Layer 3</td>
<td>3.18 ± 0.21†</td>
<td>1.76 ± 0.08‡</td>
<td>100.3 ± 2.9†</td>
</tr>
<tr>
<td>Seawater eel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer 2</td>
<td>9.01 ± 0.52*</td>
<td>2.86 ± 0.12*</td>
<td>12.0 ± 0.6*</td>
</tr>
<tr>
<td>Layer 3</td>
<td>267.6 ± 11.67†</td>
<td>70.27 ± 0.94†</td>
<td>29.8 ± 1.1†</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol·mg protein\(^{-1}\)·min\(^{-1}\); n = 4. *P < 0.001 compared with corresponding values in freshwater eel. †P < 0.001, ‡P < 0.005 between layer 2 and layer 3.

Table 2. High- and low-affinity Ca\(^{2+}\)-ATPase in the gill cells of the Japanese eel adapted to freshwater or seawater

<table>
<thead>
<tr>
<th></th>
<th>Low Affinity</th>
<th>High Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K(_{m})</td>
<td>V(_{max})</td>
</tr>
<tr>
<td>Freshwater eel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer 2</td>
<td>13.14 ± 0.90</td>
<td>11.93 ± 0.24</td>
</tr>
<tr>
<td>Layer 3</td>
<td>9.87 ± 0.28†</td>
<td>48.43 ± 0.83†</td>
</tr>
<tr>
<td>Seawater eel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer 2</td>
<td>16.9 ± 0.28</td>
<td>6.09 ± 0.06*</td>
</tr>
<tr>
<td>Layer 3</td>
<td>31.3 ± 0.34†</td>
<td>20.3 ± 0.35†</td>
</tr>
</tbody>
</table>

Values are means ± SE in \(\mu M\) Ca\(^{2+}\) (for K\(_{m}\)) and nmol·mg protein\(^{-1}\)·min\(^{-1}\) (for V\(_{max}\)); n = 4. *P < 0.001 compared with corresponding values in freshwater eel. †P < 0.001 between layer 2 and layer 3.
Table 3. Succinate dehydrogenase in gill cells isolated by a three-step Percoll gradient (layer 2 and layer 3): enzymatic activity and ratio of transport ATPases to succinic dehydrogenase activity

<table>
<thead>
<tr>
<th></th>
<th>SDH, µmol·mg protein⁻¹·min⁻¹</th>
<th>Na⁺-K⁺-ATPase/ (SDH × 0.001)</th>
<th>Mg²⁺-ATPase/ (SDH × 0.001)</th>
<th>Alkaline Ca²⁺-ATPase/ (SDH × 0.001)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshwater eel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer 2</td>
<td>173 ± 6</td>
<td>0.003 ± 0.0004</td>
<td>0.009 ± 0.0003</td>
<td>0.255 ± 0.0159</td>
</tr>
<tr>
<td>Layer 3</td>
<td>211 ± 13†</td>
<td>0.015 ± 0.0013</td>
<td>0.009 ± 0.0007</td>
<td>0.481 ± 0.0368</td>
</tr>
<tr>
<td>Seawater eel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer 2</td>
<td>444 ± 20*</td>
<td>0.020 ± 0.0020</td>
<td>0.006 ± 0.0003</td>
<td>0.027 ± 0.0007</td>
</tr>
<tr>
<td>Layer 3</td>
<td>1,700 ± 18†</td>
<td>0.158 ± 0.0064</td>
<td>0.041 ± 0.0005</td>
<td>0.018 ± 0.0009</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4. SDH, succinate dehydrogenase. *P < 0.001 compared with corresponding values in freshwater eel. †P < 0.001, ‡P < 0.005 between layer 2 and layer 3.

In conclusion, we demonstrate clearly the method of pemavement and chloride cells isolation and also illustrate the cytological and biochemical changes of the cells associated with external salinities. The consequence of increased Ca²⁺-ATPase in freshwater chloride cell or increased Na⁺-K⁺-ATPase in seawater chloride cell suggested an importance of the cell in mediating Ca²⁺ uptake and Na⁺ extrusion, respectively.

Perspectives

In the past 60 years, our understanding on the gill ion transport has relied on the electrophysiological, morphometric, and biochemical studies of the whole gill preparations. These studies provide unequivocal evidence that strengthens the view of gill transepithelial transport associated with fish osmoregulation. However, the knowledge of the mechanisms of cellular ion transport (especially Na⁺, Cl⁻, and Ca²⁺) in freshwater and Na⁺ in seawater) are still limited. The studies described here on gill cell isolation represent only the first in a series of steps needed to further understand the pavement cells and chloride cells in ionic transport. The next step is to characterize the properties of calcium channels on the apical membrane of chloride cells by using patch-clamp technique. In addition, the decrease of intracellular Ca²⁺ level of freshwater chloride cell on the addition of stanniocalcin (5) suggested that stanniocalcin receptor was expressed in the cells. RNA fingerprinting could be applied to the isolated freshwater and seawater chloride cells to clone the receptor and also to distinguish the differential expression of particular functional genes. The technique developed in this study opens up a new approach to tackle the problems that have yet to be solved.

This work was supported by the Research Grants Council, Hong Kong.

Address for reprint requests: C. K. C. Wong, Dept. of Biology, Hong Kong Baptist Univ., Kowloon Tong, Hong Kong.

Received 31 December 1997; accepted in final form 30 September 1998.
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