KINETICS OF ATP- AND Na⁺-GRADIENT DRIVEN Ca²⁺ TRANSPORT IN BASOLATERAL MEMBRANES FROM GILLS OF FRESHWATER- AND SEAWATER-ADAPTED TILAPIA

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Summary

Plasma membranes of the gills of freshwater- and seawater-adapted tilapia were analyzed for Ca²⁺-ATPase and Na⁺/Ca²⁺ exchange activity. The relative importance of ATP-driven and Na⁺-gradient-driven Ca²⁺ transport in Ca²⁺ extrusion was evaluated on the basis of kinetic analyses in vitro. The Na⁺/Ca²⁺ exchangers in branchial membranes from freshwater or seawater fish displayed similar kinetics. The ATP-driven Ca²⁺ pump, however, showed a somewhat lower affinity for Ca²⁺ in membranes isolated from seawater gills than in membranes from freshwater gills; no difference in Vₘₐₓ was found. The activity of the exchanger was estimated to be 50% of that of the ATP-driven pump at prevailing cytosolic Ca²⁺ concentrations (10⁻⁷ mol L⁻¹).

Opercular ionocyte densities and branchial Na⁺/K⁺-ATPase content were not significantly different in fish residing in fresh water or sea water. We conclude that the gills of tilapia living for prolonged periods in fresh water or sea water do not differ in the make-up of their basolateral membrane with regard to Ca²⁺-ATPase, Na⁺/Ca²⁺ exchange and Na⁺/K⁺-ATPase activity. Apparently, the densities of these carriers suffice for calcium and sodium homeostasis under these vastly different ambient conditions.

Introduction

The major fraction of calcium absorbed by freshwater fish is taken up directly from the water via the gills (Fenwick, 1989). Flux ratio analysis indicates that this uptake is transepithelial: the electrochemical potential for a passive Ca²⁺ flux is directed outwards and, at the same time, a significant Ca²⁺ influx occurs (Perry and Flik, 1988). Moreover, the branchial epithelium of freshwater fish is generally considered to be a tight epithelium with a minimal paracellular flux of ions and water (Wendelaar Bonga et al. 1983; Ogasawara and Hirano, 1984). In a current model for transbranchial Ca²⁺ uptake (Flik et al. 1985c), Ca²⁺ enters the ionocytes (also called chloride cells) through Ca²⁺ channels in the apical membrane (the rate-limiting step; Verbost et al. 1993) and is transported

Key words: Ca²⁺-ATPase, Na⁺/Ca²⁺ exchanger, Na⁺/K⁺-ATPase, plasma membranes, chloride cells, fish gills, tilapia, Oreochromis mossambicus.
across the basolateral membrane to the serosa by a high-affinity Ca\(^{2+}\)-ATPase. High-affinity Ca\(^{2+}\)-ATPase activity has been demonstrated in the basolateral membranes of the ionocytes of tilapia, eel and trout (Flik et al. 1985b,c; Perry and Flik, 1988). There is good evidence that stanniocalcin inhibits the transcellular Ca\(^{2+}\) influx by limiting the entrance of Ca\(^{2+}\) at the apical membrane (Verbost et al. 1993).

In the basolateral membrane of some Ca\(^{2+}\)-transporting epithelia, ATP-dependent Ca\(^{2+}\) transport as well as Na\(^+\)/Ca\(^{2+}\) exchanger activity have been demonstrated (Van Os, 1987; Taylor, 1989). The relative importance of exchanger-mediated and ATP-driven Ca\(^{2+}\) transport in active Ca\(^{2+}\) absorption may differ among tissues. In rat intestinal and renal epithelia, the ATP-driven Ca\(^{2+}\) pump is responsible for the major part of the transcellular Ca\(^{2+}\) transport (Ghijsen et al. 1983; Van Heeswijk et al. 1984); the exchanger appears to provide an emergency system for removal of excess Ca\(^{2+}\) from the cytosol (Van Os, 1987). In the intestine of tilapia, the species studied here, however, the exchanger forms the main driving force for the absorption of Ca\(^{2+}\) (Flik et al. 1990).

No data are available on Na\(^+\)/Ca\(^{2+}\) exchange activity in branchial Ca\(^{2+}\) uptake. We present here the first direct evidence for a basolateral Na\(^+\)/Ca\(^{2+}\) exchanger in fish branchial epithelium in studies with vesicles isolated from plasma membranes. In previous studies on the ATP-driven Ca\(^{2+}\) pump in fish gills (Flik et al. 1985c) it was shown that the capacity of this pump was sufficient to explain the whole-body uptake. The finding of exchange activity in membranes containing Ca\(^{2+}\)-ATPase encouraged us to determine its importance relative to that of ATP-driven transport.

Bearing in mind the differences in Na\(^+\) turnover in freshwater and seawater fish (Maetz, 1974; Evans, 1979) and the presence of a sodium-dependent Ca\(^{2+}\) transport mechanism in gills (in addition to the classical ATP-dependent Ca\(^{2+}\) pump), we compared these mechanisms in gills of freshwater- and seawater-adapted tilapia. We thus hoped to obtain information about the physiological importance of both transporters in freshwater and seawater gills by examining possible adaptive responses in the enzyme kinetics.

**Materials and methods**

**Holding conditions of fish**

Male freshwater tilapia *Oreochromis mossambicus* (Peters) from laboratory stock, weighing around 250 g, were held in 1001 tanks. The aquaria were supplied with running tap water (0.7 mmol 1\(^{-1}\) Ca\(^{2+}\), 25 °C) under a 12 h:12 h light:dark photoperiod. Animals were fed Trouvit fish pellets (Trouw and Co., Putten, The Netherlands) at a rate of 1.5 % body mass per day.

Artificial sea water was prepared by dissolving natural sea salt (Wimex, Krefeld, Germany) to a density of 1.022 g l\(^{-1}\). Fish were first transferred to half-strength sea water and, after 1 day, to full-strength sea water. Fish were kept in sea water for at least 8 weeks before experimentation (8.5 mmol l\(^{-1}\) Ca\(^{2+}\), 25 °C). Food was supplied at 1.5 % body mass per day.

**Plasma analysis**

For the analysis of blood plasma calcium, sodium, potassium and choride contents, a
blood sample was taken by puncture from the caudal vessels using a heparinized syringe with a 23-gauge needle. Blood cells were removed by centrifugation (1 min, 10,000 g) and the plasma was stored at −20 °C until further analysis. Plasma calcium contents were measured colorimetrically using a calcium kit (Sigma Diagnostics). Plasma sodium and potassium contents were determined with a flame photometer (FLM3, Radiometer) coupled to an autoanalyzer (SA-20, Skalar). Plasma chloride content was determined colorimetrically using the method of Zall et al. (1956).

**Analysis of chloride cell density**

One branchial operculum from each fish was removed and incubated for 1 h in a well-aerated 2 μmol l⁻¹ solution of 2-(dimethylaminostyryl)-1-ethylpyridiniumiodine (DASPEI), which stains the mitochondria-rich chloride cells. After rinsing, the inner opercular epithelium was examined in a Zeiss fluorescence microscope at a magnification of 250×. Cells were counted in 20 different squares of the opercular epithelium with a total surface area of 5 mm² per fish. The density of chloride cells is presented as the number of cells per surface area of the inner operculum.

**Membrane biology**

Plasma membranes were isolated from gill tissue scraped off the gill arches as described in detail previously (Flik et al. 1985c). In brief, after homogenization of gill scrapings, blood cells and cellular debris were separated from the membrane fraction by centrifugation. The membranes (from the supernatant) were collected by ultracentrifugation. The fluffy top layer from the resulting pellet was resuspended in buffer using a Dounce homogenizer. This membrane suspension was centrifuged differentially and the basolateral membranes in the resulting supernatant were collected by pelleting. The procedure yields membrane suspensions in which 47–51% of the membranes are resealed vesicles. Vesicles were kept cooled on ice and were used on the day of isolation. The protein content of the vesicle preparations was 1.86±0.4 g l⁻¹ (N=12), as determined with a commercial reagent kit (Bio-Rad) using bovine serum albumin as a reference. Protein recovery was 1.67±0.16 % (N=6) in freshwater preparations; in seawater preparations, protein recovery was slightly, though significantly, lower: 1.23±0.11 % (N=6; P<0.001). Na⁺/K⁺-ATPase activity in fish branchial basolateral plasma membrane vesicles (BLMVs) was determined as described previously for similar preparations of enterocytes (Flik et al. 1990). Na⁺/K⁺-ATPase activity was defined as the Na⁺- and K⁺-dependent ouabain-sensitive phosphatase activity. The orientation of the vesicles was determined on the basis of the acetylcholine esterase activity measured in intact and permeabilized vesicles (reflecting the inside-out oriented vesicles, IOVs) and the specific trypsin sensitivity of the cytosol-oriented part of the Na⁺/K⁺-ATPase (reflecting the right-side-out oriented vesicles, ROVs) as described by Flik et al. (1990). The orientation of the vesicles was similar to previously reported values (Flik et al. 1985c) and not significantly different for freshwater (19.2±4.2 % IOV; 29.8±4.4 % ROV, N=6) and seawater (22.5±4.8 % IOV; 26.8±6.4 % ROV) preparations. In the calculation of Ca²⁺ transport velocities of ATP- and Na⁺-
gradient-driven mechanisms, no correction for IOV content was made (see also Discussion). For determination of total Na\(^+\)/K\(^+\)-ATPase and Ca\(^{2+}\)-ATPase activities, the membrane vesicles were permeabilized with saponin (0.20 mg saponin per mg protein).

\(^{45}\text{Ca}^{2+}\)-transport was determined in assay media that contained 0.5 mmol l\(^{-1}\) EGTA, 0.5 mmol l\(^{-1}\) N-(2-hydroxyethyl)-ethylenediamine-\(N,N',N'\)-triacetic acid (HEEDTA) and 0.5 mmol l\(^{-1}\) nitrilotriacetic acid (NTA) as a Ca\(^{2+}\) buffering system. Free calcium and magnesium levels were calculated using a matrix computer program (Schoenmakers et al. 1992) taking into account the first and second protonations of the respective ligands (ATP, EGTA, HEEDTA, NTA). In this program, the metal–chelator stability constants are corrected for the ionic strength and the temperature of the medium. All incubations were performed at 37 °C, which is the optimum assay temperature.

The ATP-driven transport of \(^{45}\text{Ca}^{2+}\) was determined in 1 min incubations (yielding initial pump velocities; Flik et al. 1985c) of membrane vesicles (45 µg per assay) in the presence of 3 mmol l\(^{-1}\) ATP, 150 mmol l\(^{-1}\) KCl, 20 mmol l\(^{-1}\) Hepes/Tris, pH 7.4, 0.8 mmol l\(^{-1}\) Mg\(^{2+}\) and the Ca\(^{2+}\) buffers mentioned above. \(^{45}\text{Ca}^{2+}\) uptakes were corrected for uptake in the absence of ATP. Free Ca\(^{2+}\) concentrations were varied from 50 nmol l\(^{-1}\) to 1 µmol l\(^{-1}\). The uptake was quenched by a 14-fold dilution of incubate in ice-cold isotonic buffer containing 20 mmol l\(^{-1}\) Tris–HCl (pH 7.4), 150 mmol l\(^{-1}\) KCl or NaCl and 0.1 mmol l\(^{-1}\) LaCl\(_3\). Vesicles were collected by a rapid filtration technique (Van Heeswijk et al. 1984; using Schleicher & Schüll ME25 filters, pore size 0.45 µm). Filters were rinsed twice with 2 ml of ice-cold medium and transferred to counting vials. 4 ml of Aqualuma was added per vial, filters were allowed to dissolve (30 min at room temperature) and radioactivity was determined in a Pharmacia Wallac 1410 liquid scintillation counter.

\(\text{Na}^{+}/\text{Ca}^{2+}\) exchange activity was assayed as described previously (Flik et al. 1990). Briefly, 5 µl of prewarmed membrane vesicles (9–12 µg protein), equilibrated with 150 mmol l\(^{-1}\) NaCl, was added to 120 µl of medium containing \(^{45}\text{Ca}^{2+}\) and either 150 mmol l\(^{-1}\) NaCl (blank) or 150 mmol l\(^{-1}\) KCl. Ionic Ca\(^{2+}\) concentrations ranged from 250 nmol l\(^{-1}\) to 25 µmol l\(^{-1}\). After 5 s at 37 °C, the reaction was stopped by addition of 1 ml of ice-cold isotonic stopping solution (as above) containing 1 mmol l\(^{-1}\) LaCl\(_3\). Vesicles were collected as described above for the ATP-dependent Ca\(^{2+}\) transport assay. The difference in \(^{45}\text{Ca}^{2+}\) accumulation after transfer to Na\(^+\) or K\(^+\) medium was taken to represent \(\text{Na}^{+}\)-gradient-driven Ca\(^{2+}\) transport.

Calculations and statistics

The \(K_m\) and \(V_{\text{max}}\) values of the ATP-dependent Ca\(^{2+}\) pump and the \(\text{Na}^{+}/\text{Ca}^{2+}\) exchanger were derived from best fits of the curves by non-linear regression analysis (with the Levenberg–Marquardt method) of the velocities measured as a function of substrate concentration (computer program by Leatherbarrow, 1987). Values for preparations of individual fish were averaged (\(N=5–7\)) and used for statistical assessment of differences.

Statistical significance of differences between mean values was tested by the Mann–Whitney \(U\)-test. Statistical significance was accepted at \(P<0.05\).
**Results**

**Plasma analysis**

Plasma osmolarity is 6% higher in tilapia held in sea water than in those held in fresh water; plasma calcium, sodium and chloride concentrations are slightly, but not significantly, different from the values found in freshwater tilapia (Table 1), illustrating the euryhalinity of this species.

**Na+/K+-ATPase activity**

As shown in Table 2, the total and specific Na+/K+-ATPase activities in the branchial epithelial homogenate of freshwater- and seawater-adapted tilapia are not significantly different. The Na+/K+-ATPase specific activity in the basolateral plasma membrane (BLM) fraction was 46% higher in seawater preparations than in freshwater preparations but the total Na+/K+-ATPase was not significantly different for freshwater gills and seawater gills (indicating better purification of BLMs from seawater gills). Using the purification values for Na+/K+-ATPase activity in Table 2, we calculate a 15.20/9.36=1.6 times higher purification for this BLM marker of seawater gills than of freshwater gills (P<0.05). This means that the BLMs from seawater gills are better purified than those from freshwater gills when they are isolated using the same procedure.

**Chloride cells**

The numbers of chloride cells in the opercular membrane, which give a good reflection of the numbers in the gills (Wendelaar Bonga *et al.* 1990), are not significantly different for freshwater and seawater tilapia (Table 2).

**Na+/Ca2+ exchange activity**

To compare the Ca2+ transport capacity in freshwater and seawater fish, we have expressed exchange relative to Na+/K+-ATPase activity (as a more specific reference). Using this comparison, the $V_{\text{max}}$ values ($V_{\text{max,corr}}$) are not significantly different for freshwater- and seawater-derived BLMs ($V_{\text{max,corr}}=V_{\text{max,Ca}/V_{\text{spec,Na/K-ATPase}}}$: 19.56±4.80 nmol Ca2+/μmol⁻¹ Pᵢ for FW and 16.92±4.80 nmol Ca2+/μmol⁻¹ Pᵢ for seawater.

<table>
<thead>
<tr>
<th>Table 1. Plasma osmolarity and calcium, sodium and chloride contents of freshwater- and seawater-adapted tilapia</th>
</tr>
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<tbody>
<tr>
<td><strong>Fresh water (N=12)</strong></td>
</tr>
<tr>
<td>Osmolarity (mosmol l⁻¹)</td>
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<tr>
<td>[Calcium] (mmol l⁻¹)</td>
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<tr>
<td>[Sodium] (mmol l⁻¹)</td>
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<tr>
<td>[Chloride] (mmol l⁻¹)</td>
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Values are means ± s.d.

Calcium, sodium and chloride contents are not significantly different in freshwater- and seawater-adapted fish.

*Significantly different from the freshwater value, P<0.001.
Table 2. Total and specific Na⁺/K⁺-ATPase activity in freshwater- and seawater-adapted tilapia gill cell homogenates and purified basolateral membrane vesicles (BLMVs) and the derived BLM purification value and chloride cells density in the opercular membranes of these fish

<table>
<thead>
<tr>
<th></th>
<th>Fresh water (N=5)</th>
<th>Sea water (N=7)</th>
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<tbody>
<tr>
<td>Homogenates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{spec}} )</td>
<td>4.04±0.63</td>
<td>3.64±0.35</td>
</tr>
<tr>
<td>( V_{\text{total}} )</td>
<td>371±58</td>
<td>376±36</td>
</tr>
<tr>
<td>BLMVs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{spec}} )</td>
<td>37.8±5.0</td>
<td>55.3±6.5*</td>
</tr>
<tr>
<td>( V_{\text{total}} )</td>
<td>57.1±7.6</td>
<td>70.2±8.3</td>
</tr>
<tr>
<td>Percentage recovery</td>
<td>15.4±1.0</td>
<td>18.7±1.2</td>
</tr>
<tr>
<td>Purification</td>
<td>9.36±1.05</td>
<td>15.20±2.11*</td>
</tr>
<tr>
<td>Chloride cells</td>
<td>81.0±3.5</td>
<td>90.3±4.2</td>
</tr>
</tbody>
</table>

\( V_{\text{spec}} \), the specific Na⁺/K⁺-ATPase activity in \( \mu \text{mol} \text{ P}_i \text{ h}^{-1} \text{ mg}^{-1} \).

\( V_{\text{total}} \), the total Na⁺/K⁺-ATPase activity in \( \mu \text{mol} \text{ h}^{-1} \) (\( V_{\text{spec}} \times \text{mg protein} \)).

Percentage recovery, \( V_{\text{total,BLMV}}/V_{\text{total,Ho}} \).

Purification, \( V_{\text{spec,BLMV}}/V_{\text{spec,Ho}} \).

Chloride cells, number of chloride cells (DASPEI-stained cells) per mm² of opercular membrane.

Values are mean ± s.e.m.

*Significantly different from the freshwater value, \( P<0.05 \).

BLMVs, basolateral plasma membrane vesicles; Ho, cell homogenates.

gills), which means that the density of the exchanger relative to Na⁺/K⁺-ATPase activity is similar. The affinities for Ca²⁺ (\( K_m \)) of the branchial Na⁺/Ca²⁺ exchangers are also similar in BLMVs from freshwater- and seawater-adapted tilapia (Fig. 1).

**ATP-driven Ca²⁺ transport**

The \( V_{\text{max}} \) of the Ca²⁺ pump, expressed relative to Na⁺/K⁺-ATPase activity, does not differ in BLMVs from freshwater and seawater gills (Fig. 2), indicating similar pump densities (\( V_{\text{max,corr}}=V_{\text{max,calc}}/V_{\text{spec,Na/K-ATPase}} \); 7.50±1.08 nmol Ca²⁺·µmol⁻¹·Pᵢ for freshwater and 7.38±0.84 nmol Ca²⁺·µmol⁻¹·Pᵢ for seawater gills). When the values are not corrected for the difference in purification, the \( V_{\text{max}} \) in seawater BLMs appears to be higher than in freshwater BLMs (\( P<0.005 \)). The affinity of the pump for Ca²⁺ was significantly lower in BLMVs from seawater tilapia than in BLMVs from freshwater tilapia (Fig. 2).

High-affinity Ca²⁺-ATPase activity, determined as Ca²⁺-dependent ATP hydrolysis (Flik et al. 1984), amounts to 2.28±0.64 \( \mu \text{mol} \text{ P}_i \text{ h}^{-1} \text{ mg}^{-1} \) and 3.87±0.85 \( \mu \text{mol} \text{ P}_i \text{ h}^{-1} \text{ mg}^{-1} \) (\( N=6 \), ± s.e.m.) for freshwater and seawater BLMs, respectively. The ratio of this Ca²⁺-ATPase activity to Na⁺/K⁺-ATPase activity is not significantly different for BLMs obtained from freshwater and seawater gills (2.28/37.8=0.060 and 3.87/55.3=0.070, respectively), which indicates a similar density of the Ca²⁺-ATPase under both conditions.
ATP- and Na+-gradient driven Ca²⁺ transport

Fig. 1. Ca²⁺-dependence of Na⁺-driven Ca²⁺ uptake in basolateral membranes from freshwater- (FW, filled circles) and seawater- (SW, filled squares) adapted tilapia. The $V_{\text{max}}$ and $K_m$ values were the average values of five (for FW) to eight (for SW) gill membrane preparations (for further details see Materials and methods). These values are not significantly different. The $V_{\text{max}}$ values expressed relative to Na+/K+-ATPase (from Table 2), to eliminate purification differences between the FW and SW membranes, are also comparable ($V_{\text{max,corr}}$ for FW=19.56±4.80 nmol Ca²⁺ min⁻¹ mg⁻¹ and for SW=16.92±4.80 nmol Ca²⁺ min⁻¹ mg⁻¹). Mean values ± standard errors are presented.

Comparison of Ca²⁺ pump and Na⁺/Ca²⁺ exchange transport activities

The kinetic data presented in Figs 1 and 2 are used to plot the potential activities of both Ca²⁺ extrusion systems as a function of intracellular Ca²⁺ concentrations in Fig. 3. These results predict that, at resting levels of intracellular Ca²⁺ of approximately $10^{-7}$ mol l⁻¹, the Ca²⁺ pump activities exceed the Na⁺/Ca²⁺ exchange activities in freshwater and seawater BLMs by factors of 1.8 and 1.5, respectively. At $10^{-6}$ mol l⁻¹ Ca²⁺, the activity of the exchanger exceeds that of the pump by a factor of 1.2 in freshwater and in seawater BLMs.

Discussion

The activities of three ion carriers in the basolateral membrane from gill epithelium of freshwater and seawater tilapia were measured in vitro. The Na⁺/Ca²⁺ exchange activity in this membrane displayed similar kinetics ($V_{\text{max}}$, $K_m$) in freshwater and seawater gills. The ATP-driven Ca²⁺ pump showed a somewhat lower affinity for Ca²⁺ in BLMs isolated
from seawater gills than in BLMs from freshwater gills, whereas \(V_{\text{max}}\) was the same. The significance of this change in \(K_{\text{m}}\) for cellular calcium transport is discussed below (\(Ca^{2+}\) transport across the basolateral membrane). The \(V_{\text{max}}\) values were expressed relative to \(Na^+/K^+\)-ATPase activity to eliminate purification-dependent differences between BLM preparations. This correction allows a comparison of the relative density of the carriers between freshwater and seawater gills. Because the \(V_{\text{max}}\) values of the exchanger and the \(Ca^{2+}\) pump do not differ in BLMs from freshwater and seawater fish, it follows that the density of these carriers is similar in freshwater and seawater gills. Total exchanger capacities, calculated as the product of \(V_{\text{max}}\) and total protein in the homogenate (and adjusted to 100% IOV and 100% recovery), amount 2297±566 and 2294±650 \(\mu\)mol \(Ca^{2+}\)h\(^{-1}\) at 37°C in freshwater and seawater gills, respectively. Total \(Ca^{2+}\) pump capacities, calculated in the same way, amount to 877±125 and 998±113 \(\mu\)mol \(Ca^{2+}\)h\(^{-1}\) in freshwater and seawater branchial epithelium (average total protein in homogenates was 92 mg for freshwater gills and 103 mg for seawater gills). The branchial \(Ca^{2+}\) influx...
Fig. 3. Activities of the basolateral plasma membrane Ca²⁺ pump (Ca²⁺-ATPase) and the Na⁺/Ca²⁺ exchanger in gills from seawater- and freshwater-adapted tilapia as a function of intracellular Ca²⁺ concentration.

In vivo for a tilapia of the same size (approximately 7.5 μmol h⁻¹ at 25°C; Flik et al. 1985a; G. Flik, personal observations) is a factor 212 [carrier capacity (2297±877) corrected for the temperature difference assuming an activation energy of 57.9 kJ mol⁻¹, as reported for the eel gill (Flik et al. 1984) divided by 7.5] lower than the total Ca²⁺ transport capacity in the plasma membrane compartment. This indicates a significant overcapacity to transport Ca²⁺.

In this study, we have compared freshwater tilapia with fish adapted to sea water for 8–12 weeks. The plasma composition (Table 1) shows slight, insignificant, increases in calcium, sodium and chloride concentrations which, collectively, appear to be responsible for the 6% increase in plasma osmolarity in seawater tilapia. These observations indicate successful ionoregulation under these widely differing water conditions and thus illustrate the euryhalinity of this fish species.

**Membrane biology**

Mixed populations of of right-side-out (ROV), inside-out (IOV) and leaky BLMVs were used in the in vitro determinations. One could argue that the activities of the pump and the exchanger should only be compared directly if the Na⁺/Ca²⁺ exchange activity is measured in IOVs only, as is the case for the ATP-dependent Ca²⁺ transport. This has indeed been shown in several mixed-vesicle preparations, in Ca²⁺ affinity studies.
(Schoenmakers and Flik, 1992) and in an experiment in which chemical blocking of the cytosolic side of the exchanger completely inhibited Na+/Ca2+ exchange in a mixed population of IOVs and ROVs (Li et al. 1991).

We can only speculate about the reason for the difference in branchial BLM purification between seawater and freshwater gills (Table 2). It is plausible that the looser character of the branchial epithelium in sea water (Sardet et al. 1979; Foskett et al. 1981) leads to a more effective dispersion of the cells in the isolation procedure, which subsequently results in a higher yield of basolateral membranes. Changes in the lipid fraction of the gills after a shift from fresh water to sea water, as reported for eels (Hansen, 1975), could also influence the effectiveness of our BLM isolation procedure.

**Ca2+ transport across the basolateral membrane**

Although the $V_{\text{max}}$ of the exchanger is roughly 2.5 times higher than that of the Ca2+ pump, the exchanger’s relative contribution to Ca2+ excretion at a physiological cellular Ca2+ concentration will be lower than that of the pump because of its low affinity for Ca2+. Only at concentrations of $10^{-6}$ mol l$^{-1}$ or more does the Na+/Ca2+ exchanger becomes a major transport system (from cytoplasm to blood) (Fig. 3). We conclude, on the basis of kinetic data obtained from isolated plasma membranes, that Ca2+ transport may depend for a substantial part on the Na+/Ca2+ exchanger (giving the model depicted in Fig. 4), because the relative contribution of the exchanger to the transport of Ca2+ is intermediate when compared with its minor role in rat intestine and rat kidney (Ghijsen et al. 1983; Van Heeswijk et al. 1984) and its major role in tilapia intestine (Flik et al. 1990). At concentrations higher than $10^{-6}$ mol l$^{-1}$, which are unlikely to occur intracellularly, the exchanger becomes the major Ca2+ transport system. In this in vitro study, the presence of the Ca2+ transporters and their kinetic properties have been determined. Clearly, other studies with intact epithelia are needed to obtain direct evidence for the contribution to Ca2+ transport of each transporter in vivo. The effect of ouabain on net calcium transport should be evaluated, as was done for rat small intestine (Ghijsen et al. 1983) and fish intestine (Flik et al. 1990), to determine the Na+-dependency (serosal) of the Ca2+ uptake.

The lower Ca2+ affinity of the Ca2+ pump in BLMs from seawater gills compared with that of freshwater gills suggests a more important role for the exchanger in Ca2+ influx in sea water (but with no change in affinity). This may mean that, in sea water, the Ca2+ influx depends on the Na+ status to a greater extent than it does in fresh water. However, this extra contribution of exchange activity in seawater BLMs is only marginal at intracellular Ca2+ concentrations around $10^{-7}$ mol l$^{-1}$. The difference would only become manifest if the intracellular Ca2+ concentration rose (to $10^{-6}$ mol l$^{-1}$ or more; Fig. 3). We realize, however, that at present we can only evaluate the contribution of the exchanger to Ca2+ extrusion on the basis of its Ca2+ affinity. Changes in Na+ affinity may occur, raising the possibility of another mode of regulation. It would, therefore, be very interesting to evaluate the Na+-dependency of the exchanger to see whether the regulation is at the Na+ site.
ATP- and Na+-gradient driven Ca2+ transport

Fig. 4. Diagram of Ca2+ transport mechanisms present in the plasma membrane of the gills. The Ca2+-ATPase, Na+/Ca2+ exchanger and Na+/K+-ATPase contents of the membrane are similar for freshwater and seawater fish. This suggests that the difference in gill physiology between the freshwater and seawater situations is dictated by other factors. Such factors could include transepithelial potential, the permeability of the apical membrane and the presence (induction) of other carriers. Levels of ionic calcium are indicated (in contrast to Table 1, in which total calcium concentrations are given), since only the ionic form contributes to the electrophysiology. ER, endoplasmic reticulum; CaBP, calcium-binding proteins; \( \Delta \Psi \), transepithelial potential.

Capacity and function of carriers

A major conclusion drawn from these results is that the fully adapted seawater fish possesses amounts of the exchanger, the Ca2+ pump and the Na+/K+-ATPase that are comparable to those of freshwater fish. Apparently, these mechanisms suffice to accomplish Na+/Cl− extrusion in sea water and to maintain a similar Ca2+ influx to that in freshwater fish [the rates of branchial Ca2+ influx do not differ for freshwater and seawater fish; data from Mayer-Gostan et al. (1983) on killifish and G. Flik (unpublished data) on eel]. The similar levels of Ca2+ transport capacity indicate that, in both freshwater and seawater gills, Ca2+ may be absorbed transcellularly. This assumption is further supported by two findings. First, stanniocalcin, the hypocalcaemic fish hormone, circulates at higher concentrations and has a higher turnover rate in seawater fish (Hanssen et al. 1992, 1993) and it is known to exert its inhibitory control through a reduction in transcellular Ca2+ uptake, also in tilapia (Verbost et al. 1993). Second, the transepithelial potential (TEP) measured in seawater fish is never less than the resting potential for Ca2+ in the gill epithelium of fish in sea water, thus excluding a passive influx of Ca2+ in seawater fish (resting potential is 21 mV calculated according to the Nernst equation with 8.5 mmol l−1 Ca2+ outside and 1.5 mmol l−1 Ca2+ inside, whereas
the TEP in seawater fish is greater than 21 mV, based on TEP values given by Maetz and Bornancin, 1975; Young et al. 1988).

The total branchial Na+/K+-ATPase activity of freshwater tilapia was similar after adaptation to sea water, in contrast to what has been reported for killifish (Epstein et al. 1967), eels (Epstein et al. 1971; Bornancin and de Renzis, 1972; Utida and Hirano, 1973), coho salmon (Zaugg and McLain, 1970) and some other species (reviewed by Foskett et al. 1983). It is believed that the capacity of fish to tolerate hyperosmotic media is linked with their ability to increase the production of this enzyme, thereby gaining a more efficient system for ridding the body of excess salts (Maetz, 1971). When tilapia are gradually transferred to sea water (as performed in this study), the Na+/K+-ATPase activity increases only slightly (51% after stepwise acclimation to sea water in 8 weeks; Dange, 1985) or does not increase at all (this study). We did not measure a difference in Na+/K+-ATPase activity 8–12 weeks after the transfer, although there is a transient increase in the activity (2 weeks after transfer, 40±8% increase, P<0.01, N=8; G. Flik, personal observations). The densities of chloride cells in the opercular membrane of the freshwater and seawater tilapia were also alike. These cell numbers match data on enzymatic activity and are in agreement with data by Foskett et al. (1981) showing that, in tilapia opercular membranes, the number of chloride cells increases only transiently after transfer to sea water. Microscopical evaluation indicated a subsequent hypertrophy of the chloride cells, which was suggested to be correlated with the amount of chloride secretion (Foskett et al. 1981), but the corresponding Na+/K+-ATPase activity was, unfortunately, not measured. One may conclude from the available data that the differences in branchial Na+/K+-ATPase are not necessarily indicative of osmoregulatory responses during seawater adaptation in tilapia (Dange, 1985). In any case, the quantity of Na+/K+-ATPase available for Na+ uptake in freshwater gills suffices to sustain the Na+ and (indirectly) Cl− excretion in seawater gills, which is much more rapid than the Na+ uptake in fresh water (Maetz, 1971). This implies that, in freshwater conditions, an unknown but small proportion of the Na+/K+-ATPase capacity is used in vivo. This, in turn, suggests that the difference in gill physiology between the freshwater and seawater situations is dictated by properties of the epithelium other than the capacity of these plasma membrane carriers. Such other properties could include transepithelial potential (Marshall and Bern, 1980), the permeability of the apical membrane and the presence of other carriers (we have preliminary evidence that, in sea water, a basolateral bumetanide-inhibitable Na+/K+/Cl− co-transporter is activated and accounts for the high rate of Cl− extrusion, as originally proposed by Silva et al. 1977).

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