KINETICS OF ATP- AND Na$^+$-GRADIENT DRIVEN Ca^{2+} 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 Ca^{2+}-ATPase and Na$^+$/Ca^{2+} exchange activity. The relative importance of ATP-driven and Na$^+$-gradient-driven Ca^{2+} transport in Ca^{2+} extrusion was evaluated on the basis of kinetic analyses *in vitro*. The Na$^+$/Ca^{2+} exchangers in branchial membranes from freshwater or seawater fish displayed similar kinetics. The ATP-driven Ca^{2+} pump, however, showed a somewhat lower affinity for Ca^{2+} in membranes isolated from seawater gills than in membranes from freshwater gills; no difference in V_{max} was found. The activity of the exchanger was estimated to be 50 % of that of the ATP-driven pump at prevailing cytosolic Ca^{2+} 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 Ca^{2+}-ATPase, Na$^+$/Ca^{2+} 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 transcellular: the electrochemical potential for a passive Ca^{2+} flux is directed outwards and, at the same time, a significant Ca^{2+} 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 Ca^{2+} uptake (Flik *et al.* 1985*c*), Ca^{2+} enters the ionocytes (also called chloride cells) through Ca^{2+} channels in the apical membrane (the rate-limiting step; Verbost *et al.* 1993) and is transported

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.* 1985*b*,*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.* 1985*c*) 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 100 l tanks. The aquaria were supplied with running tap water (0.7 mmol l^{-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 mm^2 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.* 1985*c*). 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.* 1985*c*) 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 Ca^{2+} 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).

$^{45}Ca^{2+}$-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 $^{45}Ca^{2+}$ was determined in 1 min incubations (yielding initial pump velocities; Flik *et al.* 1985*c*) 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. $^{45}Ca^{2+}$ 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.

Na^+/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 $^{45}Ca^{2+}$ 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 $^{45}Ca^{2+}$ accumulation after transfer to Na^+ or K^+ medium was taken to represent Na^+-gradient-driven Ca^{2+} transport.

Calculations and statistics

The K_m and V_{max} values of the ATP-dependent Ca^{2+} pump and the Na^+/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 Ca^{2+} 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_{max} values ($V_{max,corr}$) are not significantly different for freshwater- and seawater-derived BLMs ($V_{max,corr}=V_{max,Ca}/V_{spec,Na/K-ATPase}$; 19.56 ± 4.80 nmol Ca^{2+} μmol^{-1} P$_i$ for FW and 16.92 ± 4.80 nmol Ca^{2+} μmol^{-1} P$_i$ for seawater

Table 1. *Plasma osmolarity and calcium, sodium and chloride contents of freshwater-and seawater-adapted tilapia*

	Fresh water (*N*=12)	Sea water (*N*=12)
Osmolarity (mosmol l^{-1})	327±3	348±5*
[Calcium] (mmol l^{-1})	2.78±0.12	2.85±0.17
[Sodium] (mmol l^{-1})	158±7	167±10
[Chloride] (mmol l^{-1})	130±3	138±5

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*

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

V_{spec}, the specific Na$^+$/K$^+$-ATPase activity in μmol P$_i$ h^{-1} mg^{-1}.
V_{total}, the total Na$^+$/K$^+$-ATPase activity in μmol h^{-1} ($V_{spec} \times$ mg protein).
Percentage recovery, $V_{total,BLMV}/V_{total,Ho}$.
Purification, $V_{spec,BLMV}/V_{spec,Ho}$.
Chloride cells, number of chloride cells (DASPEI-stained cells) per mm^2 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 Ca^{2+} (K_m) of the branchial Na$^+$/Ca^{2+} exchangers are also similar in BLMVs from freshwater- and seawater-adapted tilapia (Fig. 1).

ATP-driven Ca^{2+} transport

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

High-affinity Ca^{2+}-ATPase activity, determined as Ca^{2+}-dependent ATP hydrolysis (Flik *et al.* 1984), amounts to 2.28±0.64 μmol P$_i$ h^{-1} mg^{-1} and 3.87±0.85 μmol P$_i$ h^{-1} mg^{-1} (*N*=6, ± S.E.M.) for freshwater and seawater BLMs, respectively. The ratio of this Ca^{2+}-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 Ca^{2+}-ATPase under both conditions.
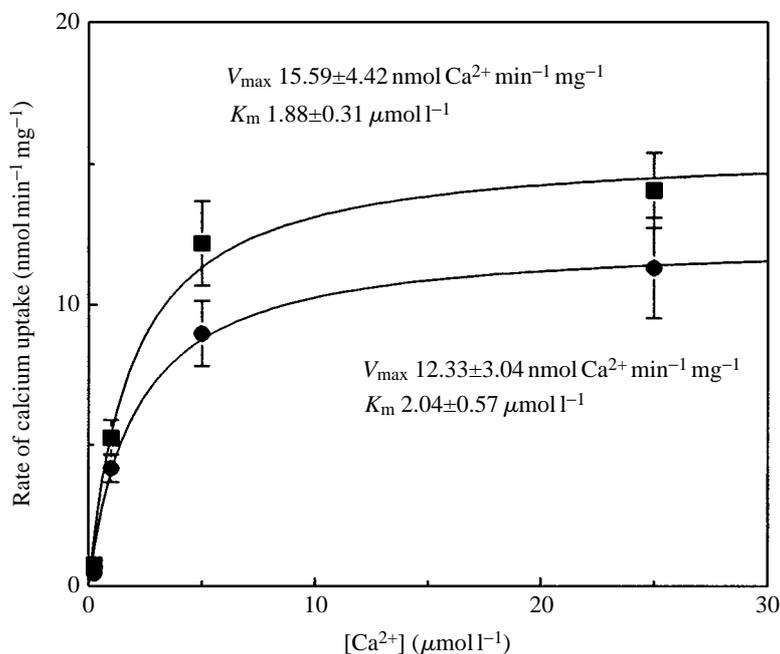
Fig. 1. Ca^{2+}-dependence of Na$^+$-driven Ca^{2+} uptake in basolateral membranes from freshwater- (FW, filled circles) and seawater- (SW, filled squares) adapted tilapia. The V_{max} and K_m values were the average values of five (for FW) to eight (for SW) gill membrane preparations (for further details see Materials and methods). These values are not significantly different. The V_{max} values expressed relative to Na$^+$/K$^+$-ATPase (from Table 2), to eliminate purification differences between the FW and SW membranes, are also comparable ($V_{max,corr}$ for FW=19.56±4.80 nmol Ca^{2+} μmol^{-1}P$_i$ and for SW=16.92±4.80 nmol Ca^{2+} μmol^{-1}P$_i$). Mean values ± standard errors are presented.

Comparison of Ca^{2+} pump and Na$^+$/Ca^{2+} exchange transport activities

The kinetic data presented in Figs 1 and 2 are used to plot the potential activities of both Ca^{2+} extrusion systems as a function of intracellular Ca^{2+} concentrations in Fig. 3. These results predict that, at resting levels of intracellular Ca^{2+} of approximately 10^{-7} mol l^{-1}, the Ca^{2+} pump activities exceed the Na$^+$/Ca^{2+} exchange activities in freshwater and seawater BLMs by factors of 1.8 and 1.5, respectively. At 10^{-6} mol l^{-1} Ca^{2+}, 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$^+$/Ca^{2+} exchange activity in this membrane displayed similar kinetics (V_{max}, K_m) in freshwater and seawater gills. The ATP-driven Ca^{2+} pump showed a somewhat lower affinity for Ca^{2+} in BLMs isolated
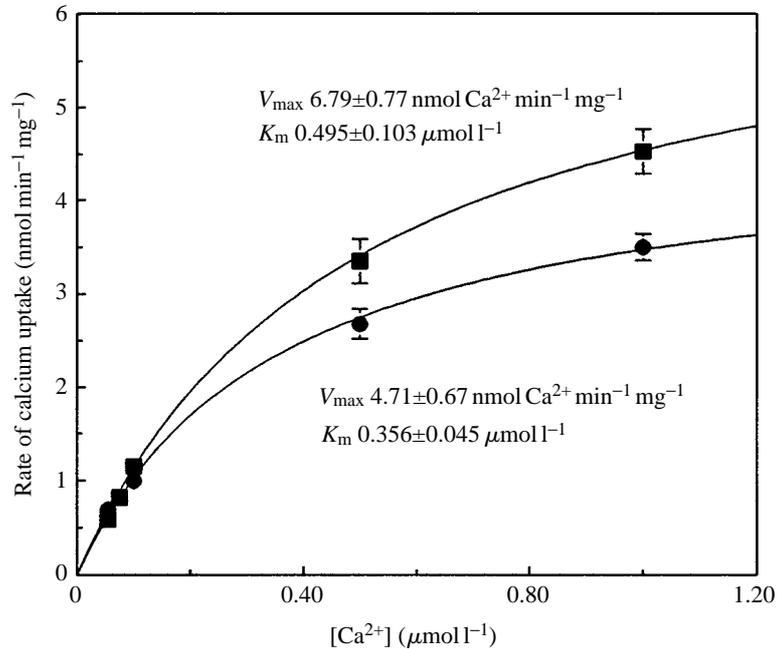
Fig. 2. Ca^{2+}-dependence of ATP-driven Ca^{2+} uptake in basolateral membranes from freshwater- (FW, filled circles) and seawater- (SW, filled squares) adapted tilapia. The V_{max} and K_m values were the average values of seven gill membrane preparations (for further details see Materials and methods). The K_m of SW gills is higher than that of FW gills ($P<0.005$). Without correction for differences in membrane purification, the V_{max} value for SW gills is significantly higher than that for FW gills. However, the V_{max} values expressed relative to Na^+/K^+-ATPase activity (from Table 2), eliminating the purification differences between the FW and SW membranes, are comparable ($V_{max,corr}$ for FW=7.50 ± 1.08 nmol Ca^{2+} μmol^{-1} P_i and for SW=7.38 ± 0.84 nmol Ca^{2+} μmol^{-1} P_i). Mean values $\pm$ standard errors are presented.

from seawater gills than in BLMs from freshwater gills, whereas V_{max} was the same. The significance of this change in K_m for cellular calcium transport is discussed below (*Ca^{2+} transport across the basolateral membrane*). The V_{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_{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_{max} and total protein in the homogenate (and adjusted to 100 % IOV and 100 % recovery), amount 2297 ± 566 and 2294 ± 650 μmol 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 μmol 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
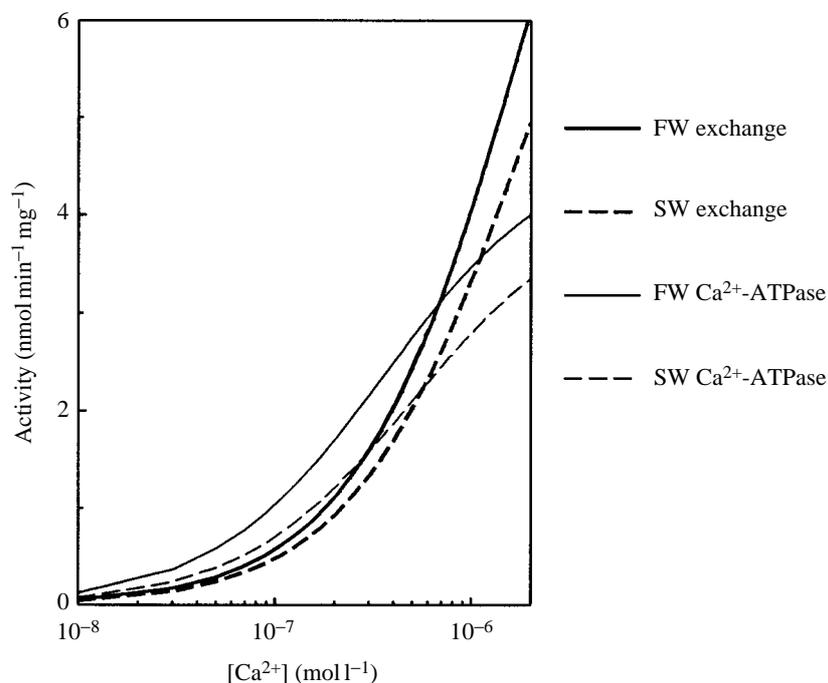
Fig. 3. Activities of the basolateral plasma membrane Ca^{2+} pump (Ca^{2+}-ATPase) and the Na$^+$/Ca^{2+} exchanger in gills from seawater- and freshwater-adapted tilapia as a function of intracellular Ca^{2+} concentration.

in vivo for a tilapia of the same size (approximately 7.5 μmol h^{-1} at 25 °C; Flik *et al.* 1985*a*; 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 Ca^{2+} transport capacity in the plasma membrane compartment. This indicates a significant overcapacity to transport Ca^{2+}.

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$^+$/Ca^{2+} exchange activity is measured in IOVs only, as is the case for the ATP-dependent Ca^{2+} transport. This has indeed been shown in several mixed-vesicle preparations, in Ca^{2+} affinity studies

(Schoenmakers and Flik, 1992) and in an experiment in which chemical blocking of the cytosolic side of the exchanger completely inhibited Na^+/Ca^{2+} 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.

Ca^{2+} transport across the basolateral membrane

Although the V_{max} of the exchanger is roughly 2.5 times higher than that of the Ca^{2+} pump, the exchanger's relative contribution to Ca^{2+} excretion at a physiological cellular Ca^{2+} concentration will be lower than that of the pump because of its low affinity for Ca^{2+}. Only at concentrations of $10^{-6}\,mol\,l^{-1}$ or more does the Na^+/Ca^{2+} 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 Ca^{2+} transport may depend for a substantial part on the Na^+/Ca^{2+} exchanger (giving the model depicted in Fig. 4), because the relative contribution of the exchanger to the transport of Ca^{2+} 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 Ca^{2+} transport system. In this *in vitro* study, the presence of the Ca^{2+} transporters and their kinetic properties have been determined. Clearly, other studies with intact epithelia are needed to obtain direct evidence for the contribution to Ca^{2+} 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 Ca^{2+} uptake.

The lower Ca^{2+} affinity of the Ca^{2+} pump in BLMs from seawater gills compared with that of freshwater gills suggests a more important role for the exchanger in Ca^{2+} influx in sea water (but with no change in affinity). This may mean that, in sea water, the Ca^{2+} 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 Ca^{2+} concentrations around $10^{-7}\,mol\,l^{-1}$. The difference would only become manifest if the intracellular Ca^{2+} 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 Ca^{2+} extrusion on the basis of its Ca^{2+} 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.

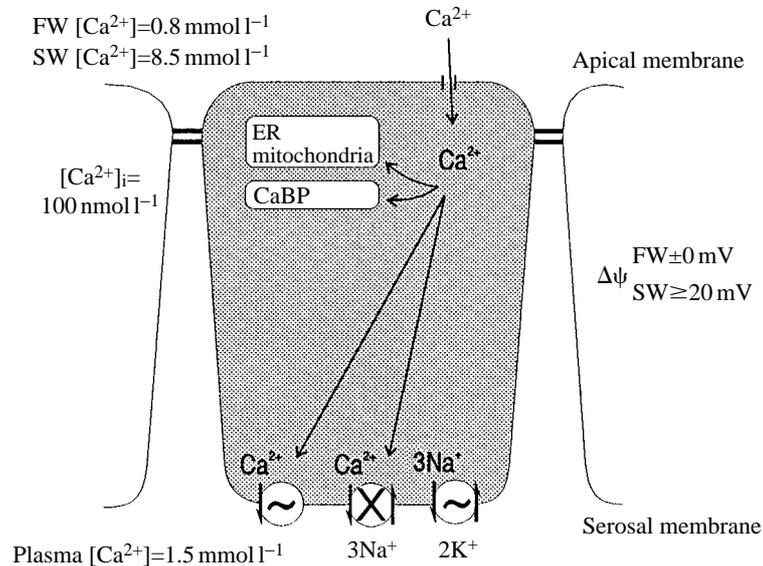Fig. 4. Diagram of Ca^{2+} transport mechanisms present in the plasma membrane of the gills. The Ca^{2+}-ATPase, Na$^+$/Ca^{2+} exchanger and Na$^+$/K$^+$-ATPase contents of the membrane are similar for freshwater and seawater fish. This suggests that the difference in gill physiology between the freshwater 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 Ca^{2+} 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 Ca^{2+} influx to that in freshwater fish [the rates of branchial Ca^{2+} 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 Ca^{2+} transport capacity indicate that, in both freshwater and seawater gills, Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} in the gill epithelium of fish in sea water, thus excluding a passive influx of Ca^{2+} in seawater fish (resting potential is 21 mV calculated according to the Nernst equation with 8.5 mmol l^{-1} Ca^{2+} outside and 1.5 mmol l^{-1} Ca^{2+} 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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