

TRANSCELLULAR INTESTINAL CALCIUM TRANSPORT IN FRESHWATER AND SEAWATER FISH AND ITS DEPENDENCE ON SODIUM/CALCIUM EXCHANGE

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Summary

Transepithelial calcium uptake and transcellular calcium uptake mechanisms were compared in the proximal intestine of freshwater- and seawater-adapted tilapia, *Oreochromis mossambicus*. Stripped intestinal epithelium of seawater fish showed a higher paracellular permeability to calcium *in vitro*. Net transepithelial calcium uptake was 71% lower, reflecting a physiological response to the increased inward calcium gradient. Na⁺/K⁺-ATPase activity was significantly enhanced in enterocytes of seawater-adapted fish, in line with the water transport function of the intestine in seawater fish. The V_{\max} and the K_m values for Ca²⁺ of the ATP-dependent Ca²⁺ pump in seawater fish enterocytes were 28% and 27%, respectively, lower than in freshwater fish. The K_m for Ca²⁺ of the Na⁺/Ca²⁺ exchanger was 22% lower, and a 57% decrease in the V_{\max} for Ca²⁺ of the exchanger was observed. Apparently, the density of exchanger molecules in the basolateral plasma membrane is reduced in seawater fish. From the correlation between the differences in net intestinal calcium uptake and Na⁺/Ca²⁺ exchange activity we conclude that Na⁺/Ca²⁺ exchange is the main basolateral effector of transcellular calcium uptake.

Introduction

The Na⁺/Ca²⁺ exchanger is a plasma membrane protein crucial to calcium homeostasis in, for example, muscles and neurones (DiPolo, 1989). During the last decade, the opinion prevailed that the protein might be typical of excitable cells (Baker, 1986). Non-excitable cells normally do not develop the high intracellular ionic calcium concentrations characteristic of the former. Therefore, it was thought that the contribution of the Na⁺/Ca²⁺ exchanger, which has a relatively low Ca²⁺ affinity, to cellular calcium homeostasis of such cells was of minor importance. In recent years, however, evidence has accrued that the exchanger can be found in quite a number of non-excitable cells. Cells in ion-transporting epithelia were repeatedly shown to display Na⁺/Ca²⁺ exchange activity involved in transcellular ion transport (Flik *et al.* 1990; Taylor, 1989). The Na⁺/Ca²⁺ exchanger can serve different purposes in non-excitable cells, as shown by its

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involvement in volume regulation and Na^+ extrusion in canine erythrocytes (Parker, 1988; Schoenmakers and Flik, 1992). Basolateral membranes of enterocytes of the freshwater fish *Oreochromis mossambicus* Peters (tilapia) have been shown to display a weak ATP-dependent Ca^{2+} pump activity and a vigorous $\text{Na}^+/\text{Ca}^{2+}$ exchange activity (Flik *et al.* 1990; Schoenmakers *et al.* 1992a).

The issue of the importance of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in non-excitable cells is interlaced with the topic of its regulation. *In vitro* the functioning of the $\text{Na}^+/\text{Ca}^{2+}$ exchange protein has been modulated in many ways: membrane composition, intracellular Ca^{2+} concentration and cellular energy status are factors known to influence $\text{Na}^+/\text{Ca}^{2+}$ exchange activity (DiPolo, 1989; Philipson and Ward, 1985). These studies were performed on cells or tissues in a steady state. Few studies have concentrated on the question of how altered physiological requirements are reflected by changes in the activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Tibbits *et al.* 1989).

We provide data on the importance and regulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in an ion-transporting cell. We used the intestine of the tilapia as a model of an ion-transporting epithelium. The tilapia is a euryhaline fish able to live in fresh water as well as in full-strength sea water. Freshwater fish are hyper-osmoregulators in which the active uptake of ions is needed for metabolism and growth. They drink little and take up most of the monovalent ions they need (such as Na^+ , K^+ and Cl^-), as well as most of their calcium, *via* their gills (Mayer-Gostan *et al.* 1987; Fenwick, 1989). Seawater fish, however, are hypo-osmoregulators, confronted in particular with osmotic water loss. Therefore, seawater fish drink large amounts of sea water and excrete, *via* the gills and kidneys, the excess ions taken up in the intestine (Maetz, 1976).

Freshwater fish need extra calcium during growth and ovarian maturation or when the calcium concentration in the water is low, as in soft fresh water (Flik *et al.* 1986). Under the latter circumstances, fish experience extra calcium loss as a result of an increased epithelial permeability to calcium (Höbe *et al.* 1984). When faced with such adverse conditions, they supplement branchial calcium uptake with an increase in intestinal uptake (Berg, 1970).

Experiments on tilapia in our laboratory have shown that the net branchial uptake of calcium, sufficient for growth and homeostasis of fish in fresh water containing around $1 \text{ mmol l}^{-1} \text{ Ca}^{2+}$, increases when the fish are transferred to sea water: branchial Ca^{2+} influx is not affected, but branchial Ca^{2+} efflux is drastically reduced (G. Flik, personal observations). Drinking of sea water containing around 10 mmol l^{-1} calcium will lead to an excess influx of calcium in seawater fish unless the intestinal uptake of calcium is strictly controlled. Paracellular influx of calcium in the intestine is counteracted by the generation of an inside-positive transepithelial potential (Maetz, 1976). Here, we concentrate on another means of decreasing net calcium uptake, i.e. the reduction of the active transcellular transport of calcium across the intestine.

We measured unidirectional calcium fluxes in stripped intestinal epithelium of freshwater- and seawater-adapted fish. The isolation of basolateral plasma membrane vesicles allowed us to test which of the two extrusion mechanisms for Ca^{2+} (the ATP-dependent Ca^{2+} pump or $\text{Na}^+/\text{Ca}^{2+}$ exchange) is more involved in transcellular calcium uptake under these conditions.

Materials and methods

Holding conditions of fish

Male freshwater tilapia from laboratory stock, weighing around 250g, were held in 100l tanks. The aquaria were supplied with running tap water ($0.7\text{mmol l}^{-1}\text{ Ca}^{2+}$, 25°C) under a photoperiod of 12h of light alternating with 12h of darkness. Animals were fed Trouvit fish pellets (Trouw and Co., Putten, The Netherlands) at 1.5% body weight 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. After 1 day, they were transferred to full-strength sea water. Fish were adapted to sea water for at least 8 weeks before experimentation. Food was supplied at 1.5% body weight per day.

Epithelial calcium flux measurements

The intestinal epithelium was stripped of its underlying muscle layers as described previously (Flik *et al.* 1990; Groot *et al.* 1979). Briefly, fish were killed by spinal transection and pithed. The peritoneal cavity was opened and the intestinal tract removed. The intestine was flushed with saline ($117.5\text{mmol l}^{-1}\text{ NaCl}$, $5.7\text{mmol l}^{-1}\text{ KCl}$, $25\text{mmol l}^{-1}\text{ NaHCO}_3$, $1.2\text{mmol l}^{-1}\text{ NaH}_2\text{PO}_4$, $1.25\text{mmol l}^{-1}\text{ CaCl}_2$, $1.2\text{mmol l}^{-1}\text{ MgSO}_4$, $5\text{mmol l}^{-1}\text{ glucose}$ and $28\text{mmol l}^{-1}\text{ mannitol}$) and attached strands of fat were carefully removed. The intestine was cut open lengthwise and dipped up and down in saline to remove any remaining food residues. Next, the intestine was put on a Parafilm-covered glass plate mucosa-side-up. The mucosa of the proximal 15cm was stripped free of underlying muscle layers (Groot *et al.* 1979). The stripped epithelium was mounted on slides (the exposed tissue area was 0.16cm^2) and incubated for approximately 20min in gassed saline (95% O_2 /5% CO_2 ; $\text{pH}7.3\pm 0.1$).

The slides were then mounted as the partition between two 4ml half-chambers filled with saline. At time zero, $^{45}\text{CaCl}_2$ (specific activity: 19GBqmmol^{-1}) was added as a tracer to either side of the tissue to yield a radioactive concentration of 0.33MBqml^{-1} . After 45min, samples ($100\mu\text{l}$) were taken from both half-chambers simultaneously every 15min, for a total period of 180min. The samples were instantly replaced by $100\mu\text{l}$ of fresh Ringer's solution. In pilot experiments it was established that unidirectional fluxes are constant over this period.

Using symmetrical conditions (saline on both sides) should keep passive fluxes of ^{45}Ca at a minimum, allowing a good assessment of active calcium transport processes (Barry and Diamond, 1984). The transepithelial potential (TEP) of stripped intestinal preparations from freshwater tilapia was $0.93\pm 0.66\text{mV}$ ($N=24$). This small TEP indicates that passive calcium flux is low in this Ussing chamber apparatus.

Unidirectional calcium fluxes were calculated as follows. Specific activity (SA) of the radioactive saline (*cis*-side) was determined from three $10\mu\text{l}$ samples distributed over the whole experimental period and expressed in $\text{disintegrations min}^{-1}\text{ nmol}^{-1}$. The amount of isotope at the *trans*-side at a given time [$q(t)$ in $\text{disintegrations min}^{-1}$] was calculated from the $100\mu\text{l}$ samples. Data were corrected for background, and cumulative counts for the whole Ussing hemi-chamber were calculated. Total calcium transported (in nmolcm^{-2}) as a

function of time was obtained by calculating $q(t)/(SA \times A)$ for each sample, where A is the exposed tissue area (in cm^2). Unidirectional steady-state Ca^{2+} fluxes were determined by linear regression of calcium accumulation over periods of at least 45 min. To calculate net fluxes (J_{net}), values for unidirectional fluxes from mucosa to serosa (J_{ms}) and from serosa to mucosa (J_{sm}) across adjacent segments from the same fish were used in data analysis. Ca^{2+} fluxes were expressed in $\text{nmol h}^{-1} \text{cm}^{-2}$.

Membrane vesicle preparation and protein activity assays

Membrane vesicles were isolated from the proximal 30 cm of the intestine as described in detail previously (Flik *et al.* 1990). Vesicles were kept cooled on ice and used on the day of isolation. The protein content of the membrane vesicle preparations was $2.0 \pm 0.4 \text{ g l}^{-1}$ ($N=12$), as determined with a commercial reagent kit (Bio-Rad) with bovine serum albumin (BSA) as a reference. Protein recovery was around 1.9% in both freshwater and seawater preparations. Na^+/K^+ -ATPase activity in fish intestinal basolateral plasma membrane vesicles (BLMV) was determined as described earlier (Flik *et al.* 1990). Na^+/K^+ -ATPase activity was defined as the Na^+ - and K^+ -dependent, ouabain-sensitive phosphatase activity.

All assay media 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). Free calcium and magnesium levels were calculated as described in detail by Schoenmakers *et al.* (1992*a,b*). All incubations were performed at 37°C .

The ATP-driven transport of ^{45}Ca was assayed using a rapid filtration technique (Flik *et al.* 1990). One-minute incubations of membrane vesicles in the presence of 3 mmol l^{-1} ATP yielded ^{45}Ca uptakes (representing initial velocities of the pump) and were corrected for uptake in the absence of ATP. Free Ca^{2+} concentrations were varied from 50 nmol l^{-1} to $1 \text{ } \mu\text{mol l}^{-1}$. A 14-fold dilution in ice-cold isotonic medium containing 0.1 mmol l^{-1} LaCl_3 stopped the uptake, and the suspension was filtered (Schleicher & Schüll ME25, pore size $0.45 \text{ } \mu\text{m}$). 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 in plasma membrane vesicles from fish enterocytes was assayed as described previously (Flik *et al.* 1990). Briefly, $5 \text{ } \mu\text{l}$ of membrane vesicles equilibrated with 150 mmol l^{-1} NaCl was added to $120 \text{ } \mu\text{l}$ of medium containing ^{45}Ca 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 $25 \text{ } \mu\text{mol l}^{-1}$. After 5 s at 37°C , the reaction was stopped by addition of 1 ml of ice-cold isotonic stopping solution containing 1 mmol l^{-1} LaCl_3 and the solution was filtered. The filter was then rinsed three times with 2 ml of stopping solution and treated as described above. The difference in ^{45}Ca accumulation 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 $\text{Na}^+/\text{Ca}^{2+}$ exchanger were derived by non-linear regression analysis of the mean velocities ($N=5-6$) measured

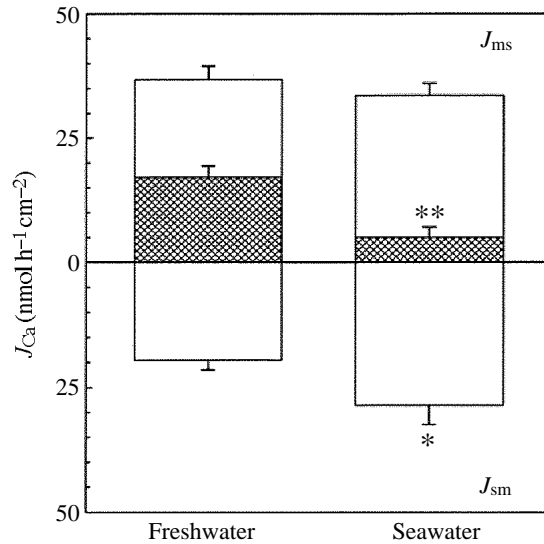


Fig. 1. Unidirectional and net calcium fluxes in stripped intestinal epithelium of freshwater- and seawater-adapted tilapia. Seawater adaptation induces an increased paracellular calcium permeability *in vitro*. Net uptake of calcium is significantly reduced in seawater-adapted fish. Open bars denote unidirectional calcium fluxes and cross-hatched bars represent net calcium flux. * indicates $P < 0.05$ and ** indicates $P < 0.001$. Mean values \pm standard error of the mean are given for 15 freshwater and 7 seawater fish.

as a function of substrate concentration. The standard deviations of the values given by the computer algorithm indicate the accuracy of the estimated values, but cannot be used for statistical assessment of differences (Press *et al.* 1986).

Otherwise, statistical significance of differences between mean values was tested using a Mann–Whitney *U*-test. Statistical significance was accepted at $P < 0.05$.

Results

Epithelial calcium flux measurements

Calcium fluxes in stripped intestinal epithelium of freshwater and seawater tilapia are shown in Fig. 1. The mucosa-to-serosa flux of Ca^{2+} (J_{ms}) in seawater epithelium was not significantly different from that through freshwater epithelium. The serosa-to-mucosa (J_{sm}) calcium flux in freshwater fish, $19.6 \pm 7.5 \text{ nmolCa}^{2+} \text{ h}^{-1} \text{ cm}^{-2}$ (mean \pm s.d.; $N=15$), was significantly ($P < 0.05$) lower than the flux, $28.6 \pm 10.0 \text{ nmolCa}^{2+} \text{ h}^{-1} \text{ cm}^{-2}$ (mean \pm s.d.; $N=7$), in seawater fish, indicating an increased permeability to Ca^{2+} of the tight junctions *in vitro*. The net uptake of Ca^{2+} ($J_{net} = J_{ms} - J_{sm}$) in seawater intestine ($5.0 \pm 5.2 \text{ nmolCa}^{2+} \text{ h}^{-1} \text{ cm}^{-2}$; mean \pm s.d.; $N=7$) was not significantly different from zero. The net uptake was significantly ($P < 0.001$) reduced with respect to the net flux in freshwater fish ($17.1 \pm 8.9 \text{ nmolCa}^{2+} \text{ h}^{-1} \text{ cm}^{-2}$; mean \pm s.d.; $N=15$).

Membrane protein activities

Using Na^+/K^+ -ATPase as a marker to assess basolateral plasma membrane purification

we found no significant ($P \geq 0.15$) difference in purification between freshwater and seawater tilapia membranes: Na^+/K^+ -ATPase activity purified 4.8 ± 1.0 ($N=5$) times for seawater tilapia membranes and 6.2 ± 1.7 ($N=6$) times for freshwater tilapia membranes. The activities of the three basolateral plasma membrane proteins we characterized are given in Table 1. Na^+/K^+ -ATPase activity in the homogenate of intestinal scrapings from seawater tilapia amounted to $26.9 \pm 2.9 \mu\text{mol P}_i \text{h}^{-1} \text{mg}^{-1}$ ($N=5$), which is significantly ($P < 0.001$) higher than the activity observed in intestinal scrapings from freshwater tilapia ($17.3 \pm 2.7 \mu\text{mol P}_i \text{h}^{-1} \text{mg}^{-1}$; $N=6$). In purified basolateral plasma membranes from seawater fish, its activity was $127.6 \pm 14.1 \mu\text{mol P}_i \text{h}^{-1} \text{mg}^{-1}$ ($N=5$), differing significantly ($P < 0.001$) from that in freshwater fish, which amounted to $102.7 \pm 10.5 \mu\text{mol P}_i \text{h}^{-1} \text{mg}^{-1}$ ($N=6$).

Seawater fish enterocytes have a greatly decreased $\text{Na}^+/\text{Ca}^{2+}$ exchange activity in the purified basolateral membrane fraction. The maximal velocity of the $\text{Na}^+/\text{Ca}^{2+}$ exchange activity in basolateral plasma membrane vesicles from freshwater-adapted tilapia [$18.21 \pm 0.40 \text{nmolCa}^{2+} \text{min}^{-1} \text{mg}^{-1}$ ($N=6$)] was reduced by 57% in vesicles from seawater-adapted tilapia [$7.85 \pm 0.21 \text{nmolCa}^{2+} \text{min}^{-1} \text{mg}^{-1}$ ($N=5$)]. The K_m of $\text{Na}^+/\text{Ca}^{2+}$ exchange in freshwater tilapia ($2.26 \pm 0.13 \mu\text{mol l}^{-1}$; $N=6$) differs only slightly from that of $\text{Na}^+/\text{Ca}^{2+}$ exchange in seawater tilapia ($1.76 \pm 0.16 \mu\text{mol l}^{-1}$; $N=5$).

The ATP-dependent Ca^{2+} pump in the basolateral plasma membrane vesicles did not show such a distinct change in maximal velocity. In vesicles from freshwater and seawater tilapia we measured a slight change in K_m , comparable in relative magnitude to the change in K_m of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. V_{max} decreased in a similar manner by 28% from $0.78 \pm 0.06 \text{nmolCa}^{2+} \text{min}^{-1} \text{mg}^{-1}$ in freshwater fish to $0.56 \pm 0.02 \text{nmolCa}^{2+} \text{min}^{-1} \text{mg}^{-1}$ in seawater fish.

Discussion

Epithelial calcium flux measurements

The net uptake of calcium in the proximal intestine calculated on the basis of unidirectional calcium fluxes approaches zero and is significantly lower in seawater fish than in freshwater fish. Apparently, seawater fish have successfully reduced active calcium transport mechanisms and keep net intestinal transcellular calcium influx low. The decrease in net uptake and the increase in paracellular calcium mobility result in an apparently unchanged unidirectional calcium influx.

The data show a manifest increase in the serosa-to-mucosa flux (J_{sm}) for calcium in the intestine of seawater-adapted fish. It is a well-known phenomenon that the intestine of seawater-adapted fish has a larger osmotic permeability to water (Skadhauge, 1969; Smith *et al.* 1975). Apparently, the looser tight junctions in seawater intestine have also become more permeable to calcium. The symmetrical conditions we used to minimize the contribution of passive fluxes to the total isotope flux (see above) may not reflect, however, the situation *in vivo*: the inside-positive transmural potential in the intact fish (around +30mV; Maetz, 1976) will inhibit passive influx of calcium. An inside-positive transmural potential of 30mV in seawater fish, when compared with the 0mV potential

Table 1. Activities of ion-transport mechanisms in freshwater and seawater tilapia enterocytes

	Na ⁺ /K ⁺ -ATPase		ATP-dependent Ca ²⁺ pump		Na ⁺ /Ca ²⁺ exchange	
	Homogenate V _{spec} (μmolP _i h ⁻¹ mg ⁻¹)	BLMV V _{spec} (μmolP _i h ⁻¹ mg ⁻¹)	K _m (nmol l ⁻¹)	V _{max} (nmol Ca ²⁺ min ⁻¹ mg ⁻¹)	K _m (μmol l ⁻¹)	V _{max} (nmol Ca ²⁺ min ⁻¹ mg ⁻¹)
Freshwater	17.3±2.7	102.7±10.5	193±34	0.78±0.06	2.26±0.13	18.2±0.4
Seawater	26.9±2.9*	127.6±14.1*	141±10	0.56±0.02	1.76±0.16	7.9±0.2
Change (%)	+55	+24	-27	-28	-22	-57

All values are mean ± standard deviation for five seawater and six freshwater tilapia. The given K_m and V_{max} values are calculated from the average values by non-linear regression analysis and cannot be tested for statistically significant differences (Press *et al.* 1986).

*Significantly different from the freshwater value, P<0.001.

BLMV, basolateral membrane vesicles; V_{spec}, specific activity; K_m, Michaelis constant; V_{max}, maximum activity.

difference seen in freshwater fish, would decrease the unidirectional passive influx of calcium by 75% compared to the value found in freshwater fish according to the formula:

$$J_{\text{sea}} = J_{\text{fresh}}(\pm zEF/RT)/(1 - e^{\pm zEF/RT}),$$

(Hodgkin and Horowicz, 1959), where z is valency, E is the equilibrium potential (for Ca^{2+}), F is the Faraday constant, R is the gas constant and T is absolute temperature. Therefore, the large inward calcium gradient in seawater fish will not result in the excessive calcium influx that one would predict on the basis of the increased paracellular permeability to calcium.

Whole-body calcium influx and efflux for a 250g freshwater tilapia can be calculated according to Flik *et al.* (1985). Influx of calcium will be around $4.3 \mu\text{mol h}^{-1}$ while calcium efflux is around $0.7 \mu\text{mol h}^{-1}$, resulting in a net whole-body calcium influx of $3.6 \mu\text{mol h}^{-1}$. The length of intestine that performs the measured net influx of $17.1 \text{ nmol cm}^{-2} \text{ h}^{-1}$ is not known. If one assumes that this transport occurs over a length of 50cm (on the basis of gut morphology), the calculated net influx (with a width of 1cm) is $0.86 \mu\text{mol h}^{-1}$. Intestinal calcium influx would then contribute significantly to the whole-body influx.

The net uptake of calcium in tilapia intestine under freshwater conditions has been demonstrated by Flik *et al.* (1990). In that study we also showed that this net uptake depended on the presence of a sodium gradient across the basolateral plasma membrane of the enterocyte. This indicated that $\text{Na}^+/\text{Ca}^{2+}$ exchange, shown to be present in these membranes, was important for transcellular calcium uptake (Flik *et al.* 1990). The lower net intestinal calcium uptake in seawater intestine shown here prompted an investigation into the molecular basis of this change.

Membrane protein activities

Seawater adaptation induced significant changes in two of the three plasma membrane ion-transporting mechanisms we studied. The increase in Na^+/K^+ -ATPase activity we found is a normal phenomenon for intestine and gills of seawater-adapted fish (Utida *et al.* 1972; Maetz, 1976; Pagliarani *et al.* 1991). Around 90% of the cells in the proximal intestine are of the absorptive cell type. Therefore, the observed increase in Na^+/K^+ -ATPase activity per milligram membrane protein represents an increase in activity per cell, not an increase in the numbers of a specific cell type. The increase in the ratio of the activities of Na^+/K^+ -ATPase to Ca^{2+} -ATPase in the purified basolateral plasma membrane vesicles, 3.80×10^3 in seawater vs 2.19×10^3 in freshwater fish [dividing the Na^+/K^+ -ATPase activity ($\mu\text{mol P}_i \text{ h}^{-1} \text{ mg}^{-1}$) by the Ca^{2+} -ATPase activity ($\text{nmol Ca}^{2+} \text{ h}^{-1} \text{ mg}^{-1}$) and assuming that 1 P_i is formed per Ca^{2+} transported], shows that the increase in Na^+/K^+ -ATPase activity is not due to a purification effect (Flik *et al.* 1984). The increase is in line with ultrastructural observations on the intestine of seawater tilapia showing more extensive basolateral membrane invaginations and more mitochondria than in freshwater fish and giving the impression of very active cells (G. Flik, personal observations). The elevated Na^+/K^+ -ATPase activity is consistent with the water transport function of the intestine of seawater fish (Skadhauge, 1969; Utida *et al.* 1972), assuming that solvent drag is the motive force for this water transport.

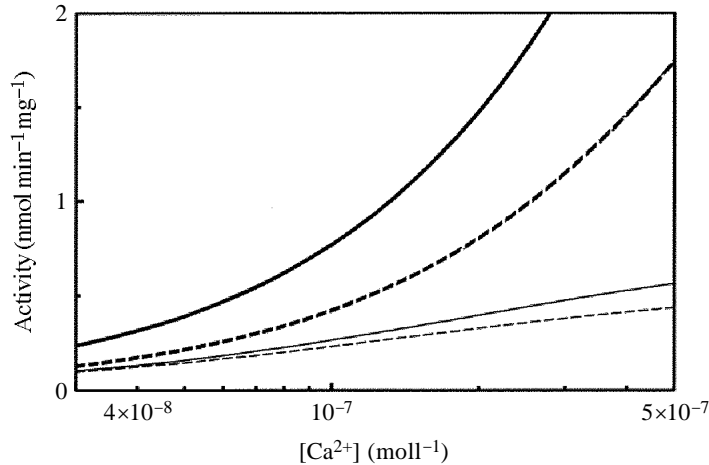


Fig. 2. Activities of basolateral plasma membrane proteins in seawater and freshwater fish as a function of intracellular Ca^{2+} concentration. Thick curves represent $\text{Na}^+/\text{Ca}^{2+}$ exchange activity, while thin curves show the activity of the ATP-dependent Ca^{2+} pump. Activities in freshwater fish are given by solid lines and dashed lines represent activities in seawater fish. $\text{Na}^+/\text{Ca}^{2+}$ exchange activity exceeds Ca^{2+} extrusion under both conditions, even when the stimulatory inside-negative cell membrane potential is disregarded.

The ATP-dependent Ca^{2+} pump activity in plasma membranes from freshwater tilapia is slightly higher than that observed in membranes from seawater tilapia. If we combine these data with the respective Na^+/K^+ -ATPase purification data, we calculate equal amounts of Ca^{2+} pump activity per milligram cell protein in the whole-cell homogenate (direct measurements of this activity in such a preparation are complicated) in freshwater ($0.13 \text{ nmol Ca}^{2+} \text{ min}^{-1} \text{ mg}^{-1} \text{ cell protein}$) and in seawater enterocytes ($0.12 \text{ nmol Ca}^{2+} \text{ min}^{-1} \text{ mg}^{-1} \text{ cell protein}$). Apparently, freshwater- and seawater-adapted enterocytes contain equal amounts of ATP-dependent calcium pump proteins.

A very significant change was found in the $\text{Na}^+/\text{Ca}^{2+}$ exchange activity. The maximum velocity of this carrier decreased by 57% in seawater tilapia. When we calculate activities in the whole-cell homogenate (as for the ATP-dependent Ca^{2+} pump above), there is a large difference between freshwater enterocytes ($2.9 \text{ nmol Ca}^{2+} \text{ min}^{-1} \text{ mg}^{-1} \text{ cell protein}$) and seawater enterocytes ($1.6 \text{ nmol Ca}^{2+} \text{ min}^{-1} \text{ mg}^{-1} \text{ cell protein}$). Seawater enterocytes clearly contain less $\text{Na}^+/\text{Ca}^{2+}$ exchange activity. The observed 57% reduction in $\text{Na}^+/\text{Ca}^{2+}$ exchange activity in purified plasma membranes is in line with the 71% decrease in net transepithelial calcium uptake. We take this observation as evidence for the involvement of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in the active transcellular uptake of calcium in both freshwater and seawater fish.

This conclusion is further supported when we plot the activities of the two Ca^{2+} -transporting membrane proteins under both conditions (Fig. 2). This graph does not take the inside-negative cell membrane potential of around -60 mV (Bakker and Groot, 1988) into account. The $\text{Na}^+/\text{Ca}^{2+}$ exchange is an electrogenic process, swapping 3 Na^+ for 1 Ca^{2+} (Flik *et al.* 1990), and would be even more active than shown here. In the

physiological intracellular Ca^{2+} concentration range (around 85nmol l^{-1} ; Schoenmakers *et al.* 1992a) the $\text{Na}^+/\text{Ca}^{2+}$ exchanger clearly dominates basolateral Ca^{2+} extrusion from the enterocytes in freshwater fish. Under seawater conditions, both the absolute calcium transport capacity of the extrusion mechanisms and the contribution of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger to this transport have decreased.

The distinct decrease in V_{max} of the $\text{Na}^+/\text{Ca}^{2+}$ exchange activity probably reflects a regulatory principle: presumably, the expression rate of the exchanger's gene has decreased and there are fewer exchanger molecules per cell. Table 1 shows that the percentage decreases in K_m of the ATP-dependent Ca^{2+} pump and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger are similar. This suggests the existence of a mechanism that has a similar effect on both membrane proteins. We suggest that seawater fish develop a more fluid plasma membrane. Increased membrane fluidity has been shown to decrease the K_m of $\text{Na}^+/\text{Ca}^{2+}$ exchange (Philipson and Ward, 1985). The lipid composition of gill membranes changes when fish adapt to seawater conditions (Hansen, 1987). The changed endocrine status of seawater fish may well underlie such a phenomenon. Cortisol and prolactin levels change upon adaptation to sea water (Utida *et al.* 1972). Actions of cortisol on calcium homeostasis appear to involve primarily a stimulation of calcium uptake mechanisms (Utida *et al.* 1972; Flik and Perry, 1989). Prolactin, however, influences plasma membrane fluidity and enzyme activity in rat tissues in a complex manner (Dave *et al.* 1981; Dave and Witorsch, 1985). Thus, the plasma membrane fluidity of the enterocytes of seawater fish may have increased as a result of the decreased prolactin levels.

We here show that seawater fish develop more intestinal Na^+/K^+ -ATPase activity, which is in line with one of the main functions of the intestine in seawater fish, i.e. water uptake by solute-linked water transport, and with the lower prolactin levels in seawater fish, as prolactin is known to exert an inhibitory control over Na^+/K^+ -ATPase activity (Pickford *et al.* 1970). Also, this study is the first to give evidence for the regulation of transepithelial calcium flux in the tilapia intestine. Seawater fish drastically reduce active intestinal uptake of calcium. The reduction in $\text{Na}^+/\text{Ca}^{2+}$ exchange activity in purified basolateral plasma membranes, which parallels the reduction in net transepithelial calcium uptake, supports the notion that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is the transport protein chiefly responsible for transcellular uptake of calcium in fish intestine.

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