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CALCIUM PUMP ACTIVITIES IN THE KIDNEYS OF *OREOCHROMIS MOSSAMBICUS*

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Summary

The mechanism that underlies transcellular Ca^{2+} reabsorption in the kidney of the euryhaline teleost *Oreochromis mossambicus* was studied. Preparations of membrane vesicles made from the kidneys of freshwater- and seawater-adapted fish were more than sevenfold enriched in the basolateral plasma membrane marker Na^{+}/K^{+}-ATPase. Significant recovery of NADH–cytochrome *c* reductase enzyme activity and of oxalate-stimulated Ca^{2+} pump activities in the membrane preparations indicated that the membrane fraction was of endoplasmic reticular origin. Indeed, thapsigargin specifically inhibited Ca^{2+} pump activity that could be attributed to oxalate-permeable endoplasmic reticular fragments. Kinetic analysis of thapsigargin-insensitive Ca^{2+} pump activity indicated the existence of a homogeneous, high-affinity, ATP-driven Ca^{2+} pump. No Na^{+}-driven Ca^{2+} transport mechanism could be demonstrated. Plasma membrane Ca^{2+} pump activity was 56 % lower in preparations from seawater-adapted fish than in preparations from freshwater-adapted fish, suggesting a physiological role for this Ca^{2+} pump activity in renal Ca^{2+} handling by euryhaline species, with an involvement in the regulation of Ca^{2+} reabsorption.

Key words: divalent cation transport, calcium transport, teleost osmoregulation, plasma membrane vesicles, thapsigargin, *Oreochromis mossambicus.*

Introduction

In euryhaline teleosts the kidney maintains Ca^{2+} homeostasis over a wide range of ambient Ca^{2+} concentrations. In sea water (SW) these fish are confronted with a constant Ca^{2+} influx from a hypercalcic environment and also with water efflux. Excess Ca^{2+} is actively excreted *via* the kidney, whilst urine flow is reduced. As a consequence, SW-adapted euryhaline teleost fish can produce urine with Ca^{2+} concentrations well above plasma levels (Björnsson and Nilsson, 1985; Schmidt-Nielsen and Renfro, 1975; Elger *et al.* 1987). In fresh water (FW), however, euryhaline fish need to minimize urinary Ca^{2+} loss, by reabsorbing filtered Ca^{2+}. Thus, the tubular epithelium of the nephron mediates both Ca^{2+} secretion (in SW) and reabsorption (in FW). It follows that transfer of the fish from FW to SW (or *vice versa*) must have a pronounced effect on the properties of Ca^{2+} transport *via* the tubular epithelium.

In mammals, Ca^{2+} reabsorption is mainly mediated through a paracellular route and is driven by the transepithelial potential (lumen positive) created by Na^{+} and Cl^{-} movement over the tubular epithelium; only in the distal nephron are hormonally controlled transcellular Ca^{2+} reabsorption mechanisms predominant (Friedman and Gesek, 1993). For freshwater fish, similar Na^{+}- and Cl^{-}-dependent transepithelial potentials have been reported (Nishimura and Imai, 1982). This would suggest that, as in mammals, Ca^{2+} reabsorption in fish follows a paracellular route and is driven by the transepithelial potential. However, the principal function of the kidneys of freshwater fish is the excretion of excess water. Reabsorption of water must, therefore, be minimized and this will limit solute-linked, paracellular reabsorption of electrolytes.

The few studies published that have examined the mechanism of renal Ca^{2+} handling in fish seem to imply a dependence on ATP-regulated or ATP-driven mechanisms for secretion as well as for absorption of Ca^{2+}. Renfro *et al.* (1982) proposed that in sea water winter flounder, *Pseudopleuronectes americanus*, intracellular ATP levels influenced Ca^{2+} secretion, whereas Na^{+} movement was not directly involved. More recently, in the kidney of *Gillichthys mirabilis,* a high-affinity Ca^{2+}-ATPase was identified, with a presumed function in Ca^{2+} reabsorption (Doneen, 1993).

In the euryhaline teleost *Oreochromis mossambicus* (hereafter called tilapia), Ca^{2+} transport in both gill and intestinal epithelium is mediated through Ca^{2+}-ATPase activity

and Na^+/Ca^{2+} exchange (Flik *et al.* 1985, 1990). The object of this study was to investigate the possible involvement of these transport mechanisms in Ca^{2+} handling in the kidneys of tilapia. Assuming that Ca^{2+} reabsorption is strongly enhanced in freshwater environments, the kidneys of euryhaline fish offer a model to study the contribution of Ca^{2+} transport mechanisms to this process.

Materials and methods

Tilapia, *Oreochromis mossambicus* (Peters), of both sexes were obtained from laboratory stock. Freshwater-adapted fish were kept in Nijmegen tap water ($[Ca^{2+}]$=0.7 mmol l^{-1}). Artificial sea water was prepared by adding Wimex sea salt (Wiegandt GMBH and Co., Krefeld, Germany) to tap water until a final concentration of 1.022 g l^{-1} was reached ($[Ca^{2+}]$=10 mmol l^{-1}). Fish were adapted to sea water over a 3-day period by the gradual infusion of sea water into tanks that were initially filled with fresh water. Once full-strength sea water was obtained, the water was constantly filtered and one-third of the volume was replaced weekly. Fish were kept for at least 3 weeks at full-strength salinity before use. The water temperature was 25 °C and the photoperiod was 12 h:12 h light:dark. Fish were fed Trouvit fish pellets (Trouw and Co., Putten, The Netherlands) at daily rations of 1.5 % of the fish total mass; the calcium content of the food was 0.34 mol kg^{-1}.

Membrane isolation

Fish were killed by spinal transection and weighed (FW: 180±43 g, mean mass ± S.D., N=13; SW mean mass: 200±31 g, N=12). The abdominal cavity was cut open lengthwise, and the intestinal tract and swim-bladder removed. Kidney tissue was gently excised, weighed (FW mean mass: 0.328±0.091 g, N=13; SW mean mass: 0.399±0.118 g, N=12) and immediately transferred to ice-cold saline containing 150 mmol l^{-1} NaCl, 1 mmol l^{-1} Hepes, 1 mmol l^{-1} 1,4-dithiothreitol (DTT) and 0.1 mmol l^{-1} EDTA, adjusted to pH 8.0 with Tris. All further steps were performed at 0–4 °C. The renal tissue was disrupted by 30 strokes with a Dounce homogenizer equipped with a loosely fitting pestle in isotonic sucrose buffer containing 250 mmol l^{-1} sucrose, 10 mmol l^{-1} Hepes, 1 mmol l^{-1} DTT and 100 trypsin inhibitor units ml^{-1} aprotinine, adjusted to pH 7.4 with Tris, with approximately 0.7 g of kidney tissue in 15 ml of buffer. This homogenization disrupts the complex renal tissue but leaves most blood cells intact. The homogenate was centrifuged for 10 min at 1400 g (Heraeus Sepatech Omnifuge 2.0RS, BS4402/A rotor, 2850 revs min^{-1}) to remove nuclei, cellular debris and blood cells. The resulting supernatant was brought to 37 % sucrose (w/w) by mixing (5 strokes with the Dounce homogenizer) with 1.25 volumes of sucrose (60 % w/w) dissolved in 10 mmol l^{-1} Hepes/Tris (pH 7.4). 9 ml of this suspension was overlaid with 3 ml of isotonic sucrose buffer and centrifuged isopycnically for 90 min at 200 000 g (Beckman L8–80, SW40 Ti rotor, 40,000 revs min^{-1}). The membranes on the interface of the sucrose block and the isotonic buffer were collected in a volume of 0.5 ml and mixed with 12 ml of isotonic buffer, containing the basic ingredients of the assay medium: 150 mmol l^{-1} NaCl (for Na^+/Ca^{2+} exchange) or 150 mmol l^{-1} KCl (for ATP-dependent Ca^{2+} uptake) and 20 mmol l^{-1} Hepes/Tris (pH 7.4). The membranes were pelleted by centrifugation for 30 min at 150 000 g (50Ti rotor, 45 000 revs min^{-1}), rinsed with isotonic buffer and resuspended by 20 passages through a 23 gauge needle in 0.5 ml of assay medium. Membrane preparations contained approximately 2.1 mg ml^{-1} protein and were used on the day of isolation without being frozen. Protein concentration was determined with a commercial reagent kit (Biorad), using bovine serum albumin as a reference.

Enzyme assays

The marker enzymes used were Na^+/K^+-ATPase for basolateral plasma membranes (Mircheff and Wright, 1976), aminopeptidase for brush border membranes (George and Kenny, 1973; Pfleiderer, 1970), NADH–cytochrome *c* reductase for endoplasmic reticulum (ER) (Omura and Takesue, 1970) and succinic acid dehydrogenase for mitochondrial fragments (Flik *et al.* 1983). Enzyme activities were assayed after treatment with a detergent, 0.2 mg ml^{-1} saponin (10 min, 25 °C), at a protein concentration of 1 mg ml^{-1}, to unmask enzyme activity that was latent as a result of membrane resealing (Flik *et al.* 1990).

Membrane orientation was determined as described previously (Flik *et al.* 1990). The percentage of inside-out orientated vesicles (IOV) was determined on the basis of acetylcholine esterase activity, using digitonin (0.1 % w/v, 10 min at 25 °C) to unmask latent enzyme activity. Determination of the percentage of rightside-out orientated vesicles (ROV) was based on the specific trypsin sensitivity of the cytosol-oriented part of the Na^+/K^+-ATPase. Trypsin was used at 4500 BAEE units mg^{-1} membrane protein, where BAEE is N-benzoyl-arginine ethyl ester, for 30 min at 25 °C. After quenching the trypsin activity with 25 mg ml^{-1} soybean trypsin inhibitor, trypsin-insensitive Na^+/K^+-ATPase activity (representing the ROV membrane fraction) was revealed by treatment with detergent. In controls, trypsin inhibitor was added before the addition of trypsin to allow assessment of total Na^+/K^+-ATPase activity.

Ca^{2+} transport

Transport of Ca^{2+} was assayed by means of a rapid filtration technique (Flik *et al.* 1990; Van Heeswijk *et al.* 1984). The composition of the assay medium was 150 mmol l^{-1} KCl, 0.5 mmol l^{-1} EGTA, 0.5 mmol l^{-1} HEEDTA, 0.5 mmol l^{-1} nitrilotriacetic acid (NTA), 0.8 mmol l^{-1} free Mg^{2+}, 3 mmol l^{-1} ATP (Tris-salt, Sigma), 20 mmol l^{-1} Hepes/Tris (pH 7.4) and 5 μg ml^{-1} oligomycin B. $CaCl_2$ was added to obtain the calculated free Ca^{2+} concentrations of 1.0×10^{-8} to 2.0×10^{-6} mol l^{-1}. $^{45}CaCl_2$ (specific activity 24 TBq mol^{-1}, Amersham) was added to the incubation medium to make up a radioactive concentration of approximately 74 kBq ml^{-1}. In order to achieve a uniform specific activity of all the Ca^{2+} species in the incubation medium, the radiotracer was added at least 30 min prior to experimentation. ATP-dependent uptake

was determined as the difference between Ca^{2+} uptake in the presence and in the absence of ATP. Pilot experiments demonstrated that Ca^{2+} uptake was linear for approximately 1 min. Therefore, 30 s incubations were used to estimate the initial rate of Ca^{2+} uptake. When Na^+/Ca^{2+} exchange was assayed, ATP was omitted from the assay medium and in some cases KCl was replaced by NaCl. Incubations were carried out at 37 °C for optimum enzyme activity. Free Ca^{2+} and free Mg^{2+} concentrations were calculated according to Schoenmakers *et al.* (1992). In experiments where oxalate (2 mmol l^{-1}) was included in the assay medium, Ca^{2+} and Mg^{2+} binding to oxalate was taken into account. Thapsigargin was added from a 1 mmol l^{-1} stock solution in ethanol. A23187 (calcimycin) was dissolved in dimethyl sulphoxide (DMSO) and added to the assay medium at 5 $\mu g\,ml^{-1}$. The concentration of solvents in the assay medium did not exceed 0.1 % (v/v). The reaction was quenched by adding 1 ml of ice-cold stop buffer: 150 mmol l^{-1} KCl (or NaCl when Na^+/Ca^{2+} exchange was assayed), 20 mmol l^{-1} Hepes/Tris, pH 7.4, 0.8 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} $LaCl_3$ to 0.15 ml of incubate. A volume of 1 ml, equivalent to 10–20 μg of membrane protein, was then filtered (Schleicher and Schuell, ME25; pore size: 0.45 μm). Filters were rinsed twice with 2 ml of stop buffer and dissolved in 4 ml of scintillation fluid. ^{45}Ca specific activity was determined by counting the radioactivity in 0.05 ml of vesicle suspension. Radioactivity was determined in a Pharmacia Wallac 1410 liquid scintillation counter.

Calculations and statistics

Values are expressed as mean ± standard deviation (S.D.). Data were analyzed with a nonlinear regression data analysis program (Leatherbarrow, 1987). Data were analyzed statistically by the Mann-Whitney *U*-test or the Student's *t*-test when appropriate. Statistical significance was accepted at $P<0.05$.

Results

Basolateral membrane isolation and orientation

In Table 1, protein recovery and the recovery and enrichment of several marker enzymes are listed for freshwater and seawater membrane preparations. Recovery and enrichment of the basolateral membrane marker Na^+/K^+-ATPase did not differ for freshwater and seawater preparations: specific enzymatic activity (SA; expressed as the rate of phosphate release) was 121±28 $\mu mol\,h^{-1}\,mg^{-1}$ protein for freshwater preparations and 91±17 $\mu mol\,h^{-1}\,mg^{-1}$ protein for seawater preparations (P=0.061). The aminopeptidase activity indicates that recovery of brush border membranes is relatively low. NADH–cytochrome *c* reductase recovery, however, was considerable and of special importance since this fraction exhibits Ca^{2+} transport activity (see next section for further details).

Plasma membrane orientation of freshwater preparations was 29±4 % IOV (N=5), 44±7 % ROV (N=7) and 27±11 % leaky membranes (calculated value). For seawater preparations, the corresponding percentages are 42±6 % (N=5), 34±7 % (N=5) and 24±13 %, respectively. The seawater preparations contained significantly more IOV orientated vesicles (P=0.004) and fewer ROV orientated vesicles (P=0.035) than the freshwater preparations.

Thapsigargin inhibition of ATP-dependent Ca^{2+} uptake

Fig. 1 shows the dose-dependent inhibition of Ca^{2+} uptake by thapsigargin. Thapsigargin partly inhibited ATP-dependent Ca^{2+} uptake. Maximal inhibition occurred at a thapsigargin concentration of 0.5 $\mu mol\,l^{-1}$. Increasing the thapsigargin concentration to 5 $\mu mol\,l^{-1}$ did not have any further effect on Ca^{2+} uptake rates. The residual, thapsigargin-insensitive, activities at a Ca^{2+} concentration of 500 nmol l^{-1} amounted to 26.1±0.4 % for freshwater preparations and 36.2±1.3 % for seawater preparations of the total Ca^{2+} uptake. These thapsigargin-insensitive activities divided by the respective IOV percentages for freshwater (29 %) and seawater (42 %) preparations yield the total thapsigargin-insensitive Ca^{2+} pump activities of the plasma membranes. These corrected thapsigargin-insensitive activities amount to, for freshwater preparations, 55 % (14 nmol $min^{-1}\,mg^{-1}$ protein) and, for seawater preparations, 57 % (5.6 nmol $min^{-1}\,mg^{-1}$ protein) of the total Ca^{2+} pump activity (consisting of the thapsigargin-

Table 1. *Relative recoveries and enrichment of marker enzymes in freshwater- and seawater-adapted tilapia kidney plasma membranes*

	Recovery (%)		Enrichment	
Marker	FW	SW	FW	SW
Protein	1.9±0.7 (13)	2.1±0.3 (5)	–	–
Aminopeptidase	3.7±1.1 (6)	2.9±0.9 (4)	2.3±0.6 (6)	1.7±0.7 (4)
Na^+/K^+-ATPase	15.5±2.9 (5)	15.6±4.6 (5)	7.5±1.0 (5)	7.2±1.1 (5)
Succinic acid dehydrogenase	2.5±1.1 (8)	2.6±0.7 (3)	1.3±0.9 (8)	1.4±0.5 (3)
NADH–cytochrome *c* reductase	5.4±2.0 (5)	6.7±2.5 (3)	2.8±1.1 (5)	2.9±0.6 (3)

Recovery was calculated as the percentage of the total activity in the plasma membrane fraction relative to that in the initial tissue homogenate.

Enrichment was calculated by dividing the specific activity in the plasma membrane fraction by the specific activity in the initial tissue homogenate.

Number of observations is indicated in parentheses.

FW, freshwater-adapted; SW, seawater-adapted membranes.

sensitive activity plus the corrected thapsigargin-insensitive activity). Thapsigargin at $1\,\mu mol\,l^{-1}$ completely abolished oxalate stimulation of ATP-dependent Ca^{2+} uptake in freshwater and seawater preparations (Fig. 2), indicating complete inhibition of endoplasmic reticulum (ER)-related Ca^{2+} uptake. Thapsigargin-insensitive Ca^{2+} accumulation was ATP-dependent and was reversed by the Ca^{2+} ionophore A23187 (Fig. 3).

Kinetics of thapsigargin-insensitive Ca^{2+} uptake

Kinetic analysis of the $[Ca^{2+}]$-dependence of ATP-dependent Ca^{2+} uptake was performed on individual preparations. Fig. 4 shows the pooled data of five freshwater and five seawater preparations. For freshwater preparations a V_m of $4.50\pm0.89\,nmol\,min^{-1}\,mg^{-1}$ protein and a K_m of $57\pm17\,nmol\,l^{-1}$ were calculated. For seawater preparations V_m decreased to $2.96\pm0.26\,nmol\,min^{-1}\,mg^{-1}$ protein (P=0.008) and K_m was $63\pm20\,nmol\,l^{-1}$. When appropriate corrections for the percentages of IOV present are made, the calculated values for V_m are $16\,nmol\,min^{-1}\,mg^{-1}$ protein for freshwater preparations and $7.0\,nmol\,min^{-1}\,mg^{-1}$ protein for seawater preparations. This amounts to a 56 % decrease of renal Ca^{2+} pump activity upon transfer to sea water.

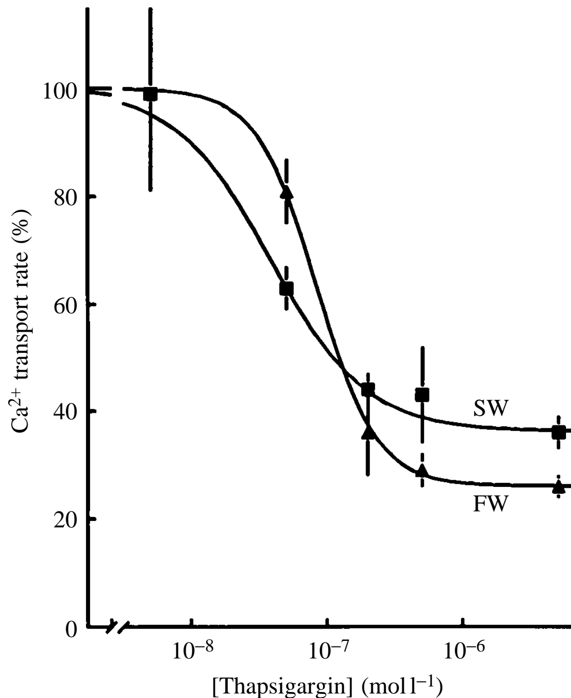

Fig. 1. Dose-dependence of thapsigargin inhibition of ATP-driven Ca^{2+} uptake by freshwater (FW, ▲) and seawater (SW, ■) membrane vesicle preparations of renal tissue. Initial rates of uptake were assayed at a free Ca^{2+} concentration of $500\,nmol\,l^{-1}$ and corrected for ATP-independent uptake. Transport rates in the absence of thapsigargin were designated 100 %. Values depict means ± S.D. of three preparations.

Na^{+}/Ca^{2+} exchange

Table 2 summarizes data on Ca^{2+} uptake in membrane vesicles from freshwater-adapted tilapia, in the absence of ATP. Stimulation of Ca^{2+} uptake by applying a Na^{+} gradient could not be demonstrated. No dependence on Ca^{2+} concentration was indicated and data did not allow further kinetic analysis.

Table 2. *Dependence of Ca^{2+} uptake on Na^{+} gradient and Ca^{2+} concentration in freshwater tilapia kidney plasma membrane vesicles*

$[Ca^{2+}]$ ($nmol\,l^{-1}$)	Ca^{2+} uptake ($nmol\,mg^{-1}$) $[Na^{+}]_o$*	$[K^{+}]_o$†
50	0.38±0.23 (7)	0.32±0.17 (7)
100	0.60±0.35 (9)	0.71±0.37 (9)
250	0.98±0.43 (7)	1.37±0.95 (7)
500	1.31±0.68 (8)	1.41±0.58 (8)
1000	1.73±0.41 (9)	2.16±0.76 (9)
2000	2.22±0.67 (7)	2.68±0.83 (7)

Values depict Ca^{2+} uptake over a 5 s period and are means ± S.D.

At all Ca^{2+} concentrations tested, $[K^{+}]_o$ values were not significantly different from $[Na^{+}]_o$ values.

*Na^{+}-loaded vesicles in $150\,mmol\,l^{-1}$ Na^{+}; †Na^{+}-loaded vesicles in $150\,mmol\,l^{-1}$ K^{+}.

Number of observations is indicated in parentheses.

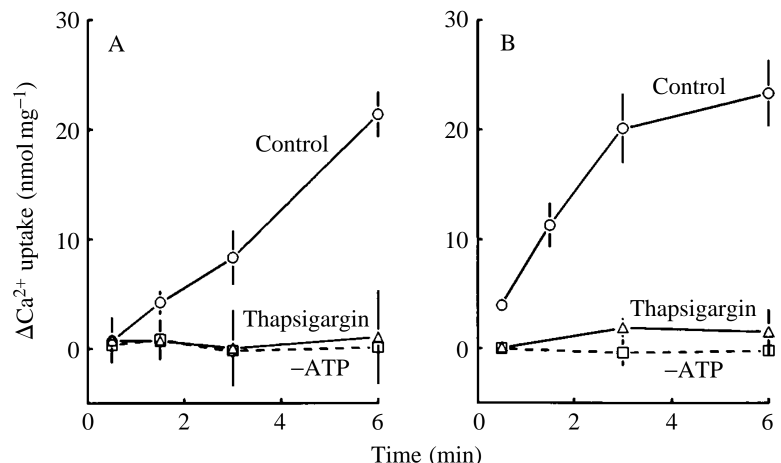

Fig. 2. Oxalate-stimulated Ca^{2+} uptake by freshwater (A) and seawater (B) tilapia membrane preparations of renal tissue. Values represent the difference (ΔCa^{2+}) between Ca^{2+} uptake in the presence and in the absence of $2\,mmol\,l^{-1}$ oxalate, at a free Ca^{2+} concentration of $500\,nmol\,l^{-1}$. Thapsigargin ($1\,\mu mol\,l^{-1}$) completely abolished oxalate stimulation of ATP-dependent Ca^{2+} uptake (△). ATP-independent Ca^{2+} uptake (□) was not stimulated by oxalate. Means ± S.D. of three preparations are given.

Discussion

To study the involvement of active transporters in Ca^{2+} reabsorption in the fish kidney, we isolated a membrane fraction from a renal tissue homogenate of tilapia. The separation techniques applied were based on studies of ion transporters in the mammalian kidney (reviewed by Mürer and Gmaj, 1986) and were successfully used in our laboratory for isolation of the basolateral membrane fraction of tilapia enterocytes (Flik *et al.* 1990). The enrichment factor and the specific enzymatic activity of the basolateral membrane enzyme marker Na^+/K^+-ATPase derived for our preparation are comparable to values reported earlier for tilapia intestinal membranes (Flik *et al.* 1990). Recovery, enrichment and specific activity of the Na^+/K^+-ATPase were not significantly different in freshwater or seawater renal membrane preparations of tilapia, although a decrease of Na^+ pump activity upon transfer to high ambient salinity has been reported for several other euryhaline species (Doneen, 1993; Trombetti *et al.* 1990; Venturini *et al.* 1992). The high recovery of the ER enzyme marker NADH–cytochrome *c* reductase indicated that our final membrane preparation still contained a considerable proportion of ER fragments. This was confirmed by the ^{45}Ca uptake studies, which showed that oxalate, which is preferentially transported into ER-derived vesicles (Ponnappa *et al.* 1981), was able to stimulate ^{45}Ca accumulation by trapping ^{45}Ca in vesicles. Attempts to enhance the purification of plasma membranes relative to the ER fraction by other initial tissue fragmentation techniques or by further centrifugation on sucrose block gradients ranging from 31 % to 43 %, or on a self-generating Percoll gradient as reported by Van Heeswijk *et al.* (1984), were unsuccessful (results not shown).

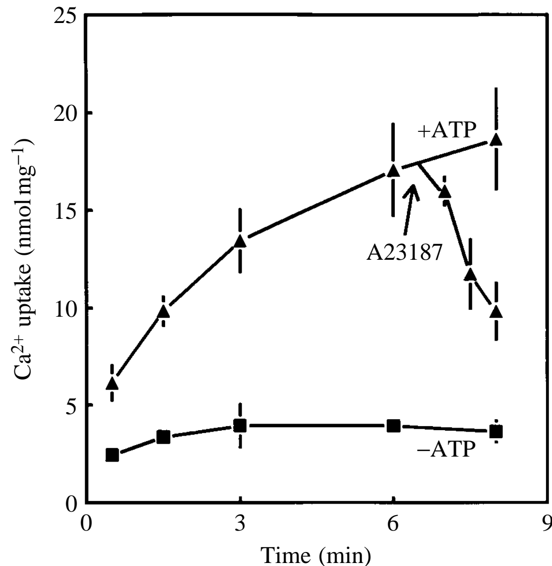

Fig. 3. Thapsigargin-insensitive Ca^{2+} uptake in renal membrane preparations from freshwater fish, assayed at a free Ca^{2+} concentration of 500 nmol l^{-1} and a thapsigargin concentration of 1 μmol l^{-1}. Thapsigargin-insensitive Ca^{2+} uptake was stimulated by ATP, and Ca^{2+} accumulation was reversed by the addition of the Ca^{2+} ionophore A23187 (arrow). Means ± S.D. of three experiments are given.

We used the highly specific ER Ca^{2+}-ATPase inhibitor thapsigargin to inhibit ^{45}Ca uptake into ER-derived vesicles (Thastrup *et al.* 1990). Our study shows that thapsigargin abolished oxalate-stimulated Ca^{2+} uptake, indicating complete inhibition of all ER-related Ca^{2+} pump activity. Maximal inhibition was reached at a thapsigargin concentration of 0.5 μmol l^{-1}. The residual Ca^{2+} uptake was not affected by an increase in the thapsigargin concentration to 5 μmol l^{-1}, indicating that at these low concentrations thapsigargin inhibits ER Ca^{2+}-ATPase activity, but does not have an ionophoretic effect on the membrane vesicles, as reported by Favero and Abramson (1994). Thapsigargin-insensitive Ca^{2+} uptake was

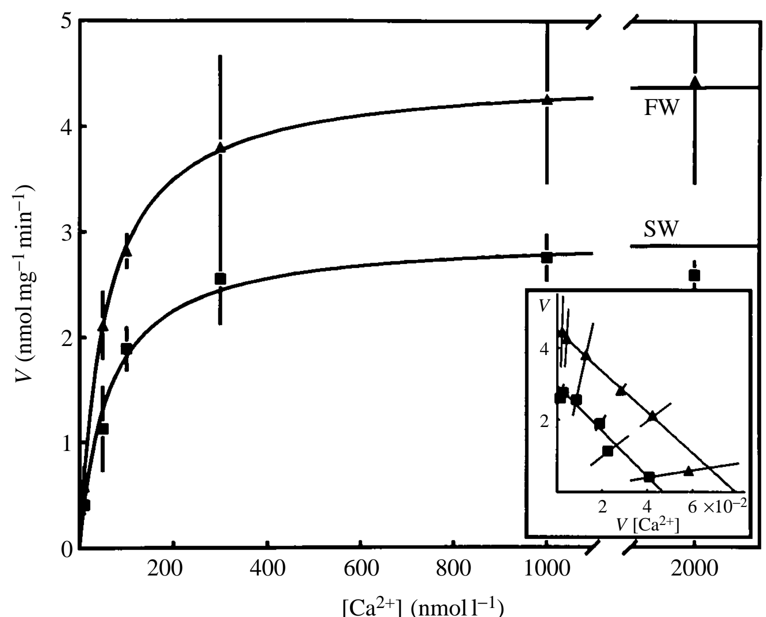

Fig. 4. Kinetics of thapsigargin-insensitive, ATP-dependent Ca^{2+} uptake in freshwater (FW, ▲) and seawater (SW, ■) membrane preparations of renal tissue. Initial rates of uptake were corrected for ATP-independent Ca^{2+} uptake. Values depict means ± S.D. of five preparations. Data of individual preparations were fitted to the Michaelis–Menten equation; for FW, V_m=4.50±0.89 nmol min^{-1} mg^{-1} protein and K_m=57±17 nmol l^{-1}; for SW, V_m=2.96±0.26 nmol min^{-1} mg^{-1} protein and K_m=63±20 nmol l^{-1}. The inset shows an Eadie–Hofstee transformation of the data. V, transport rate.

stimulated by ATP, and could be reversed by the addition of the Ca^{2+} ionophore A23187, indicating uphill Ca^{2+} transport. The K_m values derived for freshwater and seawater preparations indicate that this Ca^{2+} pump is activated at intracellular Ca^{2+} levels. This strongly suggests that this Ca^{2+} pump activity originates from the plasma membrane fraction of the membrane preparation and reflects a mechanism for Ca^{2+} extrusion.

We were unable to demonstrate Na^+/Ca^{2+} exchange activity in renal epithelium of tilapia: no significant difference was found between Ca^{2+} uptake in the absence and in the presence of a Na^+ gradient. Results obtained in our laboratory, with membrane preparations of tilapia gill and intestine, using a similar experimental setup, show that application of a Na^+ gradient stimulates Ca^{2+} uptake in these preparations by at least a factor of two. When assaying preparations of kidney and intestine from the same fish simultaneously, only in the intestine preparation could we demonstrate Na^+-driven Ca^{2+} transport, which excludes a methodological origin for our inability to demonstrate Na^+/Ca^{2+} exchange in renal preparations (results not shown). Furthermore, the addition of the K^+ ionophore valinomycin to prevent the build-up of a potential difference did not affect Ca^{2+} transport rates (results not shown). We tentatively conclude that Na^+/Ca^{2+} exchange activity is low or absent in tilapia kidney and, in the case of low activity, may be obscured by a proportionally large, non-specific Ca^{2+} uptake or Ca^{2+} binding.

Earlier studies report that euryhaline species maintain Ca^{2+} homeostasis upon transfer from fresh water to sea water by reducing renal Ca^{2+} reabsorption (Elger *et al.* 1987; Foster, 1976; Schmidt-Nielsen and Renfro, 1975). To evaluate the physiological significance of the identified Ca^{2+} pump in renal Ca^{2+} handling, we compared its activity in freshwater- and seawater-adapted tilapia. In our experiments, in which freshwater- and seawater-kidney preparations were isolated and assayed simultaneously, the renal Ca^{2+} transport capacity of freshwater-adapted fish significantly exceeded that of seawater-adapted fish, indicating that Ca^{2+}-pump activity correlates with Ca^{2+} reabsorption. At the estimated cytosolic Ca^{2+} concentration of $100\,nmol\,l^{-1}$ and at 25 °C, and correcting for the percentage of IOVs present and assuming an activation energy of $33\pm4\,kJ\,mol^{-1}$ (Van Heeswijk *et al.* 1984), we calculate a Ca^{2+} pump activity of $5.9\,nmol\,min^{-1}\,mg^{-1}$ protein for freshwater preparations. From a protein yield of 1.1 ± 0.3 mg and the percentage found for basolateral membrane recovery, it follows that the Ca^{2+} transport capacity amounts to $6.0\,\mu mol\,h^{-1}\,kg^{-1}$ fish.

This transport capacity of the Ca^{2+} pump is well in line with data on renal Ca^{2+} handling in freshwater-adapted fish. From the estimated glomerular filtration rate of $4\,ml\,h^{-1}\,kg^{-1}$ fish for freshwater-adapted species (Hickman and Trump, 1969) and an ultrafiltrable plasma Ca^{2+} concentration of $1.82\pm0.29\,mmol\,l^{-1}$ (N=3), a renal Ca^{2+} filtration rate of $7.3\,\mu mol\,h^{-1}\,kg^{-1}$ fish can be calculated. Estimating the Ca^{2+} excretion at $2.5\,\mu mol\,h^{-1}\,kg^{-1}$ fish (Butler, 1993; Elger *et al.* 1987; Oikari and Rankin, 1985; Schmidt-Nielsen and Renfro, 1975; Hickman, 1968), we calculate that a net reabsorption of $4.8\,\mu mol\,h^{-1}\,kg^{-1}$ fish occurs, which amounts to 80 % of the transporting capacity of the Ca^{2+} pump. The high capacity of the Ca^{2+} pump relative to the estimated rate for net Ca^{2+} reabsorption suggests that in fish, in contrast to mammalian species (Friedman and Gesek, 1993), the transcellular route for Ca^{2+} reabsorption prevails. Paracellular reabsorption of solutes may be relatively small because of the low water permeability of the tubular epithelium (as reabsorption of water must be minimized) in the distal part of the nephron (Nishimura and Imai, 1982).

For seawater-adapted fish, a Ca^{2+} transporting capacity of $2.7\,\mu mol\,h^{-1}\,kg^{-1}$ fish is calculated. The observation that seawater-adapted fish maintain Ca^{2+} pump activity even though they have no requirement for net Ca^{2+} reabsorption, suggests that part of the pumping activity is required for intracellular Ca^{2+} homeostasis. Consequently, the relative contribution of the Ca^{2+} pump to reabsorption may be smaller than estimated on the basis of total pump activity and, therefore, reabsorption may require additional Ca^{2+} transport mechanisms.

Ca^{2+} transport *via* the intestine and gill epithelium of freshwater- and seawater-adapted tilapia has been characterized by Schoenmakers *et al.* (1993) and Verbost *et al.* (1994), respectively. Whereas in the epithelium of the intestine Na^+/Ca^{2+} exchange is the predominant mechanism for transcellular Ca^{2+} transport, in the gill epithelium the activity of the exchanger was estimated to be only 50 % of that of the ATP-driven pump (at prevailing cytosolic Ca^{2+} concentrations). This study indicates, however, that transcellular Ca^{2+} transport in renal epithelium of tilapia is fully dependent on an ATP-driven pump. The picture that emerges is that Ca^{2+} handling by the euryhaline tilapia is controlled by the regulated and differential expression of both Ca^{2+} pump and Na^+/Ca^{2+} exchange activities in the three major Ca^{2+}-transporting epithelia, i.e. gill, intestine and kidney. It enables the euryhaline tilapia to maintain Ca^{2+} homeostasis over a wide range of ambient Ca^{2+} concentrations, which is a prerequisite for successful salinity adaptation.

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