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Na^+-dependent Ca^{2+} uptake in isolated opercular epithelium of *Fundulus heteroclitus*

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Abstract It is concluded that Ca^{2+} transport across the basolateral membranes of the ionocytes in killifish skin is mediated for the major part by a Na^+/Ca^{2+}-exchange mechanism that is driven by the (transmembrane) Na^+ gradient established by Na^+/K^+-ATPase. The conclusion is based, firstly, on the biochemical evidence for the presence of a Na^+/Ca^{2+}-exchanger next to the Ca^{2+}-ATPase in the basolateral membranes of killifish gill cells. Secondly, the transcellular Ca^{2+} uptake measured in an Ussing chamber setup was 85% and 80% reduced in freshwater (FW) and SW (SW) opercular membranes, respectively, as the Na^+ gradient across the basolateral membrane was directly or indirectly (by ouabain) reduced. Thapsigargin or dibutyryl-cAMP/IBMX in SW opercular membranes reduced Ca^{2+} influx to 46%, comparable to the effects seen in FW membranes [reduction to 56%; Marshall et al. 1995a]. Basal Ca^{2+} influx across the opercular membrane was 48% lower in membranes from fish adapted to SW than in membranes from fish adaptated to FW. Branchial Na^+/K^+-ATPase activity was two times higher in SW adapted fish.

Key words Calcium transport · Ca^{2+}-ATPase gill function · Killifish · Na^+/Ca^{2+}-exchange

Abbreviations *FW* fresh water · *SW* sea water · *BLMV* basolateral membrane vesicles · G_t transepithelial conductance · V_t transepithelial potential

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Introduction

Teleosts utilize the environment as their Ca^{2+} source and this Ca^{2+} is primarily taken up via the gills (Fenwick 1989). Ca^{2+} flux-ratio analysis shows that Ca^{2+} movement from the water to the blood is an active, transcellular process (Perry and Flik 1988; Verbost et al. 1994; Marshall et al. 1995a). Branchial Ca^{2+} influx is under inhibitory control by stanniocalcin, a rapid-acting hormone produced by the corpuscles of Stannius in both freshwater and seawater fish (Wendelaar Bonga and Pang 1986; Flik and Verbost 1993). Ca^{2+} uptake in freshwater fish is stimulated by prolactin, but the effect of prolactin takes days to develop (Pang 1981; Flik et al. 1994; Sundell et al. 1993; Flik et al. 1986). There is a wealth of data indicating that the chloride cells are the site of calcium uptake in the gills (Flik et al. 1985a; Fenwick 1989; McCormick et al. 1992; Marshall et al. 1995a; Perry and Walsh 1989). The first model that has been developed for the transcellular Ca^{2+} uptake suggested that a high affinity Ca^{2+}-ATPase in the basolateral membrane of the chloride cells drives the uptake (Flik et al. 1985b; Flik et al. 1985a; Perry and Flik 1988) by pumping Ca^{2+} from the cytosol to the blood against a steep electrochemical gradient. Since then, new membrane vesicle studies revealed the kinetics of the Na^+/Ca^{2+}-exchanger in the basolateral membranes of branchial epithelium (Flik et al. 1993; Verbost et al. 1994). It was concluded that both the ATPase and the exchanger could be equally important for the transmembrane transport of Ca^{2+}. This raises the question that forms the basis for this study: what is the contribution of each system in operational chloride cells in situ? We decided to determine the degree of sodium-dependency of Ca^{2+} influx (unidirectional) across fish gills using the opercular membrane in Ussing-style membrane chambers, which would allow us to estimate the contribution by the Na^+/Ca^{2+}-exchanger. The alternative approach, i.e. specific inhibition the high affinity Ca^{2+}-ATPase, is not presently available.

Na^+/Ca^{2+}-exchange activity has never been shown in killifish gills. Therefore, we wanted to show the presence and the capacity of the Ca^{2+} transporters (ATPase and exchange) in vitro in FW and SW gills. To this end we isolated basolateral membrane vesicles from the gills and measured ATP- and Na^+-gradient-dependent Ca^{2+} transport.

In addition, we were interested to see the effect of adaptation to either FW or SW on the second messenger regulation of the influx and the possible changes in the Ca^{2+} uptake mechanism, the ATPase and the exchanger. In SW, according to current models, Cl^- secretion is dependent on basolateral Na-K-2Cl cotransport in the chloride cells (Foskett and Scheffey 1982). This Na^+-dependent cotransporter would operate in parallel with a Na^+/Ca^{2+}-exchanger and feasibly both transport processes would utilize the same Na^+ electrochemical driving force. Hence, stimulation of Cl^- secretion after transfer from FW to SW may inhibit Ca^{2+} uptake by this indirect dependency on the Na^+ gradient. To study the possible interactions between these transport processes and to describe the transcellular uptake of Ca^{2+}, we determined the effects of the Na^+gradient across the basolateral membrane in opercular membranes from FW and SW animals.

Materials and methods

Animals

Adult killifish of both sexes were obtained from the Antigonish estuary and were transferred to indoor holding facilities and were maintained in 10% SW (3.0–3.2 ppt) at 20–25 °C under artificial light operating on natural photoperiod. Animals were transferred directly to FW or SW and were held for at least 12 days prior to flux experiments. The composition (in $\mu mol \cdot l^{-1}$) of the FW medium in which killifish were acclimated was Na^+ 1000, K^+ 20, Cl^- 1000, SO_4^{2-} 140, Ca^{2+} 100, Mg^{2+} 60 with titration alkalinity (titration to pH = 4.0) of 280 $\mu mol \cdot l^{-1}$ and pH of 6.8–7.2. The SW (30–32 ppt salinity) contained 9–10 $mmol \cdot l^{-1}$ Ca^{2+}. The fish were fed twice daily with Tetra Min Marine supplemented with frozen brine shrimp.

Fish were killed by transection of the spinal cord followed by destruction of the brain and the opercular epithelia (the epithelium lining the medial surface of the opercular bone) were removed and mounted in Ussing-style membrane chambers (see Ussing chamber tracer fluxes). The gills were removed, filaments were separated from the gill arch and immediately frozen in storage medium until further use (see membrane vesicle isolation). The storage medium contained (in $mmol \cdot l^{-1}$) sucrose 300, Na_2EDTA 20, imidazole 100, and 2 $mg \cdot l^{-1}$ aprotinin and pH of 7.1.

Ussing chamber tracer fluxes

The bathing solution was a modified Cortland's saline with the composition (in $mmol \cdot l^{-1}$) Na^+ 150, Cl^- 136, K^+ 2.6, HCO_3^- 17.7, Ca^{2+} 1.6, Mg^{2+} 0.9, PO_4^{2-} 3.0, SO_3^{2-} 0.9, and glucose 5.6, pH 7.8 when bubbled with 99% O_2/1% CO_2. When Na^+ was reduced (from 151 to 21 $mmol \cdot l^{-1}$) NaCl was replaced by *N*-methyl-*D*-glucamine chloride. In these cases one membrane of each pair had normal Cortland's on both sides, the other had low-Na^+ Cortland's on both sides. When FW bathed the mucosal surface, this solution was bubbled with 100% O_2. Ca^{2+} fluxes with FW on the mucosal side were also performed with paired membranes from one fish: one membrane served as control, having Cortland's saline on both sides, while the other was flushed extensively with FW (20 times the chamber volume, mucosal side only) to remove saline that was used in the process of mounting. Unidirectional $^{45}Ca^{2+}$ influx from mucosa to serosa (J_{ms}) was determined as published previously (Marshall et al. 1995a). Briefly, 50 $KBq\,ml^{-1}$ $^{45}Ca^{2+}$ (as $^{45}CaCl_2$; I.C.N. Radiochemicals) was added to the mucosal side and tracer was allowed to equilibrate for 30 min before the actual sampling was started. Samples were taken every 20 min and the volume taken was replaced with a solution of the same ionic composition.

The Ussing membrane chambers, 3 ml volume per hemichamber, had an exposed membrane area of 0.125 cm^2 and were water jacketed to control temperature at 22 °C. Two ports on top of each chamber were used to put in the voltage measuring bridge and current passing bridge and a third port for aeration and fluid manipulation (and sampling). Transepithelial conductance (G_t in $mS\,cm^{-2}$) and transepithelial potential (V_t in mV, mucosal side grounded and corrected for junction potentials) were monitored as described previously (Marshall 1986) using dual-channel current-voltage clamps (D. Lee Co. and W.P. Instruments DVC 1000). In experiments where saline bathed both sides of the epithelium, V_t was clamped to zero and the shortcircuit current (I_{sc} in $\mu A\,cm^{-2}$) was also measured.

Membrane vesicle isolation

Plasma membranes were isolated from gill filaments as described in detail previously (Flik et al. 1985b). In brief, after homogenization of the filament cells in hypotonic medium (25 $mmol \cdot l^{-1}$ NaCl, 1 $mmol \cdot l^{-1}$ Hepes, adjusted to pH 8.0 with TRIS), cellular debris were separated from the membrane fraction by centrifugation (550 *g* at, 4 °C for 5 min). The membranes (from the supernatant) were collected by ultracentrifugation (105 000 *g* at 4 °C for 30 min, Beckman Ti-70 rotor). From the resulting pellet the fluffy top layer was resuspended in buffer with a Dounce homogenizer (100 strokes). This membrane suspension was centrifuged differentially (1000 *g* 10 min and 10 000 *g*, 10 min at 4 °C) and the basolateral membranes in the resulting supernatant were collected by pelleting at 50 000 *g* at 4 °C for 15 min. The procedure yields membrane suspensions in which 20–25% of the vesicles are orientated inside-out, based on the acetylcholine esterase activity in intact and (Triton X-100) permeabilized vesicles. Vesicles were used on the day of isolation. The protein content of the vesicle preparations was 2.23 ± 0.50 $g \cdot l^{-1}$ (n = 12), as determined with a commercial reagent kit (Bio-Rad) with bovine serum albumin (BSA) as a reference. Protein recovery was not significantly different in FW preparations and SW preparations, amounting to 3.05 ± 0.38% and 2.81 ± 0.15% (n = 5), respectively. The purification of the basolateral membranes, expressed as the ratio of the Na^+/K^+-ATPase activity (see below) in the final pellet and that in the filament-homogenate, was 26.5 ± 4.5 times in FW preparations and 21.8 ± 3.1 times in SW preparations (n = 5, not significantly different).

Protein activity assays

Na^+/K^+-ATPase activity in fish branchial basolateral plasma membrane vesicles (BLMV) was defined as the Na^+- and K^+-dependent, ouabain-sensitive phosphatase activity. Incubation was performed in Na/K-ATPase-assay medium containing (in $mmol \cdot l^{-1}$) NaCl 100, imidazole 30, $MgCl_2$ 5, EDTA 0.1, Na_2ATP 3, adjusted to pH 7.4 with TRIS (500 µl per tube). Incubation was 10 min at 37 °C (optimum temperature) with 10 µg protein per tube. Reaction was stopped by adding 500 µl ice-cold 8.6% trichloroacetic acid. Quantification of inorganic phosphate was done with the colorometric Fiske and Subbarow method. For determination of total enzymic activity the membrane vesicles were permeabilized with saponin (by preincubation, 20–30 min on ice, with 0.2 $mg \cdot ml^{-1}$ saponin used at a protein concentration of 1.0–1.5 $mg \cdot ml^{-1}$).

$^{45}Ca^{2+}$ transport was determined in assay media that contained: 0.5 mmol·l^{-1} ethyleneglycol-*bis* (β-aminoethyl ether)*N,N′,N′,N′*-tetra-acetic acid (EGTA), 0.5 mmol·l^{-1} *N*-(2-hydroxyethyl)-ethylenediamine-*N,N′,N′*-triacetic acid (HEEDTA), and 0.5 mmol·l^{-1} nitrilo triacetic acid (NTA) as Ca^{2+}-buffering system. Free calcium and magnesium levels were calculated with a matrix computer program (Fenwick 1984) 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 optimal assay temperature.

The ATP-driven transport of $^{45}Ca^{2+}$ was determined in 30-s incubations (yielding initial velocities of the pump) 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 as mentioned above. $^{45}Ca^{2+}$ uptakes were corrected for uptake in the absence of ATP. Free Ca^{2+} concentration was 1 μmol·l^{-1}. Thapsigargin (1 μmol·l^{-1}) was present to prevent any uptake by endoplasmic reticulum contaminations that are always present to a small extent (Flik et al. 1985b). 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 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. Four ml 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 prewarmed membrane vesicles (9–12 μg protein) equilibrated with 150 mmol·l^{-1} NaCl were added to 120 μl of media containing $^{45}Ca^{2+}$ and either 150 mmol·l^{-1} NaCl (blank) or 150 mmol·l^{-1} KCl. Ca^{2+} concentrations ranged from 250 nmol·l^{-1} to 25 μmol·l^{-1}. After 5 at 37 °C the reaction was stopped by addition of 1 ml 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.

Data presentation

Data are expressed as the mean ± one SE unless indicated otherwise. For the Ussing chamber experiments control and test conditions were analysed by paired *t*-tests when the tissue served as its own control. Otherwise, the unpaired *t*-test was applied.

Results

ATP-dependent and Na^+-gradient-dependent vesicle Ca^{2+} transport

The physiological experiments with opercular membranes are a means to study the mechanism of branchial Ca^{2+} uptake. With vesicle studies the presence of the Ca^{2+} transporters in the gills was determined as the ATP-dependent and Na^+-gradient-dependent uptake of $^{45}Ca^{2+}$ in isolated basolateral membranes of killifish gills. There was no difference in Ca^{2+}-ATPase or Na^+/Ca^{2+}-exchange activity between FW and SW membranes (Table 1). Since the uptake studies were done at V_{max} conditions (at 1 and 2 μmol·l^{-1} free Ca^{2+}, respectively), and initial uptake velocities were determined, these results signify similar transport capacities for both transporters in FW and SW fish. The Na^+/K^+-ATPase activity measured in crude homogenates was two times higher in SW gills than in FW gills (Table 1).

Table 1 Ca^{2+} transport activity in basolateral membrane vesicles (*BLM*) from gills of FW- and SW adapted killifish. The Ca^{2+}-ATPase and Na^+/Ca^{2+}-exchange were determined as actual $^{45}Ca^{2+}$ transport activities (transport rates at 1 and 2 μmol·l^{-1} Ca^{2+}, respectively) and are expressed in nmol Ca^{2+}.min^{-1}.mg^{-1}. The listed Na^+/K^+-ATPase activity was determined on the initial gill homogenates as the ouabain sensitive phosphatase activity, expressed in μmol Pi h^{-1} mg^{-1} (n = 5)

	Ca^{2+}-ATPase (in BLM)	Na^+/Ca^{2+}-exchange (in BLM)	Na^+/K^+-ATPase (in homogenate)
FW	5.50 ± 1.05	4.10 ± 0.84	2.04 ± 0.48
SW	5.74 ± 1.11	4.27 ± 0.37	4.23 ± 0.61
			$P < 0.05$[a]

[a]compaired to FW group, unpaired *t*-test

Resting transport rates in FW and SW opercular membranes

J_{ms} of Ca^{2+} in SW membranes was approximately 2.2 times lower than that in FW membranes (Tables 2, 3 and 5). On average, I_{sc} is about 20 times higher in SW membranes than in FW membranes, V_t is 11 times higher and G_t is 1.7 times higher in SW membranes (all significant differences, unpaired *t*-test).

Table 2 Effects of ouabain (10 μmol·l^{-1}, serosal side) on $^{45}Ca^{2+}$ influx across the opercular epithelium of FW- (**A**, n = 6) and SW (**B**, n = 7)-adapted killifish. The membranes were bathed in symmetrical saline

1 h treatment periods	V_t[a] (mV)	G_t (mS cm^{-1})	I_{sc} (μA cm^{-2})	J_{ms} (nmol cm^{-2} h^{-1})
A. FW				
Period 1 (control)	1.17 ± 0.61	6.49 ± 1.10	10.8 ± 2.7	54.1 ± 7.0
Period 2 +ouabain	1.19 ± 0.56	2.49 ± 0.40 $P < 0.003$[b]	4.5 ± 1.8 $P < 0.04$[b]	31.1 ± 3.8 $P < 0.003$[b]
Period 3 +ouabain	1.10 ± 0.54	1.70 ± 0.30 $P < 0.005$[b]	1.7 ± 0.6 $P < 0.02$[b]	8.1 ± 1.3 $P < 0.001$[b]
B. SW				
Period 1 (control)	19.4 ± 2.5	9.53 ± 1.25	185.2 ± 34.4	28.3 ± 4.3
Period 2 +ouabain	0.87 ± 0.21 $P < 0.0001$[b]	10.54 ± 1.74	6.53 ± 3.45 $P < 0.0001$[b]	16.0 ± 3.1 $P < 0.009$[b]
Period 3 +ouabain	0.90 ± 0.27 $P < 0.0001$[b]	8.89 ± 1.66	3.54 ± 2.94 $P < 0.0001$[b]	6.1 ± 0.5 $P < 0.002$[b]

[a]Membranes were clamped to zero V_t except for 3 s every 20 min
[b]compared to control period, paired *t*-test

Table 3 Effects of low sodium (membranes symmetrically bathed in saline with 21 mmol·l^{-1} Na^+) on $^{45}Ca^{2+}$ influx across the opercular epithelium of killifish. Sodium was replaced by NMDG. Paired control membranes were symmetrically bathed with Cortland saline (151 mmol·l^{-1} sodium). For FW membranes n = 6, and for SW membranes n = 7

Treatment	V_t[a] (mV)	G_t (mS cm^{-2})	I_{sc} (μA cm^{-2})	J_{ms} (nmol cm^{-2} h^{-1})
FW control	3.45 ± 2.11	4.12 ± 0.73	5.9 ± 1.6	58.6 ± 7.4
FW low Na	2.08 ± 0.83	1.44 ± 0.37 $P < 0.008$[b]	5.0 ± 1.9	13.2 ± 1.7 $P < 0.0001$[b]
SW control	16.2 ± 2.4	9.33 ± 1.17	135.8 ± 5.7	30.2 ± 2.3
SW low Na	8.47 ± 1.14 $P < 0.01$[b]	6.36 ± 0.94	48.1 ± 7.9 $P < 0.0001$[b]	15.7 ± 2.6 $P < 0.001$[b]

[a]Membranes were clamped to zero V_t except for 3 s every 20 min
[b]compared to respective control, unpaired t-test

Effects of serosal ouabain

Ouabain inhibits Na^+/K^+-ATPase rapidly and progressively depletes the transmembrane Na^+ gradient and secondary active transport processes that are dependent on the established Na^+ gradient (Silva et al., 1977). In opercular membranes from killifish adapted to FW, ouabain (10 μmol·l^{-1} added serosally) reduced Ca^{2+} influx (J_{ms}) by 43% during the first hour and by 85% during the second hour. I_{sc} was low in these FW membranes and was further reduced by ouabain, accompanied by a decrease in tissue conductance (Table 2A).

In opercular membranes from SW-adapted killifish, ouabain reduced J_{ms} by 43% in the first hour and by 78% in the second hour (Table 2B). During the second hour in which ouabain was present the inhibition of J_{ms} was slightly but significantly smaller in SW membranes than in FW membranes (78% versus 85%; $P < 0.03$, unpaired t-test). J_{ms} reached a steady-state low value 80 min following addition of ouabain. There was no effect on G_t.

Effects of lowering serosal [Na^+]

When the concentration of Na^+ was reduced to 14% of control (from 151 to 21 mmol·l^{-1} by replacement of NaCl with NMDG-Cl) the effects were similar to those seen after addition of ouabain (Table 3). In FW membranes Ca^{2+} influx was reduced by 77% and the conductance was significantly smaller. In SW membranes Ca^{2+} influx was reduced by 48%, concomitant with a drop in I_{sc}. The reduction in Ca^{2+} influx was significantly smaller in SW membranes than in FW membranes ($P < 0.01$, unpaired t-test). In both cases, the remaining J_{ms} in low Na^+ bathing solutions was the same, 13–16 nmol·cm^{-2}·h^{-1}. Interestingly, J_{ms} in the presence of low Na^+ could be further reduced by addition of ouabain: in the second hour of ouabain exposure J_{ms} of the SW membranes was reduced by 63%, from 15.7 ± 2.6 (Table 3) to 5.8 ± 0.8 nmol cm^{-2} h^{-1} (data not presented). At the same time I_{sc} is further reduced from 48.1 ± 7.9 to 6.9 ± 3.4 μA cm^{-2}. This combination experiment was not performed on FW membranes.

The effect of reduced serosal Na^+ was also tested in the presence of FW on the mucosal side. Ca^{2+} influx in FW membranes bathed in low-Na^+ saline (serosal) and FW (mucosal) was 72% lower than in their paired membranes (from the same fish) bathed in normal saline (serosal) and FW on the mucosal side (13.2 ± 1.9 and 47.5 ± 5.7 nmol·cm^{-2}·h^{-1}, respectively; $P < 0.0001$, n = 8, unpaired t-test). This is an important observation in terms of the endeavour to reveal the in vivo situation because it indicates that Ca^{2+} uptake is also dependent on serosal Na^+ in the more natural situation with FW on the mucosal side. G_t was not significantly different (1–3 mS cm^{-2}). V_t was quite variable with the FW mucosal bath, but significantly higher in the low Na^+ (serosal) group than in the normal Na^+ (serosal) group (11.0 ± 8.6 mol·l^{-1} versus −17.5 ± 23.5 mV; $P < 0.006$, n = 8, unpaired t-test).

Effects of thapsigargin or dibutyryl-cAMP/IBMX on SW membranes

Agents that increase intracellular Ca^{2+} should increase the rate of transepithelial Ca^{2+} transport, unless intracellular Ca^{2+} itself is involved in the regulation of this transport. Serosal addition of 1.0 μmol·l^{-1} thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase that generates an elevated intracellular Ca^{2+} concentration (Thastrup et al. 1990), reduced Ca^{2+} influx by 21.1% in the first hour (not significant) and by 55.3% ($P < 0.008$) in the second hour (Table 4). G_t and I_{sc} were somewhat reduced in the second hour of thapsigargin exposure (16% and 22%, respectively), but V_t was not affected. Addition of dibutyryl cAMP and 3-isobutyl-1-methyl-xanthine (db-cAMP/IBMX), to elevate intracellular cAMP and Cl^- secretion rate (as I_{sc}), also reduced Ca^{2+} influx: by 33.3% in the first hour ($P < 0.01$) and by 52.7% in the second hour ($P < 0.003$). G_t, I_{sc} and V_t were all elevated by cAMP.

Effects of serosal vanadate

Vanadate is an inhibitor of many P-type ATPases and could inhibit both the plasma membrane Ca^{2+}-ATPase and the Na^+/K^+-ATPase. Vanadate, however, does not inhibit Na^+/K^+-ATPase activity in our system (at the concentrations used) because the increase in I_{sc} in SW membranes using db-cAMP/IBMX following a 2-h incubation with 1–10 μmol·l^{-1} vanadate (I_{sc} from 67.4 ± 18.5 to 218.9 ± 27.4 μA cm^{-2}) is of similar size compared to the increase seen in control membranes without vanadate (as in Table 4). This increase in I_{sc} (read increase in Cl^- secretion) depends on the activity of the

Table 4 Effects of thapsigargin (1.0 μmol·l^{-1}, serosal side) and db-cAMP (0.5 mmol·l^{-1}, serosal side) + IBMX (0.1 mmol·l^{-1}, serosal side) on $^{45}Ca^{2+}$ influx across the opercular epithelium of SW-adapted killifish. The membrane was bathed in symmetrical saline, $n = 8$

1 h treatment periods	V_t[a] (mV)	G_t (mS cm^{-2})	I_{sc} (μA cm^{-2})	J_{ms} (nmol cm^{-2} h^{-1})
Period 1 (control)	12.6 ± 1.5	8.33 ± 0.34	113.0 ± 13.7	35.1 ± 3.6
Period 2 + thaps.	10.5 ± 0.77	7.96 ± 0.52	85.1 ± 10.0	27.7 ± 4.4
			$P < 0.01$[b]	
Period 3 + thaps.	12.2 ± 1.1	6.96 ± 0.55	88.2 ± 11.1	15.7± 3.6
		$P < 0.03$[b]	$P < 0.01$[b]	$P < 0.008$[b]
Period 1 (control)	15.4 ± 1.55	10.1 ± 1.17	153.0 ± 20.6	37.6 ± 6.5
Period 2 + cAMP/IBMX	24.6 ± 0.88	13.5 ± 1.22	307.9 ± 38.1	25.1 ± 4.1
	$P < 0.004$[b]	$P < 0.009$[b]	$P < 0.004$[b]	$P < 0.01$[b]
Period 3 + cAMP/IBMX	23.7 ± 1.04	14.0 ± 0.93	295.6 ± 35.1	17.8 ± 3.5
			$P < 0.005$[b]	$P < 0.0025$[b]

[a]Membranes were clamped to zero V_t except for 3 seconds every 20 minutes.
[b]compared to control period, paired t-test

Table 5 Effects of vanadate (1.0 μmol·l^{-1}, serosal side) on $^{45}Ca^{2+}$ influx across the opercular epithelium of SW (**A**) and FW (**B**)-adapted killifish. The membrane was bathed in symmetrical saline, $n = 6$

A. SW 1 h treatment periods	V_t[a] (mV)	G_t (mS cm2)	I_{sc} (μA cm^2)	J_{ms} (nmol cm^2 h^{-1})
Period 1 (control)	18.4 ± 2.0	7.48 ± 1.22	141 ± 21	28.3 ± 4.5
Period 2 + vanadate	11.1 ± 1.1	6.13 ± 1.02	74.0 ± 15.2	28.0 ± 4.7
	$P < 0.01$[b]	$P < 0.004$[b]	$P < 0.003$[b]	
Period 3 + vanadate	11.0 ± 1.6	5.18 ± 1.18	67.4 ± 18.5	12.2 ± 3.3
	$P < 0.004$[b]	$P < 0.03$[b]	$P < 0.02$[b]	$P < 0.01$[b]
B. FW				
Period 1 (control)	0.47 ± 0.97	5.28 ± 0.38	2.55 ± 5.53	78.8 ± 16.1
Period 2 + vanadate	1.47 ± 1.02	6.05 ± 0.51	7.78 ± 6.41	81.9 ± 9.9
	$P < 0.005$[b]	$P < 0.02$[b]	$P < 0.01$[b]	
Period 3 + vanadate	2.13 ± 0.97	6.29 ± 0.57	12.89 ± 7.19	76.3 ± 5.6
	$P < 0.001$[b]	$P < 0.05$[b]	$P < 0.01$[b]	

[a]Membranes were clamped to zero V_t except for 3 s every 20 min
[b]compared to control period, paired t-test

Na^+/K^+-ATPase; there is absolutely no increase in I_{sc} in SW membranes treated with db-cAMP/IBMX following pretreatment with ouabain (results not shown).

Since vanadate (1 μmol·l^{-1}, serosal side) had no effect on Na^+/K^+-ATPase activity we tested vanadate as a potential inhibitor of the plasma membrane Ca^{2+}-ATPase. This is in contrast to ouabain which would affect the uptake of Ca^{2+} via the Na^+/Ca^{2+}-exchanger through the inhibition of the Na^+/K^+-ATPase.

In SW membranes vanadate did not change the influx of Ca^{2+} in the first hour but reduced the flux by 56% in the second hour of exposure. I_{sc} (and V_t) was reduced by 48% in the first hour and remained almost constant after that. G_t showed a gradual but small decrease (Table 5A). In FW membranes vanadate had no effect on Ca^{2+} influx. V_t, G_t and I_{sc} were all increased (Table 5B).

Discussion

The main conclusion of this study is that transepithelial Ca^{2+} uptake in killifish opercular membrane, that is known to be conducted by the chloride cells, is approximately 80–85% Na^+ dependent. This is based on 1) in vitro experiments with branchial basolateral membrane vesicles showing the presence of a Na^+/Ca^{2+}-exchanger and a Ca^{2+}-ATPase and 2) on $^{45}Ca^{2+}$ uptake measurements with opercular membranes from FW- and SW-adapted killifish in which the Na^+ gradient across the basolateral membrane was manipulated. Our results therefore strongly indicate an important role for the Na^+/Ca^{2+}-exchanger in the transcellular uptake of Ca^{2+}. The inhibitory effect on Ca^{2+}-uptake of low [Na^+] or ouabain in the bath medium is explained by the fact that the functioning of the Na^+/Ca^{2+}-exchanger depends on the Na^+ gradient that in situ (and in vivo) is maintained by the Na^+/K^+-ATPase.

The experiments were designed to test a new model for transcellular Ca^{2+} uptake in the gills that is based on in vitro studies with isolated basolateral membranes from tilapia gills (Flik et al. 1993; Verbost et al. 1994). According to this model, Ca^{2+}-ATPase and Na^+/Ca^{2+}-exchanges are localized in the basolateral membrane and both could contribute to the transcellular uptake of Ca^{2+}. At present little is known about the prevalence or control of the Na^+/Ca^{2+}-exchanger. For the Ca^{2+}-ATPase the kinetics have been determined in trout (Perry and Flik 1988), eel (Flik et al. 1985b) and tilapia (Flik et al. 1985a) and it has been shown that the activity can be stimulated by prolactin treatment (Flik et al. 1989; Flik et al. 1994). The high affinity of the Ca^{2+}-ATPase which enables it to operate at normal intracellular Ca^{2+} concentrations and the possibility of hormonal regulation led to the previous model (Flik et al. 1985a) in which the ATPase drives the majority of transcellular uptake. Because of the complexity of branchial epithelium it has been impossible to test this hypothesis directly in an Ussing chamber. In an attempt to approach this experimental system as closely as possible we applied the technique on opercular membranes and found evidence for an important role for the Na^+/Ca^{2+}-exchanger.

With the aim of extrapolating the data obtained with the opercular membranes to the branchial Ca^{2+} uptake we identified, apart from ATPase-driven $^{45}Ca^{2+}$ transport, a Na^+/Ca^{2+}-exchange activity in basolateral membranes of gills from FW- and SW-adapted killifish. The

full kinetics of the two transporters were not determined, but the available results give a picture comparable to that seen in tilapia (Verbost et al. 1994): the transport capacities of the ATPase and the exchanger are in the same order of magnitude and there is no difference in capacity (of either transporter) between FW and SW fish. The presence of the exchanger in the basolateral membrane, co-localized with the Na^+/K^+-ATPase (membranes were 22–27 times enriched in Na^+/K^+-ATPase activity), supports the model in which the exchanger is important for the transcellular uptake of Ca^{2+} by the gills.

Comparison of Ca^{2+} uptake in FW and SW

Experiments conducted to examine the predicted differences in the degree of Na^+-dependency of Ca^{2+} influx between FW and SW membranes are summarized in Fig. 1. SW membranes had a somewhat lower sensitivity to ouabain (7% lower inhibition) and J_{ms} in SW membranes was clearly less affected by lowering the Na^+ concentration (29% lower inhibition than in FW membranes). The combination of low Na^+ and ouabain, however, showed that 80% of the Ca^{2+} uptake in SW membranes was Na^+ gradient dependent. This means that although the Ca^{2+} uptake process in both FW and SW membranes is mainly Na^+ dependent, the uptake in SW membranes is less affected by a drop in Na^+ (i.e. a decrease in Na^+ gradient). Apparently, the affinity of the exchanger for Na^+ is higher in the SW membranes. Possibly, the "Na^+ affinity" manifests a means of regulation at the Na^+ site, an idea that deserves attention in future research.

Consistently, the influx of Ca^{2+} in FW membranes was two times higher than that in SW membranes. This may simply reflect the fact that fish adapted to 100 $\mu mol \cdot l^{-1}$ Ca^{2+} in vivo (in FW) have had their Ca^{2+} uptake upregulated in order to maintain Ca^{2+} homeostasis. It shows, however, that these membranes are intrinsically different since they were disconnected from any humoral input in the Ussing chamber. Interestingly, a similar enhancement of Ca^{2+} uptake was observed in vivo (Mayer-Gostanet al. 1983). In that study extraintestinal Ca^{2+} influx by SW and FW (0.1 $mmol \cdot l^{-1}$ Ca^{2+})-acclimated killifish was about 2.8 $\mu mol \cdot h^{-1}$ 100 g^{-1} and 5.5 $\mu mol \cdot h^{-1}$100 g^{-1}, respectively. In all cases the in vivo Ca^{2+} influx was from a 1.5 $mmol \cdot l^{-1}$ Ca^{2+} medium. Our results indicate that this difference in whole body Ca^{2+} uptake is governed by a (possibly hormonally regulated) change in the Ca^{2+} uptake pathway rather than by the acute action of a humoral factor. The results with the basolateral membrane vesicles from FW and SW killifish, showing no difference in Ca^{2+} transporter capacities, suggest that such a change in the uptake pathway lies in the apical membrane or the intracellular routing (Ca^{2+} binding proteins, second messengers). The fact that Na^+/K^+-ATPase in SW was double that in FW appears to be solely correlated with Na and Cl extrusion, not with Ca^{2+} uptake, because Ca^{2+} uptake is even lower in SW than in FW.

Electrophysiology

The electrophysiological data are in agreement with the standing model for Cl^- secretion in SW membranes that was first described by Silva (1977) and subsequently supplemented by others for the hormonal control and the second messengers involved (Foskett et al. 1983; Karnaky et al. 1984; Marshall et al.1993). According to this model, Cl^- extrusion starts with basolateral uptake into the cell through the $Na^+/K^+/2Cl^-$-cotransporter is driven by the Na^+,K^+-ATPase, using the Na^+ electrochemical gradient across the basolateral membrane. Cl^- extrusion is driven by the Cl^- electric gradient across the apical membrane via anion channels in the apical membrane (Marshall et al. 1994, 1995b). Net Cl^- secretion is fully responsible in SW membranes for the observed short circuit current (Karnaky 1986; Pequeux

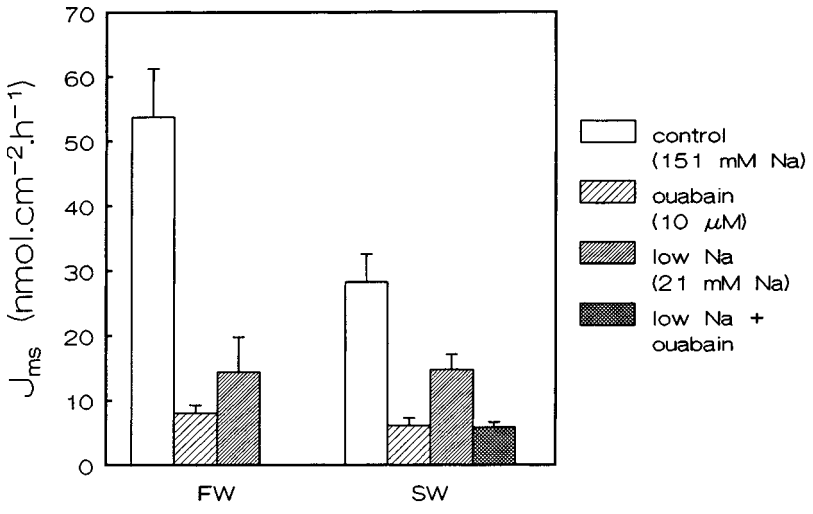

Fig. 1 Effects of ouabain and low Na^+ on Ca^{2+} influx in killifish opercular membrane. Flux values for the low Na^+ condition are from paired observations; membranes from the same fish served as control. Presented ouabain values are the fluxes in the second hour following addition of ouabain, each membrane serving as its own control

et al. 1988). The reduction in I_{sc} caused by ouabain and by low Na^+ are thus fully explained by a depletion in the Na^+ electrochemical gradient and a concomitant reduction in Cl^- secretion. In unstimulated FW membranes there is no measurable Cl^- extrusion (Wood and Marshall, 1994). With saline bathing both surfaces of the epithelium and V_t clamped to zero, the I_{sc} was slightly positive averaging 6–11 μA cm^{-2}. In the presence of ouabain the conductance was significantly reduced, consistent with the simultaneous reduction in I_{sc}. The reduction in I_{sc} represents a reduced anion secretion and/or a reduced influx of cations. The reduction in Ca^{2+} uptake cannot, however, account for all of the decrease in I_{sc}: 54.1 minus 8.1 = 46 μmol Ca^{2+} cm^{-2} h^{-1} represents 2.48 μA cm^{-2} which is only 27% of the 9.1 μA cm^{-2} drop. Thus, the origin of the change in I_{sc} in FW membranes is not revealed by these experiments.

Effects of thapsigargin or db-cAMP/IBMX

The reduction in Ca^{2+} influx in SW membranes treated with thapsigargin or db-cAMP/IBMX is comparable to that seen in FW membranes. Also in FW membranes increases in intracellular Ca^{2+} and elevations in cAMP reduced Ca^{2+} influx (Marshall et al.1995a). Although we were not equipped to measure intracellular Ca^{2+} after thapsigargin, the efficacy would strongly suggest that it was operating as normal to increase intracellular Ca^{2+} (Thastrup et al.1990). Because an increase in intracellular Ca^{2+} should by itself increase Ca^{2+} uptake because of increased substrate availability for the extrusion mechanism, an alternate explanation of the reduction in Ca^{2+} influx by thapsigargin is needed. Our results are consistent with the suggestion (van Os 1987) that Ca^{2+} transport is regulated via intracellular Ca^{2+} and that a marked rise in intracellular Ca^{2+} produces down-regulation of transcellular Ca^{2+} transport. On the basis of the present study one may conclude that not only is the mechanism of uptake similar in FW and SW membranes (Na^+dependence) but also the second messenger signals are functionally comparable.

Effects of vanadate

Vanadate had no effect on Ca^{2+} influx in FW membranes and in SW membranes it reduced Ca^{2+} influx only in the second hour of exposure (there was a non-significant lowering in FW membranes in the second hour). If we consider vanadate to be a specific inhibitor of the Ca^{2+}-ATPase in the basolateral membrane these results would suggest that the ATPase is not important for transepithelial Ca^{2+} uptake. But Ca^{2+} influx is still inhibited in the second hour of vanadate exposure in the SW membrane. Let us consider the effects of vanadate in more detail.

Our results clearly show that vanadate does not affect the Na^+/K^+-ATPase activity since activation of the Cl^- secretion through an increase in intracellular cAMP is not affected in the presence of vanadate. Electrophysiological data (Table 5) suggest an increase in intracellular Ca^{2+} following addition of vanadate because the effects are comparable to those seen with thapsigargin: in SW membranes the reduction in I_{sc} is consistent with the intracellular Ca^{2+}-mediated response reported by Marshall et al. (1993); in FW membranes vanadate caused an increase in I_{sc} (from 2.6 to 12.9 μA cm^{-2}) comparable to that seen with thapsigargin, from 5.7 to 20.6 μA cm^{-2} (Marshall et al. 1995a).

The effect of vanadate is different from that of thapsigargin in the sense that the latter reduces Ca^{2+} influx in both FW and SW opercular membranes, whereas vanadate doesn't or does so with a ≥ 2-h lag time. A logical explanation would be that vanadate increases intracellular Ca^{2+} mainly by inhibiting the plasma membrane Ca^{2+}-ATPase such that the Ca^{2+} concentration reaches a level high enough (or a certain site in the cytosol) for it to act as a second messenger resulting in the inhibition of the Ca^{2+} influx. Since the vanadate binding site is at the cytosolic side of the Ca^{2+}-ATPase (Pick 1982) entrance of vanadate into the cell is required which would explain the lag time. In any case, the FW membranes were less sensitive to vanadate (1 μmol·l^{-1}) than the SW membranes.

In summary, the results favour a model in which the plasma membrane Ca^{2+}-ATPase is active mainly in the maintainance of low intracellular Ca^{2+} concentrations, whereas the Na^+/Ca^{2+}-exchanger acts in transepithelial transport. Definitive confirmation of this proposed mechanism will require measurements of intracellular Ca^{2+} during manipulation of Na^+/Ca^{2+}-exchange.

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