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ORIGINAL PAPER

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Ca^{2+} transport by opercular epithelium of the fresh water adapted euryhaline teleost, *Fundulus heteroclitus*

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Abstract We examined transepithelial transport of Ca^{2+} across the isolated opercular epithelium of the euryhaline killifish adapted to fresh water. The opercular epithelium, mounted *in vitro* with saline on the serosal side and fresh water (0.1 mmol·l^{-1} Ca^{2+}) bathing the mucosal side, actively transported Ca^{2+} in the uptake direction; net flux averaged 20–30 nmol·cm^{-2}·h^{-1}. The rate of Ca^{2+} uptake varied linearly with the density of mitochondria-rich cells in the preparations. Ca^{2+} uptake was saturable, apparent $K_{\frac{1}{2}}$ of 0.348 mmol·l^{-1}, indicative of a multistep transcellular pathway. Ca^{2+} uptake was inhibited partially by apically added 0.1 mmol·l^{-1} La^{3+} and 1.0 mmol·l^{-1} Mg^{2+}. Addition of dibutyryl-cyclic adenosine monophosphate (0.5 mmol·l^{-1}) + 0.1 mmol·l^{-1} 3-isobutyl-1-methylxanthine inhibited Ca^{2+} uptake by 54%, but epinephrine, clonidine and isoproterenol were without effect. Agents that increase intracellular Ca^{2+}, thapsigargin (1.0 µmol·l^{-1}, serosal side), ionomycin (1.0 µmol·l^{-1}, serosal side) and the calmodulin blocker trifluoperazine (50 µmol·l^{-1}, mucosal side) all partially inhibited Ca^{2+} uptake. In contrast, apically added ionomycin increased mucosal to serosal unidirectional Ca^{2+} flux, indicating Ca^{2+} entry across the apical membrane is rate limiting in the transport. Verapamil (10–100 µmol·l^{-1}, mucosal side), a Ca^{2+} channel blocker, had no effect. Results are consistent with a model of Ca^{2+} uptake by mitochondria rich cells that involves passive Ca^{2+} entry across the apical membrane *via* verapamil-insensitive Ca^{2+} channels, intracellular complexing of Ca^{2+} by calmodulin and basolateral exit *via* an active transport process. Increases in intracellular Ca^{2+} invoke a downregulation of transcellular Ca^{2+} transport, implicating Ca^{2+} as a homeostatic mediator of its own transport.

Key words Mitochondria rich cells · Trifluoperazine · Thapsigargin · Killifish, *Fundulus* · Calcium transport regulation

Abbreviations *DASPEI* 2-(4-dimethylaminostyryl)-*N*-ethylpyridinium iodide · *db-cAMP* dibutyryl-cyclic adenosine monophosphate · *FW* fresh water · G_t transepithelial conductance · I_{sc} short-circuit current · *IBMX* 3-isobutyl-1-methylxanthine · *SW* sea water · *TFP* trifluoperazine · V_t transepithelial potential

Introduction

Teleosts utilize the environment as their Ca^{2+} source rather than bone, hence transepithelial Ca^{2+} transport is a major regulated process in Ca^{2+} balance. Calcium metabolism of euryhaline teleosts has been studied extensively to determine the means by which Ca^{2+} is taken up across the skin, gill and intestinal epithelia. Ca^{2+} transport is under multihormonal control by stanniocalcin, a rapid inhibitor of Ca^{2+} transport in SW and FW teleosts (Lafeber et al. 1988) and by prolactin (a slower stimulator of Ca^{2+} transport in FW teleosts) and possibly by other factors as well (review Flik et al. 1993; Kaneko and Hirano 1993; Cano et al. 1994). It is thought that Ca^{2+} transport by the gills occurs in the mitochondria-rich cells, based on the metabolic potential of these cells, selective Ca^{2+} uptake by secondary gill lamellae (Payan et al. 1981, 1984), the presence of Na^{+}, K^{+}-ATPase in the basolateral membranes of these cells (Karnaky et al. 1976) and the

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association of Na^+, K^+-ATPase with Ca^{2+}-ATPase in tilapia gill membranes (Flik et al. 1985). While Ca^{2+}-ATPase may be directly involved in transmural Ca^{2+} transport, Na^+, K^+-ATPase may indirectly support Ca^{2+} uptake *via* maintenance of a transmembrane Na^+ electrochemical gradient that in turn may drive basolateral Na^+-Ca^{2+} exchange (Flik et al. 1985, 1990). There has long been a suspected role of the general body surface in Ca^{2+} uptake (Mashiko and Jozuka 1964), and in FW-acclimated rainbow trout (*Oncorhynchus mykiss*) the general body surface accounts for half the total Ca^{2+} uptake (Perry and Wood 1985).

Recently two potential models for Ca^{2+} transepithelial transport have been investigated, the opercular epithelium of tilapia (*Oreochromis mossambicus*) and the cleithrum skin of rainbow trout (*Oncorhynchus mykiss*). Tilapia opercular epithelium has a net flux of Ca^{2+} in the uptake direction, but the rate of transport is very low, ca.1.0 $nmol \cdot cm^{-2} \cdot h^{-1}$ (McCormick et al. 1992). The trout skin preparation has a net loss of Ca^{2+} *in vitro*, although Ca^{2+} active transport is present by the Ussing flux ratio criterion (Marshall et al. 1993). In both preparations the number of mitochondria rich cells is low, below 100 cells per mm^2. The jaw skin of the goby *Gillichthys mirabilis* (Marshall 1977) and the opercular epithelium of the killifish *Fundulus heteroclitus* have been used extensively in studies of the mechanisms of Cl^-secretion by marine teleosts [reviews: Karnaky (1986); Péqueux et al. (1988); Wood and Marshall (1994)] and now is being used as a model for NaCl transport by FW teleosts (Wood and Marshall 1994).

In this study we introduce the killifish opercular membrane as a Ca^{2+}-transporting tissue that may aid Ca^{2+} regulation in FW-adapted animals. This preparation affords an opportunity to study Ca^{2+} transport mechanisms in asymmetrical conditions with FW bathing the mucosal surface and in symmetrical conditions with saline on both sides of the tissue. The former conditions mimic those *in vivo* while the latter allow simultaneous measurements of Ca^{2+} fluxes with electrogenic Cl^- transport by mitochondria rich cells in this epithelium.

Materials and methods

Animals

Adult killifish of both sexes were obtained from the Antigonish estuary and were transferred to indoor holding facilities that were maintained in 10% SW (3.0–3.2 $mg \cdot ml^{-1}$) at 20–25 °C under artificial light operating on natural photoperiod. Animals were transferred directly to FW and were held for at least 10 days prior to flux experiments. The composition ($\mu mol \cdot l^{-1}$) 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. Fish were killed by decapitation and the opercular epithelia were removed and mounted in Ussing style membrane chambers which had an exposed membrane area of 0.125 cm^2 and were water jacketed to control temperature at 22 °C.

Tracer fluxes

The serosal side *in vitro* bathing solution was a modified Cortland's saline with the composition (in $mmol \cdot l^{-1}$): Na^+142, Cl^-136, K^+2.6, HCO_3^-9.7, Ca^{2+} 1.6, Mg^{2+} 0.9, PO_4^{2-} 3.0, SO_4^{2-} 0.9, and glucose 5.6 with bovine serum albumin (Sigma, grade III, fraction V) 20 $mg \cdot ml^{-1}$ as a replacement for plasma protein; the solution, when bubbled with 0.3% CO_2/balance O_2 had a pH of 7.8. When FW bathed the mucosal surface, this solution was bubbled with 100% O_2. The paired membranes were flushed extensively with FW (20 times the chamber volume, mucosal side only) to remove saline and unidirectional $^{45}Ca^{2+}$ (as $^{45}CaCl_2$, I.C.N. Radiochemicals) efflux from serosa to mucosa (J_{sm}), influx from mucosa to serosa (J_{ms}), and net flux ($J_{net} = J_{ms} - J_{sm}$) were determined as published previously (Marshall 1986). Because of the paired design, observed flux ratios could be calculated for each animal. Calculation of expected flux ratios used the Ussing flux ratio equation (Ussing 1949) and care was taken to adjust the concentration terms to reflect the activity of the ions in solution (Marshall et al. 1992). Briefly, Ca^{2+} activity (measured by calcium electrode) in FW was the same as the concentration, while that in albumin-containing saline was 0.8 $mmol \cdot l^{-1}$ (versus a concentration of 1.6 $mmol \cdot l^{-1}$, above).

Electrophysiology

Transepithelial conductance (G_t, $mS \cdot cm^{-2}$) and transepithelial potential (V_t, 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}, $\mu A \cdot cm^{-2}$) was also measured. I_{sc} is used as a measure of Cl^- secretion in SW fish skin (Marshall and Nishioka 1980) and this transport can be evoked pharmacologically by cAMP even in FW killifish opercular epithelia.

Kinetics of Ca^{2+} uptake

To examine kinetics of Ca^{2+} uptake, $CaCl_2$ was added to the mucosal FW bathing solution to concentrations of 0.05, 0.10, 0.50, 2.50 and 5.0 $mmol \cdot l^{-1}$, while the serosal bathing solution Ca^{2+} was constant at 1.56 $mmol \cdot l^{-1}$. Ca^{2+} uptake was measured for three 20-min flux periods while G_t and V_t were monitored at open circuit. A non-saturable component of the flux that increased linearly with increasing Ca^{2+} concentration was assumed to be Fickean diffusion and was subtracted from the mean fluxes. The remaining saturable component results were graphically analyzed using a Lineweaver-Burk plot and the apparent $K_{\frac{1}{2}}$ and V_{max} were calculated from the regression line through the data. Necessarily, the flux measurements were performed at steady state and are not based on initial transport rates.

Fluorescence microscopy

The fluorophore DASPEI (I.C.N. Biomedicals, Costa Mesa, Calif.,) USA) is a mitochondrial vital stain that has been used routinely to identify mitochondria-rich cells (Marshall and Nishioka 1980; Karnaky 1986). DASPEI was dissolved in a stock solution of 0.2 $mg \cdot ml^{-1}$ in 0.9% (w/v) NaCl. Opercular epithelia from the

Table 1 Unidirectional influx (J_{m-s}), efflux (J_{s-m}) and net flux ($J_{m-s} - J_{s-m}$) across opercular epithelia from fresh water (1.0 mmol·l^{-1} NaCl; 0.1 mmol·l^{-1} Ca^{2+}) *Fundulus heteroclitus* bathed *in vitro* with Cortland saline (Ca^{2+} = 0.80 mmol·l^{-1}) on the serosal side and fresh water on the mucosal side (n = 7 for fluxes, 14 for electrophysiology)

Treatment period (1 h each)	V_t (mV)	G_t (mS·cm^{-2})	J_{m-s} (nmol·cm^{-2}·h^{-1})	J_{s-m} (nmol·cm^{-2}.h^{-1})	J_{net} (nmol·cm^{-2}·h^{-1})
Control (h 1)	-57.3 ± 1.45	2.01 ± 0.603	29.6 ± 4.5	0.45 ± 0.14	29.2 ± 4.45
Control (h 2)	-57.7 ± 1.37	1.65 ± 0.274	28.0 ± 4.3	0.38 ± 0.13	27.6 ± 4.18
Verapamil 10 μmol·l^{-1} (h 3)	-56.0 ± 1.22	1.88 ± 0.384	28.5 ± 4.2	0.37 ± 0.08	28.2 ± 4.19

Ussing chambers were bathed in oxygenated Cortland's saline with 10 μmol·l^{-1} DASPEI for 30 min before viewing as a wet mount on a Zeiss Photomicroscope III equipped with epifluorescence. Excitation wavelength was 485 nm (bandwidth 20 nm) and barrier filter was 540 nm (bandwidth 40 nm). Cell counts were made at three or four randomly selected areas of membrane, each count comprising 0.04 mm^2.

Pharmaceuticals

Epinephrine (1.0 μmol·l^{-1}, serosal side), the β-adrenergic agonist isoproterenol (1.0 μmol·l^{-1}, serosal side), the α_2-adrenergic agonist clonidine (10 μmol·l^{-1}, serosal side), db-cAMP (0.5 mmol·l^{-1}, serosal side), IBMX (0.1 mmol·l^{-1}, serosal side) and verapamil (10–100 μmol·l^{-1}, mucosal side), all from Sigma were dissolved fresh in saline and added to the final concentrations indicated. Thapsigargin inhibits endoplasmic reticulum Ca^{2+}-ATPase and in most cells invokes a rise in intracellular Ca^{2+} from intracellular sources. In SW killifish opercular epithelium, thapsigargin inhibits Cl^- secretion, mimicking the rise in intracellular Ca^{2+} after clonidine (Marshall et al. 1993). Ionomycin is an efficient Ca^{2+} ionophore (Liu and Hermann 1978) that is effective in teleost epithelial systems (Marshall et al. 1993), and intracellular Ca^{2+} appears to mediate the inhibition of Cl^- secretion by α_2-adrenoceptors of the SW opercular epithelium. Thapsigargin (1.0 μmol·l^{-1}, serosal, Sigma) and ionomycin (1.0 μmol·l^{-1}, Calbiochem) were dissolved fresh in DMSO, giving a final DMSO concentration of < 0.1% that has no apparent effect on ion transport by the epithelium (Marshall et al. 1993). TFP (Sigma), a calmodulin inhibitor, was dissolved in 10 mmol·l^{-1} HEPES (US Biochem. Corp.) buffer, pH 7.8, and was added to a final concentration of 50 μmol·l^{-1}, mucosal side. The ionic inhibitors of Ca^{2+} transport, La^{3+} and Mg^{2+}, were added to the mucosal side at concentrations of 0.1 and 1.0 mmol·l^{-1}, respectively, in the presence of 0.1 mmol·l^{-1} Ca^{2+}.

Data presentation

Data are expressed as the mean ± one standard error unless indicated otherwise. Generally, control and test conditions were analyzed by paired (or unpaired, as appropriate) t-tests.

Results

$^{45}Ca^{2+}$ fluxes

Opercular membranes from FW-adapted killifish had a net flux of Ca^{2+} in the uptake direction that averaged 27.6–29.2 nmol·cm^{-2}·h^{-1} (Table 1). The uptake unidirectional flux was 77.1 ± 23.6 (n = 7) times that in the efflux direction. In contrast, the flux ratio predicted on the basis of passive diffusion by the Ussing flux ratio equation (Ussing 1949) was 11.08, much smaller ($P < 0.01$) than the observed flux ratio, indicating that Ca^{2+} distribution across the epithelium was not simply by diffusion. Ca^{2+} uptake occurred against an overall transepithelial concentration gradient of 8.6:1.0 and with an electrical gradient of 50–60 mV (Table 1). In time control experiments, the net flux was in steady state for over 3 h *in vitro* (Table 1). For this reason, paired time controls were not usually necessary. Addition of the Ca^{2+} channel blocker verapamil to the apical bath had no effect on Ca^{2+} transport rates nor on electrophysiology of the epithelium (Table 1).

Kinetics of Ca^{2+} influx were examined by measuring Ca^{2+} mucosal to serosal unidirectional fluxes at 0.05, 0.10, 0.50, 2.50 and 5.00 mmol·l^{-1} Ca^{2+} in the mucosal bath. Serosal Ca^{2+} concentration was held constant at 1.56 mmol·l^{-1}. A plot of uptake against Ca^{2+} concentration in the mucosal bath (inset, Fig. 1) revealed a saturable component superimposed on a linear (presumably diffusional) component. Kinetic analysis of the saturable component after subtraction of the linear component was done using a Lineweaver-Burk plot (Fig. 1). This analysis revealed an effective $K_{\frac{1}{2}}$ of 0.348 mmol·l^{-1} and V_{max} of 94 nmol·cm^{-2}·h^{-1}. Increasing mucosal Ca^{2+} concentration in a stepwise fashion decreased the negative inside V_t from -51.6 ± 2.24 at 0.05 mmol·l^{-1} Ca^{2+} to -46.5 ± 2.67 at 0.5 mmol·l^{-1}, -34.7 ± 3.06 at 2.5 mmol·l^{-1} and -28.5 ± 3.90 at 5.0 mmol·l^{-1} ($P < 0.001$, paired t-tests to the previous period with the next lower Ca^{2+} concentration, n = 8–12). There was also a significant decrease in conductance ($P < 0.05$; paired t-tests, n = 8) at the higher Ca^{2+} concentrations (2.5 and 5.0 mmol·l^{-1}). G_t was 1.26 ± 0.19 mS·cm^{-2} at 0.05 mmol·l^{-1} Ca^{2+}, 1.27 ± 0.11 at 0.5 mmol·l^{-1}, 0.96 ± 0.08 at 2.5 mmol·l^{-1} and 0.95 ± 0.08 at 5.0 mmol·l^{-1}.

The influx of Ca^{2+} was correlated with the number of DASPEI-positive cells in the epithelium (Fig. 2). The variation in cell density was not large, nevertheless the regression of Ca^{2+} influx on cell density gave a regression of slope 1.324 ± 0.843 pmol Ca^{2+}·h^{-1} per cell

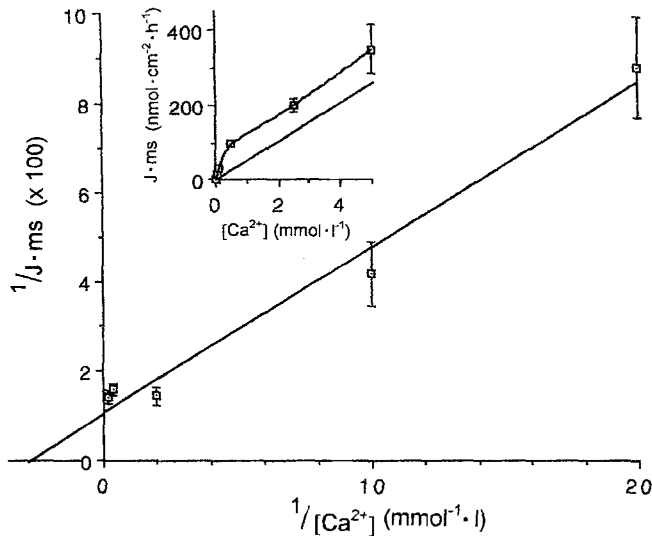

Fig. 1 The kinetic analysis of Ca^{2+} uptake by killifish opercular epithelium includes a graph of unidirectional influx of Ca^{2+} (mean ± SEM, n = 8–12) plotted against calcium concentration (uncorrected for activity) that indicates a linear portion that is additive to a saturable component (*inset*). Subtraction of the non-saturable (presumably diffusional) component and replotting of the means ± SEM on a Lineweaver-Burk diagram, yielded an apparent $K_{1/2}$ of 0.348 mmol·l^{-1} and V_{max} of 94 nmol·cm^{-2}· h^{-1}

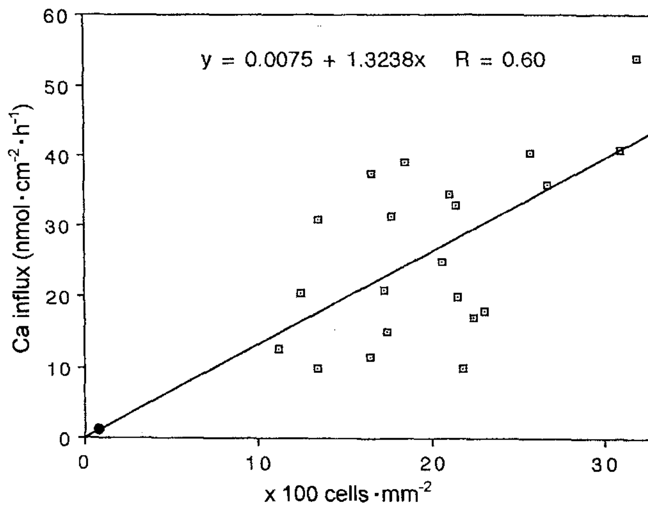

Fig. 2 Relation of Ca^{2+} unidirectional uptake to the density of DASPEI-positive cells in opercular epithelial pieces. There was a significant linear relation (regression $y = 0.0075 + 1.3238x$, $P < 0.01$, $n = 21$) with intercept that was not significantly different from zero. The *solid dot* indicates corresponding cell densities and transport rates for cleithrum skin of rainbow trout (Marshall et al. 1992)

and a y intercept of 0.0075 ± 17.5 nmol·cm^{-2}·h^{-1} that was not significantly different from zero ($r = 0.602$, $P < 0.01$). The mean of our previous results with rainbow trout cleithrum skin (139 cells·mm^{-2}, 0.073 nmol·cm^{-2}·h^{-1}) is included in the figure for comparative purposes (Marshall et al. 1992).

Inhibition by La^{3+} and Mg^{2+}

Calcium transport systems are often competitively inhibited by other multivalent cations such as Mg^{2+}, La^{3+}, Zn^{2+} and Cd^{2+} (Verbost et al. 1987, 1989; Flik et al. 1993; Hogstrand et al. 1994). Here we used La^{3+} at 0.1 mmol·l^{-1} and Mg^{2+} at 1.0 mmol·l^{-1} in the presence of 0.1 mmol·l^{-1} Ca^{2+}, the concentration to which the animals were acclimated and well below the $K_{\frac{1}{2}}$ and V_{max} for the transport (see above). Table 2 shows the effect of Mg^{2+} and La^{3+} on Ca^{2+} unidirectional fluxes and electrophysiology of the killifish opercular epithelium. Both cations partially blocked Ca^{2+} uptake by 17 and 30% for Mg^{2+} and La^{3+}, respectively. La^{3+} significantly increased the Ca^{2+} efflux so that the net Ca^{2+} flux dropped by a slightly larger amount, 31%. Mg^{2+} had no effect on the Ca^{2+} efflux. While Mg^{2+} had no effect on V_t or G_t, La^{3+} rapidly reduced the negative inside transepithelial potential by 36 mV and decreased G_t significantly (Table 2).

Effect of trifluoperazine

Opercular epithelia bathed with FW on the mucosal side were exposed to TFP, a calmodulin inhibitor, on the mucosal side at 50 µmol·l^{-1} final concentration. Ca^{2+} influx was not changed in the first hour after TFP addition, but was reduced by 49% ($P < 0.01$) in hour 2 (Table 3). This was accompanied by a modest rise in Ca^{2+} efflux but no change in epithelial conductance. There was a significant elevation of V_t from −32 to −22 mV after TFP in hour 1 but no further change in hour 2. Addition of TFP to the serosal side in hour 3 produced no further change in any parameter (data not shown).

Effects of thapsigargin and cAMP

Thapsigargin was added to the serosal side and $^{45}Ca^{2+}$ influx was measured under control conditions and for 2 h after addition. Performance of this series of experiments in symmetrical saline removes the large negative V_t when the epithelium is bathed in FW and allows use of the short-circuit technique to detect electrogenic ion transport. Thapsigargin treatment significantly inhibited Ca^{2+} uptake in hour 2 after addition of the drug (Table 4). As with TFP and serosal ionomycin, thapsigargin did not inhibit Ca^{2+} uptake until the second hour. There was a slight (1–2 mV) but statistically significant increase in V_t, connected with a marginally significant rise in I_{sc} after thapsigargin but no other electrophysiological effects.

To increase intracellular cAMP we added db-cAMP (0.5 mmol·l^{-1}) plus IBMX (0.1 mmol·l^{-1}). This treatment mimics in SW opercular epithelium the addition of isoproterenol and it is linked with stimulation of

Table 2 Effect of La^{3+} (0.1 mmol·l^{-1}, mucosal side) and Mg^{2+} (1.0 mmol·l^{-1}, mucosal side) on ^{45}Ca unidirectional and net fluxes across opercular epithelia bathed in fresh-water (muscosal side). Paired *t*-test, two tailed, compared to control period; $n = 12$ for electrophysiology, 6 for fluxes

Treatment period	V_t (mV)	G_t (mS·cm^{-2})	J_{m-s} (nmol·cm^{-2}·h^{-1})	J_{s-m} (nmol·cm^{-2}.h^{-1})	J_{net} (nmol·cm^{-2}·h^{-1})
Control	-47.4 ± 4.3	1.97 ± 0.24	20.0 ± 4.0	0.15 ± 0.03	19.8 ± 3.6
La^{3+}	-11.4 ± 2.0	0.94 ± 0.12	14.1 ± 3.1	0.43 ± 0.08	13.7 ± 3.3
P	< 0.001	< 0.001	< 0.05	< 0.01	< 0.05
Control	-54.6 ± 3.3	1.52 ± 0.24	33.3 ± 4.7	0.08 ± 0.04	33.2 ± 4.4
Mg^{2+}	-56.0 ± 3.1	1.40 ± 0.11	27.8 ± 3.6	0.09 ± 0.01	27.7 ± 3.3
P	NS	NS	< 0.02	NS	< 0.05

Table 3 Effect of trifluoperazine (TFP, 50 μmol·l^{-1}, mucosal side) on ^{45}Ca unidirectional and net fluxes across opercular epithelia bathed in fresh water (mucosal side)

Treatment period	V_t (mV)	G_t (mS·cm^{-2})	J_{m-s} (nmol·cm^{-2}·h^{-1})	J_{s-m} (nmol·cm^{-2}.h^{-1})	J_{net} (nmol·cm^{-2}·h^{-1})
Control	-32.8 ± 6.9	1.18 ± 0.42	26.5 ± 3.6	0.19 ± 0.03	26.3 ± 3.6
TFP hour 1	-21.8 ± 6.9	1.25 ± 0.48	22.9 ± 3.0	0.31 ± 0.05	22.6 ± 3.0
P	< 0.01	NS	NS	< 0.05	NS
TFP hour 2	-17.9 ± 5.6	1.14 ± 0.48	13.4 ± 3.6	0.44 ± 0.10	13.0 ± 3.5
P	< 0.01	NS	< 0.005	< 0.05	< 0.005

Paired *t*-test, two tailed compared to control period $n = 16$ for electrophysiology, 8 for fluxes

Table 4 Effects of thapsigargin (1.0 μmol·l^{-1}, serosal side), db-cAMP (0.5 mmol·l^{-1}, serosal side) + IBMX (0.1 mmol·l^{-1}, serosal side) on $^{45}Ca^{2+}$ influx across the killifish opercular epithelium bathed in symmetrical saline. Paired *t*-test, two tailed, compared to control period; $n = 6$

Treatment period	I_{sc} (μA·cm^{-2})	G_t (ms·cm^{-2})	V_t^a (mV)	J_{m-s} (nmol·cm^{-2}·h^{-1})
Control	5.7 ± 4.7	5.02 ± 0.84	1.23 ± 1.21	59.8 ± 3.8
Thapsigargin	16.1 ± 9.5	5.80 ± 0.97	2.43 ± 1.54	52.5 ± 5.4
P	< 0.05	NS	< 0.05	NS
Thapsigargin	20.6 ± 11.7	6.10 ± 1.19	2.88 ± 1.55	32.8 ± 3.8
P	NS	NS	< 0.05	< 0.005
Control	20.5 ± 6.5	6.09 ± 1.17	4.19 ± 1.37	57.2 ± 9.2
cAMP + IBMX	179.0 ± 34.9	11.43 ± 1.71	15.73 ± 2.32	31.4 ± 8.6
P	< 0.005	< 0.002	< 0.001	< 0.02
cAMP + IBMX	226.9 ± 52.7	14.68 ± 2.32	15.45 ± 2.36	32.5 ± 9.1
P	< 0.01	< 0.005	< 0.001	< 0.002

[a] Membranes were clamped to zero V_t except for 3 seconds every 20 min

Cl^- secretion and opening of an apical membrane Cl^- conductance [reviews: Karnaky (1986); Péqueux et al. (1988)]. In this case, the opercular epithelium of FW-acclimated killifish responded also by augmentation of Cl^- secretion, as the I_{sc} rose sharply from 20 to more than 200 μA·cm^{-2}, with a concomitant marked increase in G_t and V_t (Table 4). In connection with the stimulation of Cl^- secretion there was a significant inhibition of Ca^{2+} uptake of about 50%. Because these membranes were clamped to zero mV transepithelial potential, the drop in Ca^{2+} transport was not likely a result of a change in the apical membrane voltage and reduced Ca^{2+} entry. Also the increase in ion conductance during a decrease in Ca^{2+} uptake implies that the conductance change is limited to opening of channels other than those for Ca^{2+}, presumably apical membrane anion channels. This relationship between augmentation of I_{sc} and diminution of Ca^{2+} uptake also holds for membranes that have been pretreated by ionomycin (serosal or mucosal sides) and by thapsigargin. The pooled result ($n = 24$) gave a change in I_{sc} of $+94.15 \pm 12.46$ μA·cm^{-2} after db-cAMP and IBMX with a change in Ca^{2+} influx of -19.08 ± 2.61 nmol·cm^{-2}·h^{-1}.

Effect of ionomycin

Addition of ionomycin to the mucosal side of the epithelium should increase the availability of Ca^{2+} to the basolateral transport mechanisms and increase transepithelial Ca^{2+} transport, if apical membrane channels

Table 5 Effects of ionomycin (1.0 μmol·l^{-1}) on the serosal side and mucosal side of the killifish opercular epithelium on $^{45}Ca^{2+}$ unidirectional and net fluxes in symmetrical saline.

Treatment period	I_{sc} (μA·cm^{-2})	G_t (mS·cm^{-2})	J_{m-s} (nmol·cm^{-2}·h^{-1})	J_{s-m} (nmol·cm^{-2}.h^{-1})	J_{net} (nmol·cm^{-2}·h^{-1})
Serosol addition					
Control	−16.3 ± 6.5	8.79 ± 0.77	71.6 ± 10.6	1.88 ± 0.33	69.7 ± 10.6
Ionomycin (ser)	−3.5 ± 7.2	9.26 ± 0.80	56.8 ± 8.3	2.12 ± 0.24	54.7 ± 8.3
P^a	< 0.001	< 0.05	< 0.05	NS	< 0.05
Ionomycin (ser)	−3.2 ± 6.7	9.63 ± 0.77	49.1 ± 6.9	2.07 ± 0.31	47.7 ± 7.1
P^a	< 0.001	< 0.02	< 0.02	NS	< 0.05
Muscosal addition					
Control	4.6 ± 2.0	5.02 ± 0.72	51.7 ± 5.9	3.71 ± 1.08	48.0 ± 5.8
Ionomycin (muc)	3.8 ± 2.5	5.50 ± 0.81	68.5 ± 7.0	3.35 ± 0.44	65.2 ± 6.8
P^b	< 0.005	< 0.02	< 0.01	NS	< 0.01
Ionomycin (muc)	10.2 ± 3.4	6.25 ± 0.78	69.1 ± 8.8	2.43 ± 0.19	66.7 ± 8.8
P^b	< 0.001	< 0.02	< 0.02	NS	< 0.02

[a] Paired t-test, two tailed, compared to control period; $n = 7$ for fluxes, 14 for electrophysiology
[b] Paired t-test, two tailed, compared to control period; $n = 6$ for fluxes, 12 for electrophysiology

are rate limiting in the overall transport. In contrast, addition of ionomycin to the basolateral side would instead increase intracellular Ca^{2+} without affecting the influx of Ca^{2+} across the apical membrane.

Ionomycin added to the serosal side of the opercular membrane inhibited Ca^{2+} uptake by 20% in the first and second hours of exposure without affecting the rate of Ca^{2+} efflux (Table 5). In contrast, ionomycin addition to the mucosal side of the membrane significantly increased Ca^{2+} uptake rate by 31% ($P < 0.01$) again without affecting the Ca^{2+} efflux rate. I_{sc} was slightly negative initially but moved slowly positive regardless of the pharmacological treatment. There also was an associated slow rise in G_t over time.

Lack of direct catecholamine effect

There are well-recognized actions of catecholamines on ion transport in opercular membranes [reviews: Péqueux et al. (1988); Marshall et al. (1993)]. We therefore applied epinephrine, the β-adrenergic agonist (isoproterenol) and an α_2-agonist (clonidine) to membranes bathed in serosal saline and mucosal FW to determine if Ca^{2+} uptake was affected directly by these agents. Epinephrine (1.0 μmol·l^{-1}, serosal side) produced no change in Ca^{2+} uptake, with 19.6 ± 3.07 before epinephrine and 21.9 ± 3.9 nmol·cm^{-2}·h^{-1} after ($P > 0.05$, $n = 6$) and no change in G_t, V_t and Ca^{2+} efflux (data not shown). In a second series, isoproterenol (1.0 μmol·l^{-1}, serosal side) produced no significant change in Ca^{2+} unidirectional uptake that was 20.4 ± 2.9 before and 18.4 ± 2.2 nmol·cm^{-2}·h^{-1} after drug addition ($P > 0.05$, $n = 7$) with the membranes bathed in FW on the mucosal side and at open circuit. There was no significant change in G_t, V_t and Ca^{2+} efflux with isoproterenol treatment (data not shown). Isoproterenol (1.0 μmol·l^{-1}, serosal side) after epinephrine caused no significant change in any parameter measured. In another series, the α_2-adrenoceptor agonist clonidine at 10 μmol·l^{-1} (serosal side) produced no change in V_t and G_t and no apparent change in Ca^{2+} influx. Initially Ca^{2+} influx was 26.1 ± 2.5, and was 35.7 ± 5.8 nmol·cm^{-2}·h^{-1} after clonidine ($P > 0.2$, paired t-test, $n = 10$).

Discussion

Calcium transport characteristics

The most striking finding is the large, easily measured active uptake of Ca^{2+} across the FW killifish opercular membrane (Tables 1–5), an epithelium that in FW retains numerous mitochondria-rich cells. The rate of Ca^{2+} uptake varies with the density of mitochondria rich cells in the epithelium (Fig. 2). Among three species, tilapia [opercular epithelium; McCormick et al. (1992)], rainbow trout [cleithrum skin; Marshall et al. (1992)] and killifish (opercular epithelium) the relationship still holds, in that rainbow trout and tilapia have few mitochondria-rich cells and much lower Ca^{2+} transport rates. Even in those species with few mitochondria-rich cells there also exists the positive correlation between cell density and Ca^{2+} uptake rate (McCormick et al. 1992; Marshall et al. 1992). We infer therefore that mitochondria-rich cells are the major, if not the only, source of Ca^{2+} transport. If mitochondria-rich cells are the only location for Ca^{2+} transport and all the DASPEI positive cells are contributing, then the slope of the regression yields an estimate of the single cell rate of Ca^{2+} uptake, namely 1.3 pmol·h^{-1} per cell. This is not significantly different from the

previous estimate of 0.77 $pmol \cdot h^{-1}$ per cell from similar data with the trout cleithrum skin (Marshall et al. 1992). Taken together, the trout, tilapia and killifish data strongly support the conclusion that mitochondria-rich cells in the skin (and gills) of FW teleosts are responsible for Ca^{2+} transport.

Contribution of the opercular epithelium

The opercular epithelium may well be an important organ for extraintestinal uptake of Ca^{2+} in killifish. The very high density of mitochondria-rich cells, compared to the opercular epithelia and skin of rainbow trout and tilapia would suggest such an important contribution as a percentage of the whole body extraintestinal uptake. We can estimate the contribution of the opercular epithelium from a previous study that showed Ca^{2+} extraintestinal uptake *in vivo* of approximately 5.0 $\mu mol\ Ca \cdot 100\ g^{-1} \cdot h^{-1}$ (Mayer-Gostan et al. 1983) for killifish that were acclimated to a soft (0.1 $mmol \cdot l^{-1}$ Ca) FW similar to that used in our study. In FW of 0.65 $mmol \cdot l^{-1}\ Ca^{2+}$, Pang et al. (1980) found a Ca^{2+} extraintestinal uptake rate of 3.25 $\mu mol \cdot 100\ g^{-1} \cdot h^{-1}$. A 5 g adult killifish has approximately 2–3 cm^2 of opercular epithelium, or about 1.0–1.5 $\mu mol \cdot 100\ g^{-1} \cdot h^{-1}\ Ca^{2+}$ net flux (from Tables 1–3). Therefore, the opercular epithelium may account for 20–46% of active Ca^{2+} uptake by the animal in soft FW. If the opercular epithelium transport rates are compared to whole animal Ca^{2+} uptake rates for animals in hard FW, the opercular epithelium has the transport capacity to account for all of the Ca^{2+} uptake.

Calcium transport and kinetics

The $K_{\frac{1}{2}}$ we observed (0.348 $mmol \cdot l^{-1}$) for the killifish opercular epithelium is comparable to the uptake kinetics of several FW teleosts taken from *in vivo* experiments. In rainbow trout perfused head preparations, Ca^{2+} uptake had a $K_{\frac{1}{2}}$ that ranged from 0.115 to 0.245 $mmol \cdot l^{-1}$ for trout that were acclimated from 7–30 days in soft FW; control animals in 0.9 $mmol \cdot l^{-1}$ Ca^{2+} FW had a $K_{\frac{1}{2}}$ of 0.14 $mmol \cdot l^{-1}$. Previous studies with killifish *in vivo* were unable to determine $K_{\frac{1}{2}}$ values for extraintestinal Ca^{2+} transport for this species (Mayer-Gostan et al. 1983). The $K_{\frac{1}{2}}$ values *in vivo* and on intact epithelia *in vitro* are physiologically relevant to give an indication of the Ca^{2+} concentrations where the animals can be expected to be in positive Ca^{2+} balance, but the $K_{\frac{1}{2}}$ for Ca^{2+} transepithelial transport are much greater than the K_m values for active and secondary active Ca^{2+} transport systems. The Ca^{2+}-ATPase from gills has a K_m of 160 $nmol \cdot l^{-1}$ in rainbow trout (Perry and Flik 1988) and 102 $nmol \cdot l^{-1}$ in tilapia (Flik et al. 1985). Also, the Na^{+}/Ca^{2+} exchange mechanism that could account for the basolateral transport of some Ca^{2+} has been identified in teleost intestine and has a K_m of 181 $nmol \cdot l^{-1}$ (Flik et al. 1990). It is not clear for the epithelial systems which component of Ca^{2+} transport is saturating at 0.1–0.4 $mmol \cdot l^{-1}$, but clearly the basolateral pump(s) are not rate limiting. A combination of limited capacity to transfer Ca^{2+} intracellularly (presumably bound to calmodulin) and a downregulation of Ca^{2+} channels in high calcium media could account for the response.

The unidirectional efflux of Ca^{2+} across the killifish opercular epithelium, averaging 0.19–0.45 $nmol \cdot cm^{-2} \cdot h^{-1}$ (Tables 1–3), is comparable to that obtained previously under similar conditions for trout cleithrum skin [0.2–0.4 $nmol \cdot cm^{-2} \cdot h^{-1}$; Marshall et al. (1992)], indicating the low rate of passive leakage of Ca^{2+}. Tilapia opercular epithelium has a smaller leak pathway, with unidirectional efflux values of approximately 0.05–0.1 $nmol \cdot cm^{-2} \cdot h^{-1}$. The size of the Ca^{2+} leak pathway does not seem to be directly linked with epithelial conductance, as the trout opercular epithelium has a G_t that averaged 0.08–0.13 $mS \cdot cm^{-2}$ (Marshall et al. 1992), compared to the much higher conductance of killifish opercular epithelium (1.18–1.65 $mS \cdot cm^{-2}$, Tables 1, 2). In spite of the large difference in density of mitochondria-rich cells between the two tissues, the Ca^{2+} leak is very similar, hence the mitochondria-rich cells are likely not the location of the leak. Instead, the Ca^{2+} leak pathway may occur at the collective intercellular junctions.

It has been shown for SW opercular epithelia (Foskett and Scheffey 1982) that mitochondria-rich cells appear to be the localized source of both active ion transport and monovalent cation leakage. Consistent with this finding, G_t seems to be a function of the number of mitochondria-rich cells in the epithelium, inasmuch as the cell density is about 30 times higher in killifish (present study) than in tilapia opercular epithelium (McCormick et al. 1992) and G_t is 12- to 16-fold greater. This extends previous findings with the SW opercular epithelium to FW preparations; in both cases the low resistance pathway is associated with mitochondria-rich cells.

There is a significant decrease in Ca^{2+} uptake with La^{3+} and Mg^{2+} (Table 2); however this response is much smaller than the inhibition in Ca^{2+} uptake seen *in vivo* and in the isolated perfused trout head (Verbost et al. 1987, 1989). This difference may arise from the different methodologies and/or different species used. For instance, the *in vitro* flux experiments count only those labelled Ca^{2+} tracers that appear on the blood side, whereas flux studies *in vivo* include Ca^{2+} that may be adsorbed to fixed sites on the exterior of the animal and Ca^{2+} that may be sequestered intracellularly. Rinses of the whole animal with non-radioactive $CaCl_2$ solutions with or without EGTA should exclude adsorbed Ca^{2+} from uptake measurements, but La^{3+}

may reduce the availability of these sites. We have observed previously that La^{3+} can displace Ca^{2+} from fixed binding sites on the mucosal side of the epithelium (Marshall et al. 1992). Because La^{3+} has a marked effect on V_t, presumably because it occupies fixed binding sites in the tight junctions, making them less permeable to ions, it is also feasible that part of the La^{3+} response *in vivo* is indirect *via* gustatory sense and the brain. It is now recognized that neural (cholinergic) connections to the corpuscles of Stannius modulate a rapid inhibitory response on Ca^{2+} uptake (Cano et al. 1994).

Calcium involvement in calcium transport

Ionomycin added to the serosal side of the preparation inhibited Ca^{2+} uptake. This effect could be a manifestation of a Ca^{2+}-induced, downregulation of a step in transepithelial Ca^{2+} transport (i.e. either apical Ca^{2+} channels or the basolateral active step), but alternatively it could represent a recycling of non-radioactive Ca^{2+} from the serosal side. Another agent that is known to augment intracellular Ca^{2+} is thapsigargin. Thapsigargin inhibits selectively Ca^{2+}-ATPase on endoplasmic reticulum, thus allowing the depletion of this Ca^{2+} reserve and a concomitant increase in cytoplasmic Ca^{2+}. The similarity of the two responses, i.e. both thapsigargin and serosal ionomycin significantly decrease Ca^{2+} uptake with similar time-courses, suggests a commonality of the response. Of the two, serosal ionomycin is the only one that relies upon serosal (non-radioactive) Ca^{2+}. The thapsigargin response utilizes intracellular sources of Ca^{2+} that are more likely to be equilibrated with transported $^{45}Ca^{2+}$. Indeed, if there were no downregulation of Ca^{2+} transport, serosal ionomycin ought to have inhibited Ca^{2+} uptake (as observed), whereas there should have been an *increase* in transepithelial transport with thapsigargin (not as observed). For this reason, we favour the explanation involving a Ca^{2+} induced inhibition of Ca^{2+} transport. It is not clear as yet whether the effect is on the apical Ca^{2+} channels, although this would seem to be the most reasonable as Ca^{2+} homeostasis of the cell would be maintained. A less likely action would be inhibition of Ca^{2+} basolateral transport, that would result in chronically elevated intracellular Ca^{2+} with the ultimate result of cell death.

With saline bathing both surfaces of the epithelium and V_t clamped to zero, there was a large net flux of Ca^{2+} averaging 50–70 $nmol \cdot cm^{-2} \cdot h^{-1}$ (Table 5). This increase in Ca^{2+} uptake is expected on the basis of the higher Ca^{2+} content of the saline; however, the calcium concentration of the saline is within the normal range for hard FW and should not significantly alter the transport mechanism. Whereas Ca^{2+} transport is proposed to be electrogenic (inasmuch as the apical entry step is via Ca^{2+} channels), the resultant current is not within normal range of measurement. For instance, a 50 $nmol \cdot cm^{-2} \cdot h^{-1}$ Ca^{2+} net flux would produce a current of 2.7 $\mu A \cdot cm^{-2}$ and through a typical transepithelial resistance of 200 $Ohm \cdot cm^2$ this would yield a voltage change of only 0.54 mV. Cl^- transport in the SW opercular epithelium is much larger by comparison and ranges from 100 to 300 $\mu A \cdot cm^{-2}$ (Karnaky 1986; Péqueux et al. 1988). With saline on both sides of the membrane, changes in the electrophysiological parameters did not correlate with changes in Ca^{2+} transport. V_t initially was slightly negative by 0.5–5.0 mV that gave a negative I_{sc} when V_t was clamped to zero. This negative current, that could represent net gain of cations (or secretion of anions) gradually moved positively regardless of the experimental treatment and did not seem to correlate with Ca^{2+} transport rate. For instance, the increase in I_{sc} between hours 1 and 2 of ionomycin treatment on the mucosal side represents a mean increase of cation uptake of 0.175 $\mu Eq \cdot cm^{-2} \cdot h^{-1}$ (or an increase of 87 μmol $Ca^{2+} \cdot cm^{-2} \cdot h^{-1}$) while the Ca^{2+} uptake and net flux did not change. Also, the inhibition of Ca^{2+} uptake by serosally added ionomycin occurs while I_{sc} is increasing (Table 5). Clearly, if the electrogenicity of Ca^{2+} transport is to be demonstrated, other current sources will have to be inhibited and the Ca^{2+} transport will need to be maximized.

TFP complexes calmodulin and interferes with calmodulin dependent enzymes (for instance Ca^{2+}-ATPase). The drug is also known to inhibit Ca^{2+} influx in excitable cells (Clapham and Neher 1984) by apparently non-specific actions. Mucosally added TFP inhibited Ca^{2+} uptake with a time-course similar to serosal ionomycin and thapsigargin, but we cannot distinguish between a direct action on Ca^{2+} entry at the mucosal membrane or an indirect action mediated by complexing of calmodulin. TFP inhibited Ca^{2+} uptake when the drug was added to the mucosal side but was not apparently active when added to the basolateral side of the membrane. To account for this sidedness of the response, we propose that the architecture of the Ca^{2+} transport system is mostly localized to an area near the apical membrane. This is consistent with the known ultrastructural features of mitochondria-rich cells. The apical membrane is the location of presumptive Ca^{2+} channels that allow Ca^{2+} to enter into the cytoplasm. Near the apical membrane calmodulin may bind Ca^{2+} and translocate Ca^{2+} as a complex (CaM-Ca) to the basolateral side of the cell. Because the extensive tubular system of the cell is contiguous with the basolateral membrane (Philpott 1966) and ramifies to within a few micrometres of the apical crypt, the basolateral membrane is very close to the apical membrane, within 2–5 μm. Hence in spite of the large overall size of mitochondria-rich cells, the Ca^{2+} transport system needs only to operate in the most apical 5 μm of the cytoplasm.

Lack of direct catecholamine effect

There have been numerous reports regarding the regulation by catecholamines of ion transport by the fish gill and skin. Particularly there is an α_2-adrenergic response that dominates when non-specific agonists are used, and inhibits Cl^- secretion apparently *via* a rise in intracellular calcium (Marshall et al. 1993). Furthermore, there have been inconsistent results with catecholamine (epinephrine) effects on Ca^{2+} uptake: Payan et al. (1981) reported stimulation in perfused trout head, whereas Donald (1989) demonstrated inhibition of Ca^{2+} uptake in isolated gill arches and Perry et al. (1988) saw no effect on *in vivo* Ca^{2+} uptake in trout. In the same system but from SW-acclimated individuals, the opercular epithelium responds dramatically to the α_2-adrenergic agonist clonidine and the β-adrenergic agonist isoproterenol by (respectively) decreasing and increasing Cl^- secretion rate. Our results do not indicate a role for catecholamines in regulation of Ca^{2+} transport because a non-specific agonist (epinephrine) and two specific agonists, clonidine and isoproterenol, had no significant effect on Ca^{2+} uptake in FW opercular epithelium. Our results therefore support the findings of Cano et al. (1994) that target ion-transporting cells have receptors for stanniocalcin but that release of the hormone is under neural (cholinergic) control. The previous results showing effects of catecholamines on Ca^{2+} uptake in perfused gills and heads (Payan et al. 1981; Donald 1989) would seem therefore to be indirect responses perhaps mediated by the vasoactivity of these substances.

Effect of cyclic AMP

Augmentation of intracellular cAMP in opercular epithelia from FW-adapted killifish bathed in symmetrical saline results in the development of a SW-like I_{sc} that varies greatly in magnitude but was in all cases associated with a decrease in Ca^{2+} influx (Table 4). This change remained obvious even when the IBMX and db-cAMP were added after a variety of pretreatments (see results). It is not clear what hormone is connected with the stimulation of Cl^- secretion by cAMP, but there are several possibilities, including epinephrine *via* β-adrenergic receptors, urotensin I and glucagon (Karnaky 1986; Péqueux et al. 1988). To account for the change in Ca^{2+} transport, we propose an indirect action based on the generally accepted secondary active model for Cl^- secretion by chloride cells (Karnaky 1986; Péqueux et al. 1988). The stimulation of Cl^- secretion increases the rate of $Na^+,K^+,2Cl^-$-cotransport at the basal side of the chloride cell which in turn will likely reduce the Na^+ (and K^+) gradients across this membrane. Also, the increased turnover of Na^+,K^+-ATPase may reduce the supply of ATP. The former action would reduce the driving force of Na^+-Ca^{2+} exchange at the basolateral membrane [known to be present in teleost intestinal Ca^{2+} transport; Flik et al. (1990)] while the latter would reduce ATP supply for Ca^{2+}-ATPase. In either case, the indirect result would be a reduction in Ca^{2+} uptake, assuming Ca^{2+} uptake relies upon Na^+-Ca^{2+} exchange or Ca^{2+}-ATPase at the basolateral membrane. At this point we cannot distinguish this indirect response from a more direct regulation of Ca^{2+} transport by cAMP. Evidence can now be obtained using the killifish opercular epithelium to test the hypothesis of Na^+-Ca^{2+} exchange involvement in Ca^{2+} uptake by mitochondria rich cells.

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