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CALCIUM TRANSPORT BY ISOLATED SKIN OF RAINBOW TROUT

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Summary

The skin overlying the cleithrum bone of freshwater-acclimated rainbow trout contains numerous mitochondria-rich (MR) cells, as detected by DASPEI fluorescence. This tissue was mounted *in vitro* in an Ussing-style chamber with fresh water on the mucosal surface and saline supplemented with bovine serum albumin on the serosal surface. The preparation developed a high transepithelial resistance and a small transepithelial potential (V_t), positive on the serosal side. Radioisotopic flux measurements indicated that the preparation actively transported Ca^{2+} from the mucosal to the serosal surface, as assessed by the Ussing flux ratio criterion. Ca^{2+} transport was positively correlated with MR cell density. Cortisol pretreatment *in vivo* reduced MR cell density and increased V_t but did not significantly alter Ca^{2+} fluxes. Ca^{2+} transport was unaffected by adrenergic agonists (10^{-5} mol l^{-1} adrenaline, clonidine, isoprenaline) or cyclic AMP stimulants (10^{-3} mol l^{-1} dibutyryl cyclic adenosine monophosphate, db-cAMP, plus 10^{-4} mol l^{-1} isobutylmethylxanthine, IBMX) applied to the serosal surface. The Ca^{2+} ionophore ionomycin (1×10^{-6}–3.2×10^{-6} mol l^{-1} on the mucosal surface) increased both unidirectional Ca^{2+} fluxes and caused Ca^{2+} to accumulate within the epithelium. Lanthanum (10^{-4} mol l^{-1}) did not inhibit unidirectional Ca^{2+} fluxes, but apparently displaced Ca^{2+} from binding sites on the mucosal surface. Unlike Ca^{2+}, movements of Na^+ and Cl^- across the epithelium were passive, as assessed by the flux ratio criterion, and neither adrenaline nor db-cAMP plus IBMX had any effect on Na^+ or Cl^- fluxes or electrical properties. These results indicate that ion transport across the skin mediated by MR cells ('chloride cells') contributes to Ca^{2+} but not to NaCl balance in freshwater trout.

Introduction

The function of mitochondria-rich (MR) cells in the gills and related epithelial structures (jaw skin and opercular epithelium) of seawater teleosts is now very

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clearly associated with the secondary active secretion of Cl^- (Foskett and Scheffey, 1982; reviewed by Zadunaisky, 1984; Péqueux *et al.* 1988). The term 'chloride-secreting cells', first coined by Keys and Willmer (1932) and now often shortened to 'chloride cells', is an accurate description of their function in sea water. This same term is now commonly applied to ultrastructurally similar MR cells in the gills of freshwater teleosts. However, the function of these cells in freshwater fish, in particular their specific contribution to ionoregulation, remains highly controversial (reviewed by Wood, 1991). While some studies have discounted any involvement of MR cells in active Na^+ and Cl^- uptake (Girard and Payan, 1980; Payan *et al.* 1984), others have indicated that they are the principal sites of these processes (Avella *et al.* 1987; Perry and Laurent, 1989; Laurent and Perry, 1990). The most recent data suggest that the MR cells in freshwater gills are involved in Cl^- uptake, but not in Na^+ uptake (Goss *et al.* 1992*a*,*b*). The freshwater chloride cell has also been implicated in active Ca^{2+} uptake (Payan *et al.* 1981; Perry and Wood, 1985; Isihara and Mugiya, 1987; Perry and Flik, 1988; Flik and Perry, 1989).

In general, this evidence about the possible ionoregulatory role(s) of the MR cell in fresh water has been circumstantial and correlational. An important hindrance to progress has been that, in contrast to seawater teleosts, surrogate 'models' for the freshwater gill epithelium are not available – i.e. there are no related flat epithelial structures containing numerous MR cells and suitable for mounting *in vitro*. For example, the opercular epithelia of freshwater-adapted brook trout (*Salvelinus fontinalis*; Marshall, 1985) and rainbow trout (*Oncorhynchus mykiss;* present study) completely lack MR cells, based on the absence of fluorescent cells when they are stained with the mitochondrial fluorophore DASPEI. Opercular epithelia of freshwater-adapted tilapia (*Oreochromis mossambicus*; Foskett *et al.* 1981; Wendelaar Bonga *et al.* 1990) and killifish (*Fundulus heteroclitus*; Degnan *et al.* 1977) and goby (*Gillichthys mirabilis*; Marshall, 1977) adapted to 1–5 % sea water do contain some MR cells. However, when these epithelia are mounted *in vitro* with balanced saline on both sides, they either fail to transport Cl^- (tilapia) or actively secrete Cl^- (killifish, goby). The failure of these experiments to demonstrate Cl^- uptake by freshwater MR cells could be associated with the use of high mucosal NaCl concentrations *in vitro* that more closely approximate seawater conditions. Alternatively, in the case of killifish and goby, these euryhaline animals are normally seawater-resident and they may retain hormonally inhibited MR cells while in brackish water. Ca^{2+} fluxes in these preparations have not been examined except for tilapia (see note added in proof).

In the present study, we wished to examine Na^+, Cl^- and Ca^{2+} transport in a flat preparation of MR cells from a teleost that is normally resident in fresh water in an attempt to clarify the role of the freshwater chloride cell. We found that the skin overlying the cleithrum bone of rainbow trout contains numerous MR cells; this paper reports initial experiments with this preparation. The tests carefully mimicked *in vivo* freshwater ionic and osmotic conditions, hence possible artefacts associated with high mucosal NaCl concentrations were eliminated.

Materials and methods

Animals

Adult 100–300 g rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were obtained from Fraser's Mills Hatchery, Antigonish, Nova Scotia, and were maintained under natural photoperiod in flowing dechlorinated tapwater at 10–12 °C for at least 10 days prior to use. This water was soft (pH 6.8–7.2) with an average composition (in mmol l^{-1}) of Na^+, 0.16; K^+, 0.02; Ca^{2+}, 0.10; Mg^{2+}, 0.06; Cl^-, 0.17; SO_4^{2-}, 0.14; and titratable alkalinity (to pH 4.0), 0.20. At least 7 days prior to use, the trout were transferred to individual fish boxes served with flowing water of the same composition; they were not fed during this period. This confinement facilitated cortisol injections (see below) and minimized disturbance. The fish were killed by a blow to the head, and a terminal blood sample was taken by heparinized syringe from the caudal vessels for analysis of plasma Na^+, Cl^-, Ca^{2+} and cortisol. The cleithrum membranes were then immediately dissected.

In one series, fish were treated with cortisol by the method of Perry and Wood (1985) in an attempt to cause proliferation of MR cells on the cleithrum skin. Trout were injected (intramuscularly) once a day for 9–11 days with 4 mg kg^{-1} hydrocortisone hemisuccinate sodium salt (Sigma) in 140 mmol l^{-1} NaCl (0.4 ml kg^{-1}). The results were compared with those of an uninjected control group. A saline-injected control was not performed as we wished to avoid the elevation of endogenous cortisol levels that accompanies this procedure.

Bathing solutions

The *in vitro* bathing solution for the inside (serosal surface) of the cleithrum skin was Cortland saline (Wolf, 1963) modified to duplicate more closely the measured composition of trout plasma. The formulation (in mmol l^{-1}) was NaCl, 129.9; KCl, 2.55; $CaCl_2.2H_2O$, 1.56; $MgSO_4.7H_2O$, 0.93; $NaHCO_3$, 13.00; $NaH_2PO_4.H_2O$, 2.97; glucose, 5.55; NH_4Cl, 0.30 (all salts from Sigma Chemical Co.); with 20 mg ml^{-1} bovine serum albumin (Sigma grade III, Fraction V) as a substitute for plasma protein. After equilibration with a nominal gas mixture of 0.3 % CO_2, balance O_2, the measured acid–base status (pH 7.8–7.9, P_{CO_2}=0.25–0.32 kPa, HCO_3^-=5–6 mmol l^{-1}) was typical of blood *in vivo* and the P_{O_2} was greater than 65 kPa.

The bathing solution for the outside (mucosal surface) of the preparation was fresh water taken from the acclimation system; it was gassed with 100 % O_2, resulting in a P_{O_2} greater than 65 kPa. The mucosal solution was changed at hourly intervals and its composition analysed at the start and end of each 60 min experimental period. Typical values were Na^+=0.15–0.30 mmol l^{-1}, Cl^-=0.15–0.30 mmol l^{-1} and Ca^{2+}=0.08–0.12 mmol l^{-1}. Ion levels in this very dilute medium tended to change as a result of fluxes and release of bound ions from the mucosal surface (see Results). The addition of ^{22}Na and ^{45}Ca radioisotopes did not appreciably alter net mucosal concentrations, but addition of ^{36}Cl, which was of lower specific activity, typically raised the net Cl^- level to 0.40–0.60 mmol l^{-1}.

Membrane preparation

The portion of skin taken for the flux experiments overlies the cleithrum bone directly behind the posterior-ventral margin of the gill filaments. It is therefore normally irrigated with expired ventilatory water. The 'cleithrum skin' is roughly rectangular, about 2–3 cm^2 in total area, and extends dorsally and anteriorly from the pectoral fin to the ventral margin of the gill arches. It separates easily from underlying tissues. Cleithrum skin pieces from each side of the animal were moistened with Cortland saline, dissected and mounted in removable apertures of Ussing-style membrane chambers. After mounting, the mucosal surfaces were washed vigorously with fresh water to remove saline and to displace the mucus that tended to accumulate during dissection. The membranes were then placed in the chambers with saline on the serosal surface and fresh water on the mucosal surface. An initial 30 min period was allowed for the tissue to adjust to the *in vitro* conditions. At the start and end of this period, the mucosal surface was rinsed twice by the flow-through of 30 ml of fresh water (ten times the half-chamber volume) to ensure the removal of all traces of saline. Throughout the experiment, the mucosal and serosal media were continually mixed by magnetic stirrers, and the appropriate gases were passed across the surface of each media.

Electrophysiology

Experiments followed previously published methods (Marshall, 1986). In brief, the membranes were housed in water-jacketed chambers (11±1°C) of 3.0 ml volume per side with membrane apertures of 0.238 cm^2 exposed surface area. The epithelium was open-circuited throughout; current-passing and voltage-measuring bridges were of agar/150 mmol l^{-1} KCl. Transepithelial voltage was measured throughout the experiment, and resistance was estimated every 5 min from the voltage response to 1 μA current pulses from the current/voltage clamps (D. Lee Co. or WP Instruments DVC-1000). The reported membrane voltages in asymmetrical solutions were corrected for junction potentials (measured against a free-flowing 3 mol l^{-1} KCl half-cell). The membrane resistances were corrected for solution resistances. In experiments where Cl^- fluxes were measured, the bridges were removed during flux determinations to prevent alterations in mucosal Cl^- concentration resulting from bridge leaching.

Experimental protocol and radioisotopic fluxes

The two membranes from each fish were generally set up in pairs for unidirectional flux measurements, one being used for the mucosal-to-serosal unidirectional influx (J^{ms}), the other for the serosal-to-mucosal unidirectional influx (J^{sm}). After the mucosal rinse at the end of the initial 30 min settling period, ^{36}Cl ($H^{36}Cl$ from New England Nuclear, neutralized with KOH), ^{22}Na ($^{22}NaCl$ from Amersham) or ^{45}Ca ($^{45}CaCl_2$ from Amersham) was added to a final specific activity of at least 25 000 cts min^{-1} μmol^{-1} on the saline (serosal) side and 4 000 000 cts min^{-1} μmol^{-1} on the freshwater (mucosal) side. In Ca^{2+} flux exper-

iments, 10–100-fold higher specific activities were used. A further 30–60 min was allowed to elapse to ensure complete radioisotopic equilibration. Experiments typically consisted of three 60 min periods, separated by 20 min intervals used for changing over the mucosal solution and adding drugs. The first period served as a control, and the second and third were used for experimental treatments or for time controls. Within each period, samples were taken at 0 and 60 min for ionic analyses and at 0, 20, 40 and 60 min for radioactivity analyses. Unidirectional fluxes were calculated in the standard fashion from the specific activity on the labelled side and the appearance of radioactivity on the unlabelled side. Flux values for the three 20 min intervals were averaged to produce a mean for each experimental period.

Each membrane pair yielded independent measurements of J^{ms} and J^{sm}, and therefore the net flux ($J^{ms}-J^{sm}$) and the flux ratio (J^{ms}/J^{sm}). The primary criterion for the presence of non-diffusive ion fluxes was disagreement with the Ussing flux ratio equation:

$$J_i^{ms}/J_i^{sm} = (a_i^m/a_i^s)e^{(z_iFV_t/RT)},$$

where a_i^m and a_i^s are the activities of ion i on the mucosal and serosal sides respectively, z_i is its valency, V_t is the measured transepithelial voltage, and F, R and T have their usual thermodynamic meanings. The activities of Na^+ (109.5 mmol l^{-1}; using a microelectrode filled with resin, Steiner *et al.* 1979) and Ca^{2+} (0.80 mmol l^{-1}; using a WP Instruments macroelectrode) in the serosal saline were measured directly. Tests showed that Ca^{2+} activity was lowered by the presence of bovine serum albumin in the saline, but Na^+ activity was unaffected. Therefore Cl^- activity (101.6 mmol l^{-1}) was calculated using the measured Na^+ activity coefficient (0.75) multiplied by the measured Cl^- concentration, in accordance with theory for a solution of this ionic strength (Lee, 1981). The Na^+, Cl^- and Ca^{2+} activities of the mucosal fresh water were taken as equal to the measured concentrations, in view of the low concentrations (<1 mmol l^{-1}) and lack of protein.

The efflux of ions across the cleithrum skin and the release of bound ions from the mucosal surface (see Results) significantly altered the ionic concentrations in the dilute mucosal solution. If left uncorrected, mucosal concentrations, especially of Na^+ and Cl^-, would have increased greatly over the course of a 5 h experiment. To minimize this effect and to maintain concentrations as close to true freshwater values as possible, the mucosal surface was extensively rinsed initially (see above), and the mucosal solution was renewed at the end of each 60 min period. Water samples were taken immediately before and after each renewal, and the concentration of the ion of interest (Na^+, Cl^- or Ca^{2+}) was determined so that the average concentration was known for flux calculations. These measurements of change in total mucosal concentration also allowed calculation of the net loss (or gain) of ions by the membrane over each period.

At the end of some experiments, especially time controls, samples of serosal saline and mucosal fresh water were drawn from the membrane chambers into

Hamilton gas-tight syringes and analyzed for P_{O_2}, pH and total CO_2 content. This served to check the maintenance of correct acid–base status and O_2 levels.

Fluorescence microscopy

At the end of each experiment, the density of MR cells on the membranes was assessed using the mitochondrial fluorophore DASPEI [2-(4-dimethylamino-styryl)-*N*-methylpyridinium iodide; ICN Pharmaceutics; Marshall and Nishioka, 1980]. The stock was 0.2 mg DASPEI per millilitre of distilled water; this was diluted to 0.008 mg ml^{-1} final concentration with Cortland saline. The membranes were left in the chamber apertures and were incubated in oxygenated DASPEI solution for a minimum of 20 min prior to microscopic examination. A Zeiss photomicroscope III equipped with epifluorescence and a mercury/mercury vapour burner was used to observe and count the MR cells *in situ*. Cell density estimates were made by averaging the numbers of cells in 10 randomly chosen half-fields at a magnification of 200 (total counted area=3.25 mm^2). Cell counts could not be made after ionomycin treatments as DASPEI fluorescence became non-specific.

Pharmaceuticals

The adrenergic agonists adrenaline (L-epinephrine bitartrate; Sigma), clonidine (clonidine hydrochloride; Sigma) and isoprenaline (L-isoproterenol bitartrate; Sigma) were added to the serosal saline at a concentration of 10^{-5} mol l^{-1}. Cyclic AMP mobilization was duplicated by the addition of 10^{-3} mol l^{-1} db-cAMP (dibutyryl-cyclic-adenosine monophosphate, Na^+ salt; Sigma) plus 10^{-4} mol l^{-1} IBMX (3-isobutyl-1-methylxanthine; Sigma) to the serosal side. Lanthanum chloride (10^{-4} mol l^{-1}; Sigma) was added to the mucosal fresh water as a putative Ca^{2+} channel blocker. The Ca^{2+} ionophore ionomycin (ionomycin free acid; Calbiochem) was dissolved in a minimum of dimethylsulphoxide (DMSO) and added to the mucosal fresh water at concentrations of 1.0, 3.2 or 10 μmol l^{-1}. The resulting maximum DMSO concentration was 0.18%. In these experiments, the water pH was adjusted to the pK (8.3) of ionomycin with $NaHCO_3$, and the same DMSO concentration and water pH were employed in the control period prior to the addition of ionomycin.

Analytical techniques

Na^+ and Ca^{2+} in water, saline and plasma samples were determined by atomic absorption (Varian 375 AA or 1275 AA). Samples and standards for Ca^{2+} were made up in a 5000 mg l^{-1} K^+ diluent to minimize interference effects. Cl^- in water was determined colorimetrically (Zall *et al.* 1956) and in saline and plasma by coulometric titration (Radiometer CMT 10). Cortisol was determined by a commercial ^{125}I radioimmunoassay (Immuchem Cortisol kit, ICN Biomedicals Inc.) using standards diluted to the protein concentrations found in trout plasma; ^{125}I radioactivity was measured on a Packard Minaxi 5000 autogamma counter. ^{22}Na, ^{36}Cl and ^{45}Ca radioactivities were determined by counting to 1% error on a

Table 1. *Time control electrophysiology and unidirectional flux rates of the cleithrum skin*

			Na^+ fluxes		Cl^- fluxes		Ca^{2+} fluxes	
Period	V_t (mV)	R ($k\Omega\,cm^2$)	J^{ms} ($nmol\,cm^{-2}\,h^{-1}$)	J^{sm} ($nmol\,cm^{-2}\,h^{-1}$)	J^{ms} ($nmol\,cm^{-2}\,h^{-1}$)	J^{sm} ($nmol\,cm^{-2}\,h^{-1}$)	J^{ms} ($nmol\,cm^{-2}\,h^{-1}$)	J^{sm} ($nmol\,cm^{-2}\,h^{-1}$)
1. Control	2.5±1.2	11.0±1.0	0.18±0.05	134±39	3.45±1.14	310±84	0.036±0.009	0.215±0.057
2. Control	2.0±1.2	13.2±1.1	0.16±0.03	114±22	2.96±0.28	247±77	0.039±0.010	0.259±0.054
*P**	NS	<0.001	NS	NS	NS	NS	NS	NS
3. Control	2.2±1.2	13.4±1.1	0.17±0.04	137±29	4.73±1.50	198±72	0.043±0.015	0.373±0.079
*P**	NS	<0.001	NS	NS	NS	NS	NS	NS
N	33	39	12	8	5	4	6	6

Means±1 S.E.M.
* Paired *t*-test, two-tailed, compared to period 1; NS, not significant.
V_t, transepithelial potential; R, membrane resistance; J^{ms}, ion flux from mucosa to serosa; J^{sm}, ion flux from serosa to mucosa.

Table 2. *Flux ratio analysis of ion transport in the cleithrum skin under open-circuit conditions*

		Activity ($mmol\,l^{-1}$)					J^{ms}/J^{sm}		
Ion	*N*	Serosal	Mucosal	V_t (mV)	J^{ms} ($nmol\,cm^{-2}\,h^{-1}$)	J^{sm} ($nmol\,cm^{-2}\,h^{-1}$)	Observed	Predicted	*P**
Ca^{2+}	26	0.800	0.108±0.005	6.9±1.1	0.086±0.018	0.520±0.098	0.26±0.07	0.08±0.004	<0.01
Na^+	9	109.5	0.184±0.006	5.1±1.8	0.13±0.03	140±35	$1.34\times10^{-3}\pm0.39\times10^{-3}$	$1.50\times10^{-3}\pm0.15\times10^{-3}$	NS
Cl^-	8	101.6	0.583±0.068	3.4±1.3	2.06±0.75	250±98	$11.00\times10^{-3}\pm3.10\times10^{-3}$	$6.60\times10^{-3}\pm1.10\times10^{-3}$	NS

Means±1 S.E.M.
* Paired *t*-test, two-tailed, of observed *versus* predicted J^{ms}/J^{sm}; NS, not significant.

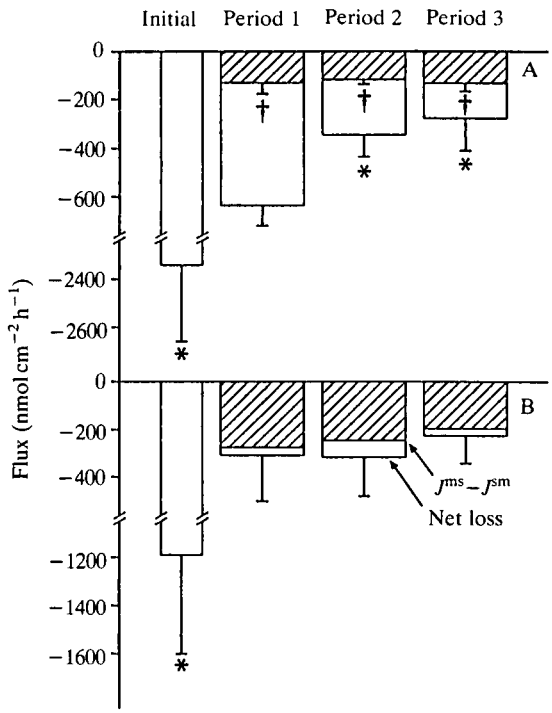

Fig. 1. Net loss rates, calculated from changes in concentration in the mucosal bath (open bars), and net flux rates (J^{net}), calculated as the difference between unidirectional flux rates ($J^{ms}-J^{sm}$; hatched bars), for (A) Na^+ and (B) Cl^- during the time series control experiments in the cleithrum skin. 'Initial' represents the 30 min settling period after initial set-up, while periods 1, 2 and 3 represent the three successive 60 min periods used in experiments. True net flux rates were not measured during the initial period. Means±1 S.E.M., N=9–13 for Na^+ net loss, 4–8 for Cl^- net loss, 8 for Na^+ net flux and 4 for Cl^- net flux. For clarity, standard error bars have been omitted for Cl^- $J^{ms}-J^{sm}$, but they were comparable to those for net Cl^- loss. * indicates $P<0.05$ relative to period 1 value; † indicates $P<0.05$ relative to the corresponding net loss rate.

Fig. 1A). In contrast, the Cl^- net loss rate stabilized at about $-300\,nmol\,cm^{-2}\,h^{-1}$ by period 1, and was never significantly different from J^{net} for Cl^- (Fig. 1B). J^{net} for both Na^+ and Cl^- did not vary significantly over the three periods. The Ca^{2+} net loss rate [not shown; overall average$=-7.1\pm7.5\,nmol\,cm^{-2}\,h^{-1}$ (13)] could not be distinguished from zero or from the J^{net} for Ca^{2+} [$-0.243\pm 0.063\,nmol\,cm^{-2}\,h^{-1}$ (6)] throughout the experiment. These results suggest that increases in Na^+ (and initially in Cl^-) concentration in the mucosal bath do not come entirely from transepithelial fluxes but instead represent washout of ions adsorbed to the epithelial surface. For this reason, the increase in mucosal ion concentration is not a good indication of net ion flux.

The P_{O_2} of the serosal bathing solution of the time controls at the end of the experiment (total incubation time approximately 5 h) was 49.5±2.6 kPa (19), the

pH was 7.844±0.019 (22), the total CO_2 was 5.51±0.08 mmol l^{-1} (20), the HCO_3^- concentration was 5.40±0.06 mmol l^{-1} (20) and the P_{CO_2} was 0.27±0.01 kPa (20). Except for a small fall in P_{O_2}, these values were unchanged from those at the start of the experiment and indicated that the *in vitro* conditions provided appropriate acid–base status and ample O_2 supply to the tissues.

Flux ratio analysis

To establish which, if any, ionic species might be actively transported by the epithelium, we compared the observed unidirectional flux ratio under open-circuit conditions for each pair of membranes (J^{ms}/J^{sm}) with that predicted by the Ussing equation from the prevailing V_t and ionic activities. Table 2 reports only data from period 1 (in the absence of drug treatments) but data from periods 2 and 3 yield the same conclusions. The analysis indicated that the observed flux ratio for Ca^{2+} was threefold greater ($P<0.01$) than that predicted on the basis of passive diffusion, i.e. there was active Ca^{2+} uptake from the mucosal to serosal sides. In contrast, the flux ratios observed for Na^+ and Cl^- fluxes were in agreement with the predicted ratios ($P=0.866$ and 0.433, respectively), indicating that the movement of these two ions across the epithelium could be explained by passive diffusion alone. The much higher flux ratios for Cl^- than for Na^+ resulted both from the positive V_t and from the higher mucosal Cl^- concentration. All observed flux ratios were far less than 1.0 in this *in vitro* preparation, reflecting the large chemical gradients favouring J^{sm} and opposing J^{ms}.

Unidirectional fluxes, MR cell numbers and the effects of cortisol

Ca^{2+} unidirectional influx (J^{ms}) was positively correlated ($r=0.63$, $P<0.01$, $N=30$) with the number of MR cells in the preparation (Fig. 2), suggesting that the MR cells are associated with Ca^{2+} uptake. There was no significant correlation of MR cell density with Ca^{2+} efflux, or with Na^+ and Cl^- influxes or Cl^- efflux, suggesting that these fluxes are not associated with MR cells. Na^+ efflux (J^{sm}) was negatively correlated ($r=-0.65$, $P<0.05$, $N=8$) with MR cell density; the meaning of this is unclear. There was no relationship between MR cell numbers and either V_t or membrane resistance.

To ascertain whether MR cell density affected the observed Ca^{2+} influx and Na^+ efflux, we applied a hormone treatment known to increase MR cell numbers on the gills of trout (Perry and Wood, 1985; Perry and Laurent, 1989; Laurent and Perry, 1990). Ten days of cortisol injections caused about a 40-fold elevation in measured plasma cortisol levels without altering plasma Na^+, Cl^- or Ca^{2+} concentrations (Table 3). The appearance of the skin changed; it became darker, more mucified and more fragile during dissection. However, contrary to expectation, this treatment caused a highly significant ($P<0.001$) 50 % decrease in MR cell density on the cleithrum skin (Table 3). In accordance with the previous correlation analyses, the decrease in MR cell numbers was accompanied by trends for decreased Ca^{2+} influx (J^{ms}) and increased Na^+ efflux (J^{sm}), though neither was

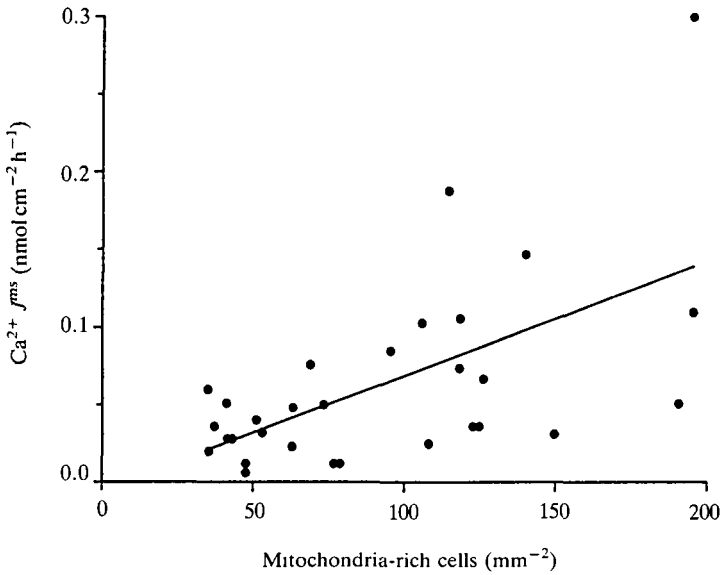

Fig. 2. The relationship between Ca^{2+} influx (J^{ms}, nmol cm^{-2} h^{-1}), measured in period 1, and the density of mitochondria-rich cells (MRC, mm^{-2}) on the cleithrum skin: J^{ms}=0.0007657MRC−0.007 (r=0.63, P<0.01, N=30).

significant. Cortisol treatment also significantly increased V_t without any effect on membrane resistance (Table 3).

Effects of lanthanum

The polyvalent cation lanthanum (La^{3+}) inhibits Ca^{2+} uptake in intact trout (Verbost *et al.* 1987, 1989; Perry and Flik, 1988). We applied 10^{-4} mol l^{-1} La^{3+} to the mucosal bath to determine whether Ca^{2+} transport by the cleithrum skin would be similarly affected. The responses to La^{3+} were followed for 2 h (periods 2 and 3) after the control period 1. There was no effect on electrophysiological variables or on unidirectional Ca^{2+} influx (J^{ms}; Table 4). However, parallel time controls normally showed an increase in the transepithelial resistance in periods 2 and 3 (see Table 1), which did not occur in the presence of La^{3+} (Table 4). The unidirectional efflux (J^{sm}) and J^{net} of Ca^{2+} were significantly increased by La^{3+} (Table 4), suggesting that La^{3+} increases the Ca^{2+} permeability of the cleithrum skin. The flux ratio criterion was satisfied (i.e. Ca^{2+} fluxes were indistinguishable from simple diffusion) after addition of lanthanum. The Ca^{2+} net loss rate, determined from changes in concentration in the mucosal medium, was markedly enhanced by La^{3+}, while the unidirectional Ca^{2+} efflux was only modestly increased. Hence, it appeared that La^{3+} displaced bound Ca^{2+} from the mucosal surface.

Effects of ionomycin

The calcium ionophore ionomycin was used in an attempt to increase the Ca^{2+}

Table 3. *The effect of cortisol treatment on MR cell numbers, unidirectional ion fluxes and electrophysiology in the cleithrum skin bathed* in vitro *with fresh water and on plasma ions and cortisol*

	Untreated control	Cortisol-treated	P*
MR cells	139±12	67±7	<0.001
(cells mm^{-2})	(58)	(20)	
Ca^{2+} J^{ms}	0.073±0.011	0.055±0.023	NS
(nmol cm^{-2} h^{-1})	(34)	(6)	
Ca^{2+} J^{sm}	0.369±0.072	0.580±0.108	NS
(nmol cm^{-2} h^{-1})	(15)	(5)	
Na^{+} J^{ms}	0.17±0.05	0.18±0.05	NS
(nmol cm^{-2} h^{-1})	(16)	(5)	
Na^{+} J^{sm}	74±22	185±51	NS
(nmol cm^{-2} h^{-1})	(7)	(7)	
V_t (mV)	+3.8±0.7	+10.8±1.6	<0.001
	(75)	(20)	
R (kΩ cm^2)	10.4±0.6	11.4±1.6	NS
	(80)	(20)	
Plasma [Na^+]	142.0±1.6	138.2±3.3	NS
(mmol l^{-1})	(33)	(13)	
Plasma [Cl^-]	127.4±1.5	122.6±3.1	NS
(mmol l^{-1})	(33)	(13)	
Plasma [Ca^{2+}]	2.73±0.05	2.65±0.10	NS
(mmol l^{-1})	(33)	(13)	
Plasma [cortisol]	49.1±9.5	2173.5±388.9	<0.001
(ng ml^{-1})	(36)	(13)	

Means±S.E.M. (N).
* Unpaired t-test, two-tailed; NS, not significant.

permeability of the apical membrane, a treatment that should increase Ca^{2+} uptake if this step were rate-limiting. Ionomycin was dissolved in a minimum of DMSO and added to the mucosal side only. In the control period, the DMSO vehicle alone was added. Results with 1.0 and 3.2 μmol l^{-1} ionomycin (with DMSO<0.06%) were identical and have been combined in Table 5. We used these lower doses because preliminary experiments with 10 μmol l^{-1} ionomycin (with 0.18% DMSO) showed marked progressive decreases in transepithelial resistance from 8.0±0.8 kΩ cm^2 in the control period (1) to 5.0±1.0 kΩ cm^2 and 2.4±0.4 kΩ cm^2 in the two successive periods (2 and 3) with ionomycin, respectively (P<0.001, N=6). This large reduction in tissue resistance is indicative of disruption of epithelial integrity. At the lower concentrations of ionomycin (and DMSO), these effects did not occur although, as with La^{3+} (Table 4), there was no increase in resistance during periods 2 and 3 (Table 5).

Ionomycin significantly augmented unidirectional Ca^{2+} influx (J^{ms}) during periods 2 and 3 and unidirectional Ca^{2+} efflux (J^{sm}) during period 3 (Table 5). However, the most dramatic effect of ionomycin during both experimental periods

Table 4. *Effects of lanthanum (10^{-4} mol l^{-1}) added to the mucosal medium on calcium fluxes and electrophysiology in the cleithrum skin*

Period	V_t (mV)	R (kΩ cm^2)	Ca^{2+} fluxes: J^{ms} (nmol cm^{-2} h^{-1})	Ca^{2+} fluxes: J^{sm} (nmol cm^{-2} h^{-1})	Net Ca^{2+} gain/loss (nmol cm^{-2} h^{-1})
1. Control	9.4±1.9	10.5±1.1	0.060±0.016	0.540±0.065	−17.4±9.6
2. Lanthanum	7.2±1.7	11.3±1.3	0.076±0.035	1.100±0.314	−63.1±20.1
*P**	NS	NS	NS	NS	<0.01
N	16	16	9	7	16
3. Lanthanum	8.2±2.0	11.9±1.7	0.091±0.045	1.180±0.171	−84.6±26.1
*P**	NS	NS	NS	<0.05	<0.05
N	11	11	5	5	9

Means±1 S.E.M.

* Paired *t*-test, two-tailed, compared to control period 1 (*N* for period 3 is smaller because, initially, membranes were followed for only 1 h in the presence of lanthanum); NS, not significant.

Table 5. *Effects of ionomycin (1×10^{-6} or 3.2×10^{-6} mol l^{-1}) added to the mucosal medium on calcium fluxes and electrophysiology in the cleithrum skin*

Period	V_t (mV)	R (kΩ cm^2)	Ca^{2+} fluxes: J^{ms} (nmol cm^{-2} h^{-1})	Ca^{2+} fluxes: J^{sm} (nmol cm^{-2} h^{-1})	Net Ca^{2+} gain/loss (nmol cm^{-2} h^{-1})
1. Control	7.2±1.5	7.6±1.0	0.096±0.028	0.520±0.120	−6.0±20.7
2. Ionomycin	7.7±1.5	8.2±1.0	0.144±0.042	0.935±0.273	59.7±19.8
*P**	NS	NS	<0.05	NS	<0.01
3. Ionomycin	6.7±1.4	7.4±0.9	0.151±0.036	0.854±0.150	64.2±20.8
*P**	NS	NS	<0.05	<0.05	<0.01
N	8	8	8	6	14

Means±1 S.E.M.

* Paired *t*-test, two-tailed, compared to control period 1; NS, not significant.

was to cause a large net gain of Ca^{2+} by the cleithrum skin, as determined from changes in concentration in the mucosal medium. This occurred despite the fact that the J^{net} indicated a modest net loss of Ca^{2+} *across* the tissue. This marked disappearance of Ca^{2+} from the mucosal solution probably represents increased apical permeability to Ca^{2+} and resultant sequestering of Ca^{2+} intracellularly in the epithelium.

Effects of adrenergic agonists and db-cAMP/IBMX

Addition of either the α_2-adrenergic agonist clonidine (10^{-5} mol l^{-1}, N=7) or

the β-adrenergic agonist isoprenaline (10^{-5} mol l^{-1}, N=7) to the serosal side had no significant effect over 2 h (periods 2 and 3) on electrophysiological variables, on unidirectional Ca^{2+} influx (J^{ms}) or on the net loss/gain of Ca^{2+} measured from changes in mucosal concentration. The unidirectional Ca^{2+} efflux (J^{sm}) was not measured in these experiments. Addition of the non-selective adrenergic agonist adrenaline (10^{-5} mol l^{-1}; N=6) alone to the serosal side in period 2, and in combination with db-cAMP (10^{-3} mol l^{-1}) plus IBMX (10^{-4} mol l^{-1}) to the serosal side in period 3, again had no effect on electrophysiological variables and no effect on the unidirectional influx or efflux, on $J^{ms}-J^{sm}$ or on the net loss/gain of Ca^{2+} by the preparation. In separate experiments, these same protocols had no effect on the comparable fluxes of Na^+ (N=4) or Cl^- (N=4). As none of these treatments had significant effects, and all data were essentially similar to the time controls (Table 1, Fig. 1), individual results have not been tabulated.

We conclude that stimulation of ion transport in this epithelium does not appear to involve adrenergic agonists or cyclic AMP mediation. Nevertheless, it is of some interest that the measured serosal P_{O_2} [40.9±1.3 kPa (14)] after epinephrine plus db-cAMP plus IBMX was significantly lower (P<0.05) than in the time controls [49.5±2.6 kPa (19)], indicating that these agents had stimulated the O_2 consumption of the tissue.

Discussion

Electrophysiology

Relative to most other teleost skin preparations, the trout cleithrum skin started with a high transepithelial resistance which progressively improved during the 5 h of *in vitro* incubation (Table 1), suggesting a progressively improved sealing of the epithelium around the edges of the chamber. The high transmural resistance is consistent with the notion that freshwater skin and gill epithelia have a low permeability to minimize diffusive ion loss. Epithelia from seawater-acclimated fish (Degnan *et al.* 1977; Marshall, 1977; Foskett *et al.* 1981) typically have resistances only a few per cent of those reported here. Even by freshwater standards, the cleithrum skin resistance (about 12 kΩ cm^2) was high: two- to threefold greater than the epithelial resistances observed previously across the opercular epithelia of freshwater-acclimated brook trout (Marshall, 1985) and tilapia (Foskett *et al.* 1981). This difference may result from the beneficial effects of serosal albumin (not used in previous studies), the use of true fresh water on the mucosal surface (cf. Marshall, 1985) and/or from the different properties of the epithelia.

The small inside-positive V_t (about +4 mV) was constant over time, indicating that ion selectivity did not change markedly during the *in vitro* incubation. The positive polarity of V_t was surprising, because a small inside-negative diffusion potential is usually recorded in intact trout and perfused trout heads in water of this Ca^{2+} concentration (McWilliams and Potts, 1978; Perry and Wood, 1985; Perry and Flik, 1988). Indeed the opercular epithelium of the brook trout mounted

under similar conditions *in vitro* developed a V_t of about −9 mV (Marshall, 1985). The reason for this difference is unknown, but it may be related to the nature of skin mucus in the freshwater rainbow trout. An inside-positive diffusion potential develops when this mucus is placed in a dialysis cell with fresh water on the outside (Handy, 1989). In this regard, it is noteworthy that cortisol pretreatment, which clearly increased mucus secretion, made V_t significantly more positive in the cleithrum skin (Table 3). Paradoxically, this same pretreatment rendered V_t significantly more negative in the perfused trout head preparation (Perry and Wood, 1985).

Calcium transport by the cleithrum skin

The cleithrum skin of the rainbow trout contains MR cells which are apparently involved in the active uptake of Ca^{2+} from the water into the fish. Evidence includes the existence of DASPEI fluorescent cells in the epithelium, the significant positive correlation between the density of these MR cells in the skin and the unidirectional Ca^{2+} influx (J^{ms}; Fig. 2) and the significantly higher flux ratio (J^{ms}/J^{sm}) than that predicted by the Ussing flux ratio equation on the basis of passive diffusion (Table 2). Because the osmotic gradient favours net volume flow from mucosa to serosa, J^{ms} for Na^+, Cl^- and Ca^{2+} could include a solvent drag component, although the flux ratios for the most permeant ions, Na^+ and Cl^-, show no evidence of this (i.e. they do not have larger than predicted flux ratios). In general, the low osmotic permeability of freshwater teleosts and the very high transmural resistance of the trout skin suggest that volume flow, and hence the solvent drag component, would be small.

The correlation between MR cell density and the rate of Ca^{2+} influx is in accord with the findings of Perry and Wood (1985) on the gills of isolated perfused trout head preparations and intact trout. The threefold higher J^{ms}/J^{sm} flux ratio than that predicted by the Ussing equation matches the fourfold difference reported by Perry and Flik (1988) for intact trout with similar water and plasma Ca^{2+} activities to those employed in the present study. These findings therefore lend considerable weight to the growing consensus that Ca^{2+} uptake is active in freshwater fish and that the MR or 'chloride' cells are the sites of this active uptake on the gills (Payan *et al.* 1981; Perry and Wood, 1985; Isihara and Mugiya, 1987; Perry and Flik, 1988; Flik and Perry, 1989).

The average MR cell density of 139 mm^{-2} on the trout cleithrum skin is similar to values of 90–230 mm^{-2} reported for the opercular epithelium of tilapia in fresh water (Foskett *et al.* 1981; Wendelaar Bonga *et al.* 1990). By way of comparison, the gills of freshwater trout contain about 400 MR cells per square millimetre on the surfaces of the secondary lamellae (Perry and Wood, 1985) and 500–2000 mm^{-2} on the filamental surfaces (Perry and Laurent, 1989; Laurent and Perry, 1990). Total gill surface area (Wood, 1974) is somewhat greater than total body surface area (Webb, 1971) and about 100-fold greater than the cleithrum skin area. In the light of these figures, one would predict that the capacity of the skin

relative to the gills for Ca^{2+} uptake would be very small if uptake were commensurate with the low density of MR cells extrabranchially.

The present data support the view that the gills are not the sole sites of Ca^{2+} uptake, and that significant uptake may also occur through the general body surface, i.e. skin and fins (Mashiko and Jozuka, 1964; Simmons, 1971; Dacke, 1979). By shielding most of the general body surface, Perry and Wood (1985) estimated that unidirectional Ca^{2+} influx through these extrabranchial routes in freshwater trout was about 7 μmol kg^{-1} h^{-1}, or about equal to the unidirectional influx through the gills. A typical 250 g fish would have a cleithrum skin area of about 6 cm^2 (both sides) with a J^{ms} of about 0.08 nmol cm^{-2} h^{-1} (Table 2), yielding an influx of about 2 nmol kg^{-1} h^{-1}. This value is negligible relative to the total extrabranchial influx (Perry and Wood, 1985). However, MR cells have been reported to be widely distributed over the general body surface in other species (e.g. Korte, 1979). Although MR cells were sparse (buccal cavity, lower jaw) or absent (opercular epithelium) on other surfaces of trout, we did not perform an exhaustive search. A 250 g trout has a surface area of about 350 cm^2 (Webb, 1971); if the average J^{ms} over this surface were the same as that across the cleithrum skin it would amount to 0.1 μmol kg^{-1} h^{-1}. If methodological differences can be ignored, the discrepancy suggests either that the *in vitro* preparation does not transport at its normal *in vivo* rate or that other extrabranchial surfaces transport at higher rates.

Sodium and chloride fluxes across the cleithrum skin

The movements of Na^+ and Cl^- could be explained by passive diffusion alone (Table 2). Again this conclusion errs on the side of caution, because Handy (1989) measured Na^+ and Cl^- activities in the skin mucus of rainbow trout which were about fivefold higher than in fresh water of comparable composition to that used here, i.e. the predicted flux ratios were probably *under*estimated (Table 2). Furthermore, there was no relationship between MR cell density on the epithelium and unidirectional Na^+ or Cl^- fluxes, apart from a curious negative correlation with Na^+ J^{sm} (of unknown explanation). Neither general adrenergic stimulation, which is implicated in the control of gill Na^+ and Cl^- transport (reviewed by Wood, 1991), nor cyclic AMP stimulation had any effect on unidirectional Na^+ and Cl^- fluxes across the cleithrum skin. Therefore, the present data provide no support for the view that the chloride cells are the major sites for the active uptake of Na^+ or Cl^- or both (see Introduction). However, they certainly do not disprove these ideas: either the *in vitro* conditions could have been less than optimal (e.g. lack of a key hormone or nutrient) or, as argued above, the MR cells on the cleithrum skin may differ from those on the gills. Indeed, based on ultrastructural evidence (e.g. Pisam *et al.* 1987), there is ample precedent for the occurrence of more than one type of MR cell in freshwater fish. In this regard, it is interesting that a cortisol injection protocol known to increase chloride cell density on the gills (Perry and Wood, 1985; Perry and Laurent, 1989; Laurent and Perry, 1990) had exactly the opposite effect on the cleithrum

epithelium (Table 3). Apparently the populations of MR cells on the gills and skin are regulated differently. Alternatively, MR cells may already have been maximally proliferated in the soft-water holding conditions used in the present study (cf. Perry and Wood, 1985; Perry and Laurent, 1989).

The mechanism of calcium uptake

The current model for Ca^{2+} uptake in freshwater fish is that Ca^{2+} enters the chloride cells from the water by passive diffusion through Ca^{2+}-selective channels on the apical membrane (Flik *et al.* 1985; Perry and Flik, 1988; Lafeber *et al.* 1988). These channels may be regulated by agents such as hypocalcin, which could limit the rate of Ca^{2+} transport. Ca^{2+}-binding proteins within the cell keep intracellular activities extremely low. At the basolateral membrane, Ca^{2+} is actively transported to the extracellular fluid by a high-affinity, calmodulin-dependent ATPase. Some of the present experiments were designed to assess whether this model is applicable to Ca^{2+} transport by the cleithrum skin.

The responses to mucosal lanthanum (10^{-4} mol l^{-1}) were tested, because this and lower concentrations of La^{3+} have been reported to inhibit immediately unidirectional Ca^{2+} influx, apparently by blocking putative apical channels in intact trout and perfused head preparations (Verbost *et al.* 1987, 1989; Perry and Flik, 1988). La^{3+} did not have this effect on the cleithrum skin but instead exerted its well-known general action of displacing bound Ca^{2+} and increasing diffusive permeability (Table 4), effects that have also been observed in intact fish (Eddy and Bath, 1979; Freda and McDonald, 1988). However, the flux ratio criterion was no longer exceeded after addition of lanthanum, suggesting passive distribution of Ca^{2+}. The discrepancy between *in vivo* results and the present data could reflect a real difference in mechanism between cleithrum skin and gill Ca^{2+} transport mechanisms. Alternatively, it may be methodological. *In vivo* measurements have generally relied on the appearance of ^{45}Ca in the whole body. The action of La^{3+} to displace superficially bound Ca^{2+} could reduce apparent whole-body uptake without affecting the true transepithelial Ca^{2+} influx.

We employed the Ca^{2+} ionophore ionomycin (Liu and Hermann, 1978) to test whether apical Ca^{2+} uptake was rate-limiting on J^{ms}. While J^{ms} was stimulated, J^{sm} was also elevated, and there was a large gain of Ca^{2+} by the epithelium based on disappearance of total Ca^{2+} from the mucosal bathing solution (Table 5). These results (and those obtained previously by monitoring disappearance of Ca^{2+} label from fresh water) must be interpreted with caution because positive transepithelial Ca^{2+} balance was not achieved, as measured by J^{net} for Ca^{2+}. It is likely that ionomycin promoted Ca^{2+} entry into both transport and non-transport cells. Ca^{2+} would diffuse into both cell types down the presumed favourable electrochemical gradient, but the ion would only be effectively translocated across the basolateral membranes of the transport cells (and hence contribute to J^{ms}). In the non-transport cells, the Ca^{2+} would instead accumulate intracellularly.

General adrenergic stimulation has been variously reported to stimulate (Payan *et al.* 1981; perfused head), to inhibit (Donald, 1989; isolated gill arches) or to have

no effect (Perry *et al.* 1988; intact animals) on Ca^{2+} uptake in freshwater rainbow trout. The reasons for these discrepancies are unclear. In the cleithrum skin, Ca^{2+} transport was unaffected by adrenaline, by selective α- and β-adrenergic agonists and by cyclic AMP stimulation. The present results are, therefore, in accord with those of Perry *et al.* (1988) and with the current model of Ca^{2+} uptake in which adrenergic regulation is not involved.

In summary, the trout cleithrum skin, mounted *in vitro* under conditions closely duplicating those *in vivo*, offers the first surrogate model for the study of transport function in the freshwater gill epithelium. The present results with this model provide strong evidence for the involvement of MR cells in active Ca^{2+} uptake. Future experiments with this preparation may cast further light on the mechanism of Ca^{2+} uptake and on the role (or lack thereof) of the MR cells in the transport of other electrolytes and acid–base equivalents.

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Note added in proof

In a simultaneous but independent study, McCormick *et al.* (1992) observed Ca^{2+} uptake by the isolated opercular epithelium of the cichlid *Oreochromis mossambicus* adapted to fresh water. As with the trout, the Ca^{2+} uptake seems to be associated with MR cells, inasmuch as proliferation of MR cells in low-calcium fresh water stimulates Ca^{2+} uptake. Circumstantially, the isolated tilapia opercular epithelium also develops the unusual positive transepithelial potential when bathed in fresh water.

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