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EFFECTS OF SEA WATER AND STANNIECTOMY ON BRANCHIAL Ca^{2+} HANDLING AND DRINKING RATE IN EEL (*ANGUILLA ANGUILLA* L.)

A. J. H. VAN DER HEIJDEN*, P. M. VERBOST, M. J. C. BIJVELDS, W. ATSMA, S. E. WENDELAAR BONGA AND G. FLIK

Department of Animal Physiology, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

*e-mail: gertflik@sci.kun.nl

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Summary

We examined the effects of seawater adaptation and extirpation of the Stannius corpuscles on branchial Ca^{2+} flows, gill plasma membrane Ca^{2+} transporters and drinking rate of European eels, *Anguilla anguilla*. Transepithelial Ca^{2+} inflow in the gills increased 2 weeks after transfer of the eels from fresh water to sea water and after stanniectomy. Neither of these treatments changed the membrane density or the affinity of the Ca^{2+}-extrusion mechanisms (Ca^{2+}-ATPase and Na^+/Ca^{2+}-exchanger) in the gill cells, as measured in basolateral plasma membrane vesicles. We conclude that the increase in the Ca^{2+}-transporting capacity observed in the gills of fish exposed to the larger transepithelial Ca^{2+} fluxes, resulting from exposure to sea water or stanniectomy, involves an increase in number and/or size of the Ca^{2+}-transporting cells, but not in the membrane density of Ca^{2+} transporters. Branchial Ca^{2+} outflow was higher in stanniectomised than in sham-operated fish. Changes in electrochemical driving forces as well as plasma stanniocalcin or teleocalcin levels may be the basis for the observed differences. Stanniectomy enhanced drinking in stanniectomised eels. Drinking was not further affected by transfer to sea water. These observations suggest that the corpuscles of Stannius are involved in the water balance.

Key words: stanniocalcin, corpuscle of Stannius, Ca^{2+} transport, drinking rate, eel, *Anguilla anguilla*.

Introduction

The main calcium-handling organs in fish are the gills, kidney and intestine (Flik et al., 1996). In contrast to terrestrial vertebrates, which obtain calcium exclusively from their food, fish extract calcium primarily from the ambient water, an effectively infinite source of ionic calcium (Fenwick, 1989; Flik et al., 1995).

Analysis of the mechanisms involved in transepithelial Ca^{2+} transport showed that transcellular processes dominate branchial Ca^{2+} uptake in diverse fish such as tilapia, eel and trout (Fenwick, 1989). Perry and Flik (1988) proposed a model in which Ca^{2+} enters the ion-transporting cells of the gills (chloride cells) at the apical side, down its electrochemical gradient, *via* a Co^{2+}- and La^{3+}-sensitive channel. Within the cytosol, Ca^{2+} is assumed to bind to specific calcium-binding proteins prior to transportion to the basolateral membrane, where extrusion takes place *via* an ATP-driven Ca^{2+} pump (Ca^{2+}-ATPase) and/or a Na^+ gradient-driven exchanger (Na^+/Ca^{2+}-exchanger). These two transporters have different kinetic characteristics. The Ca^{2+}-ATPase has an affinity for Ca^{2+} which is in the range of cytosolic Ca^{2+} concentrations, but a low Ca^{2+} transport capacity. Conversely, the Na^+/Ca^{2+}-exchanger has a low affinity and high capacity (Flik et al., 1995). Whereas it is likely that the Ca^{2+}-ATPase activity is driven primarily by cytosolic Ca^{2+} levels, the exchanger activity is assumed to be determined by the electrochemical gradients for Na^+ and Ca^{2+} (Reeves, 1985).

Hormonal control of the transcellular Ca^{2+}-transport activity can occur at the apical and basolateral sides of the chloride cells of the gills. Stanniocalcin, the dominant Ca^{2+}-regulating hormone in fish, exerts its antihypercalcemic actions by inhibiting the entry of Ca^{2+} through the apical membrane of chloride cells (Flik et al., 1995), but there are also data suggesting that the regulation of Ca^{2+} transport may be altered by controlling the activity of basolateral extrusion mechanisms. For example, prolonged treatment with either prolactin (in tilapia and eel) or cortisol (in trout) increased the number of Ca^{2+} pumps in the branchial plasma membrane in freshwater fish and also increased Ca^{2+} inflow and the proliferation of chloride cells (Flik et al., 1995).

In this study we have examined the effects of increased transepithelial Ca^{2+} flow on the kinetics of basolateral membrane Ca^{2+} extrusion mechanisms in the gills. For this purpose, the euryhaline eel offers unique opportunities. In fresh water and sea water the fish faces different levels of external Ca^{2+}, and it may be predicted for eels that branchial transcellular flow is enhanced in sea water. This could create a calcium overload and thus may be expected to stimulate antihypercalcemic control. Indeed, the synthesis, release and turnover rate of stanniocalcin is higher in sea water (containing $10\,mmol\,l^{-1}$ Ca) than in eels in fresh water (containing $0.8\,mmol\,l^{-1}$ Ca; Hanssen et al., 1992). Furthermore, eels survive the removal of the corpuscles of Stannius (stanniectomy) for several weeks, although they become lethargic from the resulting hypercalcemia (Hanssen et al., 1989). Thus with our experimental protocol, the effect of a high transepithelial Ca^{2+} flow on the extrusion mechanisms in gills can be studied in the presence (sea water) and absence (stanniectomy) of stanniocalcin as the main inhibitory control mechanism. To assess whether the treatments had an effect on branchial transepithelial Ca^{2+} flow, we measured whole-body Ca^{2+} in- and outflows, using ^{45}Ca-tracer techniques (Flik et al., 1985a). An interesting bonus with this technique is that drinking rates can be measured by assessment of intestinal ^{45}Ca content (Pang et al., 1980). We were especially interested in an effect of stanniectomy on drinking rate, as recent experiments suggest that renal water handling is affected by this treatment (Butler and Alia Cadinouche, 1995).

Materials and methods

Fish maintenance and holding conditions

Sexually immature European eels, *Anguilla anguilla* L., with a body mass of about 100 g were obtained from a local eel farm (Nijvis B.V., Nijmegen) where they were raised at 25 °C. In the laboratory the fish were kept at 20–23 °C, under a 12 h:12 h L:D photoperiod, in running Nijmegen tap water (ionic composition in $mmol\,l^{-1}$: Na, 0.9; Cl, 1.0; K, 0.09; Ca, 0.8) for 2 weeks before transfer of half of the group to full-strength artificial sea water (Na, 502; Cl, 621; K, 11; Ca, 10). Sea water was prepared by dissolving natural sea salt (Wimex) in tap water. The eels were acclimated under these conditions for 4 weeks. Stanniectomy and the sham operation were performed as described previously (Verbost et al., 1993), after anaesthesia in phenoxyethanol (Sigma, $2\,ml\,l^{-1}$). The fish were used 2 weeks after surgery, by which time the wounds were healed and a marked hypercalcemia had developed in the stanniectomised eels (Hanssen et al., 1989). The fish were not fed. Experiments were carried out in fall and winter (from October to February).

Whole body Ca^{2+} flow

Whole-body Ca^{2+} flow was determined as described previously (Flik et al., 1985a; Verbost et al., 1993). Briefly, extra-intestinal Ca^{2+} inflow was determined in individually housed eels by exposure for 3 h to water (at 20 °C) containing ^{45}Ca ($0.87\,MBq\,l^{-1}$ and $2.1\,MBq\,l^{-1}$ for freshwater and seawater fish, respectively). After the exposure, the fish were killed by an overdose of anaesthetic and rinsed in sea water (containing $10\,mmol\,l^{-1}$ Ca) to remove tracer from the integument and branchial chamber. A blood sample was taken from the caudal vessels by puncture with a 23-gauge needle fitted to a heparinised tuberculin syringe. The plasma was used to determine (total) Ca and Na concentrations. Next, the fish were quick-frozen on solid CO_2, so that the intestinal tract with its contents could be removed for separate analysis. Intact frozen urinary bladders with their urine content were also removed for measurement of urine Ca concentration. The tracer content of the fish and the intestinal tract were determined. To this end, the tissues were cooked in a microwave and homogenised in a blender with a known amount of water. Quintuple body pulp samples and triplicate intestinal samples (each weighing 0.5 g) were analysed by liquid scintillation counting (Wallac 1410TM, Pharmacia) after overnight digestion with H_2O_2. Using this method, tracer uptake as a result of drinking can be separated from branchial tracer inflow, because the Ca isotopes swallowed with the water do not leave the gut during the 3-hour period of the experiment (Pang et al., 1980). Branchial Ca^{2+} inflow was calculated on the basis of the total body (without intestine) tracer content after exposure for 3 h to the water. The specific ^{45}Ca activity of the water, which was monitored by analysis of duplicate 0.2 ml water samples taken every 60 min, did not change measurably during the experiment. The volume of water consumed was calculated from the specific ^{45}Ca activity of the water and of the gut tissue and contents.

Branchial Ca^{2+} outflow was determined on the basis of rate of appearance of ^{45}Ca in the water of fish injected with tracer (2.5 MBq and 3.3 MBq in freshwater and seawater fish, respectively) 24 h before the start of the experiment, and the plasma tracer specific activity at the end of the experiment. Release of the tracer to the water was monitored for 4 h by measuring duplicate 0.2 ml water samples at 30 min intervals. This approach allows the detection of burst release of tracer as a result of sporadic urinary and/or faecal excretions. Outflow was calculated on the basis of the steady, slow release of tracer only, and therefore reflects integumental (i.e. branchial) outflow (Flik et al., 1985a). The eels were quick-frozen for dissection of the urinary bladder for determination of urine calcium concentration. Branchial Ca^{2+} flow was expressed as $\mu mol\,h^{-1}$ per 100 g fish. Drinking rate was expressed as $\mu l\,h^{-1}$ per 100 g fish.

Plasma and urine total calcium concentrations were determined with a commercial colorimetric calcium kit (Sigma). Combined calcium/phosphate standards (Sigma) were used as a reference. Plasma sodium was determined by flame photometry on 250-fold diluted samples.

Membrane isolation and characterisation

After anaesthesia a blood sample was taken to check the success of the operation, by measuring the plasma Ca concentration, which is elevated above $3.5\,mmol\,l^{-1}$ in stanniectomised eels. Next, the eels were perfused with 40 ml heparin-containing ($20\,i.u.\,ml^{-1}$) saline to clear the gills of blood cells. The branchial tissues of three eels were pooled at the beginning of the isolation procedure. Isolation of basolateral plasma membrane vesicles of the branchial epithelium was performed by a differential centrifugation procedure as described previously (Flik et al., 1985b). Before the last centrifugation step, the preparation was split to obtain two pellets: one for resuspension in assay medium containing

NaCl (for determination of Na^+/K^+-ATPase activity, membrane orientation and Na^+/Ca^{2+}-exchange activity) and one for suspension in assay medium with KCl (for determination of Ca^{2+}-ATPase activity). Protein concentration was determined with a commercial reagent kit, using bovine serum albumin as a reference (Biorad).

Na^+/K^+-ATPase was used as a marker enzyme for basolateral plasma membranes. Na^+/K^+-ATPase specific activity and total activity of homogenates and purified membrane preparations were determined as described previously (Flik et al., 1985b). Na^+/K^+-ATPase activity was defined as the Na^+- and K^+-dependent, ouabain-sensitive adenosine triphosphate hydrolase activity. Vesicle resealing was estimated from differences in Na^+/K^+-ATPase activity of preparations treated with detergent ($0.2\,mg\,ml^{-1}$ saponin; 10 min, 25 °C), compared to the activity of unpermeabilised preparations. Saponin exposes enzyme activity of resealed vesicles. Determination of membrane orientation was based on the specific trypsin sensitivity of the cytosol-oriented part of the Na^+/K^+-ATPase. The percentage of rightside-out oriented vesicles (ROV) was determined after trypsin treatment of the preparations, which destroys the Na^+/K^+-ATPase activity of leaky and inside-out vesicles (IOV), but not of ROV. After the trypsin was inhibited with trypsin-inhibitor, the Na^+/K^+-ATPase activity in the ROVs was exposed by saponin treatment.

Ca^{2+} transport in vesicles

We assayed transport of Ca^{2+} by means of a rapid filtration technique (Heeswijk et al., 1984) adapted for fish gill preparations (Flik et al., 1985b), using assay media in which Ca^{2+} and Mg^{2+} concentrations were carefully buffered (Schoenmakers et al., 1992). In short, the Ca^{2+} dependence of the ATP-dependent Ca^{2+} transporter (Ca^{2+}-ATPase) was assessed by incubating 10–20 µg of vesicle protein with media containing calculated Ca^{2+} concentrations in a range between 0.05 and $2\,\mu g\,ml^{-1}$, in the presence or absence of ATP. The assay time was 30 s, to obtain initial velocity of the transport process. Oligomycin-B ($1\,\mu g\,ml^{-1}$) and thapsigargin ($1\,\mu g\,ml^{-1}$) were added to the incubation media to inhibit possible Ca^{2+} pumping activities of contaminating mitochondria and membrane fragments from endoplasmatic reticulum, respectively. To determine the Ca^{2+}-dependence of Na^+ gradient-dependent Ca^{2+} transport (Na^+/Ca^{2+}-exchanger), vesicles loaded with a NaCl-containing medium were exposed for 5 s to media with Ca^{2+} concentrations ranging between 0.05 and $5\,\mu g\,ml^{-1}$ in the absence or presence of a Na^+ gradient. All incubations were carried out at 37 °C for optimum enzyme activity.

The Ca^{2+} concentration dependence of Ca^{2+}-ATPase and Na^+/Ca^{2+}-exchanger activity obeyed simple Michaelis-Menten kinetics. Kinetic parameters (V_{max} and $K_{0.5}$) were derived by non-linear regression analysis of the individual experiments by use of the Enzfitter program (Leatherbarrow, 1987).

Statistics

A two-way analysis of variance (ANOVA) with acclimation water and operation as factors was employed to assess significant effects of the treatments. Differences among mean values were subsequently assessed using a Bonferroni post-hoc test for multiple comparisons. Prior to ANOVA, the data were tested for homogeneity of variance with the Levene's test. If the data did not meet this assumption of parametric statistics, they were transformed (log or square-root) and then rechecked to confirm that the assumption was justified. All data were analysed with the SPSS program and significance was accepted at $P<0.05$.

Results

Ca^{2+} flows, urinary calcium concentrations and drinking rate

2 weeks after surgery, stanniectomised eels were hypercalcemic (Table 1); this was more prominent in freshwater than in seawater fish. Additionally, freshwater-adapted stanniectomised eels were hyponatremic (Table 1).

The inflow of Ca^{2+} across the gills was significantly higher in stanniectomised eels than in sham-operated eels, and was higher in fish kept in sea water than in those kept in fresh water (Fig. 1). Stanniectomy enhanced Ca^{2+} outflow significantly, but there was no significant effect of water salinity on branchial outflow. The calculated net flow of Ca^{2+} across the gills (the difference between means for inflow and outflow) was directed inwards in all groups examined. This net branchial inflow was tenfold higher in sea water ($1.93\,\mu mol\,h^{-1}\,100\,g^{-1}$), than in freshwater sham-operated eels ($0.19\,\mu mol\,h^{-1}\,100\,g^{-1}$). The net inflow increased after stanniectomy to $3.00\,\mu mol\,h^{-1}\,100\,g^{-1}$ in fresh water, and to $5.55\,\mu mol\,h^{-1}\,100\,g^{-1}$ in sea water.

Urinary calcium concentrations (Table 1) were higher in seawater than in freshwater controls. After stanniectomy, urinary calcium concentration increased in freshwater fish. The limited data from seawater fish indicate that in our own fish urinary calcium concentration was unchanged by the operation.

Drinking rates were highly variable within groups (see Fig. 2). There was no effect of water salinity on drinking rate, whereas stanniectomy enhanced water consumption from

Table 1. *Effects of stanniectomy or sham operation on plasma Na and plasma and urinary Ca concentrations of eels adapted to fresh water and sea water*

Treatment	Plasma [Na]	Plasma [Ca]	Urine [Ca]
Fresh water			
SHAM	150.1±9.4 (12)	1.72±0.28 (12)	0.63±0.42 (8)
STX	121.4±11.7 (9)‡	7.54±1.83 (9)‡	4.22±1.24 (7)‡
Sea water			
SHAM	152.5±9.0 (11)	2.16±0.43 (11)	3.81 and 6.85
STX	147.1±6.0 (12)*	5.70±1.22 (13)*,‡	4.23 and 7.14

STX, stanniectomy; SHAM, sham operation.

Concentrations in $mmol\,l^{-1}$ (means ± S.D.). Numbers of fish examined in parentheses (there were two observations for urine samples from seawater fish).

‡Significantly different from sham-operated controls in the same medium ($P<0.05$); *significantly different from the freshwater group ($P<0.05$).

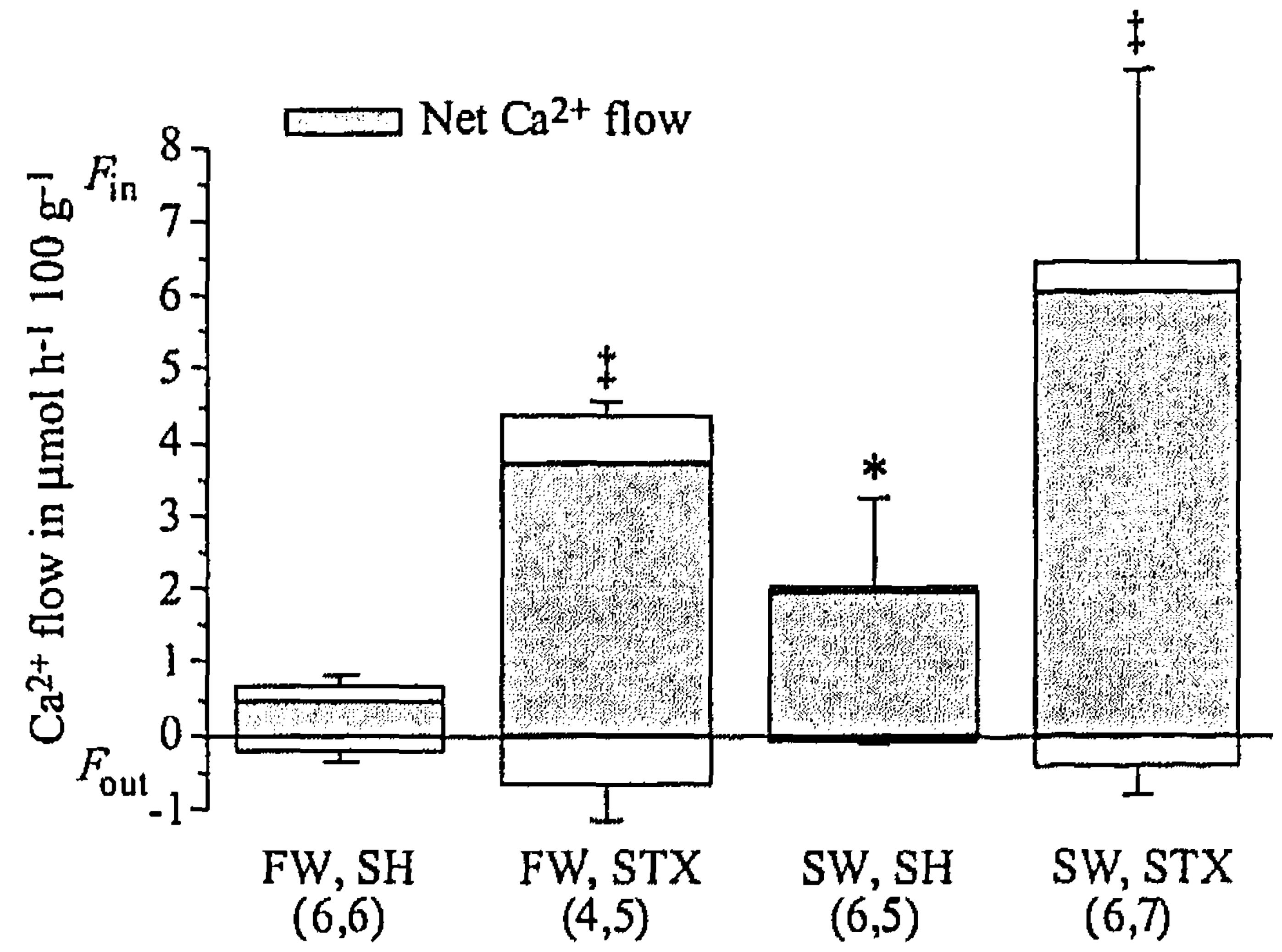

Fig. 1. Effects of stanniectomy on branchial Ca^{2+} inflow (F_{in}) and Ca^{2+} outflow (F_{out}) in eel. Calcium flows were determined with ^{45}Ca-tracer techniques in sham-operated (SH) or stanniectomised (STX) eels adapted to fresh water (FW) or to sea water (SW). Values are means ± S.D. Numbers of fish examined are indicated in parentheses (inflow, outflow). ‡Significantly different Ca^{2+} inflow from the sham-operated group in the same medium; *significantly different Ca^{2+} inflow from the freshwater group. As there were no significant effects of salinity on Ca^{2+} outflow, the data for freshwater and seawater eels were pooled for statistical analysis. Stanniectomy yielded a 3.5-fold stimulation of Ca^{2+} outflow.

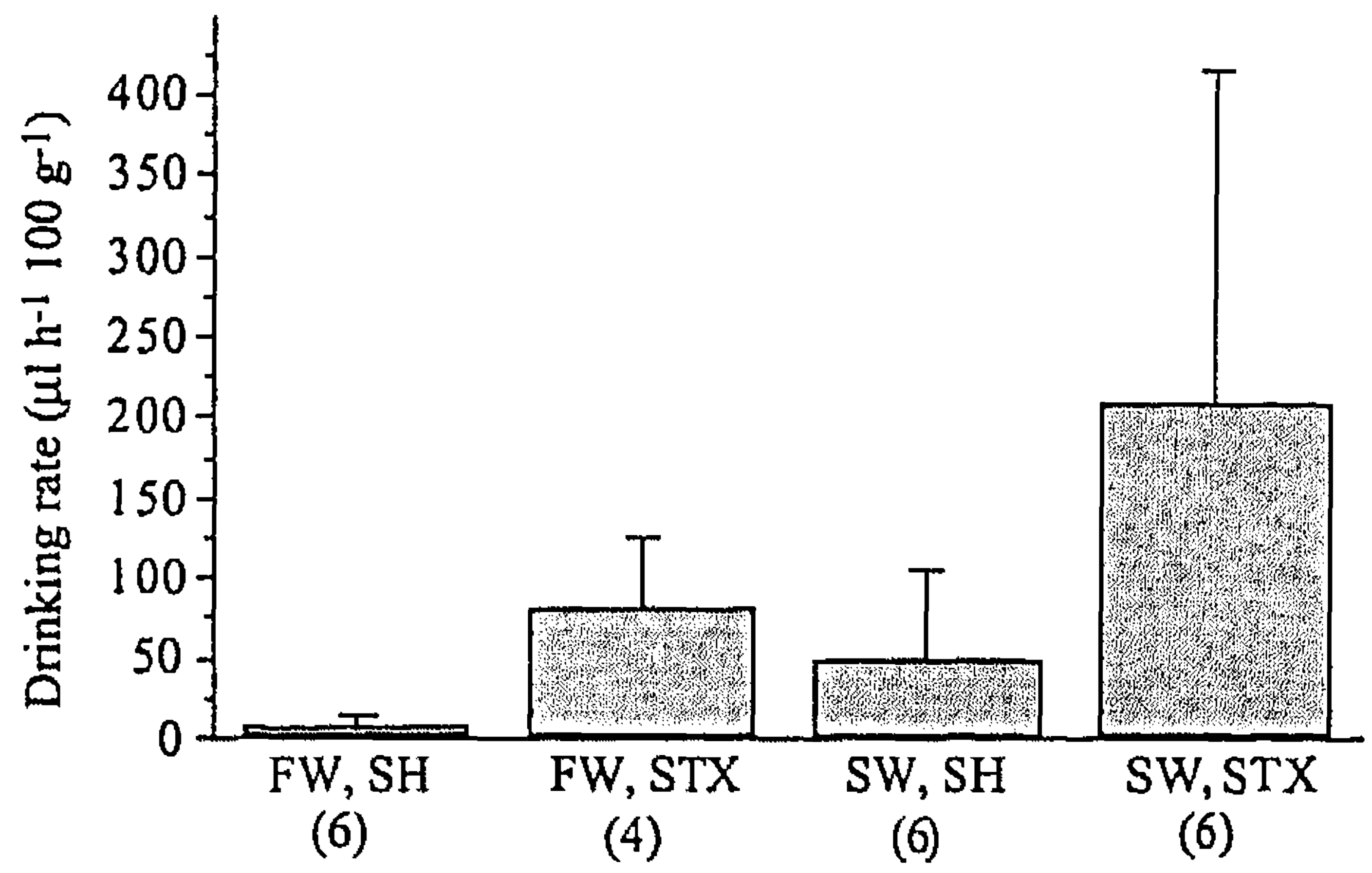

Fig. 2. Drinking rates (µl h^{-1} 100 g^{-1}) of sham-operated (SH) and stanniectomised (STX) eels, adapted to fresh water (FW) or to sea water (SW) at 20 °C. Drinking rates were calculated from intestinal ^{45}Ca content after a 3 h inflow period. Values are means ± S.D. Numbers of fish examined are in parentheses. There was no significant effect of salinity on drinking rate, and stanniectomy stimulated drinking fivefold (data from freshwater and seawater eels pooled).

30.4±12.7 µl h^{-1} 100 g^{-1} to 159±53.0 µl h^{-1} 100 g^{-1} (freshwater and seawater fish pooled, means ± S.D.).

Membrane isolation and orientation

The protein recovery of vesicle preparations was similar for sham-operated and stanniectomised fish, but there was a significantly higher recovery in seawater preparations (Table 2). The higher specific activity of the basolateral membrane marker, Na^+/K^+-ATPase, in the final preparations, as compared to the specific activity in homogenates (Table 2), was taken to indicate that the isolation procedure yielded an enriched basolateral membrane vesicle preparation. There were no differences in enrichment and recovery of Na^+/K^+-ATPase activity (Table 2) and membrane orientation between any of the groups. The plasma membrane orientation of the preparations consisted of 62±10 % leaky vesicles, 23±8 % rightside-out oriented vesicles and 15±8 % inside-out oriented vesicles

Table 2. *Protein recovery and recovery and enrichment of the basolateral membrane marker Na^+/K^+-ATPase in branchial plasma membrane vesicle preparations*

	Fresh water		Sea water	
	SHAM	STX	SHAM	STX
aProtein recovery (*N*)	1.9±0.3 (6)	1.8±0.3 (6)	2.7±0.7 (7)*	2.9±0.5 (7)
Na^+/K^+-ATPase (*N*)	(6)	(6)	(7)	(7)
Homogenates				
bV_{spec}	5.8±3.2	3.5±2.1	7.7±4.5	11.7±6.0*
cV_{total}	169±73.5	110±68.8	356±188*	615±319*
BLMV V_{spec}	43.1±29.5	31.6±20.9	36.6±18.1	58.0±33.5
dRecovery	14.2±8.9	16.3±7.0	16.2±11.4	15.8±9.6
ePurification	7.9±6.1	9.3±4.0	5.6±2.8	5.4±2.8

SHAM, sham operation; STX, stanniectomy.

Values are means ± S.D.

aProtein recovery was calculated as the percentage of total protein in the final preparation of basolateral membrane vesicles (BLMV), relative to that in the initial homogenate.

bV_{spec} is the specific Na^+/K^+-ATPase activity in µmol P_i h^{-1} mg^{-1}; cV_{total} is the total Na^+/K^+-ATPase activity in µmol h^{-1} (V_{spec}×total mg protein).

dNa^+/K^+-ATPase recovery was calculated as the percentage of total activity in the plasma membrane fraction, relative to that in the initial homogenate.

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

Numbers of observations are indicated in parentheses; *significantly different from freshwater fish after the same operation (P<0.05).

Table 3. *Effects of stanniectomy on active calcium transport in basolateral plasma membrane vesicles of branchial epithelium from eels adapted to fresh water or sea water*

	Fresh water		Sea water	
	SHAM	STX	SHAM	STX
Ca^{2+}-ATPase (*N*)	(4)	(4)	(6)	(7)
V_{max}	2.06±1.46	2.07±1.00	3.10±1.40	3.77±1.84
$K_{0.5}$	102±61	114±37	150±91	144±57
Na^+/Ca^{2+}-exchanger (*N*)	(3)	(5)	(4)	(4)
V_{max}	5.08±1.00	3.35±1.35	3.24±1.75	5.66±1.76
$K_{0.5}$	0.91±0.19	0.71±0.67	0.41±0.35	0.75±0.44

SHAM, sham operation; STX, stanniectomy.

Maximum velocities and affinity values were derived from kinetic analysis of $^{45}Ca^{2+}$-transport driven by ATP (Ca^{2+}-ATPase) or a Na^+ gradient (Na^+/Ca^{2+}-exchanger) into vesicles.

There were no significant differences between groups.

Values are means ± S.D.; *N*, number of preparations examined (three fish per preparation); V_{max}, maximum velocity in $nmol\,min^{-1}\,mg\,protein^{-1}$; $K_{0.5}$, Ca^{2+} affinity in $nmol\,l^{-1}$ for Ca^{2+}-ATPase and in $\mu mol\,l^{-1}$ for Na^+/Ca^{2+}-exchanger.

(means ± S.D. of 19 observations). Because the purification and membrane orientation of all membrane preparations were similar, comparison of Ca^{2+} kinetics of basolateral plasma membrane Ca^{2+} transporters needed no further corrections.

Specific Na^+/K^+-ATPase activity in the purified preparations was not different between groups (Table 2). The calculated total Na^+/K^+-ATPase activity for the whole gill (V_{total}=V_{spec}×total protein; Table 2) was higher in seawater than in freshwater fish, but there was no significant effect of stanniectomy.

Effects of stanniectomy on ATP- and Na^+-dependent Ca^{2+} transport

The kinetics of the Ca^{2+}-ATPase and Na^+/Ca^{2+}-exchanger in basolateral plasma membrane vesicles of the gills of freshwater- and seawater-adapted, sham-operated or stanniectomised eels are presented in Table 3. There were no significant effects of the treatments on either the capacity or the affinity of the Ca^{2+} transporters in the gills.

Discussion

Three major conclusions can be drawn from these experiments. First, the increased branchial Ca^{2+} inflow in seawater-adapted or stanniectomised eels did not change the affinity or maximum velocity of basolateral plasma membrane Ca^{2+}-ATPase and Na^+/Ca^{2+}-exchanger in the gills. Second, branchial Ca^{2+} outflow increased after stanniectomy. Third, drinking was stimulated by stanniectomy.

Branchial Ca^{2+} uptake

Seawater transfer and stanniectomy effectively induced alterations in transepithelial Ca^{2+} transport; seawater transfer caused a twofold increase in Ca^{2+} inflow, and stanniectomy enhanced the inflow three- to eightfold in seawater and freshwater eels, respectively.

Ca^{2+} inflow across fish gills is a transcellular and thus active process, as was substantiated by the deviation between the flux ratio measured and the ratio predicted on the basis of the transepithelial potential (TEP) and the Nernst equilibrium potential (Perry and Flik, 1988). This situation holds for freshwater as well as seawater fish (Perry and Flik, 1988; Flik et al., 1996) and for stanniectomised fish (Verbost et al., 1993). The chloride cell in the branchial epithelium is the mediator of the transcellular Ca^{2+} transport: extrapolation of the positive correlation between chloride cell density and Ca^{2+} inflow in the opercular membrane has suggested that this membrane becomes impermeable to Ca^{2+} in the absence of chloride cells (McCormick et al., 1992). As branchial Ca^{2+} inflow passes through the chloride cell, it may be predicted that a large increase in the inflow (as observed in the present experiment) requires adaptation in the quality and/or quantity of the Ca^{2+}-extrusion mechanisms of these cells. In previous experiments, a sixfold increase in branchial Ca^{2+} flow in stanniectomised freshwater eels did not affect the kinetic characteristics of the ATP-driven Ca^{2+} pump (Verbost et al., 1993). This left the Na^+/Ca^{2+}-exchanger as a possible target for stanniocalcin, especially in sea water where the gills may face a 15-fold higher Ca^{2+} inflow than in fresh water. Nevertheless, our study shows that affinity and density of neither of the two branchial Ca^{2+} transporters had altered after seawater transfer or after stanniectomy. As the affinity of the transporters was unchanged we conclude that the quality of the transporters was not affected by enhanced Ca^{2+} flows. However, the maximum transport rate in vitro (which was unaltered) reflects transporter density per unit of membrane, and does not give information on the total amount of transporter available to individual cells or the gills as a whole. It may be that Ca^{2+} transport capacity is enhanced by an expansion of the tubular system (which may be expressed in a larger cell size) or by an increase in cell number, while transporter density remains the same. Although we did not quantify chloride cell number and size in our present experiment, it is known from the literature that seawater transfer and also stanniectomy enhance both chloride cell size and number (Shirai and Utida, 1970; Chartier et al., 1977).

Thus it seems that increased branchial Ca^{2+} flows do indeed require a larger amount of Ca^{2+} extrusion mechanisms and that this is achieved by an increase in chloride cell number and/or size. Furthermore, we conclude that the expression of the Ca^{2+} transporters is not directly linked to stanniocalcin, as both an enhanced turnover rate of the hormone (seawater *versus* freshwater; Hanssen et al., 1992), as well as decreased amounts of the hormone (stanniectomised *versus* sham-operated eels) results in the presence of more Ca-extrusion mechanisms.

Branchial Ca^{2+} outflow

Ca^{2+} outflow *via* the gills is a passive paracellular process driven by the outwardly directed electrochemical force and depends on the permeability of the junctional complex to Ca^{2+} (Perry and Flik, 1988). Changes in Ca^{2+} outflow may therefore be explained by a change in magnitude of electrochemical force. Unfortunately, we did not measure the transepithelial potential (TEP) in our eels; thus we cannot calculate the electrochemical driving force for Ca^{2+} (which is the difference between the TEP and the calculated Nernst equilibrium potential for Ca^{2+}). However, at least for the freshwater situation, the predicted electrochemical force is larger in stanniectomised than in sham-operated eels, and thus correlates with a larger outflow (Verbost et al., 1993). Alternatively or additionally, the increased Ca^{2+} outflow after stanniectomy could result from increased junctional permeability for Ca^{2+}. For fish, little is known about the regulation of junctional permeability. It is likely though, that the permeability of junctions is influenced by cellular signalling mechanisms, as in mammals (Anderson and van Itallie, 1995). For example, in goldfish intestine, an increase in cyclic AMP level in the cytoplasm increases permeability of junctions to Cl^- (Bakker and Groot, 1989). Interestingly, two products of the corpuscles of Stannius, stanniocalcin and teleocalcin (Ma and Copp, 1978), activate different messenger systems in isolated gill cells: stanniocalcin activates the inositol phosphate cycle, whereas teleocalcin reduces adenylate cyclase activity and cyclic AMP production (Verbost et al., 1996). Whether the permeability of the branchial junctional complex for Ca^{2+} is regulated through cyclic AMP (pointing to a possible teleocalcin-controlled mechanism) awaits further experimentation.

Drinking rates

The average drinking rate of our freshwater- and seawater-adapted eels was in the same range as those determined by others (Perrot et al., 1992; Tierney et al., 1995), who used ^{51}Cr-EDTA and ^{125}I-polyvinyl pyrrolidone for determination of drinking rate in the same species. However, we did not observe a significant stimulation of drinking after seawater transfer, probably because of the large variance of our data. Such stimulation of drinking in sea water is generally accepted to be necessary to replace the water lost by dehydration of the body surface.

Drinking in eel is regulated by two hormonal systems, the renin-angiotensin system (RAS) that stimulates drinking activity, and the natriuretic peptides that work antagonistically to the RAS (Perrot et al., 1992; Tierney et al., 1995; Takei and Balment, 1993). These systems react to changes in blood pressure and/or volume and ionic concentrations. The stimulation of drinking after stanniectomy suggests an interaction of one of the products of the corpuscles of Stannius, stanniocalcin, teleocalcin or perhaps a cardio/vasoactive factor (Chester Jones et al., 1966; Butler and Oudit, 1995), with these hormonal systems. It seems unlikely to us that stanniocalcin is interacting with drinking behaviour since injection of fish with a stanniocalcin antibody did not alter drinking in *Sparus aurata* (P. Guerreiro and G. Flik, unpublished). As stanniectomised eels suffer from hypotension (Butler and Oudit, 1995) and hypotensive agents (via angiotensin II; Perrot et al., 1992; Tierney et al., 1995) stimulate drinking *via* activation of the RAS, a role for a cardio/vasoactive factor may be more probable. Interestingly, stanniectomy affects renal water balance in the eel: in stanniectomised eels renal water loss was reduced by enhanced tubular reabsorption (Butler and Alia Cadinouche, 1995). Clearly, more work is needed to characterise the possible cardio/vasoactive factor of the corpuscles of Stannius, and the mechanisms by which it seems to interact with water balance.

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