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


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Accepted 2 July; published on WWW 25 August 1999

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 transepithelial 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 transport to the basolateral membrane, where extrusion takes place via an ATP-driven Ca\(^{2+}\) pump (Ca\(^{2+}\)-ATPase) and/or a 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 transepithelial 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 transepithelial 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 Ca²⁺ 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 Ca²⁺ flow, we measured whole-body Ca²⁺ in- and outflows, using ⁴⁵Ca-tracer techniques (Flik et al., 1985a). An interesting bonus with this technique is that drinking rates can be measured by assessment of intestinal ⁴⁵Ca 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 stannsectomised eels (Hanssen et al., 1989). The fish were not fed. Experiments were carried out in fall and winter (from October to February).

Whole body Ca²⁺ flow

Whole-body Ca²⁺ flow was determined as described previously (Flik et al., 1985a; Verbost et al., 1993). Briefly, extra-intestinal Ca²⁺ inflow was determined in individually housed eels by exposure for 3 h to water (at 20°C) containing ⁴⁵Ca (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 CO₂, 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 H₂O₂. 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 Ca²⁺ inflow was calculated on the basis of the total body (without intestine) tracer content after exposure for 3 h to the water. The specific ⁴⁵Ca 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 ⁴⁵Ca activity of the water and of the gut tissue and contents.

Branchial Ca²⁺ outflow was determined on the basis of rate of appearance of ⁴⁵Ca 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 Ca²⁺ flow was expressed as μmol h⁻¹ per 100 g fish. Drinking rate was expressed as μl 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 stannectomised 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 hydrolysis 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 membrane preparations was determined after trypsin treatment of the preparations, which destroys the Na\(^+\)/K\(^+\)-ATPase activity in the ROVs (R.O.V.) was determined after trypsin treatment of the 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 assayed by incubating 10–20 jxg of vesicle protein with media containing calculated Ca\(^2+\) concentrations in a range between 0.05 and 2 μg 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 μg ml\(^{-1}\)) and thapsigargin (1 μg 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 μg ml\(^{-1}\), in the presence or absence 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\(_{\text{max}}\) and K\(_{\text{m}}\)) 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, stanninectomised eels were hypercalcemic (Table 1); this was more prominent in freshwater than in seawater fish. Additionally, freshwater-adapted stanninectomised eels were hypernatremic (Table 1).

The inflow of Ca\(^2+\) across the gills was significantly higher in stanninectomised eels than in sham-operated eels, and was higher in fish kept in sea water than in those kept in fresh water (Fig. 1). Stanninectomy 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 μmol h\(^{-1}\) 100 g\(^{-1}\)), than in freshwater sham-operated eels (0.19 μmol h\(^{-1}\) 100 g\(^{-1}\)). The net inflow increased after stanninectomy to 3.00 μmol h\(^{-1}\) 100 g\(^{-1}\) in fresh water, and to 5.55 μmol h\(^{-1}\) 100 g\(^{-1}\) in sea water.

Urinary calcium concentrations (Table 1) were higher in seawater than in freshwater controls. After stanninectomy, 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 stanninectomy enhanced water consumption from

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma [Na]</th>
<th>Plasma [Ca]</th>
<th>Urine [Ca]</th>
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<tbody>
<tr>
<td>Fresh water</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SHAM</td>
<td>150.1±9.4 (12)</td>
<td>1.72±0.28 (12)</td>
<td>0.6±0.42 (8)</td>
</tr>
<tr>
<td>STX</td>
<td>121.4±11.7 (9)†</td>
<td>7.5±1.83 (9)†</td>
<td>4.22±1.24 (7)†</td>
</tr>
<tr>
<td>Sea water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>152.5±9.0 (11)</td>
<td>2.16±0.43 (11)</td>
<td>3.81 and 6.85</td>
</tr>
<tr>
<td>STX</td>
<td>147.1±6.0 (12)*</td>
<td>5.70±1.22 (13)*</td>
<td>4.23 and 7.14</td>
</tr>
</tbody>
</table>

STX, stanninectomy; 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.

30.4±12.7 \(\mu l h^{-1} 100 g^{-1}\) to 159±53.0 \(\mu l h^{-1} 100 g^{-1}\) (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\% right-side-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

<table>
<thead>
<tr>
<th></th>
<th>Fresh water</th>
<th>Sea water</th>
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<tbody>
<tr>
<td></td>
<td>SHAM</td>
<td>STX</td>
</tr>
<tr>
<td>Protein recovery (N)</td>
<td>1.9±0.3 (6)</td>
<td>1.8±0.3 (6)</td>
</tr>
<tr>
<td>Homogenates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\psi_{spec})</td>
<td>5.8±3.2</td>
<td>3.5±2.1</td>
</tr>
<tr>
<td>(\psi_{total})</td>
<td>169±73.5</td>
<td>110±68.8</td>
</tr>
<tr>
<td>BLMV (\psi_{spec})</td>
<td>43.1±29.5</td>
<td>31.6±20.9</td>
</tr>
<tr>
<td>Recovery</td>
<td>14.2±8.9</td>
<td>16.3±7.0</td>
</tr>
<tr>
<td>Purification</td>
<td>7.9±6.1</td>
<td>9.3±4.0</td>
</tr>
</tbody>
</table>

SHAM, sham operation; STX, stanniectomy.
Values are means ± S.D.

\(\psi_{spec}\) is the specific Na\(^+/K^+\)-ATPase activity in \(\mu mol \ h^{-1} mg^{-1}\); \(\psi_{total}\) is the total Na\(^+/K^+\)-ATPase activity in \(\mu mol \ h^{-1}\) (\(\psi_{spec}\times total \ mg \ protein\)).

Na\(^+/K^+\)-ATPase recovery was calculated as the percentage of total activity in the plasma membrane fraction, relative to that 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

<table>
<thead>
<tr>
<th></th>
<th>Fresh water</th>
<th>Sea water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SHAM</td>
<td>STX</td>
</tr>
<tr>
<td><strong>Ca^{2+}-ATPase (N)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>$K_{0.5}$</td>
<td>2.06±1.46</td>
<td>2.07±1.00</td>
</tr>
<tr>
<td><strong>Na^{+}/Ca^{2+}-exchanger (N)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>(3)</td>
<td>(5)</td>
</tr>
<tr>
<td>$K_{0.5}$</td>
<td>0.91±0.19</td>
<td>0.71±0.67</td>
</tr>
</tbody>
</table>

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.

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 nmol 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 transepithelial 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 Ca²⁺ flows do indeed require a larger amount of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ outflow**

Ca²⁺ 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 Ca²⁺ (Perry and Flik, 1988). Changes in Ca²⁺ 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 Ca²⁺ (which is the difference between the TEP and the calculated Nernst equilibrium potential for Ca²⁺). 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 Ca²⁺ outflow after stanniectomy could result from increased junctional permeability for Ca²⁺. 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, R. and Groot, J. A* (1989). Further evidence for the molecular basis for regulation of paracellular permeability. Am. J. Physiol. 269, G467-G475.

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Ca\textsuperscript{2+} handling and drinking rate in eel 2511


