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Otolith growth in trout *Oncorhynchus mykiss*: supply of Ca^{2+} and Sr^{2+} to the saccular endolymph

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Summary

Kinetic and pharmacological characteristics of Ca^{2+} fluxes across the saccular epithelium of trout were studied using a perfused isolated inner ear. $^{45}Ca^{2+}$ influx from the Ringer solution to the endolymph was 3–4 nmoles $h^{-1}\mu l^{-1}$ endolymph, which corresponds to a global turnover rate of the endolymph calcium of 200 % h^{-1}. Ca^{2+} entry into the proximal endolymph was faster than into the distal fluid. Net Ca^{2+} movement across the saccular epithelium depended on the direction and intensity of the chemical gradient of calcium between the Ringer solution and the endolymph. Increasing the calcium concentration in the Ringer solution up to 4.4 mmol l^{-1} provoked an accumulation of Ca^{2+} in both proximal and distal endolymphs, and equilibrium was reached about 30 min after the beginning of perfusion. Perfusion with calcium-free Ringer partially emptied the proximal compartment of calcium, whereas the calcium levels in the distal endolymph did not vary during 70 min of perfusion. Verapamil (10^{-5} mol l^{-1}) and cyanide (CN, 10^{-3} mol l^{-1}) did not modify the accumulation of Ca^{2+} within the endolymph in the presence of a favourable calcium chemical gradient. Furthermore the relationship between Ca^{2+} net fluxes and the chemical calcium gradient across the saccular epithelium was linear, indicating a passive diffusional mechanism *via* a paracellular pathway. Similar relationships were found for Sr^{2+} fluxes across the saccular epithelium in the presence of positive chemical gradients (1, 2 and 4 mmol l^{-1} Sr^{2+}). *In vivo* experiments in which trout were intraperitoneously injected with $CaCl_2$ solution confirmed the tight relationship between the calcium levels in plasma and endolymph (both proximal and distal). Sampling proximal and distal endolymphs in trout and turbot saccules revealed a decreasing proximo–distal calcium gradient in endolymph of both fish species. The present results strongly suggest that the endolymph is supplied with Ca^{2+} and Sr^{2+} *via* a paracellular pathway located in the proximal area of the saccular epithelium.

Key words: trout, *Oncorhynchus mykiss*, calcium, flux, endolymph, otolith, perfusion, inner ear.

Introduction

Otoliths are paired calcified structures used for the maintenance of equilibrium and hearing in all teleost fishes. They are composed of more than 99 % $CaCO_3$ (Degens et al., 1969; Borelli et al., 2001) in the form of aragonite, which is deposited daily onto an organic matrix (Panella, 1980). They are generally considered to be biological archives, and as such are routinely used for age and growth estimations, stock discrimination of exploited fish populations, and characterization of events in the fish's life history (Campana, 1999; Threscher, 1999). Stock discrimination is based on the assumption that differences in physical and chemical environments will be registered by differences in otolith chemical composition, and numerous studies have focused on strontium as a marker of environmental temperature and salinity (Campana, 1999).

The pathway of ions from the environment to the otolith is a multi-stage process involving successive barriers and compartments, e.g. gill/intestine epithelia – blood – saccular epithelium – endolymph – otolith. Unlike most calcifying systems, e.g. vertebrate bones, enamel, mollusc shells and coral skeletons, otolith mineralization takes place in an acellular medium, the endolymph, which contains all the precursors for otolith formation. The endolymph is secreted by the saccular epithelium, which is composed of many cell types arranged in two zones relative to the position of the otolith. (1) A proximal zone bathing the proximal face of the otolith and

composed of the macula (sensory cells, supporting cells and secretory cells) and a crown of large ionocytes (mitochondria-rich cells) arranged in a meshwork around the macula. (2) On the opposite side, a distal zone bathing the distal side of the otolith is composed of squamous cells and small ionocytes. Between these two zones there is a transitional epithelium (Mayer-Gostan et al., 1997; Pisam et al., 1998; Takagi, 1997).

Compared with plasma, the endolymph fluid is characterized by a higher concentration of K^+ and total CO_2 corresponding to an alkaline pH, whereas the calcium level is rather low (Mugiya and Takahashi, 1985; Payan et al., 1997, 1999). Payan et al. (1999) reported the presence of proximo–distal (P–D) gradients of proteins, K^+ and total CO_2 within the endolymph of trout and turbot and proposed that this lack of chemical uniformity is involved in the otolith calcification process. Although many studies have been done on the composition of the endolymph (see review by Campana, 1999) there is little knowledge of the mechanisms of transport across the saccular epithelium for the ionic precursors of otolith formation, i.e. Ca^{2+} and HCO_3^-. Most of the studies on the transport of Ca^{2+} were carried out by Mugiya and coworkers, who used an isolated preparation of the otolith-containing sacculus from trout (Mugiya, 1984) and concluded that Ca^{2+} was transported by a transcellular route (Mugiya and Yoshida, 1995; Toshe and Mugiya, 2000). Few studies have dealt with the analysis of the concentrations of strontium in the endolymph (see review by Campana, 1999) and no published data concerning Sr^{2+} movement across the saccular epithelium are available.

In the present study, the kinetic parameters of Ca^{2+} and Sr^{2+} transport across the saccular epithelium of trout were studied on the inner ear using a perfusion technique. This original approach avoids the difficulties of an *in vivo* study and improves the isolated saccule technique by maintaining a better perfusion of the saccular epithelium and allowing for endolymph sampling at different places inside the saccule. The relationships between blood and endolymph calcium levels were investigated in particular.

Materials and methods

Biological material

The experiments were carried out on 13-month-old trout *Oncorhynchus mykiss* Walbaum reared in running tap water under a constant photoperiod of 10 h:14 h light:dark and fed daily.

In vivo *experiments*

Hypercalcemia was induced in nine trout by injection of 100 µl of 0.5 mol l^{-1} $CaCl_2$ solution into the intraperitoneal cavity. Successive blood and endolymph samples were then taken and the calcium and protein content analyzed (see above).

Collection of plasma and endolymph

The techniques of plasma and endolymph sampling have already been described (Payan et al., 1997, 1999). Briefly, blood was sampled from caudal vessels, centrifuged to obtain plasma, and kept on ice until analysis. The experiments reported in this paper complied with the Principles of Animal Care of the National Institute of Health (publication No. 86, revised 1985) and the French laws for experiments on animals (decree No. 87–848). After decapitation of the trout, 4–5 µl samples of endolymph were removed from each side of the otolith (proximal near the macula and distal at the opposite side) with calibrated capillary tubes connected to a withdrawal pump. The endolymph contained in the capillaries was kept on ice until analysis.

Plasma and endolymph analysis

Calcium and strontium contents were measured by spectrophotometry using Sigma Ca kit. The strontium standard curve was done in the presence of calcium (see Results). Protein content was measured by spectrophotometry, using Coomassie Blue with BSA (bovine serum albumin) as a standard (Bradford, 1976).

In vitro *experiments*

Heparin (5000 i.u. in 100 µl) was injected into the intraperitoneal cavity 15 min before dissection. After blood sampling and decapitation, the ventral aorta was cannulated with polyethylene tubing (Biotrol, 0.86–1.52 mm diameter) and the isolated preparation perfused with a Ringer solution at a flow rate of 1 ml min^{-1} using a peristaltic pump (P-3, Pharmacia). After 5 min of perfusion to eliminate blood from the vascular space, the dorsal aorta was cannulated with polyethylene tubing (Biotrol, 0.76–1.22 mm diameter) and the preparation retroperfused *via* the dorsal aorta at a flow rate of 1 ml min^{-1}. A diagram of the retroperfusion technique is shown in Fig. 1. At various times after the start of retroperfusion, endolymph was sampled as described above. In some experiments, after endolymph sampling from the first saccule, the composition of the Ringer solution was changed (see below) before sampling the second saccule. Experiments were performed at room temperature.

The Ringer solution consisted of: 135 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2$, 1.5 mmol l^{-1} NaH_2PO_4,

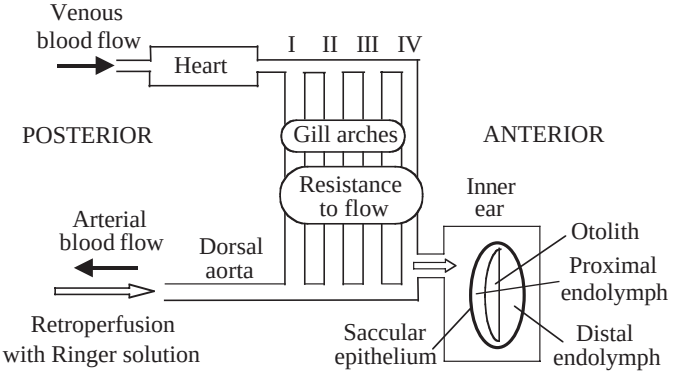

Fig. 1. Schematic drawing of the retroperfusion technique used to study Ca^{2+} fluxes across the saccular epithelium of trout. Solid arrows, *in vivo* blood circulation; open arrows, Ringer perfusion.

0.4 mmol l^{-1} KH_2PO_4, 0–4 mmol l^{-1} $CaCl_2$, 5 g l^{-1} albumin, buffered to pH 7.4 by 5 mmol l^{-1} $NaHCO_3$. In strontium experiments, $CaCl_2$ was replaced by $SrCl_2$. The solution also contained freshly added glucose (1 g l^{-1}) and was aerated before use. Pharmacological products (verapamil and cyanide) were purchased from Sigma.

The influx of Ca^{2+} through the saccular epithelium was brought about by retroperfusing the preparation with a Ringer solution containing 1.1 mmol l^{-1} of $CaCl_2$ supplemented by $^{45}Ca^{2+}$ (NEN) at a concentration of approximately 10 kBq ml^{-1}. At different times after radioactive perfusion, 4–5 µl samples of proximal and distal endolymph were taken. Radioactivity in endolymph and Ringer solution samples was measured in a vial containing 5 ml of scintillation liquid using a β counter (Kontron BETAmatic). The unidirectional Ca^{2+} influx across the saccular epithelium was evaluated by dividing the radioactivity appearing in the endolymph (cts min^{-1} ml^{-1}) by the specific radioactivity of the ^{45}Ca in the Ringer solution (cts min^{-1} $nmole^{-1}$).

Statistics

Results are expressed as means ± S.E.M. (*N* specified for individual experiments) and analyzed statistically by Statview application. Comparison of the means was done using a one-way ANOVA. Differences were considered significant at $P<0.05$.

Results

Effects of hypercalcemia on calcium levels in endolymph

Intraperitoneal injection of $CaCl_2$ was followed by a rapid hypercalcemia of up to 6 mmol l^{-1}, which provoked a significant increase in calcium levels (free+bound) of both proximal and distal sites in the endolymph (Fig. 2A). The effect was maximum in the plasma 30 min after injection and about 15 min later in the endolymph (2.7-, 2.1- and 1.8-fold increase over control values in plasma, proximal and distal endolymphs, respectively). Furthermore, the increase in calcium levels in the proximal endolymph was more rapid than in the distal. After peaking, the calcium levels decreased simultaneously in all three compartments. Protein levels in plasma and endolymph remained unchanged throughout the experiments (27.7±0.66 g l^{-1}, N=11; 14.6±0.65 g l^{-1}, N=22; 1.6±0.30 g l^{-1}, N=22, for plasma, proximal and distal endolymphs, respectively). There was a significant relationship between the calcium levels in the endolymph and those in the plasma, although the slope of the relationship was less pronounced in the distal than the proximal endolymph (Fig. 2B).

Ca^{2+} influx across the saccular epithelium

Unidirectional influx of Ca^{2+} across the saccular epithelium was measured by using $^{45}Ca^{2+}$ tracer in equilibrium conditions, where the inner ear was perfused with the same concentration of Ca^{2+} as that found in the plasma (1.2 mmol l^{-1}). $^{45}Ca^{2+}$ accumulation within the endolymph reached a plateau approximately 15 min after starting the radioactive perfusion

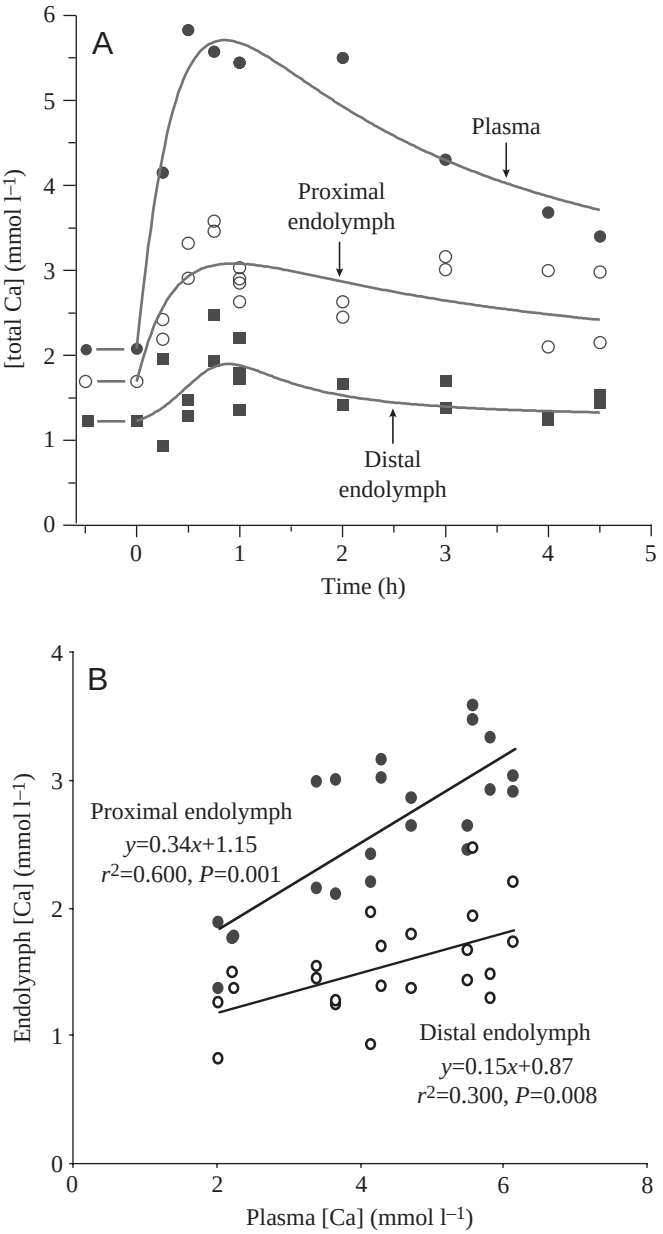

Fig. 2. Effects of plasma hypercalcemia on calcium levels in the endolymph of trout. (A) Time course of calcium concentrations in plasma (solid circles), proximal (open circles) and distal (solid squares) endolymphs after intraperitoneal injection (at time 0 min) of 100 µl 0.5 mol l^{-1} $CaCl_2$. (B) Relationships between calcium concentrations in proximal (solid symbols) and distal (open symbols) and plasma endolymph. Curve-fitting was done using Igor Pro 4.01 (Wavemetrics Inc).

(Fig. 3). A rough estimation of the Ca^{2+} influx was evaluated from the initial slope of the $^{45}Ca^{2+}$ accumulation and was approximately 2.4 and 0.6 nmoles µl^{-1} endolymph h^{-1} for proximal and distal compartments, respectively.

Net flux of Ca^{2+} across the saccular epithelium during calcium loading

In a first set of experiments, the inner ear was perfused with

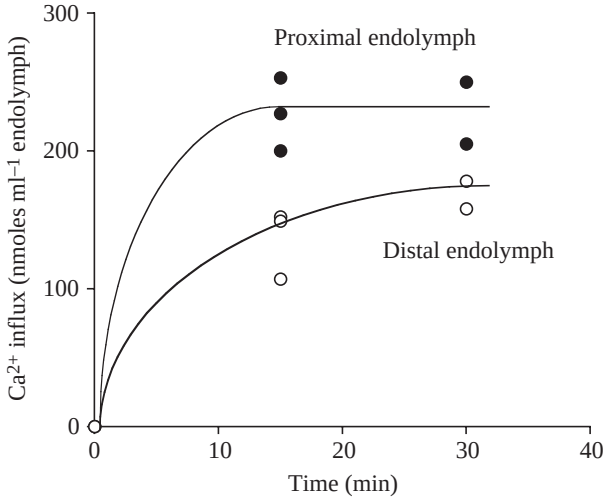

Fig. 3. Ca^{2+} influx measured by $^{45}Ca^{2+}$ appearance in proximal (solid circles) and distal (open circles) endolymphs during perfusion of the inner ear of trout with radioactive Ringer solution under equilibrium conditions.

a Ringer solution containing different calcium concentrations at time t=0 min. Samples were taken from proximal and distal endolymphs in the left saccule at t=35 min, and in the right saccule at t=70 min. Increased calcium concentration provoked increased calcium accumulation in both proximal and distal endolymphs, although the effect was less pronounced in the distal sample with 4.4 mmol l^{-1} calcium in the Ringer solution (Fig. 4A,B). Equilibrium was reached approximately 30 min after the beginning of the perfusion in both compartments. The use of a low-Ca Ringer (0.19 mmol l^{-1} Ca) partially emptied the proximal compartment whereas the calcium level in the distal sample did not vary within the 70 min of perfusion (Fig. 4A,B).

In a second set of experiments, the inner ear was perfused at time 0 with a Ringer solution containing 3.23 mmol l^{-1} of Ca. 35 min later proximal and distal endolymphs of the left saccule were sampled, then the calcium levels in the Ringer solution were changed (to 0.13 mmol l^{-1} or 4.27 mmol l^{-1}) and at t=70 min, endolymphs were sampled in the right saccule. This experimental design allowed an individual preparation to be its own control. The results, summarized in Fig. 5A,B, confirm that increased calcium levels in the Ringer solution provoke an accumulation of Ca^{2+} within the endolymph and that decreased calcium levels empty the endolymph pool of Ca. These effects are less pronounced in the distal endolymph than in the proximal one.

Net flux of Sr^{2+} across the saccular epithelium during strontium-loading

The inner ear was perfused with a Ringer solution containing different concentrations of $SrCl_2$ (Ca-free). After 35 min of perfusion, the proximal and distal endolymphs were sampled and the sum of calcium and strontium contents were measured using a Sigma kit for calcium analysis. The strontium level was estimated from a $SrCl_2$ standard curve

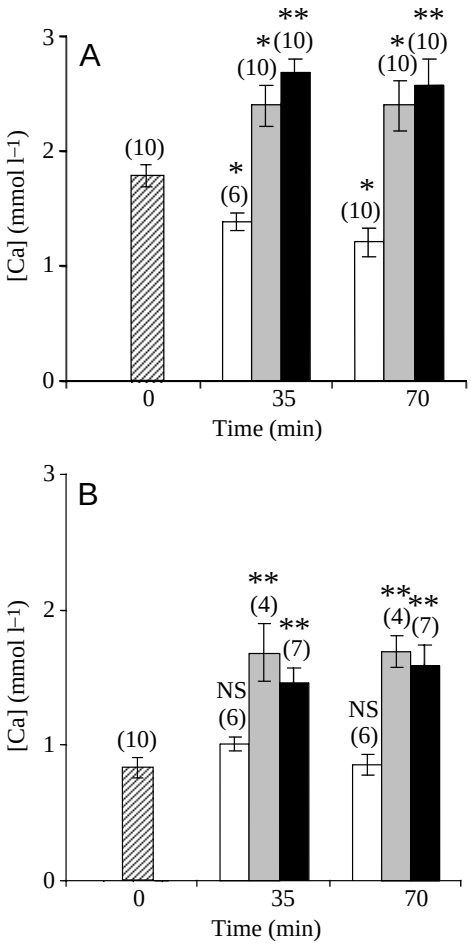

Fig. 4. Calcium levels in endolymph in the presence of chemical gradients of calcium across the saccular epithelium of perfused inner ear of trout. (A) Proximal endolymph, (B) distal endolymph. The value at time 0 min (hatched colums) is the calcium content of endolymph before starting the perfusion. The inner ear was then perfused with a Ringer solution containing different concentrations of calcium (0.19 mmol l^{-1}, white bars; 3.22 mmol l^{-1}, grey bars; 4.40 mmol l^{-1}, black bars). Endolymph was sampled in the left saccule at 35 min and in the right saccule at 70 min. N values are given in parentheses. NS, not significant; *P<0.01; **P<0.001 in comparison with control values.

done in the presence of 1.4 and 1 mmol l^{-1} of $CaCl_2$ (corresponding to the levels of calcium in proximal and distal endolymphs respectively, after 35 min perfusion with a Ca-free Ringer solution). The presence of increasing concentrations of strontium in the perfusing medium provoked an accumulation of increasing amounts of strontium in both proximal and distal endolymphs (Table 1).

Effects of inhibitors on endolymph Ca^{2+} supply

The preparation was perfused at t=0 min with a Ringer solution containing 2.8–3.2 mmol l^{-1} $CaCl_2$ and the endolymph was sampled 35 min later. 10^{-5} mol l^{-1} verapamil (a blocker of voltage-dependent calcium channels) or 1 mmol l^{-1} CN (a

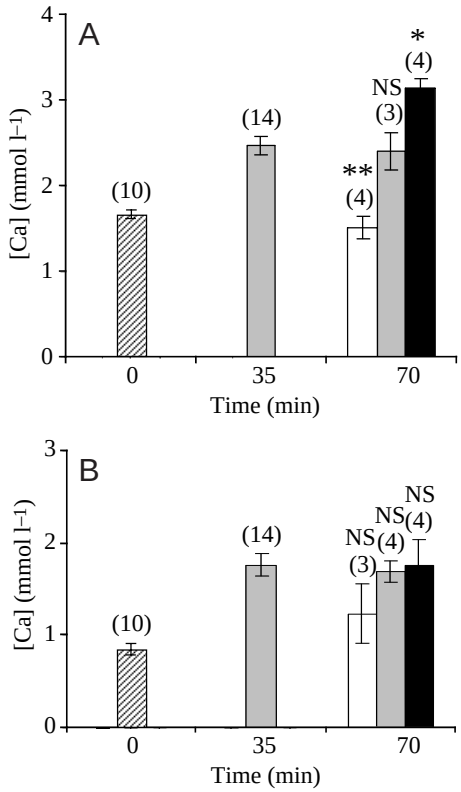

Fig. 5. Calcium levels in endolymph in the presence of chemical gradients of calcium across the saccular epithelium of perfused inner ear of trout. (A) Proximal endolymph, (B) distal endolymph. Values at time 0 min (hatched columns) are the calcium content of endolymph before starting the perfusion. The inner ear was then perfused with a Ringer solution containing 3.23 mmol l^{-1} calcium and 35 min later endolymphs were sampled in the left saccule. The perfusion was then either continued with the same [Ca]-Ringer, or with different concentrations of Ca-Ringer (0.19 mmol l^{-1}, white bars; 3.22 mmol l^{-1}, grey bars; 4.40 mmol l^{-1}, black bars) and at t=70 min the endolymph was sampled in the right saccule. N values are given in parentheses. NS, not significant; *P<0.01; **P<0.001, for comparison between t=35 min and t=70 min.

Table 1. *Sr^{2+} accumulation in the proximal endolymph in the presence of different levels of strontium in the Ringer solution*

	[Sr] (mmol l^{-1})		
Ringer solution	0.98±0.02 (6)	2.39±0.01 (6)	3.93±0.04 (6)
Proximal endolymph	0.57±0.15 (6)	1.07±0.24 (6)	1.30±0.20 (4)
Distal endolymph	0.16±0.06 (6)	0.39±0.08 (6)	0.49±0.14 (6)

Values are means ± S.E.M., number of experiments in parentheses.

The inner ear was perfused for 35 min with different [Sr] before endolymph sampling.

blocker of ATP production by the mitochondria) were added to the Ringer solution at t=0 and their effects on Ca^{2+} accumulation into the endolymph studied. Neither inhibitor modified Ca^{2+} entry into either proximal or distal endolymph (Table 2).

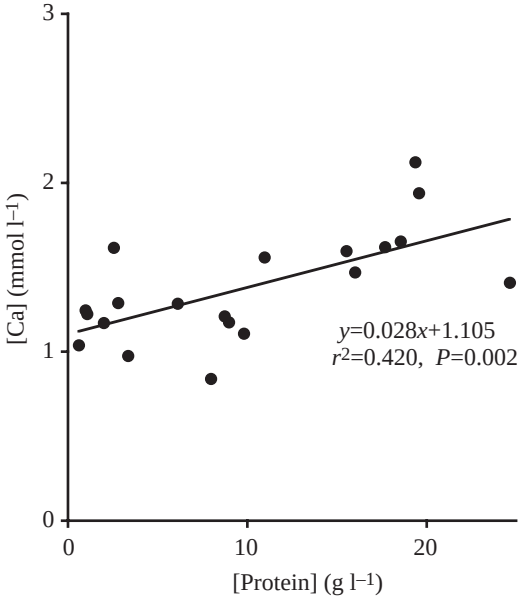

Fig. 6. Relationship between concentration of [Ca^{2+}] and [protein] in endolymph of trout.

Heterogeneous distribution of calcium within the saccule

The microtechnique described here permitted measurement of calcium and protein concentrations in each sample and thus a study of their interrelationships. In these experiments with trout, the protein content of the control samples was: 16.0±1.62 g l^{-1} (N=20) for proximal and 3.7±1.00 g l^{-1} (N=20) for distal zones, (P<0.001), and the calcium content was 1.23±0.10 mmol l^{-1} (N=10) for proximal and 0.98±0.05 mmol l^{-1} (N=10) for distal zones (P=0.0265). These results can also be presented by plotting [calcium] as a function of [protein] (Fig. 6), showing a significant positive relationship between the two parameters.

In one set of experiments performed with turbot, 4–5 µl samples of endolymph were withdrawn at different sites inside the saccule. The results show a significant positive relationship between the concentrations of calcium and protein (y=0.05x+0.76, N=64, r^2=0.137, P=0.0024; data not illustrated) similar to that found in the trout, although the protein range in turbot endolymph (1–7 g l^{-1}) was smaller than in trout (1–20 g l^{-1}).

Discussion

Functional evaluation of the perfused inner ear of trout

Perfusing the heads of trout has been successfully used to study ionic fluxes across the gill epithelium (Perry et al., 1984). It allows one to control the chemical composition of the perfusing fluid, e.g. ionic and respiratory gas levels, acid–base balance and hormonal concentration. In the present study we adapted this technique to study ionic transfers across the saccular epithelium of the inner ear of trout. The retro-perfusion of the preparation through the dorsal aorta supplies the efferent gill arteries with Ringer solution and irrigates the

Table 2. *Effects of verapamil and cyanide on endolymph Ca^{2+} supply during Ca-loading experiments*

Endolymph [Ca] (mmol l^{-1})	Proximal endolymph	Distal endolymph
Control (0 min)	1.66±0.07 (8)	0.79±0.07 (8)
Control (35 min)	2.33±0.11 (8)	1.38±0.10 (8)
Verapamil (35 min)	2.49±0.12 (8) NS	1.24±0.10 (8) NS
CN (35 min)	2.21±0.11 (8) NS	1.29±0.07 (8) NS

Verapamil, 10^{-5} mol l^{-1}; CN, 1 mmol l^{-1}.

Values are means ± S.E.M., number of measurements in parentheses.

At t=0 the inner ear was perfused with a Ringer solution containing 2.8–3.2 mmol l^{-1} [Ca] and endolymph was sampled 35 min later.

Inhibitors, where used, were added to the Ringer solution at t=0 and endolymph was sampled 35 min later.

NS, no significant difference at 35 min between values obtained in controls and with inhibitors.

inner ear complex through an artery derived from the 4th efferent gill artery (Fig. 1) (Grassé, 1958). The perfusion of the inner ear is favored by the resistance to flow offered by the retroperfused vascular gill apparatus. A few preliminary observations (not described here) confirmed the suitability of this technical approach. After about 5 min perfusion, observation of an excised saccular epithelium by transmission microscopy clearly showed that the arteries were completely cleared of red blood cells. Evans Blue, a tracer of vascular space added to the perfusing fluid, appeared within 2–3 min in the arteries of the saccular epithelium. After 2 h perfusion with a Ringer solution the K^+ content in proximal and distal endolymphs was not modified by comparison with controls, the increasing P–D gradient of K^+ being maintained. The fact that this ion was in electrochemical imbalance across the saccular epithelium (Payan et al., 1997*)* strongly suggested that the epithelium kept its capacity for active ion transport for at least 2 h. Finally, the hypercalcemia experiments performed *in vivo* and *in vitro* gave similar results (see Figs 2A, 4A), which suggests that the conclusions drawn from the *in vitro* experiments may be extrapolated to the *in vivo* ones.

Dynamics of the endolymph Ca^{2+} pool

Very few studies have been concerned with the relationship between plasma and endolymph Ca^{2+} levels and the present work is the first evaluation of the unidirectional Ca^{2+} influx through the saccular epithelium in electrochemical conditions resembling those *in vivo*. The sum of the Ca^{2+} influxes into the proximal and distal spaces is about 3–4 nmoles μl^{-1} endolymph h^{-1}, which corresponds to a global turnover rate of 230 % h^{-1} of the endolymph pool of calcium (equal to 30 nmoles, calculated for a saccule of 20 µl volume and a concentration of 1.5 mmol l^{-1} Ca). It must be noted that this value may be an underestimation because it does not take into account the Ca^{2+} incorporated into the otolith during the experiments.

This very high turnover rate suggests that the perfusion of the inner ear with different concentrations of Ca^{2+} could rapidly influence the calcium level within the endolymph, a hypothesis confirmed by the results of the hyper (*in vivo* and *in vitro*) and hypo (*in vitro*) calcemia experiments. Indeed, the presence of a positive chemical gradient of calcium between Ringer solution and endolymph provoked the accumulation of Ca^{2+} in both proximal and distal endolymphs (Figs 2A, 4A,B) and a negative chemical gradient produced an emptying of the endolymph pools (Figs 4A, 5A,B). Surprisingly, perfusion with a Ca-free solution did not empty the distal compartment (Fig. 4B), suggesting that the distal calcium was strongly bound and thus not rapidly exchangeable.

Our results should be compared with those of Toshe and Mugiya (2001), who studied the translocation of $^{45}Ca^{2+}$ from the Ringer solution to the endolymph using an isolated preparation of the sacculus. By incubating the sacculus with 3 mmol l^{-1} $CaCl_2$ for 2 h they found that Ca^{2+} incorporation into the endolymph was about 2 nmoles μl^{-1} endolymph 2 h. In fact, if the excised sacculus behaves like the perfused sacculus, after 2 h of incubation the specific radioactivites of $^{45}Ca^{2+}$ in the Ringer solution and endolymph should be identical and the results of 2 nmoles μl^{-1} endolymph 2 h^{-1} correspond to a calcium level of 2 mmol l^{-1}. This is in agreement with the value of 2.4–1.7 mmol l^{-1} (in proximal and distal spaces, respectively) obtained by perfusing the inner ear with 3.2 mmol l^{-1} Ca^{2+} concentrations (Fig. 4A,B).

The analysis of the ratio between the endolymph pool of calcium and its daily incorporation into the otolith offers a dynamic vision of the calcium needs involved in the process of otolith growth. For a 12 month-old trout the otolith mass is about 8 mg, with 0.2 % being total organic matrix and 99.8 % $CaCO_3$ (Borelli et al., 2001). In such trout, the daily otolith growth necessitates 0.23 µmoles of Ca^{2+} corresponding to a daily incorporation of eight endolymph calcium pools. These calculations indicate that the saccular epithelium must transfer huge amounts of calcium for the daily otolith growth and this reflects the high turnover rate of the endolymph calcium pool (about 55 times a day).

The endolymph Ca^{2+} is supplied mainly at the proximal area of the saccular epithelium via *a paracellular pathway*

During the hypercalcemia experiments (*in vivo* and *in vitro*) calcium levels were systematically higher in the proximal area of the endolymph than in the distal one (Figs 2A, 4A,B). This suggests that Ca^{2+} entry into the sacculus occurs mainly through the macula area into the proximal compartment. This is confirmed by the kinetics of calcium appearance in the endolymph during *in vivo* hypercalcemia, which showed that calcium levels increased more rapidly in the proximal than in the distal zones (Fig. 2A). Thus our results confirm the conclusions of Mugiya (1974) based on the analysis of an autoradiogram of the saccular epithelium in $^{45}Ca^{2+}$-injected trout.

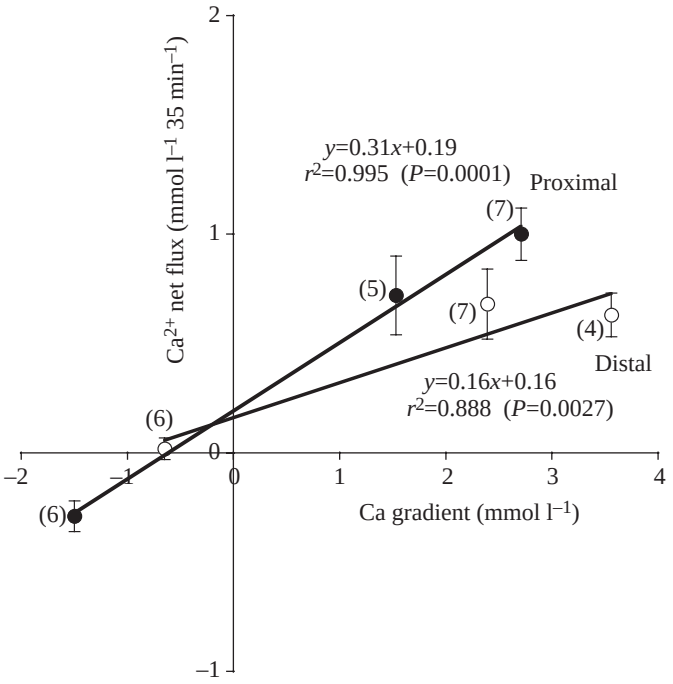

Fig. 7. Relationships between Ca^{2+} entry into the endolymph and the chemical gradient of calcium across the saccular epithelium of trout. Ca^{2+} entry (mmol l^{-1} 35 min^{-1}) corresponds to the difference in endolymph calcium levels between t=0 and t=35 min, by which time equilibrium was reached (see Results, Fig. 4). Calcium gradient (mmol l^{-1}) corresponds to the difference between calcium levels in the endolymph and the Ringer solution at the beginning of the perfusion. Regression lines are shown together with their equations and significances, which have been calculated with paired data. N values are given in parentheses.

The experiments of Ca^{2+} net flux are summarized in Fig. 7, in which the chemical gradients of calcium between the Ringer solution and endolymph at the beginning of the perfusion are plotted as functions of the resulting Ca^{2+} accumulation over a 30 min period. It is clear that these relationships (including positive and negative net fluxes) are linear in both the proximal and distal compartments, which suggests a passive mechanism for Ca^{2+} transfer across the saccular epithelium, i.e. a paracellular pathway. This is confirmed by the absence of any effect of verapamil or CN on Ca^{2+} accumulation in the endolymph in the presence of a positive chemical gradient of calcium (Table 2). Calculation of E_{Ca} by the Nernst equation applied to the results of Fig. 4A gives +4 mV (endolymph positive) if the calcium levels are used for both compartments. Mugiya (1966) found 72 % of ultrafiltrable calcium in the endolymph of trout and flatfish. Assuming that the ultrafiltrable calcium and ionized calcium are comparable, the Nernst potential is +8 mV, which is close to the only published value for teleosts, namely a saccular potential of about +10 mV (Enger, 1964). It must be noted that in vertebrates the endolymph side has always been found to be positive with respect to the perilymph, and in mammals the voltage varies from +80 mV in the cochlea to +5 mV in the utriculus (Sterkers et al., 1988).

The main conclusion to be drawn from our study is that Ca^{2+} crosses the saccular epithelium *via* a paracellular route. This is not in agreement with the model of Mugiya and Yoshida (1995), who suggested that Ca^{2+} entry into the endolymph is by a transcellular route involving a combination of a receptor-operated Ca^{2+} channel, Na^+–Ca^{2+} exchanger and ATP-dependent Ca^{2+} pump. This proposal was based on the amounts of Ca^{2+} incorporated into the otolith under different experimental conditions. Recently, Toshe and Mugiya (2001) observed that the *trans*-saccular transport of Ca^{2+} to endolymph was not affected by the usual inhibitors of the pH-regulating mechanisms (amiloride, acetazolamide, DIDS, SITS and thiocyanate). This lack of effect of inhibitors known to disrupt the acido–basic homeostasis of the intracellular medium also favours a paracellular pathway for Ca^{2+}.

Heterogeneity of the endolymph Ca^{2+} pool and otolith growth

The present study confirms the existence of the decreasing P–D gradient of proteins in endolymph that had already been observed by Payan et al. (1999), and also brings to light the presence of a lower but significant decreasing P–D gradient of calcium that had not been described. This raises the question as to whether a P–D gradient of Ca^{2+} occurs within the endolymph compartment. Attempts to evaluate Ca^{2+} levels in proximal and distal endolymphs by using a Ca^{2+}-sensitive mini-electrode (KWIK-TIP-WPI) failed, owing to difficulties in establishing a standard curve taking into account the different parameters of these two media (pH, protein, Mg^{2+}, K^+ contents). No data are available concerning the Ca^{2+} level in the endolymph of fishes and only Mugiya (1966) has measured the ultrafiltrable fraction (72 %) of calcium in this fluid. It should be noted that in higher vertebrates, vestibular endolymph contains about 3 mmol l^{-1} Ca, with one-tenth in the ionized form (Steckers et al., 1988).

The fact that perfusing with a calcium-free solution did not decrease the calcium level in the distal compartment (Fig. 4B) supports the hypothesis of a highly bound distal calcium. Recently, Borelli et al. (2001) described an increasing P–D gradient of proteoglycans whose polyanionic nature could explain this phenomenon. We therefore propose that the decreasing P–D gradient of calcium corresponds to a decreasing gradient of Ca^{2+} that is the result of two factors: a predominantly proximal entry of Ca^{2+}, combined with the position of the otolith, which presents a physical barrier to diffusion from proximal to distal compartments. It should be noted that the decreasing P–D gradient of Ca^{2+} and also that of protein concentration are clearly related to the growth axis of the otolith, as the proximal zone facing the macula corresponds to the convex shape of the otolith where the increment is greater than on the concave distal side. This situation reinforces the proposal that the proximal zone is of importance in the $CaCO_3$ deposition process as it is already held to be for the formation of organic compounds (Gauldie and Nelson, 1988; Payan et al., 1999; Borelli et al., 2001).

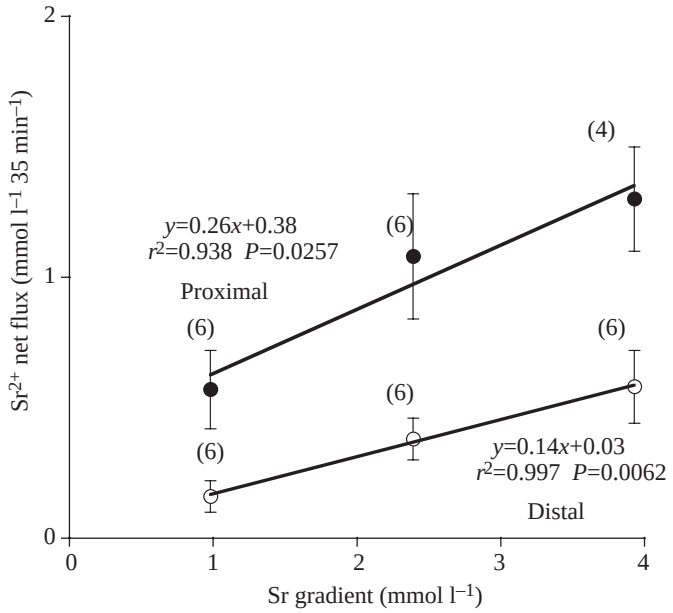

Fig. 8. Relationships between Sr^{2+} entry into the endolymph and the chemical gradient of strontium across the saccular epithelium of trout. Other details as in Fig. 7.

Endolymph Sr^{2+} homeostasis in relation to otolith strontium microchemistry

Because of their similar properties, a paracellular route across the saccular epithelium might also be expected for Sr^{2+}, as was found for Ca^{2+}. Subjecting a chemical gradient of $SrCl_2$ (0.98, 2.39 and 3.93 mmol l^{-1}) to the saccular epithelium provoked an accumulation of Sr^{2+} within the sacculus in both proximal and distal zones (Table 1). The relationship between the strontium gradient and the resulting Sr^{2+} accumulation in the endolymph over 30 min was linear and the slope comparable to that found for calcium in similar conditions (Fig. 8). The values for the strontium in endolymph may only be approximate, since we used a Ca-kit to measure the strontium level, but the data relating to the slope should still be significant. These results indicate that, under our experimental conditions, the Sr^{2+} crosses the saccular epithelium *via* a paracellular route and reinforces the hypothesis of a paracellular pathway for Ca^{2+} endolymph supply. This hypothesis is supported by Kalish (1991), who reached similar conclusions after studying the correspondence between blood plasma and endolymph strontium/calcium in bearded rock cod.

In conclusion, our results suggest that variations of Ca^{2+} and Sr^{2+} plasma levels rapidly induce corresponding variations in the endolymph *via* a passive paracellular route. This may be relevant to the calcification process as these two ions, together with HCO_3^-, are the precursors of the aragonite (which is predominant) and strontionite formations. However, within the calcification process we have to consider Ca^{2+} and Sr^{2+} activities rather than their total levels, and these depend on several parameters such as total concentration, pH of the fluid, presence, nature and concentration of binding proteins and presence of competitors (Mg^{2+}) and inhibitors (such as PO_4^{3-}, proteoglycan). The composition of the fish endolymph is not spatially uniform, showing P–D gradients for most of its components (Payan et al., 1999). Furthermore, the levels of some calcifying parameters (total CO_2, pH, proteins) vary within the proximal and distal zones as functions of environmental conditions such as circadian cycle (Edeyer et al., 2000), fasting (Payan et al., 1998) and stress (P. Payan, H. De Pontnal, A. Edeyer, G. Borelli, G. Boeuf and N. Mayer-Gostan, unpublished results), making it difficult to evaluate the ionic activities of Ca^{2+} and Sr^{2+}. These factors should be taken into consideration when studying strontium otolith microchemistry, since incorporation of strontium is directly dependent on its ionic activity at the interface between endolymph and otolith.

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