

## KINETICS OF BRANCHIAL CALCIUM UPTAKE IN THE RAINBOW TROUT: EFFECTS OF ACCLIMATION TO VARIOUS EXTERNAL CALCIUM LEVELS

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### SUMMARY

Calcium uptake ( $J_{in}^{Ca}$ ) in freshwater rainbow trout (*Salmo gairdneri*) under control conditions (external  $[Ca^{2+}] \approx 1.8 \text{ mequiv l}^{-1}$ ,  $[NaCl] \approx 0.8 \text{ mequiv l}^{-1}$ ) occurred at approximately equal rates ( $12\text{--}15 \mu\text{equiv kg}^{-1} \text{ h}^{-1}$ ) through the gills and the general body surface *in vivo*. The gut was not involved. Under the same conditions, *in vitro* branchial  $J_{in}^{Ca}$  in an isolated, saline-perfused head preparation was equal to that *in vivo*. The cells involved in  $J_{in}^{Ca}$  are mainly located on lamellae rather than on filaments since  $\sim 95\%$  of  $J_{in}^{Ca}$  occurred across the arterio-arterial circulation of the gill.  $J_{in}^{Ca}$ , *in vitro*, displayed Michaelis-Menten kinetics. Acclimation to low external  $[Ca^{2+}]$  ( $50 \mu\text{equiv l}^{-1}$ ; unchanged  $[NaCl]$ ) for 1 day caused a five-fold stimulation of  $J_{in}^{Ca}$  characterized by decreased  $K_m$  and increased  $J_{max}$ . Longer periods of low  $[Ca^{2+}]$  acclimation resulted in changes of  $J_{max}$  only.  $J_{max}$  gradually returned towards control levels as acclimation time increased, but was still elevated after 30 days. Acclimation to low ambient  $[Ca^{2+}]$  caused proliferation and increased exposure of lamellar chloride cells which were correlated with increased  $J_{max}$ . Fish exposed to high external  $[Ca^{2+}]$  ( $10 \text{ mequiv l}^{-1}$ ; unchanged  $[NaCl]$ ) displayed reduced  $J_{in}^{Ca}$ . Similar changes in  $J_{in}^{Ca}$  were observed during *in vivo* experiments. Plasma  $Ca^{2+}$  concentration remained constant regardless of external  $[Ca^{2+}]$ , while plasma  $Na^+$  and  $Cl^-$  levels were transiently reduced at 1 day low  $[Ca^{2+}]$  exposure but had recovered by 7 days. A possible role for cortisol in  $Ca^{2+}$  regulation is discussed based on observations of cortisol-stimulated lamellar chloride cell proliferation and  $J_{in}^{Ca}$ , and elevated plasma [cortisol] in low- $[Ca^{2+}]$  acclimated fish.

### INTRODUCTION

The ability of fish to maintain constant plasma  $Ca^{2+}$  levels in diverse calcium environments is well documented (see review by Pang, Griffith, Maetz & Pic, 1980). Modulation of branchial  $Ca^{2+}$  uptake ( $J_{in}^{Ca}$ ) has been implicated in this regulatory response (Mayer-Gostan *et al.* 1983; Flik, Kolar, Fenwick & Wendelaar Bonga, 1983a) yet the mechanisms involved in  $Ca^{2+}$  adjustment remain poorly understood.  $Ca^{2+}$ -ATPase is believed to play a role in gill  $Ca^{2+}$  transport but conflicting results

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have been reported with regard to  $\text{Ca}^{2+}$ -ATPase activity changes following transfer of fish to water containing low levels of  $\text{Ca}^{2+}$  (Mayer-Gostan *et al.* 1983; Flik, van Rijs, Fenwick & Wendelaar Bonga, 1983b). In the absence of observed enzyme activity changes, Mayer-Gostan *et al.* (1983) proposed that increased gill permeability was causing the stimulation of  $J_{\text{in}}^{\text{Ca}}$ .

Using an isolated-perfused trout head preparation, Payan, Mayer-Gostan & Pang (1981) presented evidence that the sites of branchial  $J_{\text{in}}^{\text{Ca}}$  in freshwater fish are chloride cells located on gill filaments. Very recently it has been shown that acclimation of trout to low ambient  $[\text{Ca}^{2+}]$  causes proliferation of chloride cells on lamellae (Laurent, Høbe & Dunel-Erb, 1985). With these two pieces of evidence in mind we decided to investigate the possibility that stimulation of  $J_{\text{in}}^{\text{Ca}}$  in low external  $[\text{Ca}^{2+}]$  is related to lamellar chloride cell proliferation. Thus, in the present investigation, fish were acclimated to low, normal or high ambient  $\text{Ca}^{2+}$  levels for varying periods of time and the effects on  $J_{\text{in}}^{\text{Ca}}$ , branchial transepithelial potential, lamellar chloride cell populations and plasma ions ( $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$  and  $\text{Cl}^{-}$ ) were evaluated. In addition, cortisol treatment was employed to cause chloride cell proliferation in the absence of external  $[\text{Ca}^{2+}]$  changes (Doyle & Epstein, 1972).  $J_{\text{in}}^{\text{Ca}}$  was monitored *in vitro*, using a perfused head preparation, as well as *in vivo*. *In vitro* measurement of  $J_{\text{in}}^{\text{Ca}}$  was vital to this study as it allows separation of  $\text{Ca}^{2+}$  uptake across cells located on lamellae from uptake across cells located on filaments (cf. Girard & Payan, 1980). Moreover, this preparation permits rapid and simple determination of the kinetic constants,  $K_m$  and  $J_{\text{max}}$ , for  $\text{Ca}^{2+}$  uptake, a procedure which would be impossible *in vivo*. By studying the kinetics of  $J_{\text{in}}^{\text{Ca}}$  following acclimation to various external  $\text{Ca}^{2+}$  levels we hoped to be able to elucidate further the mechanisms involved in  $J_{\text{in}}^{\text{Ca}}$  modulation. *In vivo* determination of  $J_{\text{in}}^{\text{Ca}}$  served to validate both the absolute values and the relative changes with acclimation condition observed *in vitro*, as well as to assess the importance of the gills relative to other possible  $\text{Ca}^{2+}$  uptake sites (skin, gut; Simmons, 1971; Dacke, 1979).

## MATERIALS AND METHODS

### *Experimental animals*

Rainbow trout (*Salmo gairdneri*) of either sex, but in non-breeding condition (mean weight =  $244.0 \pm 5.3$  g, S.E.,  $N = 131$ ), were obtained from Spring Valley Farm (Petersburg, Ontario). Fish were held indoors in large circular fibreglass tanks supplied with flowing, aerated and dechlorinated Hamilton city tap water (hardness  $\approx 140$  mg  $\text{l}^{-1}$  as  $\text{CaCO}_3$ ;  $[\text{Ca}^{2+}] \approx 1.8$  mequiv  $\text{l}^{-1}$ ;  $[\text{Mg}^{2+}] \approx 0.3$  mequiv  $\text{l}^{-1}$ ;  $[\text{Na}^{+}] \approx 0.65$  mequiv  $\text{l}^{-1}$ ;  $[\text{Cl}^{-}] \approx 0.8$  mequiv  $\text{l}^{-1}$ ;  $[\text{K}^{+}] \approx 0.05$  mequiv  $\text{l}^{-1}$ ; temperature =  $11\text{--}16^\circ\text{C}$ ). Fish were fed a daily diet of dried commercial trout pellets.

### *Acclimation conditions*

Separate groups of fish were acclimated for various fixed periods to either low ( $50$   $\mu\text{equiv l}^{-1}$ ) or high ( $10$  mequiv  $\text{l}^{-1}$ ) external  $\text{Ca}^{2+}$  concentrations at a constant NaCl level ( $0.8$  mequiv  $\text{l}^{-1}$ ) approximately equal to that in the holding water. Acclimation media were prepared by dissolving analytical grade  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  and

NaCl in distilled water in darkened 500-litre polyethylene tanks. Loading density was approximately  $0.010 \text{ kg l}^{-1}$  and acclimation water was renewed every 3–4 days. Water was aerated vigorously and filtered continuously by percolation through cotton wool and charcoal; temperature was maintained at  $14\text{--}15^\circ\text{C}$ . Control fish were kept in tanks containing normal holding water ( $1.8 \text{ mequiv l}^{-1} \text{ Ca}^{2+}$ , temperature =  $14\text{--}15^\circ\text{C}$ ) with continuous flow. An additional group of fish was kept isolated in individual black Perspex boxes in this same control water. These trout were injected (intramuscularly) once a day with  $4 \text{ mg kg}^{-1}$  body weight of cortisol (hydrocortisone hemisuccinate ( $\text{Na}^+$  salt, Sigma) dissolved in  $0.4 \text{ ml kg}^{-1}$  of  $0.9\%$  NaCl, for a period of 9–10 days.

Experiments were performed *in vitro* and *in vivo* to determine the effects of these various acclimation conditions on branchial  $\text{Ca}^{2+}$  influx ( $J_{\text{in}}^{\text{Ca}}$ ), transepithelial potential (TEP), gill morphology, plasma ion concentrations ( $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{Ca}^{2+}$ ), and plasma cortisol levels.

#### *In vitro methodology*

Isolated, saline-perfused head preparations were prepared according to Payan & Matty (1975) with various modifications. In this preparation, ionic uptake through the arterio-arterial pathway represents fluxes through the lamellae, while uptake through the arterio-venous pathway represents fluxes through the filaments (Girard & Payan, 1980).

Initially, 1 ml of blood was withdrawn from the caudal vein/artery prior to injection of 1 ml saline containing ammonium heparin (2500 USP units/fish) also *via* the caudal vein/artery. Blood samples were centrifuged and plasma frozen for subsequent analysis of  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$  and cortisol levels. The fish was placed back into acclimation water and after approximately 20–30 min, the fish was decapitated just posterior to the pectoral fins. The head was placed onto an operating table and the gills were irrigated with acclimation water *via* a tube placed into the mouth. The pericardium was cut and the ventricle severed as quickly as possible to prevent air from being pumped into the gill vasculature. A saline-filled catheter (Clay-Adams, PE 90) was inserted into the bulbus arteriosus through the severed ventricle and tied in place. Next, a heat-flared catheter (Clay-Adams, PE 200) was placed into the dorsal aorta (DA). The gills were cleared of blood by perfusing with filtered (Millipore,  $0.45 \mu\text{m}$ ) saline (Perry, Payan & Girard, 1984c) containing  $0.2\%$  bovine serum albumin (fraction V, Sigma; Perry, Booth & McDonald, 1985a) and  $10^{-6} \text{ mol l}^{-1}$  1-epinephrine bitartrate (Sigma) at a constant pressure of  $60 \text{ cmH}_2\text{O}$  ( $5.9 \text{ kPa}$ ). For the first 10–30 s, perfusion was in the retrograde direction through the DA catheter in order to remove any air bubbles trapped in the bulbus. When the bulbus catheter appeared free of air bubbles, perfusion was switched to the orthograde direction. A tightly fitting, semi-circular plastic collar (made from a disposable 50 ml syringe barrel) was placed inside the abdominal cavity and sutured into position, thereby making the body wall rigid. Next, the oesophagus was ligated and the DA catheter secured by cutting a circular slot in the muscle surrounding the DA and fastening a ligature around the DA catheter in this incision. In order to ensure a good seal of the condom on the head, so as to prevent leakage, a groove was made just behind the

operculae by tying a very tight ligature around the skin. After fitting the condom onto the head an additional ligature was tied around the condom at the level of the groove. Surgery was completed in approximately 12 min and the gills were ischaemic for periods never exceeding 2 min.

Following removal of the head from the operating table, it was briefly rinsed in distilled water to prevent ionic contamination of ensuing test media. The head was then placed into a cylindrical plastic container and held in place by the condom which prevented leakage of the recirculating external media. The oesophagus was clamped with a haemostat as a further precaution against leakage. The head was irrigated at approximately  $500 \text{ ml min}^{-1}$  with various external media by a tube placed into the mouth. Volume of the external medium was dependent on the weight of the head and was approximately 180–200 ml as determined by dye dilution upon termination of initial experiments. The external medium was maintained at acclimation temperature (mean temperature =  $14.3 \pm 0.2^\circ\text{C}$ , s.e.,  $N = 43$ ) by recirculation through coils immersed in a cooling bath. The gills were perfused using constant pulsatile flow at 40 strokes  $\text{min}^{-1}$  (mean  $\dot{Q}_{\text{in}} = 3.50 \pm 0.04 \text{ ml min}^{-1}$ , s.e.,  $N = 53$ ) from a reservoir containing gas-equilibrated saline (0.3%  $\text{CO}_2$ , 4%  $\text{O}_2$ , remainder  $\text{N}_2$ ) with a cardiac pump (Harvard model 1405) and remote pumping head as described by Davie & Daxboeck (1983).

Once the head was in place and being perfused, the refractive indices of DA perfusate and anterior-venous (A-V) perfusate (derived from the drainage of the filamental venous sinus of gill filaments and venous drainage from the general head circulation) were measured using a Goldberg refractometer (American Optical). If the refractive index of A-V perfusate was less than that of DA perfusate this indicated dilution of anterior-venous flow ( $\dot{Q}_{\text{av}}$ ) with external medium. Unless this dilution could be corrected, the experiment was terminated (approximately 10% of all preparations). Using this technique we were able to measure the dilution of  $\dot{Q}_{\text{av}}$  as low as 1%. The significance of contamination of  $\dot{Q}_{\text{av}}$  with external medium will be discussed subsequently.

Perfusion input pressure ( $P_{\text{in}}$ ) was monitored from a T-junction in the input catheter connected to a pressure transducer (Hewlett-Packard 267BC) and displayed on a Sanborn 150 chart recorder. Pulse pressure was kept constant at approximately  $10 \text{ cmH}_2\text{O}$  ( $0.98 \text{ kPa}$ ) by adjusting the size of a gas space at the top of a wide-bore side-arm (Windkessel) in the perfusion line. Mean input pressure was calculated as diastolic +  $1/3$  pulse pressure (Burton, 1972). The pressure drop across the input catheter was measured with ligatures still in place after each experiment and  $P_{\text{in}}$  corrected accordingly. Isolated heads which exhibited initial  $P_{\text{in}}$  greater than  $80 \text{ cmH}_2\text{O}$  ( $7.8 \text{ kPa}$ ) were discarded (less than 5% of all preparations). Dorsal aortic pressure ( $P_{\text{da}}$ ) was maintained between 10 and 15 cm ( $0.98$ – $1.47 \text{ kPa}$ ) above the dorsal aorta (Perry *et al.* 1985a). Upon termination of each experiment, gills were inspected visually and on average displayed approximately 95% red blood cell (rbc) clearance and never less than about 80% rbc clearance.

$J_{\text{in}}^{\text{Ca}}$  was determined by adding  $20 \mu\text{Ci}$  of  $^{45}\text{Ca}$  (New England nuclear) to the external medium and monitoring its appearance in the dorsal aortic and anterior-venous

perfusate. Following an initial 12-min isotopic equilibration period, samples of anterior-venous and dorsal aortic perfusate and the external medium were taken over 5-min intervals by one of the two experimental protocols outlined below. For each interval, influx of  $\text{Ca}^{2+}$  across the arterio-arterial pathway ( $J_{\text{in}}^{\text{Ca}_a}$ ) and the arterio-venous pathway ( $J_{\text{in}}^{\text{Ca}_v}$ ), expressed as  $\mu\text{equiv kg}^{-1} \text{h}^{-1}$ , were calculated using the following equations:

$$J_{\text{in}}^{\text{Ca}_a} = \frac{\text{c.p.m. ml}^{-1} \text{DA} \times \dot{Q}_{\text{in}} (\text{ml min}^{-1}) \times 60 \times 1000 / \text{fish weight (g)}}{\text{Specific activity of external medium (c.p.m. } \mu\text{equiv}^{-1})} \quad (1)$$

$$J_{\text{in}}^{\text{Ca}_v} = \frac{(\text{c.p.m. ml}^{-1} \text{A-V} - \text{c.p.m. ml}^{-1} \text{DA}) \times \dot{Q}_{\text{av}} (\text{ml min}^{-1}) \times 60 \times 100 / \text{weight (g)}}{\text{Specific activity of external medium (c.p.m. } \mu\text{equiv}^{-1})} \quad (2)$$

$^{45}\text{Ca}$  activity was determined using liquid scintillation counting (LKB 1217 Rackbeta; 10 ml ACS fluor, Amersham, 4.5 ml distilled water, 0.5 ml sample). Counting efficiency for saline samples was decreased by approximately 3% compared to water samples and  $^{45}\text{Ca}$  counts in saline were corrected accordingly. External  $[\text{Ca}^{2+}]$  was determined using an atomic absorption spectrophotometer (Varian model 1275).  $\dot{Q}_{\text{da}}$  and  $\dot{Q}_{\text{av}}$  were measured gravimetrically and  $\dot{Q}_{\text{in}}$  was taken as the sum of  $\dot{Q}_{\text{da}}$  and  $\dot{Q}_{\text{av}}$ . Refractive indices of DA and A-V perfusate were compared at 5-min intervals throughout the experiment and if the refractive index of A-V perfusate was less than that of DA perfusate, the experiment was terminated. Clearly, from equation 2, even slight contamination of  $\dot{Q}_{\text{av}}$  with external medium would result in significant overestimates of  $J_{\text{in}}^{\text{Ca}_v}$ .

Branchial TEPs (i.e. between the external medium, as zero, and the dorsal aortic outflow) were determined at 5-min intervals using the method of Perry, Booth & McDonald (1985b). Briefly, this was accomplished by measuring the voltage difference between the external medium and dorsal aortic perfusate by immersing a KCl-agar filled tube (Clay-Adams, PE 160) into the water while another was inserted into the DA catheter. These KCl-agar bridges were connected to Ag-AgCl electrodes (Narco) *via* 3 mol l $^{-1}$  KCl-filled catheters. The TEP (mV) was displayed on a high impedance ( $10^{-12} \Omega$ ) voltmeter (Radiometer PHM 82). Tip potentials were measured prior to and following each TEP determination, and if necessary, the TEP was corrected accordingly.

While the nominal  $\text{Ca}^{2+}$  concentration in the saline was 2.5 mequiv l $^{-1}$ , the actual measured concentration in effluent perfusate averaged about 2.0 mequiv l $^{-1}$ . This difference reflected loss of calcium precipitates during Millipore filtration. Attempts to increase the perfusate  $[\text{Ca}^{2+}]$  to *in vivo* levels (cf. Table 5) invariably resulted in precipitation after filtration, and therefore were abandoned.

### In vitro protocols

#### Constant external $[\text{Ca}^{2+}]$ experiments

This series evaluated the stability of the preparation over time, the relative  $\text{Ca}^{2+}$  uptakes through arterio-arterial and arterio-venous pathways, and the influence of acclimation to different external  $\text{Ca}^{2+}$  levels.  $J_{\text{in}}^{\text{Ca}}$  was monitored in control fish and in

fish acclimated to either low (20 days) or high external  $[Ca^{2+}]$  (50 days). The normal holding water of the control fish ( $1.8 \text{ mequiv l}^{-1} [Ca^{2+}]$ ) was used as the test medium throughout. After the initial 12-min equilibration period, dorsal aortic and anterior-venous perfusate were collected continuously over seven successive 5-min periods. A 5-ml sample of the external medium was removed at the midpoint (2.5 min) of each 5-min period for assessment of external specific activity using equations 1 and 2.

### *Ca<sup>2+</sup> transport kinetics*

This protocol was applied to control fish, cortisol-treated fish, fish acclimated to high external  $[Ca^{2+}]$  for 50 days, and fish acclimated to low external  $[Ca^{2+}]$  for 1, 7, 15 and 30 days.

$J_{in}^{Ca}$  and TEP were determined in a manner similar to that described above while the external concentration of  $Ca^{2+}$  was increased from  $\sim 50 \mu\text{equiv l}^{-1}$  to  $\sim 5 \text{ mequiv l}^{-1}$  (seven steps) in a logarithmic fashion at 5-min intervals by addition of  $Ca(NO_3)_2 \cdot 4H_2O$ . The NaCl level was maintained constant at  $0.8 \text{ mequiv l}^{-1}$ . Samples were collected only during the final 1 min of each interval. A pilot experiment demonstrated that while 4 min was sufficient time to allow  $>95\%$  re-equilibration of  $^{45}Ca$  in the dorsal aortic perfusate following a change in the specific activity of the external medium, re-equilibration of the anterior-venous perfusate was only  $\sim 65\%$ . Thus in these experiments, only  $J_{in}^{Ca}$  was determined. Before terminating the experiments, the head chamber was thoroughly rinsed with the starting water ( $50 \mu\text{equiv l}^{-1} [Ca^{2+}]$ ) and a final TEP measurement was made.  $K_m$  and  $J_{max}$  values for  $J_{in}^{Ca}$  were determined from Eadie-Hofstee plots.

### *In vivo methodology*

Trout were transferred directly from their acclimation tanks to individual flux boxes (see diagram and description in McDonald, 1983) filled with a known volume (3–6 l) of normal water ( $1.8 \text{ mequiv l}^{-1} [Ca^{2+}]$ ) at the acclimation temperature. Approximately  $15 \mu\text{Ci l}^{-1}$  of  $^{45}Ca$  was added to the water, rapidly mixed, and an initial water sample taken so that the flux determination commenced within 10 min of first exposure to the experimental conditions. Since  $^{45}Ca$  binds to fish mucus and to the walls of the flux boxes (Höbe, Laurent & McMahon, 1984), standard methods for determining unidirectional fluxes in freshwater fish could not be applied. Instead, the following procedure was used. After 6–7 h, a final water sample was taken and the fish quickly killed by an overdose of anaesthetic [ $1 \text{ g l}^{-1}$  MS-222 (ethyl *m*-aminobenzoate) Sigma]. The fish then was rinsed for 1 min with flowing well water containing a high concentration of  $Ca^{2+}$  ( $\sim 5 \text{ mequiv l}^{-1}$ ) to displace adsorbed  $^{45}Ca$  from the body surfaces. A terminal blood sample was withdrawn by caudal puncture and the carcass weighed and homogenized with an additional 100 ml of distilled water in a Waring blender. Quadruplicate weighed aliquots (0.4–0.8 g) of the homogenate for each fish were digested overnight at  $45^\circ\text{C}$  in tissue solubilizer (NCS, Amersham). The digest was then neutralized with acetic acid and diluted with 10 ml scintillation fluid (OCS, Amersham). Water (5 ml) and plasma samples (0.5 ml plus 4.5 ml distilled water)

were diluted with an aqueous fluor (10 ml ACS, Amersham) and  $^{45}\text{Ca}$  activity was measured by liquid scintillation counting. Tissue c.p.m. were corrected back to the same efficiency as water and plasma c.p.m. using the external standard ratio and a quench curve generated with actual tissue digests.  $J_{\text{in}}^{\text{Ca}}$  was calculated as:

$$J_{\text{in}}^{\text{Ca}} = \frac{\text{WBA}}{\text{SA} \times t \times W}, \quad (3)$$

where WBA (whole body activity) is the total activity accumulated by the fish (c.p.m.) as measured in the tissue digests plus the activity in the removed blood sample, SA is the mean external specific activity (c.p.m.  $\mu\text{equiv}^{-1}$ ) over the flux period,  $t$  is the time (h) and  $W$  is the body weight (kg). SA varied by less than 5 % between the initial and final water sample. When terminal internal SA exceeded external SA by more than 5 %, backflux correction of  $J_{\text{in}}^{\text{Ca}}$  was performed as described by Maetz (1956).

#### *In vivo protocols*

Whole body  $J_{\text{in}}^{\text{Ca}}$  was determined *in vivo* in control fish, in fish acclimated to high external  $[\text{Ca}^{2+}]$  for 21 days, and fish acclimated to low external  $[\text{Ca}^{2+}]$  for 1, 7 and 30 days. Several additional control series were performed to assess the relative contribution of gills, gut and skin in whole body  $J_{\text{in}}^{\text{Ca}}$  *in vivo*.

In one series, the fish were anaesthetized in 1/20 000 MS-222 on an operating table and the oesophagus was tied closed with two loops of silk suture through a small incision about 1 cm posterior to the gills and level with the pectoral fin. The wound was dusted with 100 mg oxytetracycline hydrochloride (Syndel), closed with silk suture, and the fish allowed to recover for 24–48 h prior to testing. A 'sham-ligated' series was treated identically, but the oesophageal ligations were not tightened.

In another series, the fish were lightly anaesthetized in 1/40 000 MS-222 to allow enclosure of the entire trunk posterior to the gills within a water-filled polypropylene tube. This served to isolate the major portion of the skin from the external  $^{45}\text{Ca}$ -labelled medium. The sleeve of a rubber glove secured with elastic bands made a firm seal between the tube and the fish just anterior to the pectoral fins. A 'sham-shielded' series was treated identically, but the polypropylene tube was perforated with large holes which allowed free access of the  $^{45}\text{Ca}$ -labelled external medium to the skin. As the fish periodically struggled and frequently tore themselves free from this apparatus, it was not practical to allow an extensive recovery period from anaesthesia. Instead, flux measurements were started in both sham and test groups after 2 h recovery. At the end of the flux period, a terminal water sample was taken from the sealed tubes for scintillation counting to check that leakage had not occurred.

#### *Gill histology*

At the end of selected *in vitro* experiments, gills were fixed by perfusing the head with 2.5 % glutaraldehyde (in  $0.15 \text{ mol l}^{-1}$  Na-cacodylate buffer, pH 7.4) for approximately 3 min *via* the bulbous catheter at a constant pressure of 60 cmH<sub>2</sub>O (5.9 kPa) (Perry, Laurén & Booth, 1984b). A portion of gill from the second arch (left side) was

clipped from the animal, washed in water (to remove mucus) and then placed in a Petri dish filled with ice-cold fixative. Individual filaments were then trimmed from the gill arch and fixed for 1 h. The filaments were washed twice in  $0.15 \text{ mol l}^{-1}$  cacodylate buffer and placed in a 2 % osmium tetroxide solution (in buffer) for 1 h. Next, the filaments were washed three times in distilled water, immersed for 30 min in 30 % followed by 50 % ethanol, and then transferred to 70 % ethanol where they remained until the final dehydration was accomplished in a graded ethanol series (80, 90, 95, 100 %). Filaments were then critical point dried, mounted on aluminium stubs and gold coated. Lamellae were broken off from filaments and viewed with an ISI DS130 scanning electron microscope at an accelerating voltage of 20 kV.

Within each treatment group, the gills of several fish (2–6) were selected for quantitative morphological measurements. On the basis of both their  $\text{Ca}^{2+}$  transport data and their general morphology, these fish were judged most representative of the group as a whole. Lamellar surface area was determined by tracing photomicrographs onto overlying graph paper. Apparent chloride cell density per unit lamellar surface area was assessed by counting lamellar ‘bumps’, using criteria based on scanning, light and transmission studies in our laboratory (D. J. Laurén & D. Spry, unpublished). The technique is undoubtedly conservative, for it does not detect very small chloride cells seen in sections. Chloride cell exposure refers to the area of chloride cells *not* covered by pavement cells, and is expressed as percentage of lamellar surface area.

#### *Plasma analysis*

Plasma  $\text{Ca}^{2+}$  concentrations were determined on diluted samples by atomic absorption spectrophotometry. Plasma  $\text{Cl}^-$  levels were measured coulometrically with a Radiometer CMT10 chloridometer. Cortisol concentrations were determined by using a cortisol ( $^{125}\text{I}$ ) radioimmunoassay kit (Corning).

#### *Statistical analysis*

Data shown in figures and tables are means  $\pm 1 \text{ s.e. (N)}$ . Where appropriate, paired or unpaired Student's *t*-tests were used to compare sample means and 5 % was taken as the fiducial limit of significance.

### RESULTS

#### *Haemodynamic stability of the perfused head*

A summary of haemodynamic variables in the isolated, saline-perfused head preparation is shown in Table 1. Under the conditions of our protocol there was no progressive deterioration of haemodynamic status which has often been observed in perfused trout heads. In particular, there was no change in perfusate flow distribution (i.e.  $\dot{Q}_{\text{da}}$  and  $\dot{Q}_{\text{av}}$  were stable) or branchial vascular resistance to flow ( $R_{\text{g}}$ ) during the 35-min experimental period. No haemodynamic parameter was affected by either acclimation or treatment regime; thus the data in Table 1 have been compiled from all treatment groups.



*Calcium uptake in the perfused head*

At constant external  $[Ca^{2+}]$  ( $1.8 \text{ mequiv l}^{-1}$ )  $J_{in}^{Ca}$  was stable over time throughout all experiments and flux across the arterio-arterial circulation ( $J_{in}^{Ca a}$ ) represented approximately 90–95 % of total  $J_{in}^{Ca}$  (Fig. 1). This indicates that the primary pathway

Table 1. *Summary of haemodynamic variables in the isolated, saline-perfused trout head preparation (mean  $\dot{Q}_{in} = 3.50 \text{ ml min}^{-1}$ ) at the onset ( $t = 0$ ) and completion ( $t = 35 \text{ min}$ ) of experiments*

Time (min)	$\dot{Q}_{da}$ ( $\text{ml min}^{-1}$ )	$\dot{Q}_{av}$ ( $\text{ml min}^{-1}$ )	Pin ( $\text{cmH}_2\text{O}$ )	$R_g$ ( $\text{cmH}_2\text{O ml}^{-1} \text{ min}^{-1} 100 \text{ g}^{-1}$ )
0 (57)	$2.40 \pm 0.06$	$1.06 \pm 0.06$	$30.06 \pm 1.1$	$14.9 \pm 0.8$
35 (36)	$2.36 \pm 0.07$	$1.13 \pm 0.07$	$31.1 \pm 1.3$	$15.2 \pm 0.9$

Values shown are means  $\pm$  S.E.; number of heads examined shown in parentheses.

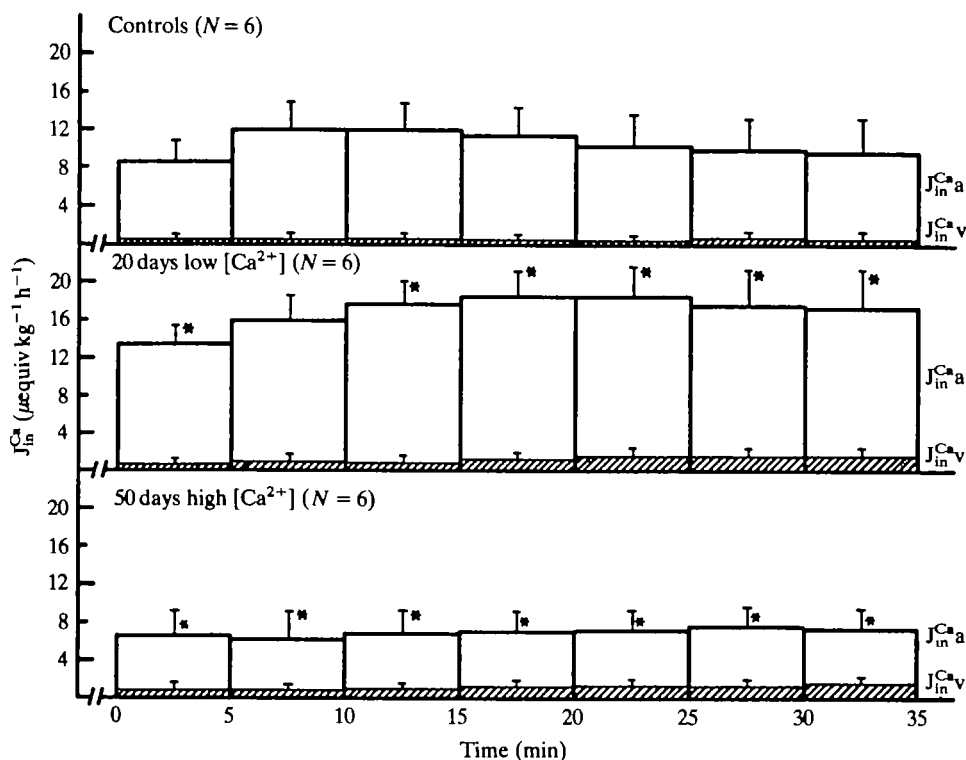


Fig. 1. The effects of low and high  $[Ca^{2+}]$  acclimation on branchial calcium uptake ( $J_{in}^{Ca}$ ) across the arterio-arterial ( $J_{in}^{Ca a}$ ) and arterio-venous ( $J_{in}^{Ca v}$ ) circulations in the isolated, saline-perfused trout head preparation. External  $[Ca^{2+}]$  was kept constant at  $\sim 1.8 \text{ mequiv l}^{-1}$  during the experiments. Values shown are means  $\pm 1$  S.E. \*, significantly different from control value at corresponding time ( $P \leq 0.05$ ).

for calcium uptake is across cells located on lamellae rather than on filaments. Under control conditions (acclimated and tested at  $1.8 \text{ mequiv l}^{-1} [\text{Ca}^{2+}]$ ), total  $J_{\text{in}}^{\text{Ca}}$  was about  $12 \mu\text{equiv kg}^{-1} \text{ h}^{-1}$ . Acclimation to low external  $[\text{Ca}^{2+}]$  acclimation resulted in increased rates of  $J_{\text{in}}^{\text{Ca}}$  while high  $[\text{Ca}^{2+}]$  acclimation produced reduced rates of  $J_{\text{in}}^{\text{Ca}}$  relative to controls. In both cases it was  $J_{\text{in}}^{\text{Ca}}$  that was affected whereas  $\text{Ca}^{2+}$  influx across the arterio-venous pathway ( $J_{\text{in}}^{\text{Ca v}}$ ) was not significantly altered (Fig. 1).

$J_{\text{in}}^{\text{Ca}}$  displayed typical Michaelis-Menten kinetics (Fig. 2). The kinetic constants,  $K_m$  and  $J_{\text{max}}$ , are displayed in Table 2. Acclimation of fish to low external  $[\text{Ca}^{2+}]$  for only 1 day resulted in a five-fold increase of  $J_{\text{max}}$ . This increase was even more pronounced following 1 week of acclimation. Longer periods of acclimation to low  $[\text{Ca}^{2+}]$  caused  $J_{\text{max}}$  to return slowly towards control levels (Fig. 2; Table 2), though even at 30 days, a 2.5-fold stimulation persisted.  $K_m$  was significantly altered only in the 1-day acclimated group.  $J_{\text{max}}$  was reduced by about 40% relative to controls in trout acclimated to high external  $[\text{Ca}^{2+}]$  for 5 days. Again  $K_m$  was not affected. Cortisol-injected fish held in control water displayed elevated  $J_{\text{in}}^{\text{Ca}}$  that was characterized by a significant increase of  $J_{\text{max}}$  but no change in  $K_m$  (Fig. 3; Table 2).

### *Transepithelial potentials*

The TEP, as measured in the perfused head preparation, was rather variable between individuals, but all fish showed similar trends. There were no significant differences between control, 1-, 7-, 15- and 30-day low  $[\text{Ca}^{2+}]$  groups, and 50-day high  $[\text{Ca}^{2+}]$  groups, so these data have been combined in Table 3. Raising external  $[\text{Ca}^{2+}]$  at 5-min intervals caused the TEP to become progressively more positive, and at a mean concentration of  $2.63 \text{ mequiv l}^{-1}$ , the TEP was approximately zero. Experiments at constant external  $[\text{Ca}^{2+}]$  ( $1.8 \text{ mequiv l}^{-1}$ ) in control fish (Table 3, from Perry *et al.* 1985b) and high  $[\text{Ca}^{2+}]$  acclimated fish (data not shown) showed that the TEP was stable over time, so this was not an artifact of deterioration. Similarly, rinsing the head with a low  $[\text{Ca}^{2+}]$  solution at the end of the experiment, so as to restore initial conditions, returned the TEP to about the original (negative) value (Table 3).

Cortisol was the only treatment to affect the TEP significantly. Values became significantly more negative at all levels of external  $[\text{Ca}^{2+}]$ , although the basic pattern remained unchanged (Table 3).

### *Calcium uptake in vivo*

Under control conditions (acclimated and tested at  $1.8 \text{ mequiv l}^{-1} [\text{Ca}^{2+}]$ ) whole body  $J_{\text{in}}^{\text{Ca}}$  measured *in vivo* was about  $26 \mu\text{equiv kg}^{-1} \text{ h}^{-1}$  (Table 4), or approximately twice the rate measured under the same conditions in the perfused head *in vitro* (Fig. 1). Oesophageal-ligation and sham-ligation had no effect on this figure (Table 4) eliminating the gut as a site of uptake *in vivo* under these conditions. However, shielding the major portion of the skin from the external radioisotope reduced whole body  $J_{\text{in}}^{\text{Ca}}$  by half, whereas the sham-shielding procedure had no

significant effect (Table 4). This indicates that approximately 50 % of whole body  $J_{in}^{Ca}$  *in vivo* normally occurs through the skin. The gill  $J_{in}^{Ca}$  *in vivo* would be about  $12\text{--}15 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ , very similar to the figure measured in the perfused head *in vitro*.

The effects of low and high  $[Ca^{2+}]$  acclimation *in vivo* (Fig. 4) were qualitatively similar but quantitatively smaller than those observed *in vitro*. Whole body  $J_{in}^{Ca}$  was

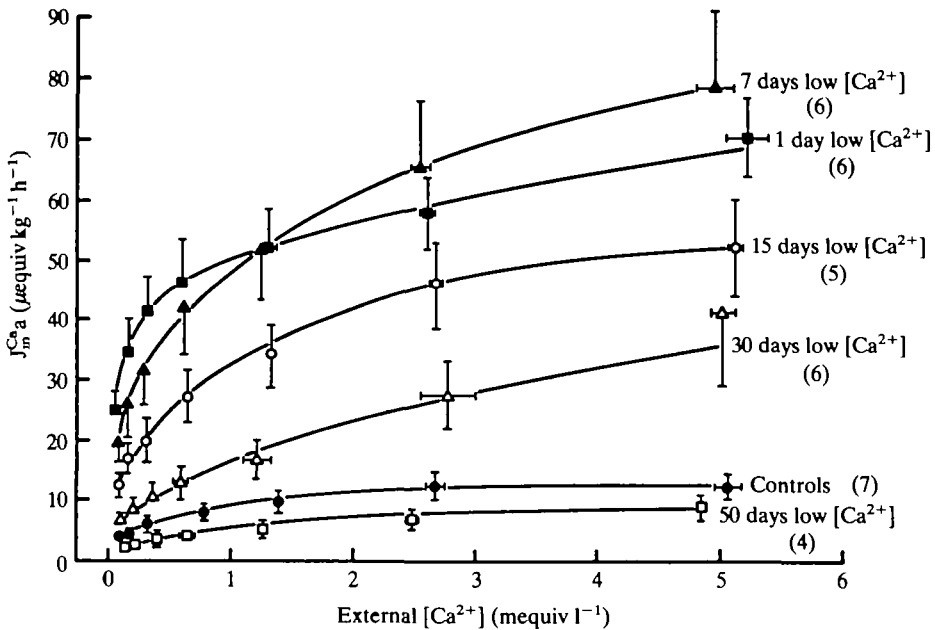


Fig. 2. The effects of low and high  $[Ca^{2+}]$  acclimation for various periods on the kinetics of  $Ca^{2+}$  transport across the arterio-arterial circulation ( $J_{in}^{Ca}$ ) in the perfused trout head during progressive increases of external  $[Ca^{2+}]$ . Numbers in parentheses indicate number of fish examined. All values are means  $\pm 1$  S.E.

Table 2. Summary of the kinetic constants,  $K_m$  and  $J_{max}$ , for  $J_{in}^{Ca}$  in the isolated saline-perfused trout head preparation for the various treatment groups

Acclimation condition	$K_m$ (mequiv $l^{-1}$ )	$J_{max}$ ( $\mu\text{equiv kg}^{-1} \text{h}^{-1}$ )
Control ( $N = 7$ )	$0.28 \pm 0.07$	$12.2 \pm 2.0$
1 day low $[Ca^{2+}]$ ( $N = 6$ )	$0.15 \pm 0.04^*$	$61.7 \pm 6.8^*$
7 days low $[Ca^{2+}]$ ( $N = 6$ )	$0.23 \pm 0.01$	$66.1 \pm 13.4^*$
15 days low $[Ca^{2+}]$ ( $N = 5$ )	$0.25 \pm 0.02$	$44.0 \pm 7.3^*$
30 days low $[Ca^{2+}]$ ( $N = 6$ )	$0.49 \pm 0.09$	$31.8 \pm 8.5^*$
50 days high $[Ca^{2+}]$ ( $N = 4$ )	$0.37 \pm 0.10$	$7.7 \pm 2.1^*$
Cortisol treated ( $N = 6$ )	$0.24 \pm 0.05$	$28.9 \pm 2.4^*$

Values shown are means  $\pm 1$  S.E.

\* Significantly different from control value ( $P \leq 0.05$ )

significantly elevated after 1 day of low  $[Ca^{2+}]$  acclimation, but unlike the results from *in vitro* experiments, longer periods of acclimation produced only non-significant

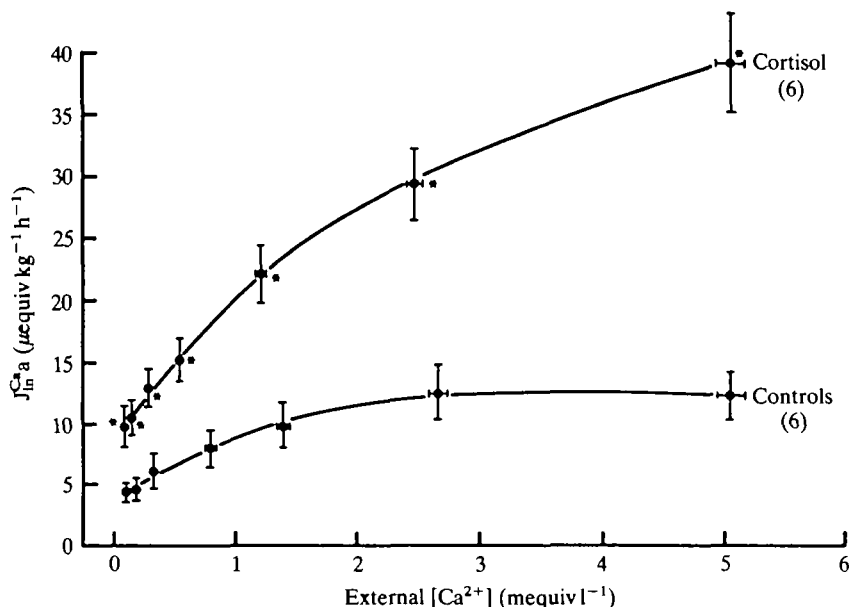


Fig. 3. The effect of prior cortisol treatment (9–10 days; fish held under control conditions) on the kinetics of  $J_m^{Ca}$  in the perfused trout head during progressive increases of external  $[Ca^{2+}]$ . Numbers in parentheses indicate number of fish examined. Values shown are means  $\pm$  1 S.E. \*, significantly different from control value ( $P \leq 0.05$ ).

Table 3. *The effect of external  $[Ca^{2+}]$  on the TEP (outside = 0 mV) in the isolated, saline-perfused trout head preparation*

Period (min)	External $[Ca^{2+}]$ (mequiv l $^{-1}$ )	All treatments† (mV) (N = 28)	Cortisol (mV) (N = 7)	Constant external $[Ca^{2+}]$ ‡ (mV) (N = 11)
0–5	0.10	$-10.2 \pm 0.9$	$-21.7 \pm 1.8^*$	$-7.4 \pm 0.8$
5–10	0.18	$-6.9 \pm 0.8$	$-18.4 \pm 1.9^*$	$-6.6 \pm 0.9$
10–15	0.33	$-4.7 \pm 0.7$	$-13.7 \pm 2.2^*$	$-6.3 \pm 1.0$
15–20	0.65	$-2.7 \pm 0.7$	$-12.1 \pm 1.9^*$	$-6.4 \pm 1.0$
20–25	1.29	$-1.3 \pm 0.7$	$-9.4 \pm 1.8^*$	$-6.6 \pm 0.9$
25–30	2.63	$+0.2 \pm 0.6$	$-8.2 \pm 1.9^*$	$-6.5 \pm 0.9$
30–35	5.04	$+1.2 \pm 0.6$	$-5.0 \pm 2.1^*$	$-6.8 \pm 0.9$
Rinse §	0.10	$-8.9 \pm 0.7$	$-24.0 \pm 2.3^*$	—

Values shown are means  $\pm$  1 S.E.

† Combined data from control group; 1, 7, 15 and 30-day low  $[Ca^{2+}]$  acclimated groups; and 50-day high  $[Ca^{2+}]$  acclimated group. There were no significant differences amongst these treatments.

‡ Data from Perry, Booth & McDonald (1985b); measurements were taken every 5 min at a constant external  $[Ca^{2+}]$  of  $\sim 1.8$  mequiv l $^{-1}$ .

§ This external  $[Ca^{2+}]$  was obtained by flushing the head chamber with a  $0.8$  mequiv l $^{-1}$   $[NaCl]$ ,  $0$   $[Ca^{2+}]$  solution for approximately 5 min.

\* Significantly different from the corresponding 'All treatments' value ( $P \leq 0.05$ ).

changes (Fig. 4). Prolonged acclimation to high  $[Ca^{2+}]$  resulted in significantly reduced  $J_{in}^{Ca}$ .

### Plasma analyses

The effects of acclimation to low and high external  $[Ca^{2+}]$  as well as cortisol treatment on plasma ion concentrations are summarized in Table 5. Plasma  $[Na^+]$  and  $[Cl^-]$  were significantly lowered after 1 day of low  $[Ca^{2+}]$  exposure, but were restored to control levels as duration of acclimation was increased. An exception was the significant reduction of  $[Na^+]$  after 15 days. Acclimation of fish to high  $[Ca^{2+}]$ .

Table 4. Rates of whole body  $J_{in}^{Ca}$  in vivo in trout tested in normal water ( $\sim 1.8 \text{ mequiv l}^{-1} [Ca^{2+}]$ ) under various treatments designed to reveal the site(s) of  $Ca^{2+}$  uptake

Treatment	$J_{in}^{Ca}$ ( $\mu\text{equiv kg}^{-1} \text{ h}^{-1}$ )
Control ( $N = 11$ )	$25.79 \pm 1.41$
Oesophageal-ligated ( $N = 8$ )	$25.58 \pm 4.64$
Sham-ligated ( $N = 8$ )	$24.74 \pm 1.34$
Skin-shielded ( $N = 6$ )	$14.54 \pm 2.79^*$
Sham-shielded ( $N = 7$ )	$29.77 \pm 3.95$

Values shown are means  $\pm 1$  S.E.

\* Significantly different from control value and appropriate sham value ( $P \leq 0.05$ ).

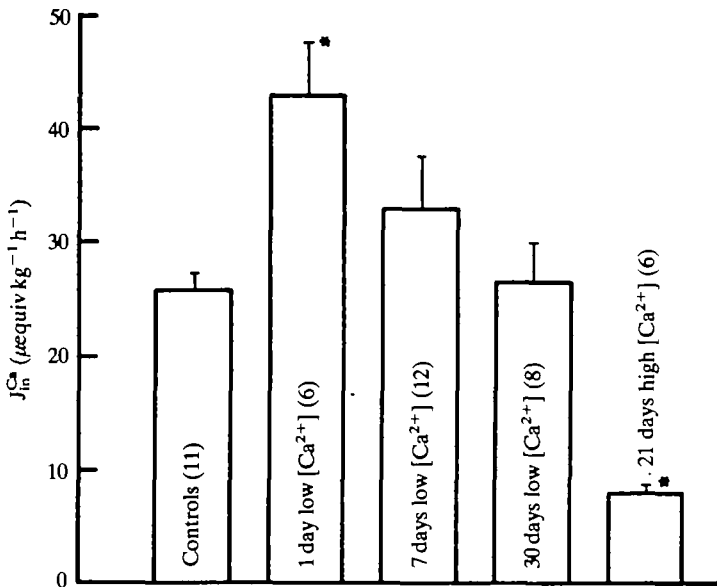


Fig. 4. The effect of low and high  $[Ca^{2+}]$  acclimation for various periods on whole body  $Ca^{2+}$  uptake ( $J_{in}^{Ca}$ ) in vivo. Values shown are means  $\pm 1$  S.E. \*, significantly different from control value ( $P \leq 0.05$ ).

produced a pronounced decrease (20 %) of  $[\text{Cl}^-]$  compared to a relatively minor reduction (5 %) of  $[\text{Na}^+]$ , perhaps reflecting a substitution of  $\text{NO}_3^-$  for  $\text{Cl}^-$  due to the high nitrate levels ( $10 \text{ mequiv l}^{-1}$ ) in this medium. Plasma  $\text{Ca}^{2+}$  concentration remained constant in all treatment groups. Cortisol-treated fish displayed no changes in any of the measured plasma ions.

Plasma cortisol levels were significantly elevated after 1 day of exposure to low  $[\text{Ca}^{2+}]$  (Fig. 5). Longer periods of low  $[\text{Ca}^{2+}]$  acclimation, as well as prolonged high

Table 5. *The effects of low and high  $[\text{Ca}^{2+}]$  acclimation and cortisol treatment on the plasma concentrations of selected ions*

Acclimation condition	$[\text{Cl}^-]$ ( $\text{mequiv l}^{-1}$ )	$[\text{Na}^+]$ ( $\text{mequiv l}^{-1}$ )	$[\text{Ca}^{2+}]$ ( $\text{mequiv l}^{-1}$ )
Control ( $N = 31$ )	$130.3 \pm 0.7$	$149.1 \pm 1.4$	$4.68 \pm 0.09$
1 day low $[\text{Ca}^{2+}]$ ( $N = 12$ )	$122.5 \pm 1.9^*$	$143.3 \pm 1.3^*$	$4.41 \pm 0.06$
7 days low $[\text{Ca}^{2+}]$ ( $N = 22$ )	$131.9 \pm 1.5$	$149.8 \pm 1.6$	$4.47 \pm 0.09$
15 days low $[\text{Ca}^{2+}]$ ( $N = 12$ )	$132.3 \pm 2.2$	$139.2 \pm 3.0^*$	$4.71 \pm 0.17$
30 days low $[\text{Ca}^{2+}]$ ( $N = 16$ )	$133.3 \pm 0.9$	$150.1 \pm 1.9$	$4.78 \pm 0.11$
21–50 days high $[\text{Ca}^{2+}]$ ( $N = 16$ )	$103.6 \pm 4.5^*$	$142.2 \pm 2.8^*$	$4.88 \pm 0.09$
Cortisol treated ( $N = 6$ )	$130.5 \pm 4.2$	$148.9 \pm 5.1$	$4.66 \pm 0.15$

Values shown are means  $\pm 1$  s.e.

\* Significantly different from control value ( $P \leq 0.05$ ).

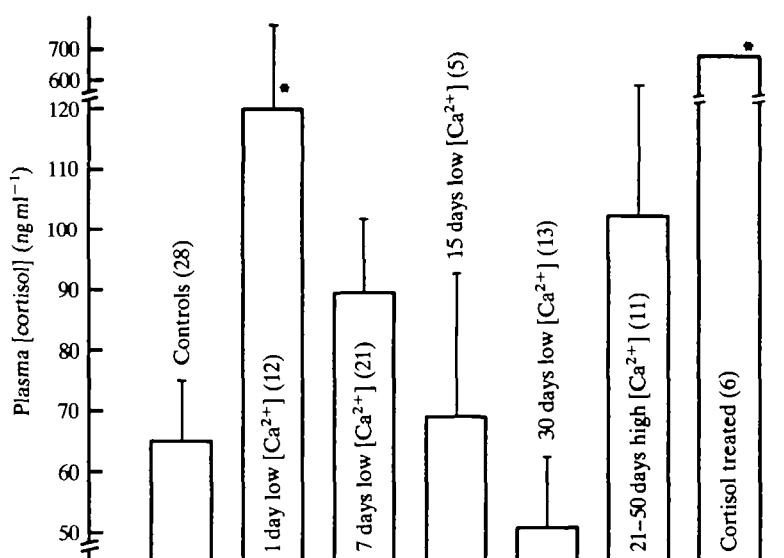


Fig. 5. The effect of low and high  $[\text{Ca}^{2+}]$  acclimation for various periods and cortisol treatment on plasma cortisol levels in rainbow trout. Values shown are means  $\pm 1$  s.e. Numbers in parentheses indicate number of fish examined. \*, significantly different from control value ( $P \leq 0.05$ ). See text for further details.

[Ca<sup>2+</sup>] acclimation, produced no significant changes in plasma [cortisol]. Not surprisingly, cortisol-injected trout displayed very large increases of plasma [cortisol].

### *Gill morphology*

Lamellae from fish kept in normal water possessed chloride cells but these cells were almost entirely covered by a layer of pavement cells (Figs 6A, 7). In only a few chloride cells was the apical membrane directly exposed to the external environment and these apical surfaces represented only a small fraction of total lamellar surface area (Figs 6A, 7B). Acclimation of fish to low external [Ca<sup>2+</sup>] water caused chloride cell proliferation (Figs 6B, 7A) on lamellae and an increase in the area of apical surfaces exposed to the external environment (Figs 6B, 7B) which was evident after 7 days (Figs 6B, 7B). There was no effect after 1 day. Longer periods of acclimation resulted in a gradual return of chloride cell density and exposure to control levels (Fig. 7). Similar increases in lamellar chloride cell density and apical exposure were observed in cortisol-treated fish as in the 7-day low [Ca<sup>2+</sup>] acclimated group (Figs 6C, 7).

### DISCUSSION

In the present investigation, two separate methods for determining calcium uptake in rainbow trout were employed. Routine methodology for measuring *in vivo* ionic uptake in freshwater fish could not be used to evaluate  $J_{in}^{Ca}$  because of <sup>45</sup>Ca adsorption to Perspex and mucus (Höbe *et al.* 1984). As we did not have access to a whole body gamma counter, a new procedure for  $J_{in}^{Ca}$  determination *in vivo* utilizing <sup>45</sup>Ca was developed as described in Materials and Methods. Control values of whole body  $J_{in}^{Ca}$  ( $\sim 26 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ ) measured using this technique are generally lower than those previously reported for freshwater fish ( $40\text{--}100 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ ; Fleming, 1968; Berg, 1968; Pang *et al.* 1980; Mayer-Gostan *et al.* 1983) but similar to the recent measurements of Höbe *et al.* (1984) in freshwater trout under comparable conditions ( $\sim 15 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ ). These lower rates may reflect species and allometric effects or elimination of adsorption artifacts. They are also consistent with measured whole body ( $\sim 15 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ ; Höbe *et al.* 1984) and renal ( $\sim 7 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ ; McDonald & Wood, 1981; Wheatly, Höbe & Wood, 1984) efflux rates measured in freshwater trout.

The oesophageal ligation experiments demonstrated that the gut was not a significant site of Ca<sup>2+</sup> uptake *in vivo* under control conditions, which is in agreement with the findings of Shehadeh & Gordon (1969) and Oduleye (1975) that drinking in freshwater trout is negligible. However, the skin-shielding experiments revealed that the skin (including fins) accounted for fully half of whole body  $J_{in}^{Ca}$  under these conditions, the other half presumably occurring through the gills, at a rate of  $12\text{--}15 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ , equal to the figure measured *in vitro*. This finding is in agreement with previous studies showing significant skin and/or fin uptake (reviewed by Simmons, 1971; Dacke, 1979), although methodical differences preclude quantitative comparison. This 'skin' component could represent Ca<sup>2+</sup> absorbed into

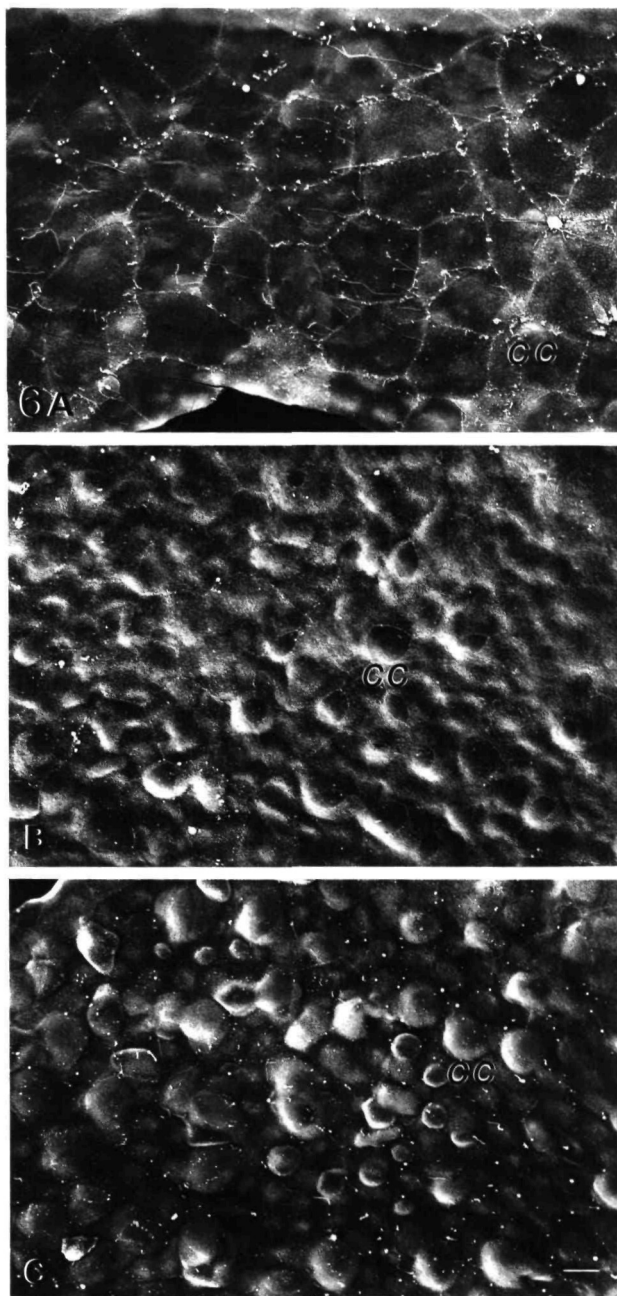


Fig. 6. Representative scanning electron micrographs of lamellae from (A) control fish, (B) 7-day low  $[Ca^{2+}]$  acclimated fish and (C) cortisol-treated fish held under control conditions. CC, chloride cell; Scale bar,  $10\ \mu m$ .



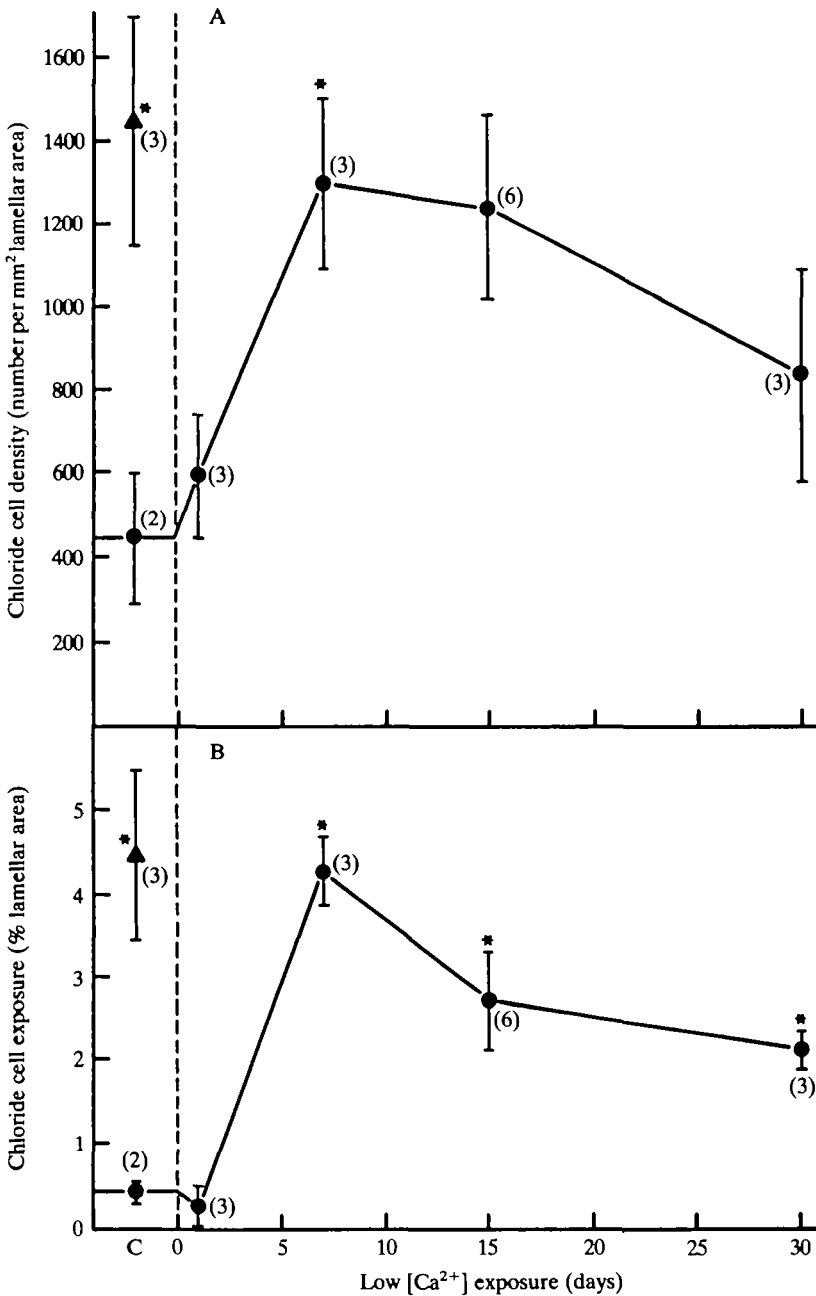


Fig. 7. Changes in (A) the density of chloride cells on lamellae and (B) lamellar chloride cell exposure in fish exposed to low external  $[Ca^{2+}]$  for various periods. The responses for trout treated with cortisol but held under control conditions are also shown (▲). C, control fish; numbers in parentheses indicate number of fish examined. Values shown are means  $\pm 1$  S.E. \*, significantly different from control value ( $P \leq 0.05$ ). See text for further details.

cutaneous blood flow, directly incorporated into scales and fin rays, or absorbed into the mucus coat (rather than adsorbed to it) so as not to be displaced by a 'cold'  $\text{Ca}^{2+}$  wash. The ratio of plasma to water specific activities at the end of the 6- to 7-h experiments in skin-shielded fish was only  $0.042 \pm 0.007$  ( $N=6$ ) relative to  $0.085 \pm 0.010$  ( $N=7$ ) in sham-shielded fish and  $0.098 \pm 0.006$  ( $N=12$ ) in control fish. As the difference is proportional to the measured difference in whole body  $J_{\text{in}}^{\text{Ca}}$  (Table 4), this suggests that  $\text{Ca}^{2+}$  entering through the skin enters the plasma compartment directly, presumably *via* cutaneous blood vessels. Further work will be needed to prove this point.

*In vitro* experiments, using the isolated, saline-perfused head preparation, were considered especially important to the present study since  $J_{\text{in}}^{\text{Ca}}$  across the arterio-arterial circulation of the gill can be distinguished from  $J_{\text{in}}^{\text{Ca}}$  across the arterio-venous circulation. Thus, influx across cells located on lamellae (bathed by the arterio-arterial circulation) can be separated from influx across cells located on gill filaments (bathed by the arterio-venous circulation, Girard & Payan, 1980). Furthermore, this preparation allows rapid kinetic analyses on the branchial component alone in individual fish, which is impossible *in vivo* (see below).

Our *in vitro* measurements with this preparation yielded true branchial  $J_{\text{in}}^{\text{Ca}}$  values almost identical to those determined *in vivo* under control conditions ( $\sim 12 \mu\text{equiv kg}^{-1} \text{h}^{-1}$ ). Furthermore, these rates were stable throughout the entire experimental period. The absolute values of the TEP and the effects of external  $[\text{Ca}^{2+}]$  on them were also very similar to those reported *in vivo* (Eddy, 1975; McWilliams & Potts, 1978). The saline-perfused trout head preparation has been criticized in recent years owing to the lack of dorsal aortic pressure (Pda), gradual deterioration of flux rates over time, steadily increasing vascular resistance to flow, and the possibility of severe and irreversible oedema (Evans *et al.* 1982; Perry *et al.* 1984a; Ellis & Smith, 1983). Using the modifications of Perry *et al.* (1985a,b) we were able to achieve a Pda of approximately 10–15 cmH<sub>2</sub>O (0.98–1.47 kPa) and more importantly, Rg, arterial/venous flow distribution, TEP and  $J_{\text{in}}^{\text{Ca}}$  were constant throughout the experimental period. Moreover, under identical conditions, trout gills have been shown to be non-oedematous (Perry *et al.* 1984b). Previous determinations of  $\text{Ca}^{2+}$  uptake rates in isolated gill preparations have been limited by a lack of physiological perfusion or irrigation rates, a lack of dorsal aortic back pressure, marked deterioration over time, an inability to measure absolute uptake rates, or a combination of two or more of these problems (Milhaud, Rankin, Bolis & Benson, 1977; Milet, Peignoux-Deville & Marletty, 1979; So & Fenwick, 1979; Fenwick & So, 1981; Mugiya & Ichii, 1981; Payan *et al.* 1981). However, where calculable, estimates of  $J_{\text{in}}^{\text{Ca}}$  appear to agree with the current values within about one order of magnitude.

In contrast to the results of Payan *et al.* (1981), we have observed that branchial  $J_{\text{in}}^{\text{Ca}}$  occurs almost entirely across the arterio-arterial circulation and therefore represents movement of  $\text{Ca}^{2+}$  across cells located on lamellae rather than on gill filaments. Reasons for the discrepancy between the two studies are unclear but may include differences in water quality and arterio-venous flow rates. Furthermore, the

possibility and consequences of isotopic contamination of A-V effluent, though unlikely, cannot be discounted. Two sources of A-V contamination were identified in our preliminary experiments; these were seepage of external medium through the ligated oesophagus and leakage due to improper sealing of the condom on the head. Invariably, both types of leaks caused contamination of A-V perfusate leading to artifactual elevation of  $^{45}\text{Ca}$  activity, thereby resulting in overestimates of  $J_{\text{in}}^{\text{Ca}}$ . The majority of leaks could be prevented by the new procedures outlined in Materials and Methods. However, occasionally extremely small leaks persisted and only in these instances was  $^{45}\text{Ca}$  activity greater in A-V perfusate than in DA perfusate (results from these experiments were discarded). The only reliable method for detecting leakage was by comparing the refractive indices of DA and A-V perfusate at regular intervals throughout all experiments. Using this technique, we were able to detect dilution of venous perfusate as low as 1 %. As a consequence of these additional precautions, we are confident that our results reflect true rates of  $J_{\text{in}}^{\text{Ca}}$  across the arterio-arterial and arterio-venous pathways.

Although our results indicate that the primary pathway for branchial  $\text{Ca}^{2+}$  uptake in rainbow trout is across cells located on lamellae, this does not imply that only respiratory cells are involved in calcium transport in freshwater fish. Indeed, results of this study and others (Laurent *et al.* 1985; D. J. Laurén & D. Spry, unpublished observations) have shown that chloride cells are not restricted to interlamellar regions of gill filaments but also occur on lamellae. While the density of filamental chloride cells is great compared to lamellar chloride cells, the vast surface area of lamellae (approximately 27 times the filamental surface area; Girard & Payan, 1980) might provide sufficient numbers of chloride cells to satisfy calcium transporting requirements. The extremely low rate of  $J_{\text{in}}^{\text{Ca}}$  across filamental chloride cells may reflect the reduced surface area of the filamental epithelium, low pressure and flow of the arterio-venous circulation or improper chloride cell orientation. Clearly, we cannot rule out the involvement of respiratory cells in  $J_{\text{in}}^{\text{Ca}}$  but the lack of mitochondria, surface microvilli and cytoplasmic tubulovesicular extensions (Laurent *et al.* 1985) characteristic of ion-transporting cells makes the respiratory cells unlikely sites for  $\text{Ca}^{2+}$  uptake, assuming that this is an active process. However in the absence of unidirectional efflux and/or net flux measurements, we cannot be certain that branchial  $\text{Ca}^{2+}$  uptake is an active process (Koch, 1970). Nevertheless the current demonstration of saturable Michaelis-Menten kinetics suggests at least that carrier-mediation, and/or a selective channel is involved. Furthermore, a comparison of the calculated Nernst equilibrium potentials for  $\text{Ca}^{2+}$  and the measured TEPs shows that the net electrochemical driving force for passive diffusion is directed outwards over most of the concentration range studied (Table 6). The observed kinetic curves for  $J_{\text{in}}^{\text{Ca}}$  are certainly not explained by the changes in TEP.

To date, no data exist on the activities of high-affinity  $\text{Ca}^{2+}$ -ATPase (Doneen, 1981) in chloride cells *versus* respiratory cells, but considering that  $\text{Ca}^{2+}$  must be absorbed against a sizeable electrochemical gradient across the basolateral membrane, it seems likely that the cell type responsible for  $J_{\text{in}}^{\text{Ca}}$  would display higher activities of

this enzyme. Naon & Mayer-Gostan (1983) reported that the activity of chloride cell low-affinity/non-specific  $\text{Ca}^{2+}$ -ATPase (assayed using  $5 \text{ mmol l}^{-1} \text{ Ca}^{2+}$ ) was approximately twice that of respiratory cell low affinity  $\text{Ca}^{2+}$ -ATPase. Because of the high  $K_m$  for this enzyme ( $0.88 \text{ mmol l}^{-1}$ , Doneen, 1981) it is unlikely to be involved in branchial  $\text{Ca}^{2+}$  transport since the upper limit of  $\text{Ca}^{2+}$  concentration in cells is approximately  $1 \mu\text{mol l}^{-1}$  (Godfraind-DeBecker & Godfraind, 1980).

Reduction of ambient calcium levels is known to increase the permeability of the gill epithelium to ions (Eddy, 1975; McWilliams, 1982; McDonald, Walker & Wilkes, 1983), leading to plasma ionic imbalance (McDonald, Høbe & Wood, 1980). In the present study trout were exposed to low environmental  $[\text{Ca}^{2+}]$  without alteration of ambient NaCl levels. While plasma  $\text{Ca}^{2+}$  levels were maintained constant, there was a short-lived reduction in plasma  $\text{Na}^+$  and  $\text{Cl}^-$  levels on day 1 which was corrected by day 7. These data demonstrate a specific effect of external  $[\text{Ca}^{2+}]$  on  $\text{Na}^+$  and  $\text{Cl}^-$  balance and support the findings of McDonald *et al.* (1983) that  $\text{Na}^+$  and  $\text{Cl}^-$  efflux eventually return to normal during chronic exposure to low  $[\text{Ca}^{2+}]$ . The gill TEP also is affected by external  $[\text{Ca}^{2+}]$ , becoming more positive as  $\text{Ca}^{2+}$  levels are elevated (Table 3) due to  $\text{Ca}^{2+}$  exerting a greater effect on  $\text{Na}^+$  permeability relative to  $\text{Cl}^-$  permeability (Eddy, 1975; McWilliams & Potts, 1978). Interestingly, while the branchial TEP varied in the standard fashion with external  $[\text{Ca}^{2+}]$  during the rapid kinetic experiments, there was no evidence of a shift in position of the TEP *versus* external  $[\text{Ca}^{2+}]$  relationship as a result of exposure to low or high  $[\text{Ca}^{2+}]$  regimes. This would suggest that these regimes had no differential effect on the relative  $\text{Na}^+$  and  $\text{Cl}^-$  permeabilities of the gill epithelium and demonstrates clear differences between acute and chronic effects of ambient  $[\text{Ca}^{2+}]$  on gill ionic permeability. In contrast, cortisol treatment clearly resulted in more negative TEPs at any  $\text{Ca}^{2+}$  level, suggesting an increase in the  $\text{Na}^+$  to  $\text{Cl}^-$  permeability ratio.

Acclimation of fish to low external  $[\text{Ca}^{2+}]$  caused marked stimulation of  $J_{\text{in}}^{\text{Ca}}$ , whereas acclimation to elevated external  $[\text{Ca}^{2+}]$  caused inhibition of  $J_{\text{in}}^{\text{Ca}}$  when

Table 6. *Concentration gradients, measured transepithelial potentials, calculated Nernst potentials (E) and driving forces (F) for  $\text{Ca}^{2+}$  in the isolated, saline-perfused trout head preparation*

External $[\text{Ca}^{2+}]$ (mequiv $\text{l}^{-1}$ )	Internal $[\text{Ca}^{2+}]^*$ (mequiv $\text{l}^{-1}$ )	TEP† (mV)	E‡ (mV)	F§ (mV)
0.10	2.0	-10.2	-37.7	+27.5
0.18	2.0	-6.9	-30.3	+23.4
0.33	2.0	-4.7	-22.7	+18.0
0.65	2.0	-2.7	-14.2	+11.5
1.29	2.0	-1.3	-5.5	+4.2
2.63	2.0	+0.2	+3.4	-3.2
5.04	2.0	+1.2	+11.6	-10.4

\* Average measured value.

† Averaged 'All treatments' value from Table 3; inside relative to outside as zero.

‡  $E = \frac{RT}{zF} \ln \frac{[\text{Ca}^{2+}]_{\text{int}}}{[\text{Ca}^{2+}]_{\text{ext}}}$ .

§  $F = \text{TEP} - E$ .

measured in normal water both *in vivo* and *in vitro*. Similar stimulation of  $J_{in}^{Ca}$  following low  $[Ca^{2+}]$  exposure has been observed in *Fundulus heteroclitus* (Mayer-Gostan *et al.* 1983) and tilapia, *Sarotherodon mossambicus* (G. Flik & J. C. Fenwick, personal communication). In contrast, Høbe *et al.* (1984) reported that  $J_{in}^{Ca}$  was largely independent of ambient  $[Ca^{2+}]$  in rainbow trout and bullhead catfish (*Ictalurus nebulosus*). In our studies, the relative *in vivo* changes were somewhat smaller than the *in vitro* changes (compare Figs 1, 2 and 4). This may reflect the fact that the *in vivo* determinations took 6 h (*versus* 5 min *in vitro*), during which time the fish were held in normal water ( $1.8 \text{ mequiv l}^{-1} [Ca^{2+}]$ ). It is quite possible that elevated or depressed  $J_{in}^{Ca}$  tended to return towards the control value over this extended period. Secondly, skin or gut uptake components *in vivo*, which were assessed only under the control conditions, may have varied independently of branchial uptake. Both of these problems, together with adsorption, effectively precluded kinetic studies *in vivo*.

A time-dependent pattern of  $J_{in}^{Ca}$  adjustment was observed during low  $[Ca^{2+}]$  acclimation;  $J_{in}^{Ca}$  was stimulated to the greatest extent during the first 1–7 days and slowly returned towards control levels as acclimation times were increased, yet plasma  $[Ca^{2+}]$  remained constant. This pattern suggests that modulation of branchial  $J_{in}^{Ca}$  is an important adjustment maintaining plasma  $[Ca^{2+}]$  over the short term, but that other factors (e.g. efflux modulation) are involved in the long-term regulatory response to low external  $[Ca^{2+}]$ . Based on the kinetic analyses, it would appear that at least two separate mechanisms are involved in the stimulation of  $J_{in}^{Ca}$ . The acute response (1-day exposure) is an increase in  $J_{max}$  associated with a decrease in  $K_m$ , while the chronic response (7–30 days exposure) is an increase in  $J_{max}$  with no significant change in  $K_m$ .

We speculate that the chronic response is due to increased activity of  $Ca^{2+}$ -ATPase caused by proliferation of lamellar chloride cells, stimulated by the mobilization of cortisol (Doyle & Epstein, 1972) and perhaps other hormones, such as prolactin (Wendelaar Bonga & van der Meij, 1981). Fenwick (1979) and Flik *et al.* (1983b) have demonstrated increases in both low- and high-affinity  $Ca^{2+}$ -ATPase activity during chronic exposure to reduced ambient  $[Ca^{2+}]$  in eels and tilapia respectively, though this was not seen by Mayer-Gostan *et al.* (1983) in the killifish. This idea is supported by the excellent correlation between lamellar chloride cell exposure and  $J_{in}^{Ca}$  across the lamellar epithelium for day 7 onwards, the stimulation of both  $J_{in}^{Ca}$  and lamellar chloride cell exposure following cortisol treatment, and significantly elevated plasma cortisol levels in 1-day acclimated fish. While plasma cortisol levels subsequently declined, it must be remembered that lamellar chloride cell proliferation is a slow process which takes several days and is thought to result from differentiation of resident stem cells (Laurent *et al.* 1985). The initial response, a temporary increase in the affinity of the calcium transporting system, can therefore be viewed as an acute adjustment to regulate plasma  $[Ca^{2+}]$  during the early stages of cell development. It could reflect modification of pre-existing calcium transport sites and/or an increase in the permeability of the apical membranes, as suggested by Mayer-Gostan *et al.* (1983). In turn, these could represent direct effects of initial cortisol or prolactin surges, or the consequence of an initial loss of membrane-bound  $Ca^{2+}$ .

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